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Characterization of the Herpes Simplex Virion-Associated Factor Responsible for the Induction of α Genes

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Herpes simplex virus (HSV) genes form three groups, α, β, and γ, whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion. Earlier studies by Post et al. (Cell 24:555–565, 1981) have shown that chimeric genes constructed by fusion of 5′ noncoding leader and upstream sequences of α genes to the 5′ noncoding leader and structural sequences of the viral thymidine kinase (TK), a β gene, are regulated as α genes upon recombination into the viral genome. In cells converted from TK^- to TK^+ phenotype, these chimeric genes are induced by infection with homologous TK^- virus. The induction of the resident chimeric gene does not require viral protein synthesis and is independent of the presence of functional α gene 4 product required for the expression of β genes. In this paper, we report on the properties of the α-TK gene chimera resident in converted TK^+ murine (L316) and human (I316) cells. Our results were as follows. (i) The pattern of induction of L316 cells exposed to 0.1, 1.0, and 10 PFU per cell suggested that exposure to competent virus is required for induction and that in untreated preparations this virus corresponds to infectious virus. (ii) UV light-irradiated virus was just as effective as untreated virus in inducing α-TK chimeras. (iii) HSV-1(HFEM)*ts*B7 induced the α-TK gene chimeras at the nonpermissive (39°C) temperature; at 39°C the parental HSV-1(HFEM)*ts*B7 capsids accumulate at nuclear pores and do not release viral DNA. (iv) The α-TK gene chimeras were not induced by infection with spontaneous TK^- mutants of pseudorabies virus and bovine mammillitis virus or with human cytomegalovirus or adenovirus type 2 or by exposure to lysates of HSV-1-infected cells from which the virus was removed by centrifugation. These results indicate that the α gene inducer is a virion component located outside the capsid and that its function might be to stimulate the transcription of α genes by recognizing regulatory sites on viral DNAs or host cell products or both.

A characteristic of the reproductive cycle of herpes simplex virus 1 (human herpesvirus 1, HSV-1) is that its gene expression is tightly regulated. Specifically, the HSV-1 genes form three groups, α, β, and γ, whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion (10, 11). The five known α genes are expressed first, and at least one, α gene 4 (6, 16, 31), is required for the expression of β genes. The β genes turn off the synthesis of α genes and enable the expression of γ genes. On the basis of studies on viral gene expression in sensitive and resistant cells exposed to α-amanitin, Costanzo et al. (5) concluded that cellular RNA polymerase II is involved in the transcription of viral DNA throughout the reproductive cycle. Central to our understanding of the regulation of HSV gene expression is the mechanism by which the cell transcriptional machinery differentiates between the α, β, and γ genes.

In an attempt to deal with this question, we mapped the site of initiation of transcription of four α genes, i.e., α0, α4, α22, and α27 (19), and constructed chimeric genes consisting of a portion of the 5′ noncoding leader sequences and the sequences upstream from the initiation of transcription of α genes fused to the 5′ noncoding sequence and the structural sequences of the HSV-1 thymidine kinase (TK), a β gene (19–22, 26). The α4-TK gene chimera, when recombined into the viral genome, was regulated like an α gene (26). Of particular interest, however, was the observation that in cells converted from TK^- to TK^+ phenotype, the α4-TK gene chimera was induced by infection with HSV-1 TK^- in the presence of cycloheximide and at 39°C in cells infected with temperature-sensitive (*ts*) vi-

ruses with mutations in α gene 4 (26). These observations suggested that unlike the induction of the natural β-TK gene (13, 17), the induction of the chimeric α-TK gene can occur in the absence of viral protein synthesis and specifically does not require the presence of a functional α4 gene product. Similar results were obtained with cells converted to the TK^+ phenotype with α0- and α27-TK chimeras (21, 22).

In this paper, we report that the induction of the chimeric α-TK gene in TK^+ converted cells requires exposure of the cells to virus and that the chimeric gene is induced by UV light-irradiated virus and by a *ts* mutant defective in the release of viral DNA from capsids at the nonpermissive temperature, but not by several other herpesviruses or by adenovirus type 2 (Ad-2).

