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Activation of early adenovirus transcription by the herpesvirus immediate early gene: Evidence for a common cellular control factor

(adenovirus 5 *E1A* gene/herpesvirus temperature-sensitive mutant/transcriptional control)

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ABSTRACT **Adenovirus mutants carrying a defective *E1A* gene, such as dl312, are unable to express any of the early viral genes upon infection of HeLa cells. However, efficient expression of the other early adenovirus genes was obtained when dl312-infected HeLa cells were coinfected with pseudorabies virus, a herpesvirus. By employing a temperature-sensitive pseudorabies mutant (tsG1) it was demonstrated that the herpesvirus function responsible for the induction of adenovirus transcription was the immediate early gene, a gene required for the activation of herpesvirus early gene expression and the maintenance of early and late herpesvirus transcription. Specifically, HeLa cells coinfected with dl312 and tsG1, when shifted to the nonpermissive temperature, lost their capacity to express the early adenovirus genes. Furthermore, activation of early adenovirus gene expression in herpesvirus coinfection occurred earlier and at a higher level than in wild-type adenovirus infection. Therefore, the herpesvirus immediate early protein not only activates the early adenovirus transcription units but apparently does so more efficiently than the adenovirus E1A gene product. Because of this fact, we argue that the activation, either by the E1A protein or the herpesvirus immediately early protein, most likely occurs indirectly through interaction with a cellular protein rather than by a direct recognition of regulatory sequences at the adenovirus promoters.**

The induction of gene expression through transcriptional activation is surely an important aspect of development and differentiation. Various genes are silent until some particular stimulus results in the activation of transcription of the gene. Clear examples of such positive transcriptional control include the genes for hemoglobin (1) and the various genes subject to hormonal control (2–7), as well as the *Drosophila* heat shock genes (8).

There is also an induction of gene expression through transcriptional activation during the normal course of adenovirus infection of HeLa cells. The activity of the various early viral transcription units depends on the action of the product of the viral *E1A* gene (9–11). We have previously postulated (11) that the function of the E1A protein may involve the inactivation of a cellular factor rather than a direct positive interaction with the early viral promoters. For example, early viral genes can be expressed in the absence of E1A function if cellular protein synthesis is blocked (11, 12). These results suggest that a short-lived cellular factor that might have a normal role in controlling the expression of some group of cellular genes must be inactivated to allow the expression of the early adenovirus genes. If such a mechanism were indeed operating, then one might expect to find circumstances, independent of the action of the adenovirus *E1A* gene product, whereby this cellular factor was rendered inactive or reduced in functional concentration. Such a condition should then allow the growth and expression of the adenovirus mutants deficient in the E1A function.

Our approach, then, has been to search for a condition, independent of adenovirus infection, leading to the inactivation of the postulated cellular controlling factor. The study of the regulation of herpesvirus gene expression suggested that infection of cells by herpesvirus might afford one such possibility. The expression of the herpesvirus genes during a productive lytic infection is temporally controlled (13–15). Immediate early (α) genes are first to be expressed, followed by the early (β) genes and then the late (γ) genes. During herpes simplex virus (HSV) infection, the expression of one of the α genes is required for the transcription of the β genes, including the gene for thymidine kinase. Under nonpermissive conditions, mutants specifying a thermolabile *ICP4* gene product (VP175) are unable to induce the transcription of the early viral genes during a lytic infection (16–18), and, unlike wild-type (WT) HSV, these mutants are unable to stimulate the expression of the viral thymidine kinase gene in DNA-transformed cells (19–23). Furthermore, when mutant-infected cells maintained at the permissive temperature are shifted to the nonpermissive temperature, the expression of the early and late viral genes ceases (17, 18). A direct activation of the herpesvirus genes by the immediate early protein through an interaction with viral DNA regulatory sequences is certainly supported by the available data. However, it does remain possible that the activation is indirect, mediated through the inactivation of a negative effector in the cell, as has been postulated for adenovirus. In this report, we demonstrate that herpesvirus coinfection of HeLa cells can in fact substitute for the requirement of the adenovirus *E1A* gene in the activation of the early adenovirus genes, prompting us to suggest that the action of the two proteins, the adenovirus *E1A* gene product and the herpesvirus immediate early gene product, is mediated through a common cellular control factor.

