Stimulation of simian virus 40 late gene expression by simian virus 40 tumor antigen

(transcription/auto regulation/RNA induction/DNA binding)

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ABSTRACT The early simian virus 40 (SV40) gene product, large tumor (T) antigen, is responsible for the initiation of viral DNA replication and the autoregulation of early gene expression through direct protein-DNA interactions. We investigated the role of T antigen in late viral gene expression, independent of its function in amplifying templates through DNA replication. SV40 DNA was transfected into BSC-1 and COS-1 cells and cultured in the presence of inhibitors of DNA replication. Electrophoretic immunoblot analysis indicated that both the onset and the extent of SV40 late gene expression is increased in COS-1 cells, which constitutively express SV40 T antigen. Blot hybridization analysis of poly(A)-selected RNA demonstrated that the level of synthesis of the major late structural protein VP-1 in COS-1 cells was due to increased transcription. Similar results were obtained when plasmids that contain the SV40 late gene but lack both the origin for viral DNA replication and the early gene coding region were transfected onto COS-1 cells. Using lines of SV40-transformed monkey kidney cells that express altered T antigens, we found that enhanced expression of the late gene product is correlated with the ability of T antigen to bind SV40 DNA. These results indicate that large T antigen plays a role in the stimulation of late viral gene expression.

The simian virus 40 (SV40) lytic cycle is expressed in two temporally regulated phases. Early gene expression begins shortly after infection and continues in a regulated fashion throughout the lytic cycle. Expression of the late SV40 genes is generally delayed until after the onset of viral DNA replication, a process that depends on the expression of the early SV40 gene product, tumor (T) antigen (reviewed in ref. 1). The factors responsible for the early-to-late transition in SV40 gene expression has been the subject of a number of reports (2–6). This transition results, at least in part, from autoregulation by the SV40 large T antigen, which inhibits early gene transcription by binding to the region of the early promoter (7–14). We and others have proposed previously that T antigen can directly enhance the expression of late SV40 genes (9, 15, 16). Such an interpretation is obscured, however, by the amplification and modification of SV40 DNA templates through viral DNA replication. It has been suggested that either or both of these factors contribute directly to the early-to-late shift (5, 9, 17). Therefore, an analysis of the effects of SV40 T antigen on late gene expression is complicated by the need both to inhibit completely viral gene replication and, at the same time, to assay the low levels of gene activity that derive from input DNA in the absence of genome amplification.

In this study we assayed the level of SV40 late gene expression after infection with intact SV40 virus, with viral DNA in the presence of a DNA synthesis inhibitor or with plasmid molecules that lack an origin for SV40 DNA replication (18, 19). Because the SV40 late promoter is not well-defined and deletion of either promoter or RNA leader sequences results in alternate RNA initiation events and aberrant RNA splicing patterns (20, 21), we chose to keep the entire late SV40 transcription unit intact. Thus, late viral gene expression was assayed directly by a sensitive electrophoretic immunoblot analysis using monospecific antibody to the major late structural protein, VP-1, or by blot hybridization analysis of late SV40 RNA. Our results indicate that SV40 T antigen has a direct stimulatory effect on late viral gene expression.

MATERIALS AND METHODS

Viral Infection, DNA Transfection, and Preparation of Protein Extracts. SV40 virus infection was carried out as described (22). Where indicated, cycloheximide was added to the culture media at 25 μg/ml. Transfection of SV40 DNA or plasmid pSVs-L18 was carried out by the calcium phosphate precipitation method (23). Whole-cell protein extracts were prepared after aspiration of growth media and washing of the cell monolayer once with phosphate-buffered saline. Buffer (0.5 ml) containing 50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, aprotinin (25 μg/ml), and L-1-tosylamido-2-phenylmethyl chloromethylketone (25 μg/ml; TPCK) was added to each 10-cm culture dish. DNase I was added to a final concentration of 50 μg/ml, and the dishes were incubated for 10 min at 25°C. One-tenth volume of 10% NaDodSO4/1.4 M 2-mercaptoethanol was added, and incubation was carried out at 25°C for an additional 3 min. The soluble cell extract was transferred to a 1.5-ml Eppendorf tube and incubated at 95–100°C for 5 min. Protein extracts were stored at −20°C.