MATERIALS AND METHODS

Virus and cells. The procedures for the propagation of virus and cells and the composition of growth and maintenance media have been published elsewhere (7, 29). The construction and properties of HSV-1(F)*ts*502Δ305, temperature sensitive in the α4 gene and containing a 700-base pair deletion in the TK gene, have been previously described (26). HSV-1(HFEM)*ts*B7 syn^+ was isolated by bromodeoxyuridine mutagenesis of HSV-1(HFEM)-infected cells (14). Previous studies have shown that in cells infected with HSV-1(HFEM)*ts*B7 and maintained at 39°C, viral capsids accumulate at the nuclear membrane and no viral gene expression can be detected (1). Ad-2 was obtained from M. Green, Institute for Molecular Virology, St. Louis, Mo. Human cytomegalovirus (human herpesvirus 5, CMV), strain AD 169, was provided by F. Rapp, The Pennsylvania State University, Hershey. HSV-2(333-B3) TK^- stock was obtained from R. B. Tenser, The Pennsylvania State University. TK-negative variants of bovine mammilitis virus (bovine herpesvirus-2, BMV) and of pseudorabies virus (Suid herpesvirus-1, PRV) were selected by previously described methods (26).

Ltk^- cells (12) were obtained from S. Kit, Medical College of Baylor University, Houston, Tex. The 143(TK^-) human cell line (4) was obtained from C. Croce, The Wistar Institute, Philadelphia, Pa. L316 cells and I316 cells were prepared by converting Ltk^- and 143(TK^-) cells, respectively, to a TK^+ phenotype with pRB316, which contains DNA sequences encoding the α4 gene promoter-regulator fused to DNA sequences coding for the structural portion of the TK gene (19–22, 26). L103 cells prepared by transfection of Ltk^- cells with the HSV-1(F) *Bam*HI Q fragment were as previously described (26).

TK^+ cells were selected and maintained in medium containing 10^{-5} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4.4×10^{-7} M methotrexate.

TK assays. Procedures for superinfection of transformed TK^+ cells and for extraction and assay of TK activity in these cells were as previously described (19–22, 26).

Preparation of supernatant fluid from HSV-1-infected cell lysates. The preparation of infected cell lysate stocks has been described elsewhere in detail (7, 29). Specifically, Vero cells infected with HSV-1(F)*ts*502Δ305 and maintained at 33°C were disrupted in a Dounce homogenizer and then sonicated at the maximum intensity with a Branson Cell Disruptor (model 200). The infected cell lysate was cleared of cell debris by centrifugation, the titer was determined to be 1.9×10^9 PFU/ml in Vero cells, and the lysate was stored at −70°C. The virus stocks were diluted to concentrations of 4×10^7 PFU/1.5 ml and 4×10^8 PFU/1.5 ml with phosphate-buffered saline containing 1% (wt/wt) glucose and centrifuged in a Beckman SW41 rotor for 90 min at 30,000 rpm and 4°C to pellet the virions. The supernatant fluids were then exposed to L316 cells as described above.

UV irradiation of virus. Stock HSV-1(F)*ts*502Δ305 virus was suspended in phosphate-buffered saline containing 1% glucose to yield a concentration of 2×10^7 PFU/1.5 ml. A 2-ml amount of virus suspension in a 10-cm-diameter petri dish was shaken on a rotary platform and exposed to a germicidal lamp (wavelength, 254 nm). A total dose of 1,000 ergs/mm^2 was delivered at the rate of 15 ergs/s per mm^2 to the surface of the fluid. Samples (1.5 ml) of irradiated virus were then used to infect L316 cells. A portion of the UV light-irradiated virus was assayed for infectivity.

RESULTS

Effect of multiplicity of infection on induction of the α-TK chimeric gene in L316 cells. The purpose of this series of experiments was to determine the effect of the multiplicity of infection on the induction of the α-TK chimeric gene resident in L316 cells.

In these experiments, replicate cultures of L316 cells were exposed to HSV-1(F)*ts*502Δ305 at a multiplicity of infection of 0.1, 1, and 10 PFU per cell for 2 h. Viral inocula were then removed, and the infected monolayers were washed, replenished with fresh medium, and additionally incubated at either permissive (33°C) or nonpermissive (39°C) temperatures. At appropriate times, the cultures were harvested, and cell extracts prepared from the infected cultures were assayed for TK activity. The results of these experiments (Fig. 1) demonstrate that at both permissive and nonpermissive temperatures, induction requires infection of the cell carrying the resident α-TK gene chimera. As has been previously observed, the extent of α-TK induction was greater at 39°C than at 33°C in L316 cells infected with HSV-1(F)*ts*502Δ305 (26).

