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Activation of the Human Thymidine Kinase (TK) Promoter by Simian Virus 40 Large T Antigen Requires both the T Antigen pRb Family-Binding Domain and TK Promoter Sequences Resembling E2F-Binding Sites

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Infection of quiescent cells with the DNA tumor virus simian virus 40 induces expression of the cellular thymidine kinase (TK) gene a minimum of 10- to 20-fold, and this induction depends upon the viral protein large T antigen (T-Ag). To define both human TK promoter elements and T-Ag functional domains required for transcriptional induction, we have established a system in which stable Rat-1 transfectants harboring TK promoter-luciferase hybrid genes are infected with recombinant adenoviruses expressing either wild-type or mutant forms of T-Ag and luciferase expression is measured as an indicator of promoter activity. The results show that (i) a 135-bp TK promoter fragment is activated 10- to 15-fold by viral infection; (ii) this activation is the result of both T-Ag-dependent and -independent mechanisms; (iii) the T-Ag pRb family-binding domain, but not the p53-binding, helicase, or ATPase domain, is required for activation; and (iv) activation is severely diminished with a TK promoter fragment in which E2F-like-binding sites have been removed. These data demonstrate a requirement for both an E2F-related factor and a pRb family member in activation of the TK promoter by T-Ag. This contrasts with the promiscuous activation of many cellular and viral genes by T-Ag, which is independent of its ability to bind pRb.

When quiescent, serum-starved cells are infected with the DNA tumor virus simian virus 40 (SV40), they reenter the cell cycle and progress to S phase (47). During this mitogenic stimulation, a number of cellular genes involved in DNA synthesis, including the thymidine kinase (TK) gene, are induced (34). Both the induction of cellular DNA synthesis and TK gene expression depend upon the viral protein large T antigen (T-Ag) (43). T-Ag is a multifunctional protein involved in the initiation of viral DNA replication and the regulation of viral transcription. It is also required for the immortalization and transformation of cells and has been shown to be a potent *trans* activator of a number of viral and cellular promoters (1, 20, 31, 52, 54, 55). The domains of T-Ag required for many of these activities have been identified. They include DNA-binding, ATPase, and helicase domains that are required for SV40 replication (6, 11, 40, 50) and several domains which interact with cellular proteins, such as DNA polymerase α (13, 14), the pRb family of proteins (12, 16), p53 (36), and TEF-1 and TBP (2, 23, 28). The alteration of host cell regulatory pathways by T-Ag is generally believed to be due to its ability to interact with these cellular proteins (10).

Although a great deal is known about the activities of T-Ag, it is not clear which ones are responsible for the induction of genes such as TK at the onset of S phase. Most studies on transcriptional activation by T-Ag have utilized transient-cotransfection assays (20, 31, 54), and, under these conditions, it has been shown that T-Ag is a promiscuous activator of many viral and cellular promoters. Analyses of these promoters have revealed that the only requirements for activation by T-Ag are either a TATA or initiator element and an upstream factor-binding site of variable nature. These findings suggest that the mechanism of activation might be via direct interaction of T-Ag with the basal transcription apparatus, and they are consistent with its documented ability to bind factors such as TBP and TEF-1 (2, 23, 28). By using a truncated form of T-Ag, the ability to *trans* activate simple promoters in transient assays was localized to the N-terminal 138 amino acids of the protein (54). An analysis of mutations within this region identified several, including *in*A2803 and *dl*2831 (see Fig. 5), which encode proteins that were defective in *trans*-activation assays. The K1 mutation, which eliminates pRb binding (12, 18, 30), did not affect the activity, indicating that pRb binding is not required for the general *trans*-activation function of T-Ag.

Several lines of evidence suggest that the specific activation of cellular genes at G_1/S phase by T-Ag might occur by a mechanism different from the one identified in the transient-cotransfection assays described above. First, although T-Ag activates many different promoters in transient assays, only a small subset of cellular genes are activated during viral infection. Second, the pRb-binding function of T-Ag seems likely to be involved in the induction of cellular genes involved in DNA replication. pRb, and the related proteins p107 and p130, act by binding to members of the E2F family of transcription factors and inhibiting their *trans*-activation functions (7, 9, 19, 25). Viral proteins such as T-Ag bind to pRb family proteins, thereby releasing E2F, which then activates target genes (8). Potential E2F-binding sites have been identified in the promoters of many S-phase-regulated genes, including the TK gene, and in some cases have been shown to be critical for regulation in serum-stimulated cells (37, 46). In addition, an E2F-binding site within the murine TK promoter has been

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found to be a target for activation by polyomavirus large T-Ag (41, 42). It therefore seems possible that SV40 T-Ag utilizes multiple mechanisms to activate transcription of target genes, some that are specific to S-phase-regulated genes and some that are more general and act on a variety of promoters. The general mechanisms may function only when the target gene is episomal. This is suggested by the example of the Rous sarcoma virus long terminal repeat, which is activated by T-Ag in transient transfections in CV1 cells (54) but not during viral infection when it is stably integrated into the CV1 cell chromosome (44).

In this study, we have investigated the mechanism by which SV40 T-Ag activates the human TK promoter, a natural target of T-Ag during viral infection. Previous studies showed that TK mRNA is highly induced during SV40 infection (49) and that at least some of this induction occurs at the level of transcription (44). By studying the expression of hybrid genes, we and others demonstrated that a human TK promoter fragment containing 135 bp upstream and 30 bp downstream of the transcriptional start site was sufficient to confer regulation to linked heterologous genes in both SV40-infected and serum-stimulated cells. The *cis*-acting elements required for regulation in serum-stimulated cells were initially mapped to a region between bp −135 and −67 relative to the transcriptional start site (32, 45), and it was later shown that replacement of sequences between bp −88 and −113 with nonhomologous sequences abolished serum regulation (33). The sequences contained within this 25-bp segment include several potential E2F-binding sites, implicating E2F or a related factor in serum-mediated regulation. E2F has been shown to activate human TK gene expression in transient-transfection assays (27), further indicating its potential importance. Gel shift mobility studies with the 25-bp fragment mentioned above identified G_1/S-phase-specific formation of a complex containing both cyclin A and p107 (37), proteins which have been shown to interact with E2F (5). In addition, E2F-containing protein complexes have been reported to bind to the mouse TK promoter (15). However, oligomers containing a single consensus E2F site were ineffective at competing with the human TK promoter fragment for complex formation, and anti-E2F antiserum was inefficient at affecting complex formation (37), thereby putting in doubt the role of E2F in human TK promoter regulation.

In this study, we analyzed both human TK promoter elements and SV40 T-Ag protein domains required for activation. We have developed a system in which Rat-1 cell lines stably transfected with TK promoter-luciferase (TK-Luc) hybrid genes are infected with recombinant adenoviruses expressing either wild-type or mutant forms of SV40 T-Ag. Our results indicate that both the pRb family-binding domain of T-Ag and the putative E2F-binding sites within the TK promoter are critical, suggesting an activation mechanism involving associations between T-Ag, a pRb family member, and an E2F-related protein.

MATERIALS AND METHODS

Cell culture. Rat-1 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (HyClone Laboratories, Logan, Utah). Stably transfected cells containing hygromycin resistance (Hgm^r) genes were maintained in medium containing 50 μg of hygromycin B (CalBiochem-Novabiochem Corporation, La Jolla, Calif.) per ml. Cells were grown in medium without hygromycin B for all virus infection experiments. Human 293 cells (22) were cultured in DME containing 5% calf serum.

