Effects of a Temperature-Sensitive Mutation in the Immediate-Early Gene of Pseudorabies Virus on Class II and Class III Gene Transcription

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The pseudorabies virus immediate-early protein activates transcription of both class II and class III genes. At present it is not known whether the activation of class II genes occurs through an activation of cellular factors or by a direct interaction of the immediate-early protein with factors or DNA. It is also not known whether the activation of class II and class III genes occurs by a similar or different mechanism. We utilized *tsG*, a temperature-sensitive mutation in the immediate-early gene of pseudorabies virus, to study the activation of viral genes transcribed by RNA polymerases II and III. Previous studies have shown that *tsG* inhibits wild-type adenovirus early gene transcription in coinfections at the nonpermissive temperature (L. T. Feldman and S. E. Ahlers, J. Virol. 57:13-17, 1986). Using this system of mixed infections as an assay, we obtained several results which allowed us to draw certain conclusions about the mode of action of the pseudorabies virus immediate-early protein (IEP). First, the *tsG* mutation inhibits the formation of new transcription complexes on class II genes, but does not affect transcription from preestablished transcription complexes formed in the presence or absence of the adenovirus E1A protein. Second, *tsG* does not inhibit transcription from class III genes, suggesting that the activation by IEP of class II and class III genes occurs by different mechanisms. Third, activation of transcription by the adenovirus E1A protein is not dominant to inhibition by *tsG*, suggesting that the temperature-sensitive IEP is involved in some physical interaction which actively inhibits transcription of viral genes.

The role of viral immediate-early proteins in the *trans*-activating transcriptional induction of genes known as *trans*-activation has been investigated in a number of studies (for a review, see reference 16). Several different studies have provided circumstantial evidence that *trans*-activators may work, in part, by interacting with cellular factors. The pseudorabies virus immediate-early protein (IEP) normally functions to activate transcription of pseudorabies virus early genes, but it can also activate other unrelated genes. In coinfections of pseudorabies virus with the adenovirus E1A deletion mutant *dl312*, the pseudorabies virus IEP not only activated the adenovirus early genes but was a stronger activator of the adenovirus genes than E1A, the adenovirus immediate-early protein, which is normally responsible for activating adenovirus early genes (5). Furthermore, plasmids encoding IEP or E1A are able to induce transcription from viral and cellular genes in cotransfection assays (8, 12). Mutational analyses of sequences upstream of several inducible promoters suggest that there is no specific binding site for *trans*-activating proteins (3, 13, 17, 19, 21). Other studies have shown that both E1A and IEP can activate the transcription of class III genes (7, 9, 23).

Gaynor and Berk (6) have demonstrated that mutants deleted in the adenovirus E1A gene are transcribed in the absence of E1A at long times after infection and that this is a *cis*-acting induction. Recently, exonuclease III protection assays have shown that E1A can induce the binding of some factor to the promoter of the adenovirus E2 gene, but this same protection is seen in E2 transcribed in the absence of the E1A protein (18). Abmayr et al. (1) have recently shown that extracts of pseudorabies virus-infected cells stimulate

transcription from various promoters *in vitro* when added to a HeLa cell extract. When these extracts are prepared from cells infected at the permissive temperature with *tsG*, a temperature-sensitive mutation in the IEP gene, the stimulatory activity is heat sensitive, whereas the activity from wild-type-infected cells is not heat sensitive. This indicates that IEP interacts directly with some component of the HeLa cell extract to stimulate transcription. Recently, we have also shown that IEP is not continuously required to maintain induced transcription through temperature shift experiments with *tsG* (1a). These data strongly suggest that IEP interacts with one or more cellular factors in order to activate transcription.

We have been studying the interaction between IEP and host cell transcription factors by examining the effect of a temperature-sensitive mutation in the IEP gene, *tsG*, on viral transcription. These studies have shown that not only does *tsG* fail to activate adenovirus early genes at the nonpermissive temperature, but it can actually inhibit wild-type adenovirus transcription in coinfections. The *tsG* IEP is therefore not inert at the nonpermissive temperature, suggesting as did the *in vitro* studies of Abmayr et al. (1), that IEP interacts with cellular components directly to activate transcription. In the present study we utilized this system of mixed infections with *tsG* and adenovirus to explore the mechanism of *trans*-activation. We drew three conclusions from our results. First, transcription complexes which are formed on viral genes prior to *tsG* infection are not inhibited by *tsG*, suggesting that IEP is involved only in the formation of transcription complexes. Second, class III gene transcription, unlike class II gene transcription, is not inhibited by coinfection with *tsG*, suggesting that class III genes are *trans*-activated by a different mechanism than class II genes.