For analysis of SV40 DNA synthesis, cells (10-cm plate) were pulsed for 2 hr with ortho[32P]phosphate (500 μCi per ml) in Dulbecco’s minimal essential medium (phosphate-free). SV40 DNA was extracted (24), and half of the sample was analyzed by electrophoresis in agarose gels. In the DNA transfection experiments, SV40 DNA was extracted at indicated times and analyzed by Southern blot analysis (25).

Immunoblot Analysis of Protein Extracts. Synthesis of the SV40 late gene product, VP-1, was determined either by “dot-blot analysis” or by the conventional electrophoretic analysis (26). Antiserum to either NaDodSO4-denatured SV40 virions or purified VP-1, isolated from acrylamide gels, was produced in rabbits by following the protocol of McMullen and Consiglio (27).

Cell Cultures and Plasmid Constructions. BSC-1 cells are a continuous line of monkey kidney cells. COS-1 cells are SV40-transformed monkey kidney cells obtained by the transformation and stable integration with an origin-defec-

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen.

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tive replication-negative SV40 DNA (28). The integration pattern of the SV40 DNA allows the stable expression of both SV40 large T and small t antigens but not VP-1, C2, C6, and C11 are SV40-transformed monkey kidney cell lines obtained after infection of CV-1 cells with UV-irradiated SV40 virus (29). These cell lines constitutively synthesize altered SV40 T antigens, which do not complement SV40 tsA mutants (30). The integration pattern in each of the transformed cell lines interrupts the SV40 late genes, thus preventing VP-1 expression.

Plasmid pSVs-L18 was constructed as follows: SV40 DNA was digested with restriction endonucleases Bgl I and Bcl I. The larger restriction fragment [2770 base pairs (bp)] containing the SV40 late promoter and coding sequences was purified and cloned into a pBR322 plasmid derivative pML-2 at the BamHI restriction site.

RESULTS

Synthesis of VP-1 After SV40 Virus Infection. BSC-1 monkey kidney cells and transformed CV-1 monkey kidney cells (COS-1 cells) were infected in parallel with 10 plaque-forming units of SV40 per cell. At the indicated times, whole-cell protein extracts were prepared and analyzed by dot-blot analysis. Because this method detects both newly synthesized VP-1 and VP-1 from the input virions, it was necessary to determine the increase in levels of VP-1 during the course of infection. Thus, VP-1 was assayed in parallel infected cultures either with or without the protein synthesis inhibitor cycloheximide. Cultures containing cycloheximide reveal the level of input viral protein. Cultures without cycloheximide treatment measure the total VP-1 in the infected cells. The difference between these two values provides an indication of the net amount of VP-1 protein.

In this experiment (Fig. 1A), SV40 VP-1 synthesis was detectable in BSC-1 cells 3–4 hr after infection. This is consistent with earlier findings that low levels of SV40 late RNA are synthesized early in the lytic cycle (2–4, 31, 32). In COS-1 cells, which constitutively produce T antigen, both the onset (1 hr after infection) and extent of VP-1 synthesis are significantly enhanced (5-fold by 4 hr after infection).

Because COS-1 cells contain SV40 T antigen, it was important to determine whether the enhanced VP-1 synthesis in this cell line was simply due to accelerated template amplification. The earliest point at which SV40 DNA synthesis (form I) was observed in BSC-1 or COS-1 cells was 8–12 hr after infection (Fig. 1B). Thus, SV40 late gene expression was detected 5–6 hr before the onset of viral DNA replication. The similarity in kinetics of DNA synthesis in BSC-1 and COS-1 cells indicates that the increased level of SV40 late gene expression in COS-1 cells (Fig. 1A) is not simply due to template amplification; it also confirms previous work by others that indicates that the delay in DNA replication after infection does not result from a lack of T antigen but requires some form of template "maturation" (33).

SV40 Late Gene Expression After Transfection of BSC-1 and COS-1 Cells with SV40 DNA. The efficiency of SV40 late gene expression also was determined after transfection of BSC-1 and COS-1 cells with purified form I SV40 DNA. This protocol allows one to directly measure VP-1 levels in the absence of input protein. To exclude template amplification, cytosine arabinoside (25 μg/ml) was added to the culture media. After transfection of BSC-1 and COS-1 cells, whole-cell extracts were prepared and analyzed by immunoblot analysis with anti-SV40 VP-1 antisera. Consistent with the results observed after SV40 virus infection, the level of SV40 late gene expression 30 hr after DNA transfection was dramatically enhanced in COS-1 cells compared to control BSC-1 cells (Fig. 2). We estimate that VP-1 synthesis was increased by at least 20–50-fold in COS-1 cells. No SV40 DNA replication could be detected in the presence of cytosine arabinoside, indicating that enhanced late gene expression is not due to template amplification.

