Transcriptionally Active Immediate-Early Protein of Pseudorabies Virus Binds to Specific Sites on Class II Gene Promoters

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Received 8 November 1988/Accepted 27 January 1989

In the presence of partially purified pseudorabies virus immediate-early protein, multiple sites of DNase I protection were observed on the adenovirus major late and human hsp 70 promoters. Southwestern (DNA-protein blot) analysis demonstrated that the immediate-early protein bound directly to the sequences contained in these sites. These sequences share only limited homology, differ in their affinities for the immediate-early protein, and are located at different positions on these two promoters. In addition, the site-specific binding of a temperature-sensitive immediate-early protein was eliminated by the same heat treatment which eliminates its transcriptional activating function, whereas the binding of the wild-type protein was unaffected by heat treatment. Thus, site-specific binding requires a functionally active immediate-early protein. Furthermore, immediate-early-protein-dependent *in vitro* transcription from the major late promoter was preferentially inhibited by oligonucleotides which are homologous to the high-affinity binding sites on the major late or hsp 70 promoters. These observations suggest that transcriptional stimulation by the immediate-early protein involves binding to *cis*-acting elements.

The immediate-early (IE) proteins of several double-stranded DNA viruses, including the E1A protein of adenovirus, the ICP4 protein of herpes simplex virus, and the IE protein of pseudorabies virus (PrV), activate subsequent early viral gene expression in *trans* (for reviews, see references 5, 15, and 22). While the mechanisms by which these proteins increase transcription are not clearly understood, the observation that different IE proteins can substitute for each other (11, 30) suggests that these mechanisms have features in common.

No specific promoter sequences have been identified which are uniquely required for stimulation by IE proteins. Indeed, transcription is stimulated from multiple promoters of both cellular and viral origin which have no upstream elements in common (for a review, see reference 5). This observation suggests that stimulation might be mediated through core promoter elements (TATA and cap sites) which interact with basic transcription factors. This suggestion is supported by reports indicating that mutations in the TATA elements of E1B and hsp 70 promoters result in a loss of the ability of the E1A protein to stimulate transcription (28, 34) and that mutations in the TATA box of the herpes simplex virus thymidine kinase promoter reduce stimulation by ICP4 much more than do other promoter mutations (8).

Both the pseudorabies IE protein and E1A have been shown to activate transcription *in vitro* (1, 29). Further studies have indicated that the pseudorabies IE protein increases *in vitro* transcription by facilitating the promoter interactions of the TATA box-binding factor TFIID (2) and that TFIID is the only basic factor required during IE protein-mediated promoter activation (33). In addition, the

stimulatory activity in extracts from adenovirus-infected cells cofractionates with TFIID (20), consistent with the supposition that E1A may activate TFIID *in vivo*.

Others have investigated the possibility that *trans*-activation is mediated through interactions of IE proteins with promoter sequences. Recent studies have shown that the 289-amino-acid E1A protein, the primary form required for *trans*-activation (for a review, see reference 24), is able to bind directly to DNA, albeit weakly and without sequence specificity (6). In contrast, site-specific binding has been observed with a partially purified ICP4 protein by immunoprecipitation, and a weakly conserved consensus sequence was derived (9, 10). Southwestern (DNA-protein blot) analysis has demonstrated that ICP4 binds directly to these sequences and has further illustrated the degeneracy of the consensus (ATCGTnnnnnYnR) (23).

We have previously used a temperature-sensitive mutation in the pseudorabies IE protein (14) to demonstrate that *in vitro* transcriptional stimulation is dependent on the presence of an active IE protein (1). In this study, we show that the IE protein binds directly to multiple specific sites on class II gene promoters and that site-specific binding also requires a functionally active IE protein. Furthermore, IE protein-dependent transcription from the major late promoter is inhibited by competing IE protein-binding sites, indicating that the promoter-binding properties of the IE protein are related to its transcriptional activating function.

MATERIALS AND METHODS

Preparation of IE protein-containing fractions. HeLa cells were infected with wild-type and *tsG* pseudorabies viruses, and nuclear extracts were prepared from infected and mock-infected cells as previously described (2). Chromatographic fractionation of nuclear extracts through DEAE-cellulose and phosphocellulose was as previously described (2; S. M.

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DNA probe preparation. The major late-promoter-containing probes used in the experiments shown in Fig. 1 and 3 were prepared as follows. The *Sma*I fragment of Ad2 (containing the major late promoter) cloned into pBR313 was linearized with *Hind*III or *Xho*I (Boehringer Mannheim) and 3' end labeled with [³²P]dCTP and [³²P]dATP (3,000 Ci/mmol; ICN Pharmaceuticals Inc.) by using T4 polymerase. Following isolation by gel filtration, the *Hind*III- and *Xho*I-cut plasmids were digested with *Xho*I or *Hind*III, respectively. The resulting 452-base-pair (bp) promoter-containing fragments (labeled at opposite ends) were isolated on 1% agarose gels, purified, and concentrated on Elutipis (Schleicher & Schuell, Inc.), ethanol precipitated, and suspended in 10 mM Tris hydrochloride (pH 8.0) and 1 mM EDTA. The specific activities were 6,000 to 8,000 cpm/ng of isolated probes.