These results thus affirm and extend previous observations that induction of the α-TK chimeric gene resident in L316 cells is a consequence of viral infection.

Ability of UV light-irradiated virus to induce the α-TK gene. Inasmuch as induction of transcription of the α-TK gene can take place in cells infected in the presence of cyclohexmide (26), the purpose of these experiments was to determine whether UV light-irradiated virus retained its ability to induce TK activity in L316 cells.

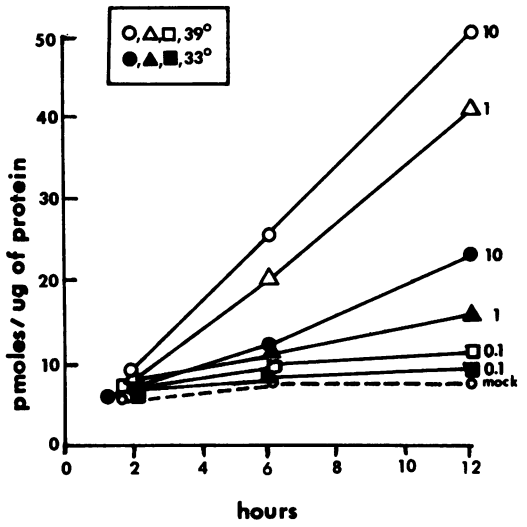

FIG. 1. Effect of multiplicity of viral infection on induction of TK activity from α4-TK gene chimeras in L316 cells. Replicate cell cultures were infected with 0.1, 1.0, or 10.0 PFU of HSV-1(F)*ts*502Δ305 virus per cell, and TK activity was measured as previously described (20–22) in cells harvested at the times shown. TK activity is expressed as picomoles of TMP formed per microgram of protein. After adsorption of virus to cells at 10°C, the cells were transferred to either 33 or 39°C. Time 0 represents the time at which cells were placed at 33 or 39°C.

L316 cells were infected with 5 PFU of HSV-1(F)*ts*502Δ305 per cell or with an equal amount of virions that had been exposed to UV-light irradiation as described above. After 2 h of adsorption, the cells were treated as described above. The UV-light dosage was sufficient to reduce the effective input multiplicities of the inocula from 5 PFU per cell in the untreated viral suspensions to 0.002 PFU per cell in the UV light-irradiated samples. The L316 cells were extracted and assayed for TK activity at 2, 6, and 12 h after exposure to untreated or UV light-irradiated virus.

The results (Fig. 2) demonstrate that virus exposed to UV-light irradiation retained its ability to induce TK activity in L316 cells at levels comparable to those induced by untreated virus, notwithstanding the loss in infectivity. These results are consistent with the conclusion that the factor responsible for induction of TK activity in L316 cells is a structural component of the virion.

Effect of HSV-1(HFEM)*ts*B7 on TK activity in L316 cells. The purpose of this series of experiments was to determine whether HSV-1(HFEM)*ts*B7 could induce the resident α-TK chimeric genes at the nonpermissive temperature.

In these experiments, replicate cultures of L316 cells were infected with 5 PFU of HSV-1(HFEM)*ts*B7, or of HSV-1(F)*ts*502Δ305, per cell or were mock infected, and the cultures were then incubated at the permissive (33°C) or nonpermissive (39°C) temperature. At various times after infection, the cultures were harvested, and cell extracts were assayed for TK activity. The results (Fig. 3A) indicate that at 39°C, HSV-1(HFEM)*ts*B7 was as capable of inducing α-TK activity as was HSV-1(F)*ts*502Δ305 and that at 33°C, HSV-1(HFEM)*ts*B7 induced somewhat more α-TK activity than did HSV-1(F)*ts*502Δ305. Since HSV-1(HFEM)*ts*B7 is not a TK^- mutant, its ability to express TK activity was assayed in 143(TK^-) cells (Fig. 3C). The results of this experiment showed that HSV-1(HFEM)*ts*B7 expressed a small amount of TK activity at 33°C, but none at 39°C.

These results indicate that the α-TK gene chimera can be induced at the nonpermissive temperature by HSV-1(HFEM)*ts*B7. Previous studies have shown that in cells infected with HSV-1(HFEM)*ts*B7 parental virus, capsids accumulate at 39°C at the nuclear membrane and do not release the viral DNA into the nucleus until the temperature is shifted to the permissive range. Consistent with this observation, no viral gene expression was detected at 39°C (1, 14). Thus, the data presented here demonstrate that the induction of the chimeric α-TK gene in L316 cells must be mediated by an extra capsid virion component.