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TABLE 1. *tsG* repression in the absence of functional E1A protein^a

Infection	cpm in RNA		
	E1A	E2	E4
<i>dl1500</i>	986	692	549
<i>dl1500</i> + <i>tsG</i>	58	57	31
<i>dl1500dl1500</i> + <i>tsG</i> ratio	17.1	12.2	17.2

^a HeLa cells were infected with *dl1500* at 150 PFU per cell with or without *tsG* at 50 PFU per cell at 40°C for 17 h in the presence of araC at 25 µg/ml. Cells were labeled for 30 min at 40°C with [³H]uridine at 200 µCi/ml. Infected cells were harvested. RNA was extracted and hybridized to plasmid filters.

Third, at the nonpermissive temperature, the *tsG* IEP is involved in some physical interaction with a cellular factor which actively prevents the formation of transcription complexes, since E1A protein is not dominant to inhibition by *tsG*.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension culture in Joklik minimal essential medium containing 6% newborn calf serum. Adenovirus type 5 (Ad5), *dl309*, *dl312*, and *dl1500* were obtained from A. Berk. The genetic properties of *dl309*, *dl312*, and *dl1500* have been described previously (14, 15, 20). Ad5 and *dl309* were grown on HeLa cells in spinner culture. The mutants *dl312* and *dl1500* were grown on 293 cells in spinner culture. The *tsG* and wild-type pseudorabies virus were obtained from T. Ben-Porat and A. S. Kaplan. The genetic properties of *tsG* have been described (2, 11). *tsG* and wild-type pseudorabies virus were grown on rabbit skin cells and plaqued on rabbit kidney cells maintained in minimal essential medium plus 5% newborn calf serum.

Infections and hybridizations. Infection of cells, labeling of RNA with [³H]uridine, and preparation of labeled RNA were as described previously (5). Plasmid filters were prepared as described earlier (4). Subsequent DNA-RNA hybridizations were performed as described by Feldman et al. (5).

S1 nuclease analysis. S1 nuclease analysis of 100 µg of cytoplasmic RNA was performed as described earlier (6). The M13 single-stranded DNA clone complementary to adenovirus E3 region mRNA was kindly supplied by A. Berk and has been described previously (6).

Nuclear runoff transcription. HeLa cells were infected as described by Feldman et al. (5). Nuclear runoff transcription from isolated nuclei from 5×10^7 cells was performed as described by Hofer et al. (10) by using a 150-µCi/200-µl reaction mixture. Where indicated, α-amanitin was added to the isolated-nucleus reaction mixture to a final concentration of 0.5 µg/ml. Reaction mixtures were incubated for 15 min at 37°C. Labeled nuclear RNA was extracted and hybridized to plasmid filters as described previously (5).

RESULTS

We had previously studied the inhibition of wild-type adenovirus early gene transcription by the temperature-sensitive mutation in the pseudorabies virus IEP, *tsG*, in coinfections (4). In these experiments, we postulated that either productive transcription complexes were never formed due to interference by the *tsG* IEP or that the reduction in E1A protein levels was the cause of the lack of adenovirus early gene transcription. To test the hypothesis

that the lack of E1A protein was not the cause of inhibition, we coinfecting *dl1500*, an adenovirus mutant which produces a non-*trans*-activating E1A protein, with *tsG* for 17 h at 150 PFU per cell at the nonpermissive temperature. These conditions allow early genes to be transcribed in the absence of functional E1A protein. This experiment enabled us to determine whether all early genes are inhibited to the same extent in the absence of functional E1A, despite vastly different types of promoters. We found that all adenovirus early genes examined were inhibited to approximately the same extent (Table 1). These results reinforce the hypothesis that, at the nonpermissive temperature, *tsG* inhibits several different adenovirus promoters and is not exclusively inhibiting the E1A promoter.