Plasmid constructions. PGL2-Basic, a promoterless luciferase vector, was obtained from Promega Corporation (Madison, Wis.). pY3 (3) is a plasmid conferring Hgm^r. 135-Luc consists of TK promoter sequences between bp −135 and +30, relative to the transcriptional start site, subcloned upstream of the firefly luciferase gene (*Luc*) in PGL2-Basic. HTK-Luc contains 131 bp of the herpes simplex virus TK promoter (bp −75 to +56) (39) linked to *Luc*. 135(E2F)-Luc contains TK promoter sequences between bp −135 and +30, with a substitution of nonhomologous sequences for promoter sequences between bp −98 and −113, as shown in Fig. 1. This mutation, which was generated via oligonucleotide-mediated mutagenesis in M13 (35), is identical to one which abolishes regulation during serum stimulation (33). The presence of the mutation was confirmed by DNA sequencing, and the mutated promoter fragment was excised from M13 and subcloned upstream of *Luc* in the PGL2-Basic vector.

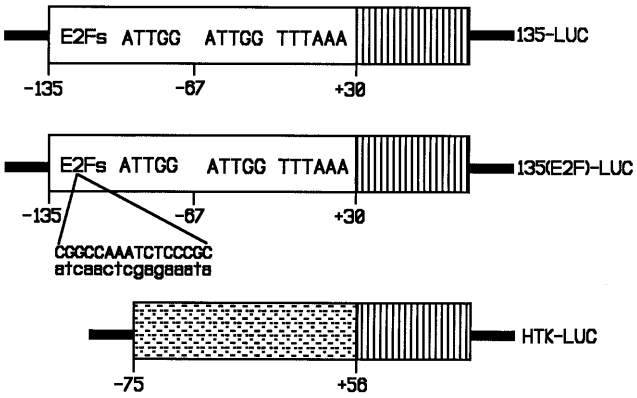


FIG. 1. Structure of human TK-Luc hybrid genes. Open bars, human TK promoter region; stippled bar, herpes simplex virus TK promoter sequences; striped bars, luciferase reporter gene; solid bars, prokaryotic vector sequences. Putative regulatory elements within the human TK promoter region are indicated. Numbers refer to sequence locations within the respective TK promoter. Sequences mutated in 135(E2F)-Luc are indicated; the wild-type sequence is shown in uppercase, and the nonhomologous substitution sequence is shown below it in lowercase.

T-Ag-coding sequences were derived from the vector wtTag/pACSE, which was a gift of Y. Gluzman. This plasmid contains a wild-type SV40 T-Ag gene flanked by parts of the E1A and E1B regions of the adenovirus genome. Mutants of T-Ag (*in*A2803, *dl*2831, K1, *in*A2809, *in*A2811, and *in*A2827) were transferred into pACSE as *Bam*HI-*Kpn*I fragments. These mutants include point mutations and small, in frame insertions or deletions and are described elsewhere (30, 53). (Their locations within T-Ag are shown in Fig. 5.)

Construction and growth of recombinant viruses. The recombinant adenoviruses used in these studies contain wild-type or mutant T-Ag-coding sequences, under the control of the SV40 promoter, inserted in place of the E1A and E1B genes in adenovirus *dl*309 (Ad T/*dl*309) (29). To construct these recombinant adenoviruses, adenovirus *dl*309 (a gift of T. Shenk) was propagated on 293 cells, and viral DNA was prepared. This DNA was digested with *Xba*I plus *Cla*I, which gave rise to one fragment encompassing 96% of the genome plus two small (2%) fragments from the left end of the genome. The 96% fragment was isolated by gel filtration on a Sephacryl S-1000 column (1.5 by 80 cm) (Pharmacia) in a buffer containing 109 mM Tris-Cl (pH 7.5), 2 mM EDTA, and 100 mM NaCl. The desired fragment eluted in the void volume and was well separated from the two 2% fragments.

To introduce T-Ag-coding sequences into the Ad T/dl309 vector, 293 cells were seeded subconfluently on 60-mm-diameter dishes and cotransfected the following day with 2 μg of a pACSE plasmid containing wild-type or mutant T-Ag-coding sequences, 2 μg of the 96% adenovirus *dl*309 fragment, and 14 μg of salmon sperm DNA per plate. In each case, the plasmid DNA was linearized by digestion with *Sal*I before transfection. Six to nine hours after the addition of DNA, the medium was removed, cells were washed twice with Tris-buffered saline, and 4 ml of overlay (DME containing 4% fetal bovine serum and 0.4% agarose) was added. The plates were incubated for 8 to 10 days (with feeding every 3 days), at the end of which time individual plaques were isolated. The recombinant viruses were plaque purified twice before amplification for use in experiments. The identities of the recombinant viruses were verified by isolating viral DNAs and analyzing their restriction endonuclease digestion patterns and by staining infected cells with a panel of anti-T-Ag monoclonal antibodies known to recognize distinct regions of the protein and to discriminate among various mutant T-Ags (9a).

Large-scale viral stocks were prepared by infecting monolayers of 293 cells with single-plaque-derived virus resuspended in 5 ml of DME plus 2% calf serum. Cytopathic effects were observed 2 to 3 days following infection, at which point cells were scraped from the plate, lysed by two rounds of freezing and thawing, and then centrifuged at 2,000 rpm for 30 min in an SS34 rotor at 4°C to pellet cell debris. The supernatant was passed through a 0.45-μm-pore-size filter and stored at −70°C. Viral titers were determined by plaque assays on 293 cells and were generally between 5×10^8 and 1×10^9 PFU/ml. The absence of wild-type adenovirus was verified by the inability of the stocks to form plaques on

HeLa cells. Mock virus is a 293 cell lysate prepared by a procedure identical to that used for generating viral stocks except that the cells received 5 ml of DME plus 2% calf serum without virus.

To prepare UV-irradiated virus, 2.5 ml of virus was transferred to a 60-mm-diameter dish, placed uncovered in a UV Stratalinker 1800 cross-linker (Stratagene, La Jolla, Calif.), and irradiated with six 2-min pulses of 1,200 μJ of UV light. The virus was irradiated just prior to use and then placed on ice. To minimize light exposure and prevent light-induced repair mechanisms, irradiated stocks were kept covered with aluminum foil and infections were performed in the absence of fluorescent lights.

Ad T/*dl*309 infections of rat cells. To synchronize cultures in G_0, cells were plated at a density of 10^5 cells per 60-mm-diameter plate, grown to confluence, and then serum starved for 24 h in medium containing 0.5% calf serum. For infection, the medium was removed at the end of the serum starvation and saved. Virus was diluted in DME plus 0.5% calf serum, and cells were infected at a multiplicity of infection (MOI) of 200 in a total volume of 0.6 ml. Infections were done for 1.5 h at 37°C. Following infection, the original low-serum medium was returned to the cultures, which were then incubated at 37°C until harvest.

DNA transfections. Stable transfections into Rat-1 cells were performed by using Lipofectin reagent (BRL Life Technologies, Gaithersburg, Md.) according to the procedure supplied by the manufacturer. Each transfection (60-mm-diameter dish) contained 8 μg of TK-Luc DNA and 2 μg of pY3 DNA. Approximately 48 h after transfection, cells were split into medium containing 200 μg of hygromycin B per ml. When resistant colonies emerged, several colonies were picked and propagated as clonal cell lines. After the initial selection, transfected cells were maintained in medium containing 50 μg of hygromycin B per ml.

Preparation of whole-cell extracts and luciferase assays. To prepare extracts, plates were rinsed with phosphate-buffered saline (PBS), and cells were scraped into an Eppendorf tube in 1.5 ml of PBS. Cells were pelleted and then resuspended in 100 μl of lysis buffer (125 mM Tris [pH 7.8] with H_3PO_4, 10 mM CDTA (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′,-tetraacetic acid), 10 mM dithiothreitol, 50% glycerol, and 5% Triton X-100) and allowed to lyse for 15 min at room temperature. Extracts were centrifuged for 1 min at room temperature to pellet cell debris, and supernatants were stored at −70°C until assayed.