In addition to analysis of the late gene product, VP-1, we analyzed the level of SV40 late mRNA synthesized in BSC-1 and COS-1 cells after transfection with SV40 DNA in the presence of cytosine arabinoside. Blot hybridization analysis showed that the level of SV40 late mRNA synthesis is dramatically increased in COS-1 cells (data not presented). Therefore, we conclude that SV40 T antigen regulates late gene expression at the transcriptional level.

SV40 Late Gene Expression After Transfection with Replication-Negative Plasmids Containing the SV40 Late Promoter and VP-1 Coding Sequences. In order to absolutely exclude DNA replication or its initiation as a basis for the enhanced level of SV40 late gene expression in COS-1 cells, we constructed a plasmid containing the presumed SV40 late promoter and the VP-1 coding sequences but lacking the origin for DNA replication and the coding sequences for T antigen. The SV40 sequences cloned into plasmid pBR322 consist of

![Fig. 1. SV40 late gene expression and DNA replication after infection of BSC-1 (●) and COS-1 (□) cells. (A) SV40 virus infection at a multiplicity of infection of 10 plaque-forming units per cell was carried out as described. Cycloheximide (25 μg/ml) was added to half of the infected cell dishes (60 μm). At the indicated times, the culture medium was aspirated, and the cell monolayer was washed once with phosphate-buffered saline. Whole-cell protein extracts were then prepared. Two to five microliters of the protein extract was spotted onto nitrocellulose paper, allowed to dry 1 hr at 25°C, and analyzed by immunoblot analysis (26). The radioactive spots were then cut from the blot and the cpm were determined. The level of SV40 late gene expression was determined by subtracting [35S]-labeled protein A radioactivity bound to cycloheximide-treated samples from untreated samples. (B) For SV40 DNA synthesis analysis, infected cell monolayers (10-cm plate) were pulsed for 2 hr with ortho-32P phosphate (500 μCi per ml) in Dulbecco's minimal essential medium (phosphate-free). SV40 DNA was then extracted by the procedure of Hirt (24), and half of the sample was analyzed by electrophoresis in a 1.5% agarose gel at 50 V (25 mA) for 16 hr with 40 mM Tris acetate pH 7.2/20 mM sodium acetate/1 mM EDTA. After electrophoresis, gels were fixed in 1% streptomycin sulfate for 30 min at 25°C, dried, and exposed to x-ray film. Lane numbers indicate hours after infection. DNA replication is evidenced by the appearance of form I SV40 DNA.](image-url)
the larger (2770 base pairs) Bgl I–Bcl I restriction fragment. The 300 bases of upstream promoter sequences include the SV40 21-base-pair repeats, the 72-base-pair enhancer element, and the ~25 late transcriptional control sequence, all of which have been shown to be important domains of the SV40 late promoter (20, 34–37). This construction allowed us to evaluate the effect of T antigen on VP-1 expression in the absence of inhibitors of DNA replication. Immunoblot analysis of whole-cell protein extracts prepared from BSC-1 and COS-1 cells at 42 hr after transfection with plasmid pSVs-L18 is shown in Fig. 3. Again, SV40 late gene expression was significantly enhanced in COS-1 cells (Fig. 3), consistent with the results obtained with both SV40 virus infection and SV40 DNA transfection. These experiments confirm that enhanced SV40 late gene expression is not dependent upon DNA replication.

Although the SV40 sequences present in plasmid pSVs-L18 were sufficient to demonstrate a differential level of SV40 late gene expression in COS-1 cells versus BSC-1 cells, the efficiency of late gene expression from this deletion mutant compared to intact SV40 DNA was significantly reduced. This may suggest an auxiliary requirement for late promoter and/or T-antigen binding sequences upstream from the Bgl I site. Similar results have been obtained by using mutants with small deletions in T-antigen binding site II, perhaps supporting the latter conclusion (unpublished data).