This experiment, as well as our previous studies, was a coinfection in which adenovirus transcription complexes were unable to form due to inhibition by *tsG*. To investigate further the nature of inhibition by *tsG*, we conducted studies on the effect of *tsG* on previously formed and already productive transcription complexes. To ensure that pseudorabies virus can superinfect an adenovirus-infected cell, we first scored for the production of IEP in a superinfection by Western blot assay for IEP. In these experiments the same level of IEP was produced whether or not the HeLa cells had been preinfected with adenovirus (data not shown). Therefore, *tsG* was able to superinfect adenovirus-infected cells and produce normal levels of the *tsG* IEP. We then examined the effect of a *tsG* superinfection on adenovirus early gene expression (Table 2). We found that *tsG* cannot inhibit ongoing adenovirus transcription. In fact, there is a stimulatory effect of the *tsG* superinfection on adenovirus transcription, which is probably due to a virion protein of pseudorabies virus (4). However, it is clear that *tsG* does not inhibit ongoing adenovirus early gene transcription. This could mean either that *tsG* cannot inhibit preformed stable transcription complexes or that E1A protein is dominant to the temperature-sensitive form of IEP for activation of transcription.

To determine why *tsG* can inhibit virus templates which are coinfecting with *tsG* but not templates which are being actively transcribed in the presence of E1A, we conducted a time course experiment of superinfection with two different adenoviruses. This experiment was designed to test the hypothesis that as transcription complexes are formed on the adenovirus template they become resistant to inhibition by *tsG*. In addition, the experiment enabled us to determine whether E1A can activate a second adenovirus template in the presence of *tsG*. The first adenovirus template used was wild-type Ad5. The second adenovirus template was *dl309*, which has a wild-type phenotype but an altered E3 gene whose transcripts can be distinguished from wild-type E3

TABLE 2. Superinfection of adenovirus by *tsG* or pseudorabies virus^a

Infection	cpm in RNA		
	E1A	E2	E4
Ad5 (6 h)	4,664	16,564	13,222
Ad5 (9 h)	3,698	14,827	11,943
Ad5 + <i>tsG</i>	10,382	53,373	16,251
Ad5 + wild-type pseudorabies	18,670	49,451	16,657

^a HeLa cells were infected with Ad5 at 5 PFU per cell at 40°C for 6 h, and then portions were superinfected with *tsG* or wild-type pseudorabies virus at 40 PFU per cell or not superinfected at 40°C. After 3 h of superinfection, cells were labeled for 30 min at 40°C with [³H]uridine at 200 µCi/ml. RNA was extracted and hybridized to plasmid filters.

FIG. 1. Adenovirus E3 transcription from preinfected Ad5 is protected from *tsG* repression. HeLa cells were infected with Ad5 at 20 PFU per cell for 0 h (lanes 1 and 2), 2 h (lanes 3 and 4), 4 h (lanes 5 and 6), or 6 h (lanes 7 and 8) at 40°C before being superinfected with *dl309* at 20 PFU per cell (lanes 1, 3, 5, and 7) or *dl309* at 20 PFU per cell plus *tsG* at 40 PFU per cell (lanes 2, 4, 6, and 8). After superinfection, cells were incubated at 40°C for 6 h. Cells were harvested by centrifugation and cytoplasmic RNA was extracted. S1 nuclease analysis was performed on the RNA isolated from these infections and probed with ³²P-labeled E3 M13 single-stranded probe. This autoradiograph shows S1-protected fragments run on a 5% acrylamide denaturing gel.

transcripts by S1 nuclease analysis. Because the half-life of adenovirus early RNAs is only 20 to 30 min (22), the amount of RNA present in the cell represents the transcription rate of these genes. As the first adenovirus assembled more active transcription complexes prior to *tsG* superinfection over 6 h, more early gene transcription was resistant to inhibition by *tsG* (Fig. 1). The second template, *dl309*, was largely inhibited by the *tsG* coinfection, even though wild-type levels of E1A protein were present from the first infection. Therefore, while some activation of the second template was observed, probably due to the presence of E1A protein in the infected cells, the second adenovirus template was largely inhibited by *tsG*.

