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Identification of a factor in HeLa cells specific for an upstream transcriptional control sequence of an EIA-inducible adenovirus promoter and its relative abundance in infected and uninfected cells

(adenovirus type 5 EIIA-early promoter/*trans*-activation/protein factor)

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ABSTRACT **Utilizing the gel electrophoresis/DNA binding assay, a factor specific for the upstream transcriptional control sequence of the EIA-inducible adenovirus EIIA-early promoter has been detected in HeLa cell nuclear extract. Analysis of linker-scanning mutants of the promoter by DNA binding assays and methylation-interference experiments show that the factor binds to the 17-nucleotide sequence 5′ TGGAGATGACGTAGTTT 3′ located between positions −66 and −82 upstream from the cap site. This sequence has been shown to be essential for transcription of this promoter. The EIIA-early-promoter specific factor was found to be present at comparable levels in uninfected HeLa cells and in cells infected with either wild-type adenovirus or the EIA-deletion mutant *dl*312 under conditions in which the EIA proteins are induced to high levels [7 or 20 hr after infection in the presence of arabinonucleoside (cytosine arabinoside)]. Based on the quantitation in DNA binding assays, it appears that the mechanism of EIA-activated transcription of the EIIA-early promoter does not involve a net change in the amounts of this factor.**

In human cells infected with adenovirus (Ad) type 2 or 5, a set of five early viral promoters are coordinately expressed (1). Efficient RNA polymerase II-mediated transcription of these viral promoters is dependent on the 32-kDa phosphoprotein encoded by the viral pre-early EIA gene (1–3). The stimulation of transcription in *trans* by the EIA gene product is not restricted to adenoviral promoters; nonviral promoters introduced into the cell by transfection (4–8) or by viral vectors (5) are also stimulated. Promoters of endogenous genes such as the hsp 70 heat shock gene (9) and the β-tubulin gene (10) are also transcriptionally activated by the EIA gene product. Finally, EIA-dependent transcriptional activation also occurs with the RNA polymerase III promoters (11–14).

Several lines of evidence suggest that the mechanism of EIA-mediated transcriptional stimulation of both viral and nonviral promoters is indirect and most probably through a cellular intermediate. The lack of DNA binding properties of the EIA protein isolated from virus-infected cells (14) and of the EIA protein expressed in *Escherichia coli* (15), the activation of adenoviral promoters by the immediate early gene product of the unrelated pseudorabies virus (16), and the failure to identify by extensive mutational analysis DNA sequence elements upstream from the cap site of the EIIA-early (E) promoter that can be specifically recognized by the EIA gene in a variety of transcription assays (17–21) suggested that direct interaction between the EIA gene product and the promoter is not required for transcriptional stimulation.

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We have analyzed the EIIA-E promoter by a linker-scanning (LS) mutagenesis procedure (20). These studies not only helped us to rule out the direct interaction of the EIA gene product with the EIIA-E promoter sequences during the EIA-stimulated transcription but also allowed us to identify two transcriptional control sequences upstream from the cap site, namely, regions I and II (see Fig. 1). Region I is located closest to the cap site and appears to be analogous to the "TATA" box, and its sequence is 5′ CTTAAGAGT 3′. Region II is 17 nucleotides long and maps upstream from the cap site, and its sequence is 5′ TGGAGATGACGTAGTTT 3′. Mutations in either region I or II result in a drastic reduction of transcription (20). Using the gel electrophoresis/DNA binding assay (22, 23), we have now detected a factor (EIIA-EF) in HeLa cell nuclear extracts that specifically binds to the upstream control sequence (region II). Surprisingly, this factor in DNA binding assays was found to be present at similar levels in uninfected HeLa cells, or in HeLa cells infected with wild-type Ad5 or with the EIA deletion mutant *dl*312. This suggests that perhaps the mechanism of EIA-activated transcription does not involve a net change in the amount of this factor.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. HeLa cell suspension culture was a gift from R. Weinmann (Wistar Institute). Wild-type (wt) Ad5 and the Ad5 deletion mutant *dl*312 were obtained from T. Shenk (Princeton University). Construction and characterization of the LS mutants of the EIIA-E promoter is described (20). Probes used in the DNA binding assays are described in the figure legends.