Effect of supernatant fluids of infected cell lysates on the α-TK gene. To test the hypothesis

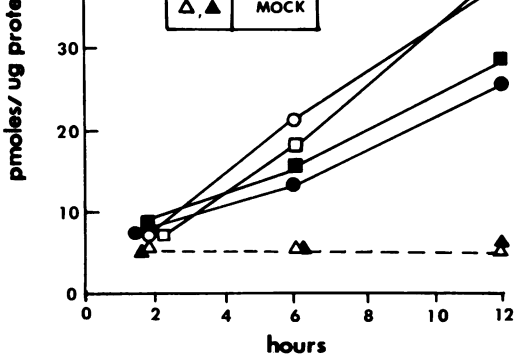

FIG. 2. Induction of α4-TK gene chimeras in L316 cells by UV-irradiated virus. Cells were infected with 5 PFU of HSV-1(F)*ts*502Δ305 virus per cell or with an equivalent amount of HSV-1(F)*ts*502Δ305 virus that had been UV irradiated as described in the text. Infected cultures were incubated at the temperatures and for the times shown.

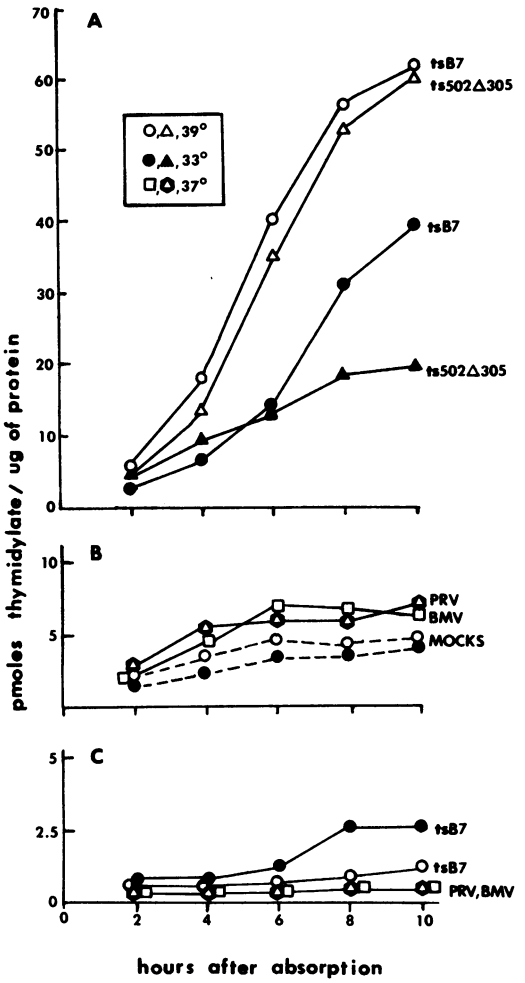

FIG. 3. Induction of TK activity by TK⁻ HSV-1, BMV, and PRV herpesviruses. The experimental protocols are described in the text. The reference box symbols in A denote the temperature of incubation and type of virus used in the experiments illustrated in A, B, and C. (A) Induction of TK activity in L316 cells infected with HSV-1(HFEM)*ts*B7 and by HSV-1(F)*ts*502Δ305. (B) Induction of TK activity in L316 cells by TK⁻ mutants of BMV and PRV. Selection of these TK⁻ viruses is described in the text. (C) TK activity in 143(TK⁻) cells infected with HSV-1(HFEM)*ts*B7, BMV(TK⁻), or PRV(TK⁻) viruses. Note that HSV-1(F)*ts*502Δ305 carries a 700-base pair deletion in the TK gene (26).

that the α-TK gene in L316 cells is induced by a nonvirion-associated factor accumulating in infected cell lysates, L316 cells were exposed to supernatant fluids obtained after high-speed centrifugation of lysates of Vero cells infected with HSV-1(F)*ts*502Δ305 as described above. The L316 cells were not induced after exposure to supernatant fluids containing the equivalent of 10 or 100 PFU per cell (Table 1). In light of the observation that induction of TK activity is demonstrable after exposure of L316 cells to 1 PFU per cell (Fig. 1), the data indicate that the supernatant fluids are devoid of inducing activity and that the inducing activity partitions with the virus.