We also wanted to investigate whether transcription complexes formed in the absence of any *trans*-activating protein would also be resistant to *tsG* superinfection. We infected HeLa cells for 33 h with the E1A deletion mutant *dl312*, which yields the same E3 RNA pattern as *dl309* by S1 analysis, in the presence of cytosine arabinoside (araC), which prevents DNA replication. Under these conditions *dl312* has been found to transcribe early genes (6). When these cells were superinfected with *tsG*, no inhibition of adenovirus transcription was observed (Fig. 2, lanes 1 to 3). Superinfection with either wild-type pseudorabies virus (lane 4) or wild-type adenovirus (lane 5) led to increased levels of transcription of the *dl312* E3 gene, indicating that the cells infected with *dl312* were superinfected by the second virus. Superinfection of wild-type Ad5 and *tsG* resulted in continued transcription from the first template (*dl312*), but complete inhibition of the second template (Ad 5) (lane 6). This indicates that stable transcription complexes are completely resistant to inhibition by *tsG*, whereas newly introduced templates are inhibited completely by *tsG*.

Having found that the formation of class II transcription complexes appears to be inhibited by *tsG*, we wished to examine the effect of *tsG* on class III transcription. The VA gene of adenovirus was used to measure RNA polymerase

FIG. 2. Transcription from *dl312* early genes at long times after infection is protected from *tsG* repression. HeLa cells were infected for 33 h at 37°C with *dl312* at 30 PFU per cell in the presence of 25 µg of araC per ml. RNA was extracted from one portion at 33 h postinfection (lane 1). Infected cells were incubated for 3 h at 40°C, and RNA was extracted at 36 h postinfection (lane 2). Further portions were superinfected at 33 h post-*dl312* infection with *tsG* at 40 PFU per cell for 3 h at 40°C (lane 3), wild-type pseudorabies virus at 40 PFU per cell for 3 h at 40°C (lane 4), Ad5 at 10 PFU per cell for 6 h at 40°C (lane 5), and Ad5 at 20 PFU per cell plus *tsG* at 40 PFU per cell for 6 h at 40°C (lane 6). A lighter exposure of lane 4 is shown in lane 7. RNA was extracted from cells at the end of these infections. S1 analysis was performed on 100 µg of cytoplasmic RNA by using the ³²P-labeled M13 single-stranded E3 probe. S1-protected fragments are visualized on this autoradiograph of a 5% acrylamide denaturing gel.

III transcription. It was apparent that at 3 h postinfection there was detectable VA gene transcription even in the absence of E1A protein (Table 3). The level of VA gene transcription was increased greatly by coinfection with wild-type pseudorabies virus, but was not increased by *tsG* coinfection at the nonpermissive temperature. Significantly, *tsG* coinfection did not inhibit VA gene transcription. This is in contrast to the inhibition of class II transcription observed in the *dl1500* infections (Table 1). Thus, coinfection with *tsG* inhibits the formation of class II transcription complexes but not the formation of class III transcription complexes. These results suggest that the activation mechanisms of class II and class III genes by IEP may be different.

To ensure that the labeled RNA hybridizing to filters containing the VA I gene was in fact a product of RNA polymerase III transcription, we determined the rate of transcription of RNA hybridizing to the VA gene in isolated

TABLE 3. VA gene transcription is stimulated by pseudorabies virus but not repressed by *tsG*^a

Infection	cpm in RNA		
	E2	E4	VA
<i>dl312</i>	25	102	2,067
<i>dl312</i> + PRV	14,428	1,777	18,783
<i>dl312</i> + <i>tsG</i>	73	81	2,845
Ad5	1,856	1,695	7,564

^a HeLa cells were infected with *dl312* at 50 PFU per cell alone, or with wild-type pseudorabies virus (PRV) or *tsG* at 40 PFU per cell, or with Ad5 alone at 50 PFU per cell at 40°C for 3 h in the presence of 25 µg of araC per ml. Cells were labeled for 30 min at 40°C with [³H]uridine at 100 µg/ml. RNA was extracted from the infected cells and hybridized to plasmid filters.

TABLE 4. Nuclear runoffs of *d1312* plus wild-type pseudorabies virus in the presence of α -amanitin^a

Condition	cpm in RNA		
	E2	E4	VA
Expt 1			
– α -Amanitin	985	380	106
+ α -Amanitin	9	0	91
Expt 2			
– α -Amanitin	1,529	546	431
+ α -Amanitin	140	81	396

^a HeLa cells were infected with *d1312* at 50 PFU per cell and wild-type pseudorabies virus at 40 PFU per cell for 3 h at 40°C. Nuclei were prepared as previously described (10). Nuclear runoff transcriptions were performed with 150 μ Ci/5 $\times 10^7$ cells. Where indicated, α -amanitin was added to the nuclear transcription reaction mixture to 0.5 μ g/ml. After a 15-min incubation at 37°C, ³²P-labeled RNA was extracted and hybridized to plasmid filters.