Luciferase assays were done according to the protocol supplied by Promega Corporation. Twenty microliters of extract was placed in a polypropylene tube (8 by 50 mm) (Turner Designs, Sunnyvale, Calif.) in a Turner TD-20E luminometer (Turner Designs). Substrate solution (100 μl) (270 μM Coenzyme A, 470 μM luciferin, and 530 μM ATP) was added, and activity was measured during a 1-min reaction. The luciferase activity (in light units) in each sample was normalized to the total protein content to calculate the specific activity of the sample (light units per microgram of protein). Fold inductions in luciferase expression were determined by dividing the specific activity at 72 h by the corresponding activity at 0 h. D-luciferin (sodium salt), acetyl coenzyme A, $MgCO_3$, and Tricine were obtained from Sigma (St. Louis, Mo.).

TK enzyme assays. Assays for endogenous TK enzyme activity were performed as previously described (49), except that the extracts used were those prepared for luciferase assays. TK activities (counts per minute) were normalized to total protein content.

Protein quantitation, SDS-polyacrylamide gel electrophoresis, and Western blotting (immunoblotting). The amount of protein in cell extracts was determined by using the Bio-Rad protein assay system (Bio-Rad, Richmond, Calif.), which is based upon the method of Bradford (4). To verify the presence of SV40 T-Ag, 25 to 30 μg of protein from whole-cell extracts were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to polyvinylidene difluoride polyscreen membranes (Dupont-NEN, Boston, Mass.), and probed with the T-Ag-specific monoclonal antibody L19 (24) or Pab416 (a gift of E. Harlow). Bands were visualized by using the Lumiglow chemiluminescent reagents (Kirkegaard & Perry, Gaithersburg, Md.).

RESULTS

Generation of stable TK-Luc cell lines and use of T-Ag-encoding viruses. The TK-Luc constructs used in these studies are diagramed in Fig. 1 and are described in detail in Materials and Methods. These hybrid genes were stably transfected, along with a plasmid conferring Hgm^r, into Rat-1 cells. In previous experiments, we have shown that transfected human TK minigenes are regulated in these cells (45). Stable transfections were performed because T-Ag is able to activate most promoters tested in transient assays (1, 54), while only a very limited subset of chromosomal genes are activated following SV40 infection. We therefore concluded that studying the activation of stably integrated genes would be the most appropriate way in which to identify mechanisms used to induce cellular genes following infection with SV40.

Following transfection, Hgm^r colonies were selected, individual colonies were expanded into clonal cell lines, and cycling populations of each clonal cell line were tested for luciferase expression. Those colonies expressing no luciferase activity were discarded, as were those expressing extremely high levels of activity (i.e., thousands of light units). Colonies chosen for analysis contained moderate levels of luciferase activity in cycling cells. All experiments were conducted with four to six independent colonies for each construct tested.

Recombinant adenoviruses (Ad T/*dl*309) containing T-Ag-coding sequences in place of the adenovirus E1A and E1B genes were used to introduce mutant and wild-type T-Ags into cells. These viruses were used because several of the T-Ag mutants to be studied could not be propagated as SV40, since they are replication defective and also show *trans*-dominant interference with wild-type T-Ag (53). The recombinant adenoviruses are replication defective in the absence of E1A and E1B but can be propagated on 293 cells, which constitutively express these proteins. Infection was chosen as the best method for introducing T-Ag into cells because it is a highly efficient process and more closely parallels the natural sequence of events occurring upon infection of a host cell by SV40 than does transfection.

Preliminary experiments were conducted to establish conditions for the efficient infection of rat cells containing stably integrated copies of 135-Luc with Ad T/*dl*309. Cells were serum starved and infected with virus as described in Materials and Methods. Infections were done at MOIs of 0, 10, 25, 50, 100, and 200, and cells were harvested for both luciferase and endogenous TK enzyme assays at various times from 0 to 72 h postinfection. In addition, infected cells were processed for detection of T-Ag by immunofluorescence at each of the time points. The number of cells expressing T-Ag increased with both increasing MOI and time of incubation, with 100% of the cells staining positive for T-Ag by 72 h postinfection at an MOI of 200. Likewise, expression of both the transfected luciferase gene and the endogenous rat TK gene increased throughout the time course, with the highest levels of induction being seen at 72 h postinfection at an MOI of 200 (data not shown). Subsequent experiments were therefore carried out by infecting cells for 72 h at an MOI of 200.

Induction of TK-Luc hybrid gene constructs in cells infected with wild-type Ad T/*dl*309. Clonal cell lines containing stably integrated 135-Luc were synchronized in G_0 and infected with the Ad T/*dl*309 recombinant adenovirus, and luciferase induction was determined as described in Materials and Methods. To verify that any induction detected was due to viral gene expression, cells were also infected in parallel with UV-irradiated virus. Figure 2 shows the average luciferase induction for four clonal cell lines, determined in multiple experiments using several different viral stocks. Induction levels ranged from approximately 10- to 15-fold, indicating that the 135-bp human TK promoter fragment was highly induced this system. For each clonal line, luciferase induction was reduced approximately 70% by UV irradiation.

As shown in a representative immunoblot in Fig. 3A, no T-Ag was detected in extracts from cells infected with UV-irradiated virus, indicating that UV irradiation effectively abolished viral gene expression. Figure 3B and C compare the patterns of luciferase and endogenous TK enzyme inductions in the same experiment shown in Fig. 3A and demonstrate that UV irradiation of the virus reduced induction of the endogenous TK gene to a similar extent as for the transfected 135-Luc gene. These results indicate that activation of the TK promoter is largely dependent on the expression of viral early genes. They also suggest the existence of a T-Ag-independent mechanism of induction, since UV-irradiated virus preparations ac-

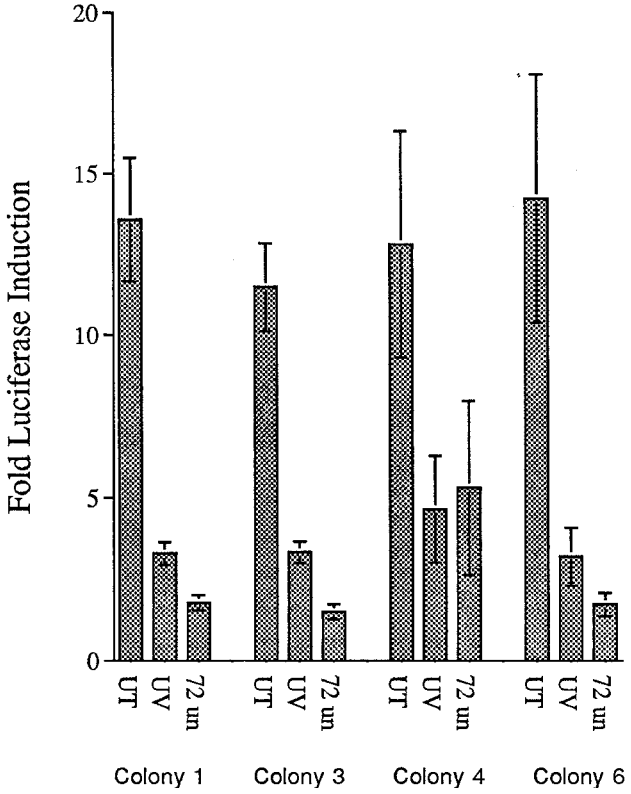


FIG. 2. Induction of luciferase in 135-Luc cells infected with untreated (UT) or UV-irradiated (UV) wild-type virus. Results for four independent clonal cell lines are shown. Extracts were prepared for luciferase analysis at both the time of infection (0 h) and 72 h after infection. Fold luciferase induction indicates the increase in luciferase expression at 72 h relative to expression at 0 h and is calculated as described in Materials and Methods. 72 un refers to uninfected cells maintained in low-serum medium throughout the infection. For each sample, the number of experiments (n) is as follows. Colony 1: UT, $n = 10$; UV, $n = 9$; 72 un, $n = 9$. Colony 3: UT, $n = 10$; UV, $n = 10$; 72 un, $n = 9$. Colony 4: UT, $n = 5$; UV, $n = 5$; 72 un, $n = 5$. Colony 6: UT, $n = 5$; UV, $n = 5$; 72 un, $n = 4$. Error bars reflect the standard error of the mean.

tivated 135-Luc approximately twofold compared with uninfected controls.