Effect of infection with HSV-2(333-B3) TK⁻ on the α-TK gene. In this series of experiments, L316 cells were infected with HSV-2(333-B3) TK⁻ virus at a multiplicity of infection of 10 PFU per cell. The results (not shown) indicate that HSV-2 induces the HSV-1 α-TK gene chimeras in the same fashion as the homologous virus. Inasmuch as the studies on HSV-1 × HSV-2 recombinants failed to reveal forbidden juxtapositions of HSV-1 and HSV-2 DNA sequences (23–25), the results were not unexpected.

Effect of other viruses on the α-TK gene. The purpose of these experiments was to determine whether other viruses, particularly PRV, BMV, CMV, and Ad-2, were able to induce resident α-TK gene chimeras in transformed cells. Since BMV and PRV each specify a TK, we selected mutants deficient in this function, as described above.

The experimental procedures for infection and maintenance of cells with these viruses were the same as those described above. The multiplicities of infection were 10 PFU of PRV and BMV per cell, 100 PFU of Ad-2 per cell, and 50 PFU of CMV per cell. The results (Fig. 3) show the following.

TABLE 1. TK activity of L316 cells exposed to supernatant fluids of lysates of Vero cells infected with HSV-1(F)*ts*502Δ305 at 33°C

Hours post-exposure or -infection[a]	pmol of thymidylate per μg of protein in:		
	Infected cells	Cells exposed to supernatant fluid	Mock-infected cells
Expt 1			
2	9.7	7.1	6.3
6	26.2	5.2	7.4
12	49.4	6.9	6.5
Expt 2			
2	ND[b]	5.3	5.8
6	ND	6.1	4.9

[a] The supernatant fluids were adjusted to yield a virus-equivalent concentration of the cell lysate of 10 PFU (experiment 1) or 100 PFU (experiment 2) per cell. The procedure for preparation of the supernatant fluid is described in the text.

[b] ND, Not done (preliminary experiments have established that at high multiplicities of infection [e.g., 100 PFU per cell], the induction of the α-TK gene in L316 cells was generally not as high as at lower (10 PFU per cell) multiplicities of infection).

(i) None of the viruses selected for this study induced TK activity in human 143(TK$^-$) cells. The results obtained for BMV and PRV mutants are shown in Fig. 3C.

(ii) Neither BMV nor PRV significantly induced the resident α-TK gene chimera in L316 cells (Fig. 3B).

(iii) Neither CMV nor Ad-2 induced the resident α-TK gene chimera in I316 cells (Fig. 4). Attempts to induce the enzyme in L316 cells with these viruses also failed.

DISCUSSION

In this paper, we affirm and expand the previous report (26) from this laboratory that (i) α4-TK gene chimeras are induced in converted, TK$^+$ cells by infection with TK$^-$ virus, (ii) the induction is independent of the synthesis of α4 gene product, and (iii) the induction does not require the synthesis of viral proteins directed by the DNA of infecting virus. The results presented in this report indicate that the induction is dependent on exposure to virus, that UV light-inactivated virus is as effective as untreated virus, and that the chimeric gene is induced at the nonpermissive temperature by an HSV-1 *ts* mutant defective in release of viral DNA from capsids, but not by other herpesviruses nor by Ad-2.

The discovery that α4-TK gene chimeras are inducible by infection (26) was unexpected inasmuch as the infectivity of the deproteinized DNA suggested that the introduction of competent viral DNA is all that is required for a programmatic execution of the reproductive cycle in permissive cells. The observations that α-TK gene chimeras are inducible (26), that the inducibility is conferred by a movable "regulatory" sequence located at least 100 nucleotides upstream from the site of initiation of transcription of α genes (20), and that all α genes contain within the regulatory regions homologs of a consensus sequence (22) suggest that α genes are indeed induced and raise questions regarding the nature of the α gene inducer and its function in the reproductive cycle of the virus.