Preparation of Nuclear Extracts from Infected and Uninfected HeLa Cells. HeLa cells were infected at 20 plaque-forming units per cell using plaque-titered cell lysates prepared from infected cells. Infection was continued for either 7 or 20 hr in the presence or absence of arabinonucleoside (cytosine arabinoside, Ara-Cyt) at 25 μg/ml. When the infection was carried out for 20 hr in the presence of Ara-Cyt, cells were again suspended in fresh media containing Ara-Cyt at 10 hr after infection. Cells were then harvested and nuclear extracts were prepared as detailed by Dignam *et al.* (24). Protein concentrations were determined as described by Bradford (25).

Gel Electrophoresis/DNA Binding Assay. Approximately 0.2 ng of the 3′-end-labeled (26) DNA fragments were mixed with 2.0 μg of poly(dI)·poly(dC) (Pharmacia) in a buffer

Abbreviations: EIIA-E, EIIA-early; Ad, adenovirus; LS, linker scanning; wt, wild type; Ara-Cyt, arabinonucleoside; bp, base pair(s); SV40, simian virus 40.

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containing 10.0 mM Tris·HCl (pH 7.5), 60.0 mM NaCl, 1.0 mM dithiothreitol, 1.0 mM EDTA, 5% (vol/vol) glycerol, and 12.0–15.0 μg of protein in a total volume of 25.0 μl. The samples were incubated for 30 min at 30°C, mixed with 2.5 μl of 50% (vol/vol) glycerol that contained 0.1% of the marker dyes bromophenol blue and xylene cyanol, and loaded on to 4.0% acrylamide gels [150.0 mm × 120.0 mm × 1.5 mm; acrylamide–bis ratio, 30:1 or 60:1 (wt/wt)]. The gels were electrophoresed for 2 hr at 4°C at 150 V with constant circulation of the electrophoresis buffer. The electrophoresis buffer contained 6.7 mM Tris·HCl (pH 7.5), 3.3 mM sodium acetate, and 1.0 mM EDTA. The gels were then dried and autoradiographed.

RESULTS

Detection of Proteins that Bind to the Ad5 EIIA-E Promoter. We have employed the gel electrophoresis/DNA binding assay (22, 23) as modified by Carthew *et al.* (23) to detect the proteins from HeLa cell nuclear extract that specifically bind to the upstream transcriptional control sequences of the EIIA-E promoter. Fig. 1*A* depicts the landmark restriction sites located in the EIIA-E promoter region of the plasmid used in these studies and diagrams two transcriptional control regions (I and II) upstream from the cap site. The nucleotide sequence of these two control regions and the LS mutants, which overlap these two control sequences, are shown in Fig. 1 *B* and *C*, respectively.

Nuclear extracts were prepared from infected or uninfected HeLa cells as described by Dignam *et al.* (24) and probed with a 3′-end-labeled 81-base-pair (bp) *Bss*HII–*Hin*dIII DNA fragment (positions −17 to −98; see Fig. 1) in the DNA binding assay. This DNA fragment contains the two transcriptional control sequences of the EIIA-E promoter mentioned earlier. With increasing concentrations of protein, a fine doublet band with increasing intensity, migrating slower than the free probe, was detected (data not shown). This band was due to a DNA–protein complex because when the extract was treated with proteinase K before the DNA binding assay, the band disappeared (data not shown).

To determine whether the band detected in these experiments is due to specific interaction of proteins with the promoter, several DNA fragments from pBR322, and DNA