To establish that the T-Ag-mediated induction described above was specific to the human TK promoter, clonal cell lines containing the control plasmid HTK-Luc (Fig. 1) were infected with Ad T/*dl*309 and assayed for luciferase expression at 0 and 72 h. Since the herpes simplex virus TK promoter is not proliferation regulated, it should not respond to T-Ag in these assays. Figure 4A shows the average levels of luciferase induction for four independent cell lines containing HTK-Luc. Expression in three of the four clonal lines was induced only two- to threefold upon infection with the untreated Ad T/*dl*309 virus. This level of activation is consistent with the low level of T-Ag-independent induction detected as described above. The induction of HTK-Luc colony 9 was more substantial, approximately sixfold.

Induction levels for three HTK-Luc colonies infected with unirradiated and UV-irradiated virus are shown in Fig. 4B. Luciferase induction levels following infection with the UV-irradiated virus were similar to those attained upon infection with untreated virus, indicating that the two- to threefold induction of HTK-Luc (sixfold induction for colony 9) was indeed the result of T-Ag-independent events.

Induction of 135-Luc in cells infected with mutant Ad T/*dl*309 viruses. The T-Ag protein, diagrammed in Fig. 5, contains a number of functional domains, several of which are involved in binding to cellular proteins. To assess the requirement for these functional domains in TK promoter induction, a series of Ad T/*dl*309 viruses expressing mutant T-Ag proteins were examined. As shown in Fig. 5, three of the mutations (*in*A2809, *in*A2811, and *in*A2827) are located within the overlapping C-terminal ATPase, helicase, and p53-binding domains of the protein. T-Ags harboring the *in*A2809 and *in*A2811 mutations are defective in the ability to bind p53, while *in*A2827 T-Ag binds p53 with wild-type affinity (52, 55). These C-terminal mutants previously showed wild-type levels of activation of simple promoters in transient-transfection assays (54). The remaining three mutations (*in*A2803, *dl*A2831, and K1) localize to N-terminal domains utilized for interaction with cellular factors. T-Ags containing either the *in*A2803 or *dl*A2831 mutation were defective for *trans* activation of some promoters in transient assays (54), while T-Ag containing the K1 mutation, which is defective for binding to pRb family members (12, 18), retained wild-type activity in these assays (54).

Serum-starved Rat-1 cells containing 135-Luc were infected with the mutant viruses described above, and luciferase induction was measured. The C-terminal mutations, *in*A2809, *in*A2811, and *in*A2827, had little or no effect on the activation of 135-Luc. Induction levels with *in*A2827 were consistently at or above the levels attained with wild-type virus. Induction levels with the p53-binding mutants *in*A2809 and *in*A2811 were also at or near wild-type levels in most experiments but were low in some experiments. We noted, however, that the level of T-Ag in infected cells was also sometimes low with these viruses. Figure 6 shows the results of one experiment in which T-Ag levels were near wild type and demonstrates that luciferase induction levels were also near or above wild type for both *in*A2809 and *in*A2811. The *in*A2827 virus in this experiment showed greater-than-wild-type levels of luciferase induction, as described above. Therefore, the C-terminal ATPase, helicase, and p53-binding activities are not required for T-Ag-mediated activation of the human TK promoter.

In contrast to the results with the C-terminal mutants, luciferase induction levels were consistently decreased to near background (UV-irradiated) levels in cells infected with viruses expressing the N-terminally mutated T-Ags *in*A2803, *dl*2831, and K1. Western blots of extracts from cells infected with viruses containing the *in*A2803 and *dl*2831 mutations, however, showed very low-level or no detectable T-Ag. This was true in a number of experiments and with several antibodies, including a polyclonal antiserum (data not shown). It therefore appears that the T-Ags encoded by these two mutants are very unstable in rat cells, and the role of these mutations in TK promoter activation could not be evaluated. In contrast to *in*2803 and *dl*2831, the K1 mutation encoded a stable T-Ag. Figure 7 compares the K1 mutant with the wild type in several independent experiments and shows that although T-Ag levels were equivalent with the two viruses, luciferase induction levels were reduced 50 to 70% with the K1 virus. This strongly suggests that the ability to bind a pRb family member is required in order for T-Ag to activate the human TK promoter.

Regulation of 135(E2F)-Luc in cells infected with Ad T/*dl*309 viruses. The results presented above indicate the importance of a pRb family member in activation of the TK promoter by T-Ag. One mechanism by which pRb family members exert their antiproliferative effect is via binding to the E2F family of transcription factors, resulting in repression of the transcriptional activity of these factors. This effect is abrogated in the presence of DNA tumor virus proteins such as SV40 T-Ag and adenovirus E1A. To determine the importance of the putative E2F-binding sites in activation of the human TK promoter by