MATERIALS AND METHODS

Cells and Virus. HeLa cells were maintained either in suspension culture in Joklik's modified minimal essential medium containing 5% fetal calf serum or in monolayers in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The growth and preparation of both WT adenovirus type 5 (Ad-5) and dl312 have been described (11, 24). WT pseudorabies virus and the immediate early mutant, tsG1, were both generously provided by T. Ben-Porat.

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Abbreviations: HSV, herpes simplex virus; Ad-5, adenovirus type 5; WT, wild type; PFU, plaque-forming units; kDal, kilodalton(s); DBP, DNA-binding protein.

Infection of Cells and Preparation of Labeled RNA. Procedures for the infection of cells, labeling of RNA with [^{3}H]uridine, and the preparation of labeled nuclear RNA have been described (24).

DNA Procedures. The Ad-5 plasmid pE2-Ad5 was constructed by inserting the *Eco*RI/*Bam*HI fragment of Ad-5 (59.5–75.9 map units) into the *Eco*RI/*Bam*HI sites of pBR322. The derivations of the other recombinant plasmids used in these experiments have been described (11). Plasmids were purified as described by Clewell and Helinski (25). Procedures for the growth of bacteria and the handling of recombinant DNAs were in accordance with the National Institutes of Health guidelines.

Hybridizations. Plasmid DNAs were linearized by heating to 100°C in 0.1 M NaOH for 5 min, diluted with 20 vol of 2 M NaCl (26), and loaded onto nitrocellulose filters. The procedures employed for DNA·RNA hybridizations have been described (23).

Preparation of Protein Extracts. Samples of washed cells were suspended in 0.01 M Tris·HCl, pH 7.4/0.01 M NaCl/0.0015 M $MgCl_2$ and sonicated for 10 sec on ice. Proteins were then precipitated with 2 vol of cold acetone. Precipitates were collected by centrifugation, dried, dissolved in gel sample buffer [0.125 M Tris·HCl, pH 6.8/5% (vol/vol) 2-mercaptoethanol/3% sodium dodecyl sulfate] (27), and heated to 100°C for 3 min. Protein samples were loaded on 10% acrylamide/sodium dodecyl sulfate gels (27) and separated by electrophoresis at 150 V for 5 hr.

Protein Transfer and Detection of the 72-Kilodalton DNA-Binding Protein (72-kDal DBP). After electrophoresis, the gels were soaked for 30 min in transfer buffer [Tris base, 3 g/liter; glycine, 14.4 g/liter; 20% (vol/vol) methanol] and the proteins were then transferred electrophoretically to nitrocellulose (28). The resulting protein blots were first incubated in RIPA buffer (29) (0.05 M Tris·HCl, pH 7.4/0.15 M NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate) containing 0.5% bovine serum albumin for 30 min, then incubated in 10 ml of RIPA buffer containing 15 μl of rabbit antisera to 72-kDal DBP (a gift of A. Babich) for 3 hr in a sealed plastic bag and then washed in RIPA buffer. Specifically bound antibody was detected by incubation with ^{125}I-labeled staphylococcal protein A (New England Nuclear) in 10 ml of RIPA buffer for 3 hr. After washing with RIPA buffer and then 0.05 M Tris·HCl, pH 7.4, the blots were used to expose x-ray film.

RESULTS

Adenovirus early gene expression involves first the expression of the E1A gene, then the expression of the five other early viral transcription units (30) (see Fig. 1). Transcription from each of these early promoters requires a functional *E1A* gene product (9–11). The dependence of early gene expression on E1A function is illustrated by the experiment shown in Fig. 2. HeLa cells were infected with either WT Ad-5 or the *E1A* mutant dl312. At various times after infection samples were removed and protein extracts were prepared, and the proteins were then separated in an acrylamide/sodium dodecyl sulfate gel. After transfer of the proteins to nitrocellulose, the product of the *E2* gene, the 72-kDal DBP, was visualized by using antisera to it and ^{125}I-labeled protein A. The protein was first detectable at 7 hr after infection in the WT adenovirus-infected cells and it increased in abundance thereafter. However, there was no detectable protein in the dl312-infected cells, even at 10 hr after infection, in agreement with various previous results that measured expression of the gene (9–11). Thus, in the absence of a functional E1A gene there is no early viral gene expression and this assay for the formation of the 72-kDal DBP provides a convenient and accurate method for the assay of the activation function of the E1A gene.