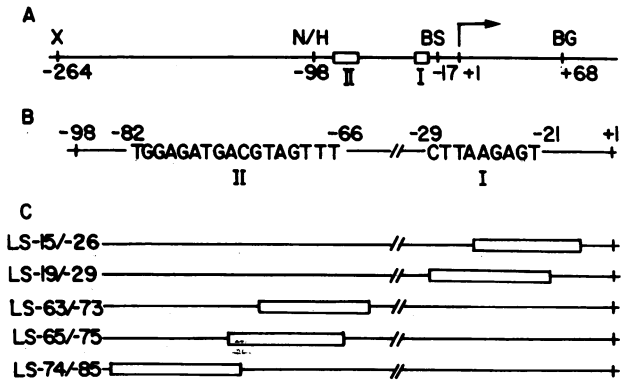

FIG. 1. Physical map of the region of the plasmid that contains the EIIA-E promoter and the details of the transcriptional control sequences. (*A*) Location of the restriction endonuclease sites in the region of the DNA segment that contains the EIIA-E promoter. The open boxes represent the transcriptional control regions. (See ref. 20 for further details.) X, *Xho* I; N, *Nar* I; H, *Hin*dIII; BS, *Bss*HII; BG, *Bgl* II. The *Nar* I site present in the original plasmid has been converted to a *Hin*dIII site using *Hin*dIII linkers (26). The arrow shows the direction of transcription. (*B*) Positions and nucleotide sequences of the two transcriptional control regions of the EIIA-E promoter. (*C*) Diagrammatic representation of the LS mutants of the EIIA-E promoter that overlap the two control elements (20).

fragments upstream and downstream from the two transcriptional control regions of the EIIA-E promoter were tested in the DNA binding assay. As shown in Fig. 2*A*, proteins from the HeLa cell nuclear extract bound to the *Bss*HII–*Hin*dIII DNA fragment (band due to the DNA–protein complex is shown by an arrow), whereas the three *Hae* III fragments from pBR322 DNA (see Fig. 2 for details) failed to form the DNA–protein complex. Similarly, neither an 85-bp DNA fragment stretching from *Bgl* II to *Bss*HII sites, which contained the downstream sequences from positions +68 to −17, nor a 166-bp DNA fragment from the *Hin*dIII to *Xho* I sites, which contained the upstream sequences from positions −98 to −264, were able to bind to proteins from the nuclear extract (Fig. 2*B*). In several of our experiments we have detected one or two additional faint bands migrating faster than the major band, but have not yet characterized them.

Specificity of the Factor for the Upstream Transcriptional Control Region. To determine which of the two transcriptional control sequences contained within the *Bss*HII–*Hin*dIII fragment is responsible for the formation of the DNA–protein complex, we have used a series of LS mutants that systematically mutate the EIIA-E promoter (20). These mutants are particularly useful in this study because they contain a clustered set of point mutations without altering the spacing of the important control signals. We have demonstrated by a transient transfection assay that LS mutants −15/−26 and −19/−29 that overlap the control sequence closer to the cap site and LS −63/−73, −65/−75 and −74/−85 that overlap the upstream control sequence transcribed with a much reduced efficiency (20). Ability of these LS mutants to bind the EIIA-E factor in the DNA binding assay was compared with that of the wt promoter. The 166-bp DNA fragments from the *Bgl* II to the *Hin*dIII sites from wt and LS mutants were end-labeled at the 3′ end and tested in a DNA binding assay. As shown in Fig. 3*A*, LS mutants −63/−73 and −65/−75, which have an altered region II, were drastically reduced in their ability to form the DNA–protein complex. The LS mutant −19/−29 bound the

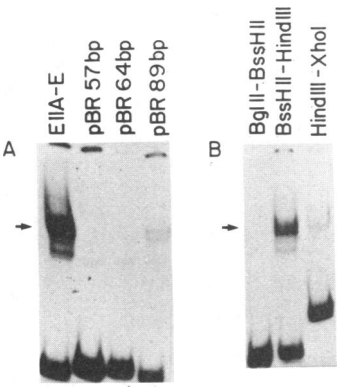

FIG. 2. Detection of EIIA-E promoter-specific DNA binding proteins. Approximately 0.2 ng of suitable end-labeled DNA fragments were incubated with 10–15 μg of protein from uninfected HeLa cell nuclear extract in DNA binding assays. (*A*) Binding of the factor to EIIA-E promoter and pBR322 DNA fragments. An 81-bp *Bss*HII–*Hin*dIII DNA fragment of the EIIA-E promoter was used as the wt probe. The three pBR322 *Hae* III DNA fragments were derived from positions 99 to 1048 (57 bp), 532 to 596 (64 bp), and 830 to 919 (89 bp) of the pBR322 DNA sequence (27). The DNA fragments were inserted into pUC18 and then digested with appropriate restriction endonucleases to generate fragments ≈100 bp long that are then 3′ end-labeled. (*B*) Factor binding to the EIIA-E promoter and to regions upstream and downstream regions of the EIIA-E promoter. DNA fragments (0.2–0.3 ng) from *Xho* I to *Hin*dIII, *Hin*dIII to *Bss*HII, and *Bss*HII to *Bgl* II (Fig. 1*A*) were end-labeled at the 3′ ends and used in the DNA binding assays.