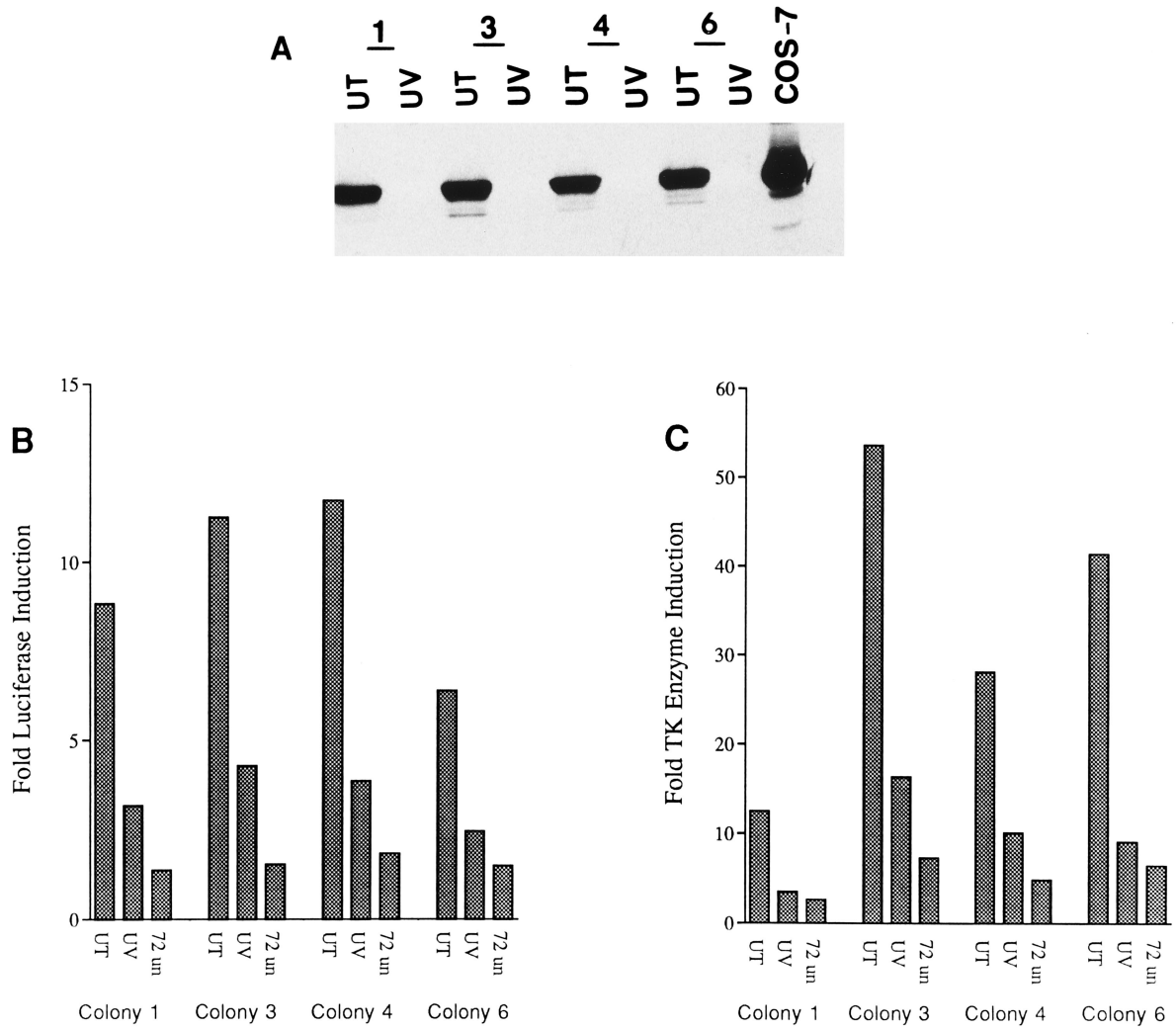
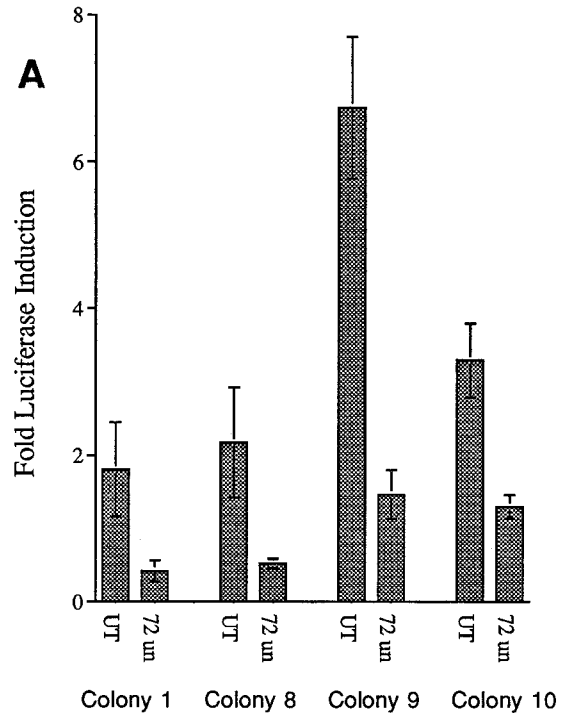


FIG. 3. T-Ag protein, luciferase, and endogenous TK enzyme expression in 135-Luc cells infected with untreated (UT) and UV-irradiated (UV) viruses. Western blots and TK assays were carried out as described in Materials and Methods. (A) Immunoblot of T-Ag protein in infected-cell extracts. For each colony (1, 3, 4, and 6), 25 μg of protein from 72-h extracts was analyzed. Ten microliters of an extract prepared from continuously cycling COS7 cells (SV40-transformed simian CV1 cells which stably express T-Ag) was used as a marker for T-Ag. T-Ag was detected by using the monoclonal antibody Pab416. (B) Increase in luciferase expression at 72 h for cells infected with untreated or UV-irradiated virus or for uninfected cells (72 un). (C) Increase in endogenous TK enzyme expression at 72 h after infection for cells analyzed as in panel B. For both panels B and C, values reflect normalization to total protein content.

T-Ag, the regulation of 135(E2F)-Luc (Fig. 1), which contains a mutation substituting nonhomologous sequences for the putative E2F-binding sites located between bp −98 and −113, was examined.

Six independent clonal cell lines containing 135(E2F)-Luc were tested for their abilities to be activated by T-Ag. In addition, two 135-Luc cell lines were included to provide a wild-type promoter control in the same experiments. Cells were infected with either untreated or UV-irradiated wild-type Ad T/*dl*309 virus or were treated for 72 h with an equivalent volume of a 293 cell lysate (mock infection). Average luciferase induction levels are depicted in Fig. 8. Western blot analyses were performed to confirm the presence of T-Ag in all experiments whose results are shown in Fig. 8 (data not shown). The parental, unmutated 135-Luc constructs exhibited characteristic inductions of 11- to 14-fold with wild-type virus, and this was decreased approximately 70% by UV irradiation. Luciferase expression in mock-infected cells was decreased to 40% of the level attained with the UV-irradiated virus and was no higher than that seen in untreated cells at 72 h. This suggests that the induction seen with UV-irradiated virus was not due to the presence of a 293 cell-derived factor(s) but was attributable instead to virus-dependent, but T-Ag-independent, events.

Luciferase induction in five of the six 135(E2F)-Luc transfectants was severely diminished relative to that with the wild-type promoter. Specifically, induction levels with untreated wild-type virus ranged from 1.5- to 4-fold. Moreover, luciferase expression was not greatly decreased by UV irradiation of the virus, indicating that the mutant TK promoters in these transfectants were not specifically activated by T-Ag. This is the predicted result if the E2F-binding sites are required for activation by T-Ag. The pattern seen with the remaining colony, 1-2, is more puzzling. With the untreated Ad T/*dl*309 virus, luciferase expression in colony 1-2 was induced to a level more than twice that attained with the parental 135-Luc constructs. In addition, expression in colony 1-2 cells infected with the UV-irradiated virus was approximately one-third of that of the untreated control. Expression in mock-infected and 72-h un-



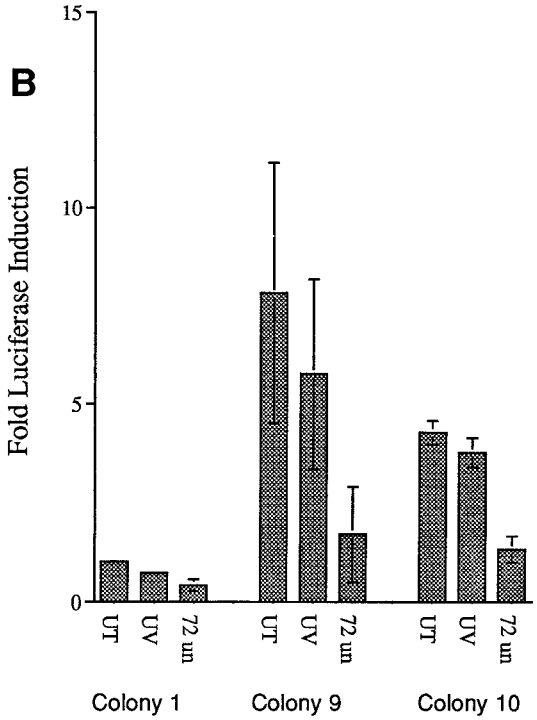


FIG. 4. Luciferase induction in HTK-Luc cells infected with untreated or UV-irradiated virus. (A) Luciferase induction following infection with untreated (UT) wild-type virus and in uninfected cells (72 un). Results are shown for four independent clonal cell lines. For each sample, the number of experiments (n) is as follows. Colony 1: UT, $n = 4$; 72 un, $n = 2$. Colony 8: UT, $n = 5$; 72 un, $n = 4$. Colony 9: UT, $n = 7$; 72 un, $n = 6$. Colony 10: UT, $n = 5$; 72 un, $n = 6$. (B) Luciferase induction in cells infected with UV-irradiated (UV) or UT wild-type virus and in uninfected cells. Results are shown for three independent colonies. For all samples, $n = 2$. Error bars reflect the standard error of the mean.