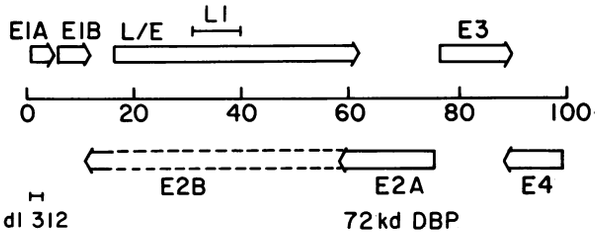

FIG. 1. Genomic map of the early adenovirus transcription units. One map unit equals 350 base pairs. The 72-kDal (kd) DBP is a product of the *E2A* transcription unit (31–33). The location of the *L1* sequences in the late transcription unit and the position of the dl312 deletion (34) are shown.

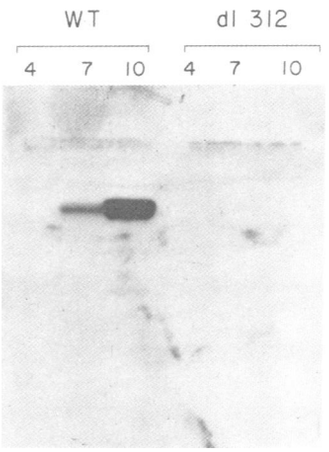

FIG. 2. Requirement of the *E1A* gene for formation of the 72-kDal DBP during adenovirus infection. Monolayers of HeLa cells were infected with 20 plaque-forming units (PFU) per cell of either WT Ad-5 or dl312. Samples were taken at the indicated times (hr) and protein extracts were prepared. The 72-kDal DBP in the gel was visualized by using specific antisera and ^{125}I-labeled protein A.

To assess the ability of herpesvirus (in this case pseudorabies virus) to provide the E1A function, HeLa cells were coinfected with WT adenovirus and pseudorabies virus or coinfected with dl312 and pseudorabies virus. Once again, the assay for gene activation was the formation of the adenovirus 72-kDal DBP. As shown in Fig. 3, there was no detectable formation of the 72-kDal DBP in dl312-infected HeLa cells, whereas the protein was readily detectable in WT-infected cells, consistent with the data of Fig. 2. However, when the dl312 infection was carried out in the presence of pseudorabies virus, there was a dramatic change: the adenovirus 72-kDal DBP was produced. Therefore, it was quite clear from this result that a lack of E1A function in dl312-infected HeLa cells could be overcome by coinfection with pseudorabies virus.

Furthermore, the presence of pseudorabies virus greatly enhanced the production of the E2 protein in the WT infection. The protein was clearly visible at 4 hr after infection, whereas in an infection with WT adenovirus alone, the protein was barely detectable at 6 hr. In fact, the production of 72-kDal DBP after 4 hr of infection with pseudorabies virus equalled the amount produced by adenovirus alone after 8 hr of infection. The even more startling result was the finding that the level of production in the dl312/pseudorabies virus coinfection equalled that of the WT/pseudorabies virus infection and exceeded that of the WT adenovirus alone. In essence, pseudorabies virus

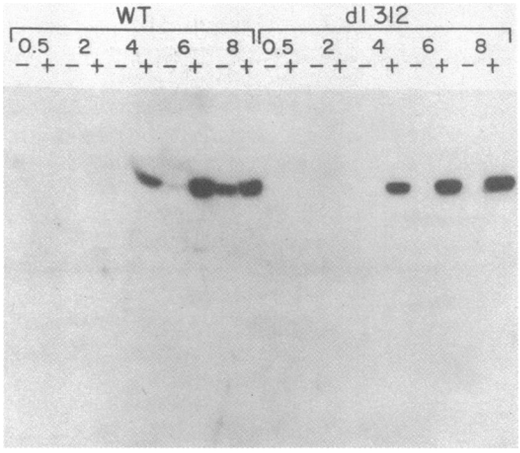

FIG. 3. Formation of the 72-kDal DBP during pseudorabies virus coinfection of HeLa cells with WT adenovirus or dl312. Infections were carried out as in Fig. 2 in the presence or absence of pseudorabies virus at 10 PFU per cell. Samples were taken at the indicated times (hr) and assayed for the formation of the 72-kDal DBP. The − and + refer to the absence or presence of pseudorabies virus.

infection appeared to stimulate early adenovirus gene expression much more efficiently than did adenovirus itself.