Signal for induction of α genes. The observations reported in this and previous publications indicate that the signal for the induction of α genes is HSV-1 and HSV-2 specific and most likely a structural component located outside the capsid. Specifically, the effect of the multiplicity of infection suggests that induction of α-TK gene chimeras requires exposure to competent virus and that the concentration of this virus in the inoculum approximates that of the infectious virus. However, the experiment with UV light-irradiated virus as well as the results of induction of the α4-TK gene chimera in the presence of inhibitory concentrations of cycloheximide

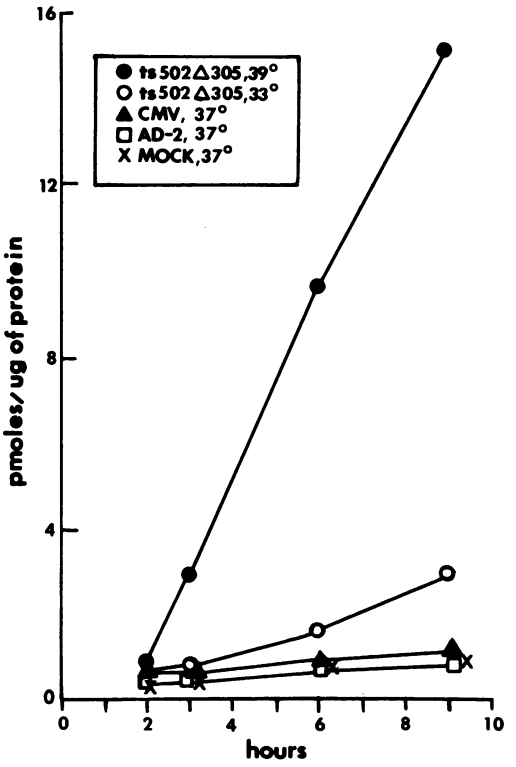

FIG. 4. Induction of TK activity in I316 cells [143(TK−) cells transformed to TK$^+$ phenotype with an α-TK gene chimera contained in plasmid pRB316] (26) by HSV-1(F)*ts*502Δ305, Ad-2, and CMV.

(26) indicate that the synthesis of viral proteins by the infecting virus is not required. The studies with the HSV-1(HFEM)*ts*B7 mutant are consistent with this conclusion and suggest that the structural component responsible for the induction of the virus is located outside the capsid. It is of interest to note that the HSV-1 virion contains at least 24 structural proteins (9, 27), of which no more than half are either at the surface of the envelope or in the capsid. The function of the noncapsid, nonenvelope proteins is unknown. Conceivably, among these proteins are those responsible for the induction of α genes and for other functions such as the virion-associated shutoff of host macromolecular synthesis (8) and the putative virion protein kinase (18).

Function of α gene inducer. Inasmuch as viral mutants lacking α regulatory regions have not been constructed, the function of the regulatory regions and the requirement for induction of the α genes after infection have not been explored. It is conceivable that the function of the α gene inducer is to enhance transcription of the α genes by binding either to the viral genome or to host gene products. We cannot differentiate between these alternatives. However, BMV and

PRV share many biological properties with HSV-1 and HSV-2 and belong to the same subfamily of herpesviruses (28); the observation that neither PRV nor BMV, a herpesvirus structurally and genetically related to HSV-1 and HSV-2 (2, 3, 30), induced the α-TK gene chimera suggests that the α gene inducer has a recognition site for specific sites in the viral DNA in addition to any putative recognition sites for host gene products. The observation that the α-TK gene chimeras are induced at a higher level at 39 than at 33°C is of considerable interest and potential help in determining the function of the inducer but does not differentiate between the alternatives listed above.

A central question is whether, in the absence of the α gene inducer or in the absence of regulatory regions, the α genes contained in the viral DNA would be transcribed at a level sufficiently high to sustain viral multiplication. The observation that α-TK gene chimeras are transcribed in the absence of the α gene inducer is not compelling evidence that they would be transcribed as part of the DNA in the infected cell; thus, in the absence of functional α4 gene product, the natural β-TK gene is transcribed when it is a resident gene in cells converted to TK^+ phenotype but not when it is part of the genome of an infecting virus.

Our results would have predicted that deproteinized HSV-1 DNA would not be infectious; in fact, the specific infectivity of HSV-1 DNA is of the order of 10^6 molecules per PFU (15). Among the possible explanations are that (i) the reproductive cycle initiated by deproteinized DNA depends on the participation of functional host analogs of α proteins and is therefore very inefficient or (ii) the reproductive cycle begins with the synthesis of the α gene inducer and continues with α proteins rather than with the α proteins as is the case in the reproductive cycle initiated by virions. Additional studies should reveal the nature and function of the inducer and its site of action.

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