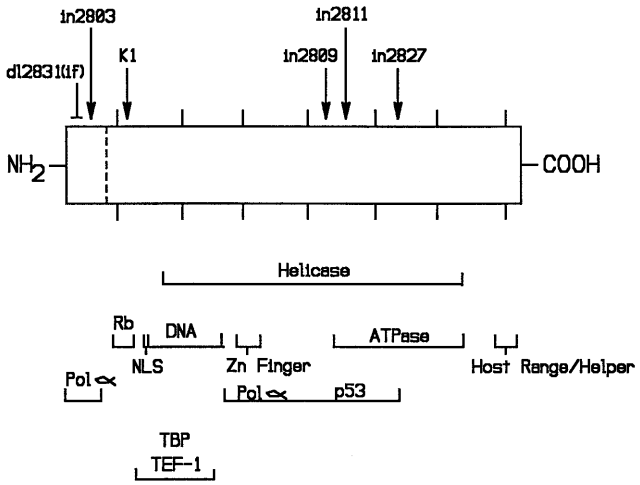


FIG. 5. SV40 T-Ag protein domains and mutations. Vertical bars on the map represent every 100 amino acids. The exon I-exon II boundary at amino acids 82 and 83 is indicated by a vertical dashed line. Labeled brackets below the map indicate domains of known function. T-Ag mutations analyzed in this study are indicated above the diagram. in, insertion; dl, deletion; if, in-frame mutation.

infected extracts was not significantly higher than that at 0 h. Thus, except for being overinduced, colony 1-2 exhibits a wild-type response to T-Ag. While we do not understand this result, it may be due to position effects, such as integration next to a T-Ag-responsive enhancer element. In any case, the fact that five of the six mutant cell lines tested show severe defects in activation by T-Ag suggests that the E2F-binding sites are indeed important for activation.

Since both the pRb-binding domain of T-Ag and the putative E2F-binding sites within the TK promoter were required for activation, a mechanism for the regulation of TK promoter

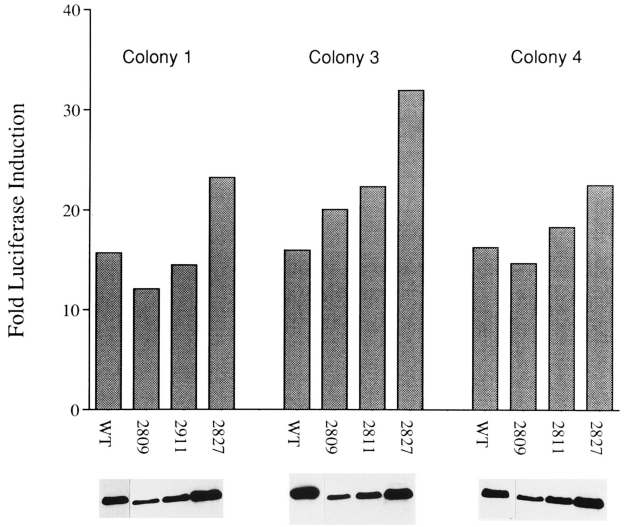


FIG. 6. T-Ag protein and luciferase induction levels in 135-Luc cells infected with wild-type (WT) virus and viruses carrying C-terminal mutations (2809, 2911, and 2827). Results are shown for three independent clonal cell lines. The 72-h infected-cell extracts used for luciferase assays were also analyzed for T-Ag expression by immunoblotting with a monoclonal antibody (L19) specific for the N terminus of the T-Ag protein. For each sample, the corresponding immunoblot depicting T-Ag protein expression at 72 h after infection is shown below the graph.

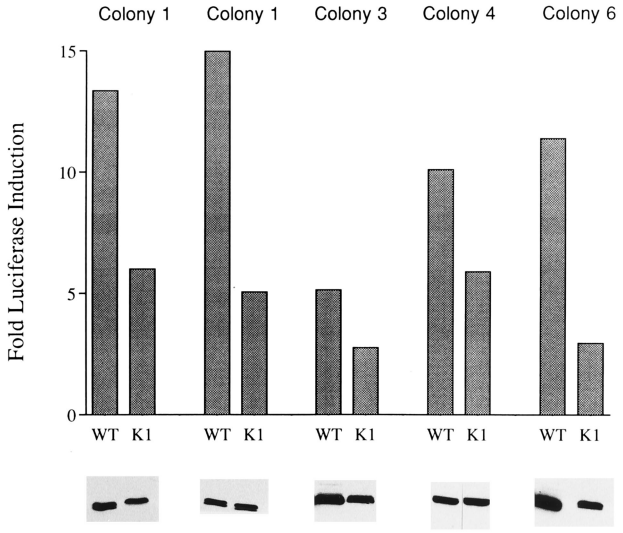


FIG. 7. T-Ag protein and luciferase induction levels in 135-Luc cells infected with wild-type (WT) and K1 mutant viruses. Results are shown for four clonal cell lines, with two independent experiments shown for colony 1. The 72-h infected-cell extracts were analyzed for T-Ag protein expression by immunoblotting with the monoclonal antibody Pab416. For each sample, the corresponding immunoblot is shown below the graph.

activity involving an interaction between a pRb family member and an E2F-like protein seemed likely. To further establish the existence of such an interaction, the 135(E2F)-Luc cell lines were infected with either untreated wild-type virus, untreated K1 virus, or UV-irradiated K1 virus. If activation is due to an interaction between a pRb family member and a protein binding to the E2F sites, the K1 virus should be no more defective than the wild type in its ability to activate the promoter lacking E2F-binding sites. If, however, the protein recognizing the E2F sites does not associate with a pRb family member, the deficits represented in the promoter and viral mutations should be independent and additive, and infection of the 135(E2F)-Luc transfectants with the K1 virus should result in induction levels lower than those observed upon infection of either wild-type promoter constructs with mutant virus or mutant promoter constructs with wild-type virus. UV-irradiated K1 virus was included for the purpose of assessing whether the T-Ag pRb-binding domain is the only T-Ag domain required for stimulation of the TK promoter; if the pRb-binding domain is the only region involved, luciferase induction levels should be equivalent with both untreated and UV-irradiated K1 virus preparations. In contrast, if other domains of T-Ag are also involved, UV irradiation of the K1 virus should result in further decreases in luciferase expression.