Activation of Adenovirus Transcription Is Due to the Herpesvirus Immediate Early Gene. The results presented thus far indicate that the function of the adenovirus E1A gene can be replaced by herpesvirus infection but do not reveal the herpesvirus function that is responsible. Of course, our premise in these experiments has been that the herpesvirus immediate early gene might function as does the adenovirus *E1A* gene. To investigate this possibility we made use of a temperature-sensitive pseudorabies virus mutant defective in the immediate early gene function. At the nonpermissive temperature, the mutant continues to express the immediate early gene but the product is defective and thus no early or late genes are expressed (T. Ben-Porat, personal communication). Furthermore, it has been shown that during HSV-I infection the protein is required continuously to maintain transcription of the early and late genes (17, 18). That is, if mutant-infected cells are allowed to proceed into late infection at the permissive temperature and then shifted to nonpermissive temperature, the transcription reverts to the immediate early stage.

To determine if in fact the pseudorabies virus immediate early gene was responsible for early adenovirus transcription in the absence of the adenovirus E1A gene, HeLa cells were infected with dl312, dl312 along with WT pseudorabies virus, or dl312 along with tsG1 (the pseudorabies virus immediate early mutant). Each culture was maintained at 32°C for 3 hr and then half of each was shifted to 41°C and half maintained at 32°C. One hour later, each culture was pulse labeled for 10 min with [^{3}H]uridine. The labeled nuclear RNA was prepared and hybridized to filters bearing adenovirus DNA fragments specific for early regions *E2*, *E3*, and *L1*. As we have previously reported (11), there was no early viral transcription in the dl312-infected HeLa cells (Table 1). Thus the activation of *E2* gene expression as shown in Fig. 3 must be at the level of transcription. This was verified by the coinfection with WT pseudorabies virus, because greatly enhanced transcription from each of the early adenovirus transcription units was obtained. In addition, coinfection with the pseudorabies virus immediate early mutant under permissive conditions also resulted in an enhancement of the early adenovirus transcription, although not quite to the same level as in the WT infection. When the WT pseudorabies virus-infected culture was shifted to 41°C, there was, as expected, no drop in adenovirus transcription, and in fact there was an increase in the transcription from each of the early adenovirus transcription units. In contrast, when the dl312-infected culture containing the pseudorabies virus immediate early mutant was shifted to the nonpermissive temperature, there was a marked drop in transcription from each of the early adenovirus transcription units. In fact, the difference between a coinfection with WT pseudorabies virus or with tsG1 in terms of adenovirus transcription at 32°C versus 41°C was a factor of 8. The same results were obtained in a separate experiment also presented in Table 1. Once again, shifting the culture containing WT pseudorabies virus to 41°C resulted in an increase in transcription from each early adenovirus region; shifting the culture containing the tsG1, however, resulted in a decrease in transcription of each of the early adenovirus transcription units. Furthermore, a decrease in transcription of the early adenovirus genes was also found when WT Ad-5-infected cells coinfected with tsG1 were shifted to 41°C (data not shown). Thus, the stimulation of WT Ad-5 gene expression as shown in Fig. 3 was also the result of the action of the herpesvirus immediate early gene. Therefore, it would appear that the herpesvirus function that stimulates early adenovirus transcription

Table 1. Activation of adenovirus transcription by the herpesvirus immediate early gene

		RNA, hybridized cpm							
		dl312		dl312 + pseudorabies virus			dl312 + tsG1		
Exp.	Adenovirus transcription unit*	32°C	41°C	32°C	41°C	41°C/32°C	32°C	41°C	41°C/32°C
1†	*E2*	11	7	522	845	1.62	273	56	0.21
	E3	0	0	330	417	1.26	165	31	0.19
	L1	6	0	180	371	2.06	145	42	0.29
2‡	*E2*			3,815	4,335	1.14	1,389	351	0.25
	E3			1,525	2,161	1.42	728	226	0.31
	E4			2,793	3,734	1.34	8,498	1,482	0.17
	L1			1,443	1,792	1.24	504	301	0.60

* DNA probes employed for hybridization were pE2-Ad-5 (see *Materials and Methods*) for *E2*, pEco D (*Eco*RI D fragment of Ad-2) for *E3*, pEco C (*Eco*RI C fragment of Ad-2) for *E4*, and pHind I (*Hin*dIII I fragment of Ad-2) for *L1*.