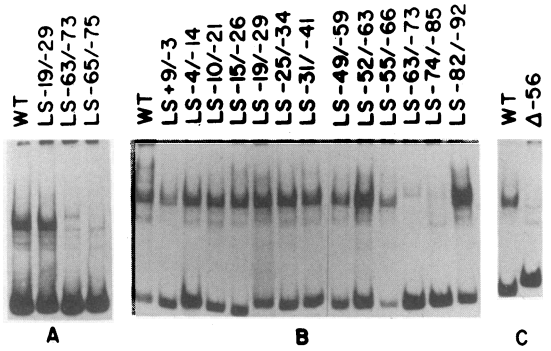

FIG. 3. Effect of LS and deletion mutations in the formation of DNA–protein complex in DNA binding assays. The 166-bp DNA fragments from *Bgl* II to *Hin*dIII were used as probes in experiments shown in *A*. The 81-bp DNA fragments from *Bss*HII to *Hin*dIII were used as probes for experiments shown in *B*. (*C*) Effect of a deletion mutation (deletion of sequences up to position −56 from the 5′ end) in the formation of the DNA–protein complex. A DNA fragment from *Bgl* II to *Bam*HI sites from Δ −56 plasmid was used as a probe. The DNA fragment from *Bss*HII to *Hin*dIII sites was used as the wt probe.

factor at levels comparable to those of a similar fragment from the wt promoter.

Next, our entire bank of LS mutants was tested in the DNA binding assay using DNA fragments from *Bss*HII to *Hin*dIII sites. As shown in Fig. 3*B*, all the LS mutants except LS −63/−73 and −74/−85 bound to EIIA-E factor with a reasonable efficiency. LS mutant −65/−75 could not be tested in this experiment because of a new *Bss*HII site generated at position −64 as a result of linker substitution. A deletion mutant of the EIIA-E promoter that contained only 56 bp upstream from the cap site was also tested in the DNA binding assay for its ability to form the DNA–protein complex. As shown in Fig. 3*C*, factor binding was drastically reduced for this mutant. The results presented in Fig. 3 suggest that the factor binding requires the 17-nucleotide sequence present between positions −66 and −82 of the EIIA-E promoter.

The finding is supported by methylation-interference experiments. In these experiments a 122-bp DNA fragment containing the promoter sequences from positions −17 to −139 (obtained from an upstream deletion mutant) was labeled at the 3′ end and methylated randomly by dimethyl sulfate using the standard DNA sequencing protocol (28). The methylated probe was purified and used in the DNA binding assay. The bound and unbound fragments were extracted from the gel, and further processed to produce the sequence ladders (28) and analyzed on DNA sequencing gels. If methylation of the guanosine or adenosine residues interfered in factor binding, the bound fraction in the DNA binding assay will be enriched only with the molecules that are not methylated within the target sequence. In DNA sequence ladders, those guanosine residues from the DNA fragment isolated from the bound fraction (Fig. 4, lane B) and that are involved in factor binding will be absent or much reduced when compared to those from the unbound fraction (Fig. 4, lane UB). Results of such an experiment are shown in Fig. 4. Lane B, corresponding to the bound fraction, shows the guanosine residues at positions −80, −78, −75, −72, and −69 with a much reduced intensity as compared to that of the unbound fraction, while the remaining guanosine residues in the sequence ladder are unaffected. The results of the factor binding to LS mutants and methylation-interference experiments suggest that the factor makes contact with the 17-nucleotide sequence of the promoter present between −66 and −82.