nuclei in the presence or absence of α -amanitin at concentrations which inhibit RNA polymerase II transcription but not transcription by RNA polymerase III. We found that virtually all transcription from E2 and E4, adenovirus class II early genes, was inhibited in the presence of 0.5 μ g of α -amanitin per ml (Table 4). On the other hand, close to the same number of counts per minute hybridized to the VA gene in the presence or absence of α -amanitin. This indicates that the labeled RNA hybridizing to this gene is in fact the VA class III gene product.

DISCUSSION

There is substantial evidence for the existence of stable complexes from which RNA synthesis is initiated for the production of multiple transcripts by RNA polymerase II and probably other factors as well. Gaynor and Berk (6) showed that transcription from adenovirus E1A mutants at long times after infection is a *cis*-acting induction, which suggests that some change in the template occurs which allows transcription to be reinitiated from that template. They further showed that transcripts from these genes expressed in the absence of E1A did not differ from transcripts induced by E1A. Thus, E1A appears to catalyze the change in the template which allows transcription to occur from that template. Recently, Kovesdi et al. (18) have shown by *in vivo* exonuclease III protection assays that adenovirus genes transcribed in the presence or absence of E1A are protected at the same site upstream of the promoter from exonuclease III digestion. This suggests that E1A (and IEP) are catalyzing the binding of some factor(s) to the DNA which then forms a stable complex from which transcription may be initiated.

Our studies of the mechanism of transcriptional activation by IEP have utilized a temperature-sensitive mutation in pseudorabies virus IEP, *tsG*. *tsG* inhibits transcription of adenovirus early genes in coinfections at the nonpermissive temperature (4). It is, however, unable to inhibit stable transcription complexes, whether they are formed in the presence or absence of E1A. Our earlier temperature shift studies showed that pseudorabies virus genes are transcribed at the same rate in infections shifted to the nonpermissive temperature as in infections maintained at the permissive temperature, and are still transcribed for hours after the shift to the nonpermissive temperature (1a). These data suggest that IEP is required for the formation, but not the maintenance, of stable transcription complexes on viral genomes for the transcription of class II genes.

Our results suggest that IEP probably acts to stimulate class II transcription by either modifying a transcription factor or physically interacting with one or more transcription factors to facilitate the formation of a stable transcription complex. In either of these cases, IEP is required only for the formation of the transcription complex.

Our results have also shown that E1A is not dominant over the *tsG* IEP for activation of genes. In Fig. 1 it is clear that even at 6 h postinfection, although some of the second adenovirus template is activated by E1A, E1A is not dominant in action to *tsG*. This suggests that the *tsG* IEP is involved in some physical interaction which prevents the formation of stable transcription complexes, since otherwise the actions of E1A would be dominant to *tsG* inhibition. This is consistent with both of the models presented above, since *tsG* could irreversibly bind to the transcription factor or factors with which it normally interacts, thus preventing the formation of any new transcription complexes.

According to the data presented here, genes transcribed by RNA polymerase III are probably activated by a different mechanism than genes transcribed by RNA polymerase II. First, we have shown that a class III viral gene, the VA gene of adenovirus, is transcribed very early in infection, even in the absence of E1A. Furthermore, although VA transcription is stimulated by E1A or IEP, it is not inhibited by the *tsG* IEP, which does inhibit class II gene transcription. This is consistent with the recent results of Yoshinaga et al. (23), who showed by *in vitro* transcription that E1A stimulation of class III genes causes an effective increase in the activity of transcription factor IIIC. Antibody depletion studies also suggest that E1A is not directly involved in the activation of class III genes. In view of this as well as our evidence that the *tsG* IEP is unable to inhibit the formation of class III transcription complexes, it is likely that IEP acts only to increase the intracellular pool of one or more polymerase III transcription factors in order to increase transcription from class III genes.

In summary, we have demonstrated the following. (i) IEP is involved in the formation, but not the maintenance, of stable transcription complexes. (ii) Since E1A is not dominant to the *tsG* IEP, at the nonpermissive temperature, the *tsG* IEP is probably involved in some physical interaction with some transcription factor(s). (iii) Activation of class II and class III genes by IEP probably occurs by different mechanisms.

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