The results of these experiments are shown in Fig. 9. For all experiments, the levels of T-Ag were equivalent in wild-type-

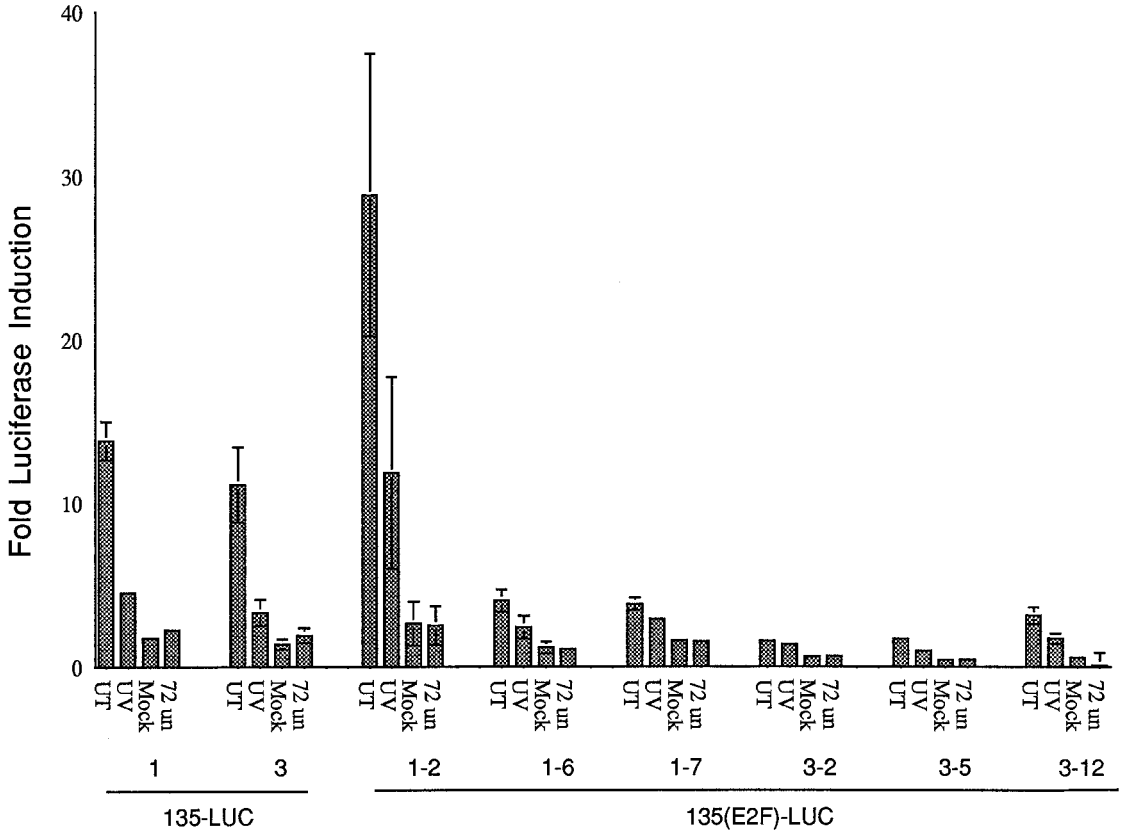


FIG. 8. Induction of luciferase by untreated (UT) and UV-irradiated (UV) wild-type virus in cells containing 135(E2F)-Luc. Results are shown for two clonal cell lines carrying the wild-type 135-Luc hybrid gene and six clonal lines carrying the mutant 135(E2F)-Luc hybrid gene. Some cells were left uninfected and either maintained in low-serum medium (72 un) or treated with a lysate from uninfected 293 cells (Mock). The viruses used are given directly below the graph, and the identities of the colonies tested are listed below the viruses. High levels of T-Ag expression were confirmed in all experiments. The numbers of experiments (n) are as follows. 135-Luc: UT, for both colonies $n = 6$; mock, for both colonies $n = 4$; 72 un, for both colonies $n = 4$; UV, for colony 1 $n = 3$ and for colony 3 $n = 4$. 135(E2F)-Luc for colonies 1-2, 1-6, and 3-12: UT, $n = 6$; UV, $n = 4$; mock, $n = 4$; 72 un, $n = 4$. 135(E2F)-Luc for colonies 1-7, 3-2, and 3-5: UT, $n = 4$; UV, $n = 2$; mock, $n = 2$; 72 un, $n = 2$. Error bars reflect the standard error of the mean.

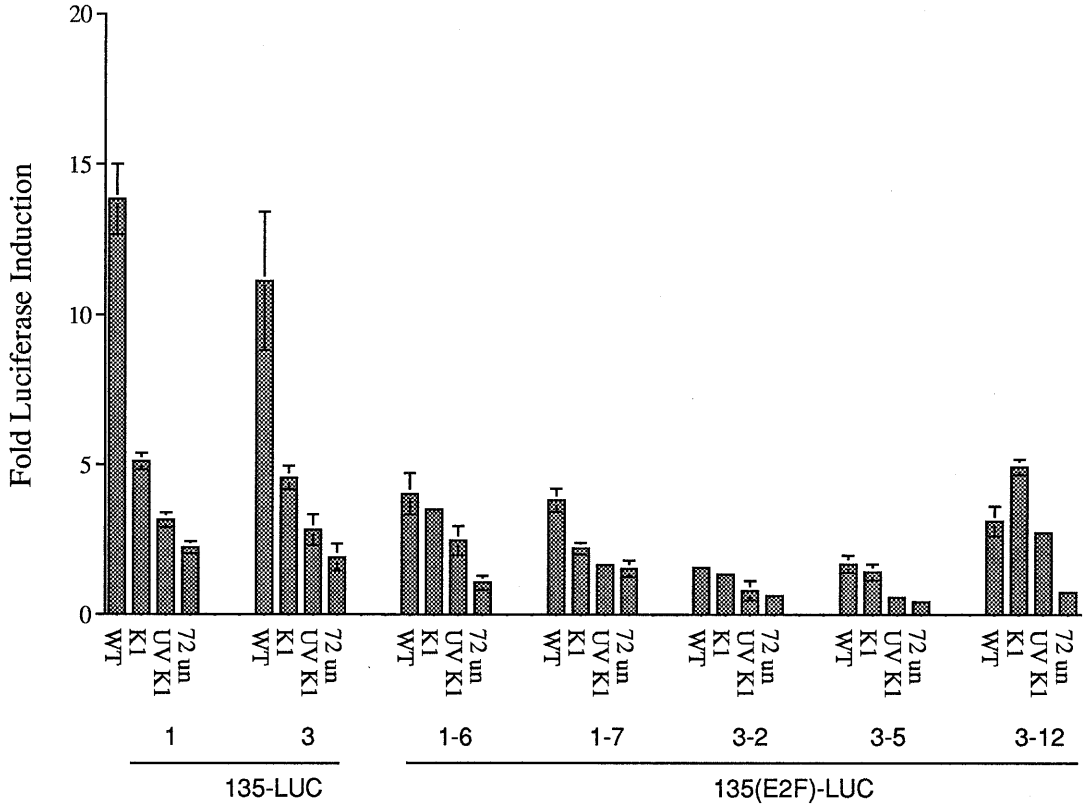


FIG. 9. Induction of luciferase by wild-type (WT), K1, and UV-irradiated K1 (UV K1) viruses in cells containing 135(E2F)-Luc and in uninfected cells (72 un). Viruses are given directly below the graph, and the identities of the colonies tested are listed below the viruses. High levels of T-Ag expression were confirmed in each experiment by Western blotting. The numbers of experiments (*n*) are as follows. 135-Luc (both colonies): WT, $n = 6$; K1, $n = 4$, UV K1, $n = 2$; 72 un, $n = 4$. 135(E2F)-Luc (colony 1-6): WT, $n = 6$; K1, $n = 4$; UV K1, $n = 2$; 72 un, $n = 4$. 135(E2F)-Luc (colony 1-7): WT, $n = 4$; K1, $n = 3$; UV K1, $n = 2$; 72 un, $n = 2$. 135(E2F)-Luc (colony 3-2): WT, $n = 4$; K1, $n = 4$; UV K1, $n = 2$; 72 un, $n = 2$. 135(E2F)-Luc (colony 3-5): WT, $n = 4$; K1, $n = 2$; UV K1, $n = 2$; 72 un, $n = 2$. 135(E2F)-Luc (colony 3-12): WT, $n = 6$; K1, $n = 4$; UV K1, $n = 1$; 72 un, $n = 4$. Error bars reflect the standard error of the mean.

and K1-infected extracts (data not shown). As in previous experiments, induction of 135-Luc colonies with the K1 virus was reduced approximately 60% relative to the levels achieved with the wild-type virus. Induction levels with UV-irradiated K1 virus were decreased only slightly more, indicating that this protein is almost completely defective for TK promoter activation. The 135(E2F)-Luc colonies exhibit the response predicted if T-Ag is activating the TK promoter by disrupting an association between an Rb family member and E2F. Induction levels with the K1 virus were at or near those produced with the untreated wild-type virus and were not greatly decreased by UV irradiation. These results are consistent with SV40 activating the TK promoter via the E2F sites, with the T-Ag pRb-binding domain being primarily responsible for this effect.