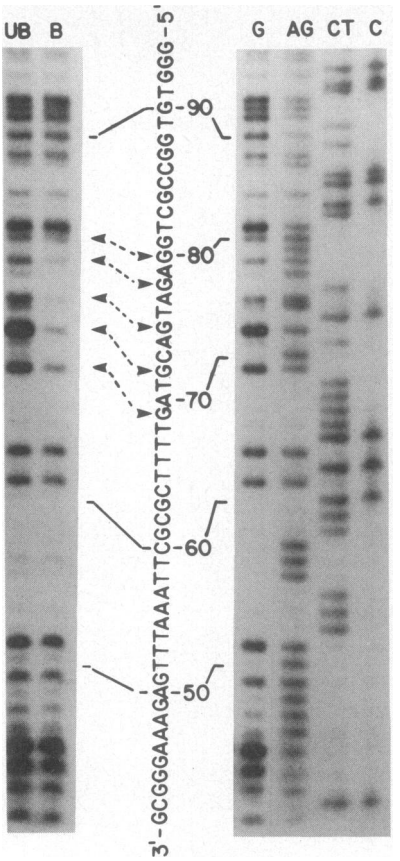

FIG. 4. Effect of methylation of DNA on EIIA-E factor binding. A plasmid that contained the upstream DNA sequences up to position −139 from the cap site of the EIIA-E promoter was 3′ end-labeled at the *Bss*HII site (at position −17) and redigested with *Bam*HI (at position −139). The 122-bp labeled DNA fragment was methylated by dimethyl sulfate (28). The probe was then incubated with nuclear extract in the DNA binding assay. The bound (B) and unbound (UB) DNA fragments were extracted from the gel and processed further to generate sequence ladders (28). Equal amounts of radioactivity from the bound and unbound fractions were loaded on the gel along with the sequence ladders generated from the probe. The guanosine residues at positions −80, −78, −75, −72, and −69 that are involved in factor binding are shown by arrows.

Affinity of the EIIA-E Factor to Other Ad Promoters. Specificity of the factor to the upstream transcriptional control sequence of the EIIA-E promoter suggests that it may be a transcription factor. As all the Ad early promoters are stimulated by the EIA gene during virus infections, we have examined by competition experiments the ability of this factor to interact with other Ad promoters. DNA fragments that contain EIA, EIB, EIIA-late, EIII, EIV promoters and the major late promoter and also the simian virus 40 (SV40) early promoter were tested for their ability to compete with the EIIA-E promoter fragment in the DNA binding assay. The results of these experiments are shown in Fig. 5. EIA and EIV promoters competed as efficiently as the EIIA-E promoter itself (Fig. 5) whereas only a slight competition was observed for the EIII and EIIA-late promoters (Fig. 5 *B* and *C*). The EIB, major late, and the SV40 early promoters did not compete even at a 50-fold molar excess (Fig. 5*C*). These results suggest that among the promoters tested this factor is likely to be utilized by only EIA and EIV.

Relative Abundance of the EIIA-E Promoter-Specific Factor in Infected and Uninfected Cells. Numerous studies have shown that the effect of the EIA gene product on the EIA-inducible promoters is indirect and probably through a cellular intermediate. While the nature of this cellular intermediate is not known, it could be one or more transcription

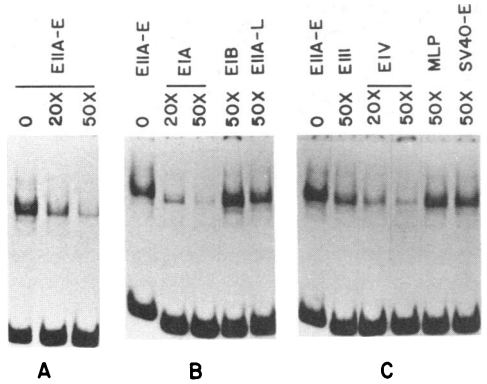

FIG. 5. Competition of various promoters with the EIIA-E promoter DNA for factor binding. Various amounts of unlabeled competitor DNA fragments were added to the reaction mixture containing 0.1 ng of labeled EIIA-E probe (*Bss*HII–*Hin*dIII). (*A*) Competition with EIIA-E promoter. (*B*) Competition with EIA, EIB, and EIIA-late promoters. For the EIA promoter, 20 and 50 ng of a 910-bp DNA fragment (positions −497 to +419 relative to the cap site) were used. For the EIB promoter, 11 ng of a 200-bp DNA fragment (positions −131 to +68) was used. For the EIIA late promoter 11 ng of a 194-bp DNA fragment (positions −164 to +30) was used. (*C*) Competition with EIII, EIV, MLP, and SV40 early promoters. For the EIII promoter, 14 ng of a 262-bp fragment (positions −232 to +26) was used. For the EIV promoter 12 and 31 ng of a 580-bp fragment (positions −323 to +251) were used. For the MLP 16 ng of a 290-bp fragment (positions −262 to +30) was used. For the SV40 early promoter, 17 ng of a 158-bp fragment (positions −93 to +69) was used. The numbers above the lane indicate the molar excess of the unlabeled competitor DNA.