† Samples of 2×10^7 HeLa cells were infected with dl312, dl312 and pseudorabies virus, or dl312 and tsG1. The multiplicity of infection for dl312 was 10 PFU per cell; for pseudorabies virus or tsG1, the multiplicity was 5 PFU per cell. Each culture was incubated at 32°C for 3 hr and then divided in half; half was maintained at 32°C and half was shifted to 41°C. After 1 hr, each culture was labeled for 10 min with [^{3}H]uridine (200 μCi/ml; 1 Ci = 3.7×10^{10} becquerels). Nuclear RNA was prepared from each and hybridized to filters bearing the appropriate plasmid DNAs. RNase-resistant hybrids were scored.

‡ Infections were carried out as in Exp. 1 except that the labeling with [^{3}H]uridine was for 15 min.

is indeed the immediate early gene product. Furthermore, the data indicate that the transcription of the adenovirus genes depends on the continued presence of the herpesvirus gene product, as is the case for the herpesvirus genes during herpesvirus infection.

DISCUSSION

We have demonstrated that the product of the pseudorabies virus immediate early gene, a herpesvirus protein that activates herpesvirus transcription (T. Ben-Porat, personal communication), as does the product of the *ICP4* gene of HSV (16–23), can in addition activate the early adenovirus transcription units. During the normal course of adenovirus infection, the various early viral transcription units are activated as a result of the action of the product of the viral *E1A* gene (9–11). In the absence of a functioning *E1A* gene, there is very little detectable transcription from the five other early viral transcription units. The lack of *E1A* function can be overcome, however, in several ways. Infection of cells with a high multiplicity of an *E1A* mutant results in early gene expression (11) and the production of progeny mutant virus (34). This result seems most likely to be due to very inefficient transcription occurring from a greater number of mutant DNA templates (11). Thus the *E1A* gene product is not absolutely essential but rather greatly enhances the level of viral transcription. Furthermore, inhibition of cellular protein synthesis prior to and during infection with an *E1A* mutant results in the partial activation of early viral gene expression (11, 12). This result led to the proposal that there was a cellular factor that, in the absence of the E1A protein, prevents early viral transcription (11). Finally, it now appears that herpesvirus coinfection of cells will allow early adenovirus gene expression to occur in the absence of the *E1A* gene. And, more importantly, it is shown to be the herpesvirus immediate early gene product that is responsible for the activation of adenovirus transcription.

By what mechanism, then, might the herpesvirus immediate early gene induce the transcription of the early adenovirus genes? During herpesvirus infection, the product of the immediate early gene (the *ICP4* gene of HSV) is responsible for the activation of early and late herpesvirus genes at the level of transcription, a situation reminiscent of the role of the adenovirus *E1A* gene function. The most straightforward explanation for the action of either the E1A protein or the herpesvirus immediate early protein in inducing transcription is through a direct interaction of the activator protein with regulatory sequences of the inducible genes. If this were the case then it would imply that the herpesvirus activator protein would also be able to interact with adenovirus regulatory sequences. This seems unlikely because the two viruses are not known to be biologically similar or to share sequence homology. In addition, the clear implication from the experiments reported here is that the herpesvirus activator was *more effective* in inducing the adenovirus genes than was the adenovirus activator, the *E1A* gene product. This finding would appear to make the notion of a direct activation very unlikely because it would imply not only that the herpesvirus activator could interact with adenovirus regulatory sequences but in addition that the interaction was functionally more productive than a homologous interaction of the adenovirus E1A protein with putative adenovirus regulatory sequences. Furthermore, the available data suggest that the activation of early adenovirus transcription by the E1A protein is not a simple direct activation, because activation of transcription occurs in the absence of protein synthesis [thus in the absence of the E1A protein (11, 30, 35)] and a high multiplicity of infection with an *E1A* mutant results in full virus yields (34).

These results suggest an alternative mechanism for the activation process, an alternative that must invoke a factor shared by the herpesvirus activator and the adenovirus activator. Such a common denominator most likely must be a component of the host cell. We have previously suggested the presence of a short-lived transcriptional control factor in cells that is responsible for the control of the expression of certain cellular genes and that also limits the expression of the adenovirus genes (11). In support of this hypothesis, we have recently shown that at least one cellular gene, a gene specifying a HeLa cell heat shock protein, is induced as a result of the action of the adenovirus *E1A* gene product (36). Such a cellular transcriptional control factor might also limit the transcription of the herpesvirus genes. The function, then, of the herpesvirus immediate early protein, as well as the adenovirus E1A protein, would be to inactivate or titrate out such a factor so as to allow the expression of the viral genes. Obviously, the proof of such a hypothesis will require the use of the two viral proteins in pure form to determine if they in fact interact with a common cellular component.

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