DISCUSSION

In this study, we have examined activation of a chromosomal human TK promoter by SV40 T-Ag. This promoter is activated during the infection of quiescent cells with SV40 and therefore represents a true cellular target of T-Ag. Our results indicate that a 135-bp TK promoter fragment was induced an average of 10- to 15-fold in the presence of wild-type T-Ag protein. Infections with UV-irradiated virus established that the majority of this induction (approximately 70%) was dependent upon the expression of T-Ag and was specific to the human TK promoter. Approximately 30% of the effect was T-Ag independent but virus dependent. This T-Ag-independent activation was not promoter specific, since it occurred with the herpesvirus TK promoter and with a human TK promoter mutant [135(E2F)] that no longer responds to T-Ag. The T-Ag-independent, nonspecific activation could be derived from several sources. One possibility is signal transduction events triggered by virus binding to cell surface receptors. Precedent for this is found in the work of Zullo et al. (56), who detected increases in c-*myc* and c-*fos* mRNA levels following exposure of cells to either polyomavirus empty virions or recombinant VP1 protein. Alternatively, the induction seen with UV-irradiated virus might be due to residual, yet undetectable, T-Ag expression. We think it unlikely that this induction reflects the presence of a 293 cell-derived factor in the virus preparations, since virtually no induction of the 135-Luc construct occurred in cells mock infected with a 293 cell lysate (Fig. 8).

Using a series of adenoviruses expressing mutant forms of T-Ag, we examined the activities of the protein that are required for efficient induction of the TK promoter. The mutants examined included ones with mutations in the C-terminal helicase, ATPase, and p53-binding domains; in an N-terminal region previously shown to be required for activation of the SV40 late promoter in transient transfections; and in the pRb family-binding domain. The only T-Ag mutant that was specifically defective in TK promoter activation was K1, which is unable to bind pRb and related proteins. Luciferase induction levels in K1-infected cells were 30 to 50% of wild-type levels and just above that seen in cells infected with UV-irradiated virus (Fig. 7 and 9). The finding that the pRb family-binding

domain of T-Ag was required for TK promoter activation suggested that the virus mediates its effect via the putative E2F-binding sites within the promoter. To test this hypothesis, we studied the expression of a hybrid gene construct [135 (E2F)-Luc] in which the E2F-binding sites were replaced with nonhomologous sequences. Luciferase induction levels for five of six 135(E2F)-Luc containing cell lines were severely decreased relative to that for unmutated controls and were near the background level defined by UV irradiation experiments. The remaining 135(E2F)-Luc transfectant displayed a wild-type phenotype, which we propose may be due to integration of the transfected gene in the vicinity of a strong enhancer or regulatory element that responds to T-Ag.

The fact that both T-Ag mutants defective for binding to pRb family members and TK promoter mutants with alterations in sequences resembling E2F-binding sites are defective for TK promoter activation leads us to conclude that T-Ag is activating this promoter via its interactions with the pRb family of proteins. Since the activation of other promoters, such as the SV40 late promoter and the Rous sarcoma virus long terminal repeat, are independent of this function (54), we propose that the specific activation of proliferation-dependent genes during viral infection and the activation of many promoters by T-Ag in transient-cotransfection assays occur by different mechanisms. The pRb-dependent mode of activation appears to be selective for growth-regulated, natural targets of T-Ag such as TK. A more general, pRb-independent mechanism appears to account for the promiscuous activation of both viral and cellular promoters that is observed in transient-cotransfection assays. This second mechanism may function only on genes in an episomal state and is likely to be responsible for the activation of the SV40 late promoter during viral infection. That T-Ag might utilize several different mechanisms to activate transcription is not surprising, given the numerous activities of this protein, including the ability to bind DNA (6), the ability to bind to elements of the cellular transcription apparatus such as TBP and TEF-1 (2, 23, 28), and the ability to bind to cellular regulatory molecules such as p53 (36) and the pRb family of proteins (12, 16).

Several interesting questions are raised by these studies. The first is whether the pRb family-binding domain is the only T-Ag domain required for TK promoter activation. As shown in Fig. 9, UV irradiation of the K1 virus lowers luciferase induction slightly, suggesting that this T-Ag is not completely defective. There are several possible explanations for this finding. First, the K1 mutation may not totally abolish interactions between T-Ag and pRb family members (55). Alternatively, other T-Ag domains, such as the TBP- or TEF-1-binding domains, may be conferring part of the activity. We attempted to investigate whether the T-Ag domains that are required for the activation of simple promoters are also required for TK promoter activation by examining mutants (*in*A2803 and *dl*A2831) that are defective in this general *trans*-activation function. Unfortunately, the proteins encoded by these mutants were unstable in rat cells, and we were therefore unable to determine their effect on TK promoter activation. Finally, some activity might be contributed by SV40 small t antigen (t-Ag). t-Ag has been reported to activate some promoters and may therefore have some activity in our system (38). It may also be contributing to TK promoter activation indirectly, since it has been shown to stimulate cell proliferation by inhibiting the actions of regulatory phosphatases such PP2A (48, 51). t-Ag does not play a major role in TK promoter activation, however, since the K1 mutant, which encodes a wild-type t-Ag, is almost completely defective.

A second interesting issue is raised by our finding that the E2F sites in the TK promoter were required for T-Ag-mediated activation. This was somewhat surprising, since we previously demonstrated that a human TK promoter fragment from bp −67 to +30, which lacks the putative E2F-binding sites, was activated in CV1 cells following SV40 infection (44). We therefore reexamined our previous finding by constructing a hybrid gene (67-Luc) containing the TK promoter fragment from bp −67 to +30, stably transfecting it into Rat-1 cells, and infecting with Ad T/*dl*309. 67-Luc was modestly induced (two- to four-fold) in this system, but the induction was not dependent on T-Ag, as determined by UV irradiation experiments (1a). Since our earlier experiments with CV1 cells did not employ UV-irradiated virus, it is likely that the induction detected was due to T-Ag-independent effects, such as binding of virus to the cell surface. It is also possible that the levels of T-Ag were higher in the infected CV1 cells, since SV40 replicates in that system.

A final issue is the identity of the cellular proteins involved in TK promoter activation. Gel shift analyses have detected constitutive binding of the $p33^{cdk2}$ protein kinase, and G_1/S-phase-specific binding of a complex dependent upon cyclin A and p107, to the human TK promoter (37). Interestingly, pRb was not detected as a component of any of the complexes formed, suggesting that it may be the ability of T-Ag to bind p107, rather than pRb, which is involved in TK *trans* activation. In addition, both an E2F antiserum and an oligomer containing a single E2F consensus site were ineffective at altering the formation of any of the detected complexes, suggesting that E2F-1, at least, is not the protein binding these sites. Since there are at least five members of the E2F transcription factor family, including two which interact specifically with p107 and/or p130 rather than pRb (17, 21, 26), the protein involved in human TK promoter regulation may be one of these related forms. Determination of the specific proteins involved will be the focus of future studies.

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