factors. If the factor identified in our studies is involved in transcriptional activation, then HeLa cells infected with Ad may show an increase in the effective concentration of this factor as a result of a net increase in synthesis or of modification of the preexisting factor. Therefore, experiments were performed to determine the levels of the factor present in nuclear extracts prepared from uninfected HeLa cells and cells infected with either wt Ad5 or the EIA deletion mutant *dl*312. Cells were infected with wt Ad5 or *dl*312 at 20 plaque-forming units per cell, and Ara-Cyt was added to prevent DNA replication. They were then harvested at 7 and 20 hr after infection, and nuclear extracts were prepared as described (24). The extracts were then tested in the DNA binding assays with various concentrations of protein and constant amounts of probe. The assays were carried out under conditions in which the probe was not limiting. Nuclear extracts were also prepared from cells infected with wt Ad5 and *dl*312 but without Ara-Cyt and assayed as described earlier. The results are presented in Fig. 6.

The EIIA-E factor was detected at comparable levels in nuclear extracts derived from cells infected with wt Ad5 or *dl*312 at 7 hr after infection (Fig. 6*A*). This level was comparable to that of uninfected cells. Similarly, nuclear extracts prepared from cells infected with wt Ad5 or *dl*312 for 20 hr in the presence of Ara-Cyt also contained almost identical levels of this factor (Fig. 6*B*), again comparable to the level in uninfected cells (Fig. 6*A*). Treatment of infected cells with Ara-Cyt for prolonged periods prolongs the early phase of infection and induces the EIA proteins to high levels (29). However, in our hands, such treatment did not increase the level of this factor even 20 hr after infection. In fact, under this condition, we have observed a slight decrease in the level of this factor in wt-infected cells compared to that of *dl*312 or uninfected cells. Results obtained for cells infected with wt Ad5 or *dl*312 for 20 hr in the absence of Ara-Cyt are strikingly different. wt Ad5-infected cells contained this factor at much reduced levels compared to that of *dl*312 (Fig. 6*C*). This reduction in cells infected with wt Ad5 incubated in the

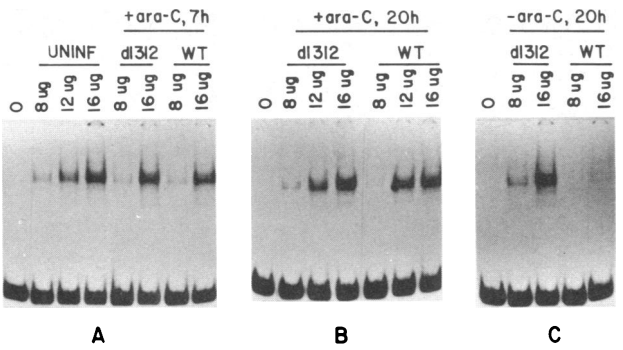

FIG. 6. Relative amounts of the EIIA-E factor in uninfected HeLa cells and HeLa cells infected with wt Ad5 or *dl*312. Cells were infected in the presence and absence of Ara-Cyt for 7 or 20 hr, and nuclear extracts were prepared and analyzed in DNA binding assays. The DNA fragment from *Bss*HII to *Hin*dIII of the wt plasmid (Fig. 1) was used as the probe in these experiments.

absence of Ara-Cyt is probably due to sequestering of a majority of the factor with replicated viral DNA templates. Since the viral DNA remains associated with the nuclei, the factor probably was not released during the extraction procedure.

DISCUSSION

We have detected a protein factor(s) in HeLa cell nuclear extracts that binds *in vitro* to the EIA-inducible Ad EIIA-E promoter. Two transcriptional control elements were identified earlier for the EIIA-E promoter by LS mutagenesis studies (20); one closer to the cap site with a sequence 5′ CTTAAGAGT 3′, and the second upstream from the cap site with a sequence 5′ TGGAGATGACGTAGTTT 3′. Both sequences are required for efficient transcription. Two lines of evidence suggest that the factor described here is specific for the upstream control sequence. First, LS mutants −63/−73, −65/−75, and −74/−85 do not bind to the factor efficiently, whereas binding proceeds efficiently in LS mutants located elsewhere in the promoter.