SV40 Late Gene Expression in SV40-Transformed Cells Expressing Altered T Antigens. The above experiments show that a function associated with the early phase of a lytic infection, present in COS-1 cells but not in BSC-1 cells, is able to stimulate SV40 late gene expression. Although the obvious candidate for this function is T antigen, the lack of a temperature-sensitive COS-1 cell makes this point difficult to rigorously demonstrate. To confirm and extend our hypothesis, we transfected SV40 DNA in the presence of cytosine arabinoside into three additional monkey kidney cell lines. C2, C6, and C11 cells are SV40-transformed monkey kidney cell lines obtained after inoculation of CV-1 cells with UV-irradiated SV40 virus (29). Each of these cell lines constitutively synthesizes a mutant SV40 T antigen, which does not complement SV40 tsA mutants (29, 30). The ability of the mutant T antigens to bind specifically to SV40 DNA has been analyzed by Prives et al. (38). Briefly, C2 and C11 T antigens, but not C6 T antigen, bind specifically to the SV40 DNA. The efficiency of SV40 late gene expression in these lines is directly comparable because we determined by immunoprecipitation that all three transformed cell lines synthesize approximately equal quantities of SV40 T antigen, comparable to levels observed 14–20 hr after SV40 virus infection (our unpublished data).

Synthesis of VP-1 after transfection of the cell lines C2, C6, and C11 was compared with results in COS-1 cells (Fig. 4). Whereas none of these lines supported the same level of VP-1 synthesis as did COS-1 cells (Fig. 4, lane 2), C2 and C11 (lanes 3 and 5) expressed at least five times as much VP-1 as did C6 (lane 4). This result correlates well with the relative ability of the mutant T antigens to bind specifically to SV40 DNA (30, 38). Similar transfection experiments with SV40 plasmids containing deletions within the early T-antigen coding sequences yielded results qualitatively similar to those presented above (data not shown). We conclude, therefore, that the observed SV40 late gene expression in cell lines C2, C6, and C11 depends on the mutant T antigen present in the transformed cell line.

DISCUSSION

In this study, data are presented that indicate that SV40 late gene expression is stimulated by the early gene product, T antigen. The use of DNA synthesis inhibitors indicates that this stimulation does not depend on DNA replication. Experiments with origin-deleted molecules, which were ampli-
These experiments, they completely exclude and ing mutant plate amplification, experiments do enhance mouse polyomavirus essential (25 arabinoside Lanes: that of the properties late VP-1 Prives, cally VP-1 C6, insig ht after T antigen stimulates, that early transcriptional complexes with sarkosyl, which releases most proteins other than preinitiated RNA polymerase, results in the synthesis of substantial amounts of late RNA (2, 31). Finally, Aloni and his colleagues have presented data suggesting that late SV40 transcription is attenuated (47). If such a repressor exists, it is likely to be cellular in origin and not associated with infecting virus (32). One might conceive of T antigen's role in late transcription to be the removal of this putative repressor.

Alternatively, T antigen may stimulate SV40 VP-1 expression by positively activating the late transcriptional unit. The viral protein may function by directing RNA polymerase molecules to a late promoter that, in its absence, is unre sponsive. This is not unlike the mechanisms proposed for the adenovirus E1A protein in its activation of the other adeno viral and cellular transcription units (48-54).

These two alternative explanations for T antigen-induced activation of the late SV40 transcriptional unit are similar to those presently under consideration for the function of the mouse mammary tumor virus (MMTV) control elements. The long terminal repeat of this virus contains the sequences responsible for glucocorticoid-regulated viral transcription. Data presently available suggest that these hormone-responsive regulatory signals may function as an inducible enhancer or as a hormone–receptor binding site that mediates release of repressor protein(s) upon specific induction (refs. 55 and 56; unpublished data).

The ability of T antigen to function both as a repressor of the early viral genes and an activator of the late genes is reminiscent of action of the bacteriophage λ repressor (cl gene product). In binding to the operators o1 and o2, repressor prevents the expression of lytic functions from promoters pα and pφ. In addition, binding at oφ stimulates transcription from promoter Pφ, leading to maintenance expression of the cl gene itself (57). It will be important to determine both the DNA site at which T antigen exerts its affect on late gene expression and the critical domain(s) in the polypeptide that mediate this activation. The detailed studies of protein–DNA interactions in prokaryotic systems should provide a useful model. The way in which T antigen activates VP-1 expression, in turn, may provide an insight into the specific T antigen induction of cellular genes (58, 59).

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