Immediate-Early Protein of Pseudorabies Virus Is Not Continuously Required To Reinitiate Transcription of Induced Genes

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We examined the role of herpesvirus immediate-early proteins in inducing and maintaining transcription by using *tsG*, a temperature-sensitive mutant in the immediate-early gene of pseudorabies virus. Cells infected at the permissive temperature were shifted to the nonpermissive temperature. Initially, early gene transcription rates were similar at both temperatures. Early gene transcription subsequently decreased slowly over several hours. Our results suggest that immediate-early protein function is required only for the initial activation of early gene transcription and is not required for multiple reinitiation events from activated early genes.

The pseudorabies virus immediate-early protein (IEP) functions to activate the transcription of pseudorabies virus early genes. In this respect, it is analogous to the herpes simplex virus immediate-early protein, ICP4. Both IEP and ICP4 are nuclear phosphoproteins of 175 to 180 kilodaltons and are encoded in the short repeat region of their respective viruses (4, 9). Mutant viruses which are temperature sensitive in these loci are temperature sensitive for the activation of early gene transcription. In addition to these proteins, the adenovirus E1A protein, the simian virus 40 large T antigen, and the pX protein of human T cell lymphotropic virus types II and III have been shown to activate early gene transcription, and like the pseudorabies virus IEP, many of these proteins can activate nonhomologous viral and even cellular genes (5). Thus, the activation of transcription by viral proteins may occur by a mechanism which is shared among several viral groups.

Watson and Clements used a mutant with a temperature-sensitive mutation in the ICP4 gene of herpes simplex virus, *tsK*, to study the role of ICP4 in the viral life cycle (11). Cells infected at the permissive temperature were shifted to the nonpermissive temperature for 1 h and then labeled for 3 h with ³²P_i. The labeled RNAs were hybridized to viral DNA in Southern blots. The pattern of hybridization obtained from these studies indicated that the virus-infected cells reverted to synthesizing mainly immediately-early RNA after the shift to the nonpermissive temperature. This led to their conclusion that ICP4 is continuously required to maintain both early and late gene transcription.

We wished to reexamine the nature of the requirement for a functional immediate-early protein. Specifically, we investigated whether the immediate-early protein function is required for a transcriptionally active early gene to reinitiate RNA synthesis. Our results show that a functional immediate-early protein is not required for reinitiation from a transcriptionally active template. In fact, early RNA synthesis continues for several hours in the absence of a functional protein. The requirement for an immediate-early protein appears to be restricted to the formation of a transcriptionally active DNA template, i.e., a stable transcription complex. However, an immediate-early protein is required to maintain normal levels of early RNA for long periods, since

stable transcription complexes appear to have a limited half-life in vivo.

The current study differs from that of Watson and Clements in several respects. First, we used *tsG*, a mutant with a mutation in the immediate-early gene of pseudorabies virus. Unlike herpes simplex type 1, which encodes five immediate-early proteins, at least two of which appear to have a role in transactivation, (8, 10), pseudorabies virus encodes only one immediate-early protein. In temperature shift assays in our laboratory, *tsG* was the only virus among several temperature-sensitive IEP or ICP4 mutants tested whose immediate-early protein denatured immediately upon a shift to the nonpermissive temperature. These properties made a direct study of the role of IEP in transactivation possible. Second, in contrast to Watson and Clements, we examined the transcription rate of an early gene at the time of the shift rather than over period of 3.5 h. Third, the RNAs were labeled by short pulses and hybridized to discrete plasmids encoding early genes.

Table 1 shows the results of two different experiments in which cells infected with *tsG* at 32°C for 3 h were shifted to 41°C for various times and pulse-labeled. The levels of RNA hybridizing to early genes (*Bam*HI clones 10 and 12) indicated that early gene transcription continued at almost identical rates at the nonpermissive temperature and the permissive temperature. The rate of transcription of early genes was highest at 30 min after the shift to the nonpermissive temperature and thereafter slowly declined in the *tsG*-infected cells. This increase was most likely due to an increase in the activity of transcription factors and the use by polymerase of the stable transcription complexes at the higher temperature. In the wild-type-infected cells, there was a greater increase in the rate of transcription of early genes after the shift up; this was perhaps due to the fact that denatured IEP prevents the formation of new transcription complexes (3). Although the rate of transcription was not exactly the same as that in the wild type at the time of the shift, it was most significant that IEP function did not appear to be required to reinitiate transcription from an induced early gene. This result is related to the mechanism of IEP action and is separate from the issue of whether IEP is required over the course of infection to maintain high levels of transcription complexes. Because appreciable early gene transcription was still obtained 3 h after the shift, IEP

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TABLE 1. Temperature shift of *tsG* from the permissive to the nonpermissive temperature^a

Expt	Time after shift	Temp (°C) of pulse	RNA (cpm) hybridizing to:		
			<i>Bam</i> HI clone 8	<i>Bam</i> HI clone 10	<i>Bam</i> HI clone 12
1	0 h	32	648	1,190	1,781
	0 h	41	8,844	1,352	2,030
	30 min	41	13,061	1,700	2,345
	1 h	41	9,092	1,564	1,910
	2 h	41	6,912	1,172	1,433
2	0 h	32	827	2,522	1,688
	0 h	41	15,030	2,426	1,636
	30 min	41	12,910	6,429	5,508
	1 h	41	8,476	4,288	4,909
	2 h	41	3,589	3,251	2,979
	3 h	41	2,735	2,138	1,478
3 (Wild type)	0 h	32	100	608	267
	0 h	41	389	1,197	1,193
	30 min	41	663	1,778	1,169
	3 h	41	2,512	1,602	1,989

^a HeLa cells were infected with *tsG* or the wild type at 40 PFU per cell for 3 h at 32°C. One aliquot was labeled with [³H]uridine at 100 µCi/ml for 20 min at 32°C, while the other aliquots were labeled at the times indicated at 41°C for 20 min. Labeled cells were harvested by centrifugation. RNA was extracted and hybridized to plasmid filters as described elsewhere (9), except that labeled RNA hybridizing to plasmid filters was washed in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The wild-type counts are lower because uridine from a different vendor was used.

function is not required for a reinitiation event from a transcriptionally active template.