Further evidence that the protein makes contact with the upstream control element comes from methylation interference experiments. Methylation of guanosine residues of the coding strand at positions −80, −78, −75, −72, and −69 affected the binding of the factor. These five guanosine residues are within the upstream control sequences 5′ TGGAGATGACGTAGTTT 3′ (guanosine residues shown with asterisks are at positions −80, −78, −75, −72, and −69, respectively). Since we know that mutations in this sequence negatively affect both binding and transcription, we infer that this factor plays an important role in transcription of the EIIA-E promoter. Determination of the exact boundaries of the factor binding sequence and of the stimulatory effect of this protein in transcription of the EIIA-E promoter will require the purification and functional analysis of this factor.

The factor detected in these experiments appears to be different from NF-1 (30), Sp1 (31), factor B (32), USF (33), or MLTF (23), IgNF-A (34), the "TATA" box-specific factors (35), or the nuclear protein that binds to the "CCAAT" box (36). Lack of nucleotide sequence homology of the region II with the DNA binding domains of the other factors and the inability of the DNA fragments containing the major late promoter and the SV40 early promoter to compete supports this suggestion. A partial sequence homology of the upstream control sequence of the EIIA-E promoter with the EIA and EIV promoter sequences has been noted earlier (20). Consistent with this observation, these two promoters efficiently compete with the EIIA-E promoter in the DNA binding

assay. It will be interesting to determine if transcription of these two promoters shows an absolute requirement for the same factor detected in our experiments. A part of the upstream transcriptional control sequence (5′ GGNNNTGACG 3′; N, any nucleotide) shows homology with the long terminal repeat sequence of the human T-cell leukemia virus type II (HTLV-II) (37). Ad5 EIIA-E promoter can be activated by HTLV-II *x* gene product in transient transfection assays (38) raising the possibility that the factor detected in our studies may also be involved in the transcriptional activation of the HTLV-II promoter.

The presence of EIIA-E factor in uninfected HeLa cell extracts indicates that it is a host factor and, therefore, almost certainly utilized by cell for the expression of some host genes. In this regard, it is similar to USF (33) or MLTF (23) and Sp1 (31). Promoter mutagenesis studies have shown that DNA sequences needed for basal level transcription are inseparable from those required for EIA-induced transcription in that both require the same DNA–protein contacts (17–21). Therefore, the EIIA-EF factor is very likely to be essential for both basal as well as EIA-induced transcription. We have not detected an increase in the levels of this factor in cells infected with wt virus, suggesting that there may not be a net increase in the amounts of this factor during virus infection. However, the possibility of the presence of this factor at higher levels in infected cells that escaped detection because of inefficient extraction cannot be entirely ruled out. *In vivo* exonuclease III mapping studies have suggested that a factor binds to the upstream sequences of the EIIA-E promoter in wt but not in *dl*312-infected cells (39). It is not clear if this factor is identical to the one detected in our studies. If the EIIA-E factor is involved in EIA-dependent *trans*-activation, the mechanism may involve modification of this factor during virus infections. Indeed, *in vitro* studies of Abmayr *et al.* (40) suggest that the *trans*-acting protein of pseudorabies virus acts by directly or indirectly altering the activity of transcription factors rather than by increasing their amounts. We also cannot rule out the role of factors specific to the transcriptional control sequences closer to the cap site (region I) of the EIIA-E promoter in EIA-stimulated transcription. Alternatively, the effective concentration of this factor may increase in virus-infected cells by releasing the factor from host promoters where they are sequestered. These changes are not detected in the extracts used here, where bound and unbound factors might be present. Purification, physical, and functional characterization of this or other factors from uninfected and infected HeLa cells should allow us to discriminate among these possible mechanisms.

Note Added in Proof. When this paper was in press, Kovesdi *et al.* (41) described a factor similar though not identical to the one reported here. Kovesdi *et al.* report that the factor is present in much reduced amounts in uninfected cells, which contradicts our results.

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