Transcription of the immediate-early gene itself (*Bam*HI clone 8) increased dramatically upon a shift to the nonpermissive temperature. Thus, both our study and that of Watson and Clements revealed a greatly increased synthesis of immediate-early RNA, but our study further showed that early gene transcription continues at a similar rate after the shift.

To determine whether the immediate-early protein was immediately denatured upon a shift to the nonpermissive temperature, we superinfected cells at the time of the shift with the adenovirus E1A deletion mutant *dI312*. Early genes from this virus (E2 and E4) were not activated in *tsG*-infected cultures shifted to 41°C but were activated in *tsG*-infected cultures at 32°C or in wild-type-infected cultures at both temperatures (Table 2). This result indicates that the immediate-early protein formed at 32°C was functionally inactivated upon the shift to 41°C. The fact that *tsG* shifted from the permissive to the nonpermissive temperature was unable to transactivate newly introduced templates (Table 2) is in agreement with the in vitro transcription results of Abmayr et al. (1), who showed by denaturation of *tsG* IEP at high temperatures in vitro that a functional IEP is required at the time of stimulation of transcription.

The increased transcription of the immediate-early gene upon a shift to the nonpermissive temperature was also observed by Watson and Clements. Although they interpreted this to be a shift in the transcription program from early to immediate-early, this increase in immediate-early gene transcription is likely to result from regulation of the ICP4 promoter by ICP4. Two recent studies have demonstrated autoregulation of the ICP4 gene in transient assays (2, 8), and the 20-fold increase in immediate-early gene transcription upon a shift in our experiments indicated that a functional IEP is required to repress immediate-early gene

transcription. In addition, Kristie and Roizman (7) have recently shown that ICP4 binds to two sites upstream of its own promoter. Therefore, IEP appears to act like the simian virus 40 large T antigen and ICP4 in that it represses its own transcription.

The temperature shift data in Table 1 strongly suggest that immediate-early protein function is not required to reinitiate RNA synthesis from those early genes which are previously induced. These data are in agreement with the results of Kovesdi et al. (6), who showed that E1A and IEP may act by increasing the binding of a cellular factor to the promoter region of an induced gene. IEP probably acts only at the initial step of gene activation, perhaps by facilitating the formation of a stable transcription complex. Such a complex, once formed, would not require either the presence or the function of IEP to allow reinitiation of RNA synthesis. The data in Table 1 indicate that early gene transcription continues for over 3 h in the absence of a functional IEP. The decrease in the rate of transcription of early genes after the shift of *tsG* to the nonpermissive temperature may indicate a gradual decay of active transcription complexes. New transcription complexes would be unable to form after the shift because of the lack of a functional IEP.

Our experiments, together with the results of Abmayr et al. (1) and Kovesdi et al. (6), suggest that the important step in the activation of transcription by IEP is the initial interaction of IEP with one or more cellular proteins. Kovesdi et al. (6) showed that a cellular factor was bound to the upstream region of a transcribed gene, whether or not the promoter was activated by E1A or IEP. Thus, transactivating proteins may increase the binding of a factor which will bind at a much slower rate in their absence. Abmayr et al. (1) have shown that IEP in vitro does not act solely by increasing the level of a transcription factor. Therefore, IEP might act either by modifying a transcription factor or by forming a complex with a factor to promote its binding to a specific DNA sequence. In either case, we would interpret our results to mean that IEP promotes the binding of a cellular factor to an upstream region to form a stable transcription complex. Once formed, this stable transcription complex is transcriptionally active for several hours in the absence of a functional IEP. It is therefore possible that IEP catalyzes the formation of a stable transcription complex and is not an actual component of that complex. However, since the

TABLE 2. *dI312* superinfection of wild-type- or *tsG*-infected cultures at the time of the temperature shift^a

Expt	Primary infection	Temp (°C) of <i>dI312</i> superinfection	RNA (cpm) hybridizing to:	
			E2	E4
1	Wild type	32	3,281	196
	Wild type	41	6,471	472
	<i>tsG</i>	32	713	257
	<i>tsG</i>	41	0	32
2	Wild type	32	2,110	494
	Wild type	41	10,160	2,215
	<i>tsG</i>	32	297	90
	<i>tsG</i>	41	44	6

^a HeLa cells were infected with either wild-type pseudorabies virus or *tsG* at 40 PFU per cell at 32°C in the presence of 25 µg of 1-β-D-arabinofuranosylcytosine per ml. After 3 h at 32°C, the cells were superinfected with *dI312* at 50 PFU per cell at either 32 or 41°C. After 3 h of superinfection, infected cells were labeled for 30 min at the same temperature with 100 µCi of [³H]uridine per ml. RNA was extracted and hybridized to plasmid filters as described elsewhere (3).

transcription complexes appear to decay in the absence of a functional IEP, it is likely that IEP is required continuously in an infection to induce the formation of new complexes.

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