The hsp 70 probes used in the experiments shown in Fig. 2 were prepared as above. The 376-bp promoter-containing *Nco*I-*Sal*I fragment from plasmid pHC (28) was labeled at either end, with resulting specific activities of 10,000 to 12,000 cpm/ng. The hsp 70 probe used in Fig. 3 was internally labeled at the *Hpa*II site (−3 relative to the cap site) by digestion of the *Nco*I-*Sal*I fragment with *Hpa*II, followed by dephosphorylation with alkaline phosphatase. The resulting fragments were end labeled with [γ -³²P]ATP (4,500 Ci/mmol) and T4 polynucleotide kinase and religated with T4 ligase. The specific activity of this probe was 7,800 cpm/ng.

Oligonucleotide probes. Oligonucleotides were synthesized, and the complementary strands were annealed, leaving 3 to 4 bases single-stranded at each 5' end. Annealed oligonucleotides were labeled by filling in the 3' ends with [³²P]dCTP, [³²P]dATP, and [³²P]dGTP (3,000 Ci/mmol) by using T4 polymerase. All oligonucleotides used in Southwestern analysis were at the same specific activity (1,300 cpm/ng). The 3' ends were not filled for the unlabeled oligonucleotides used in the transcriptional analysis. Non-specific oligonucleotides utilized for Southwestern and/or transcription analysis included a mutated octamer-containing sequence (33 bp) from the mouse immunoglobulin heavy chain enhancer, the adenovirus VA1 gene B-block (39 bp), and a heptamer- and octamer-containing sequence (43

type- or tsG-infected cells or the corresponding fraction from mock-infected cells for 15 min at 30°C in a 20- μ l reaction mixture containing BC100 buffer (1) with 1.67 μ g of sonicated salmon sperm DNA per ml and 3 mM MgCl₂. Partial digestions were initiated by the addition of DNase I and CaCl₂ to final concentrations of 0.02 μ g/ml and 0.1 mM, respectively, and allowed to proceed for 2 min at 20°C. Digestion products were separated on 8% acrylamide-48% urea sequencing gels. Heat treatment of fractions prior to the binding reaction was done at 45°C for 20 min.

Southwestern and Western (immunoblot) analysis. The binding of promoter sequences to immobilized IE protein was analyzed by a modification of the method of Michael et al. (23). Briefly, 75 to 80 μ g of protein in 7.5 μ l of nuclear extract (prepared from wild-type PrV- or mock-infected cells) was electrophoretically separated on 6% sodium dodecyl sulfate-polyacrylamide gels, followed by electroblotting to nitrocellulose. Immediately following transfer, bound proteins were renatured by washing the nitrocellulose sheets in three changes (1 h each) of a buffer containing 10 mM Tris hydrochloride (pH 7.2), 5% powdered skim milk, 10% glycerol, 2.5% Nonidet P-40, 0.1 mM dithiothreitol, and 150 mM NaCl. The nitrocellulose was cut into strips and placed in Seal and Save bags (Sears and Roebuck) containing BC100 buffer with 1.67 μ g of salmon sperm DNA per ml, 3 mM MgCl₂, and labeled probe as indicated in the figure legends. After overnight incubation at room temperature, the strips were washed in three changes (1 h each) of the same buffer without labeled probe. Detection of bound probe was determined by autoradiography. Following autoradiography, the same nitrocellulose strips were washed according to the procedure of Kramer and Roeder (17). The strips were subsequently incubated with antibody to the IE protein as previously described (1) for 18 h at 37°C, followed by incubation for 5 h with a 1:7,500 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Promega Biotec). The strips were then washed for 30 min in 150 mM NaCl–10 mM Tris hydrochloride (pH 8.0)–1% Triton X-100 and then for 30 min in the same buffer with 5% Triton X-100. Following a 5-min wash in 100 mM Tris hydrochloride (pH 9.5)–100 mM NaCl–5 mM MgCl₂, the bound antibody was detected by color staining using a Protoblot kit from Promega.

Transcription assays. Runoff transcription assays were

5' GATCCGGCCTAATTTGCATGTCCTTCTGCTATATCATTAGCTCTAGG
GCCGGATTAAACGTACAGAAAGACGATATAGTAATCGAGATCCCTAG 5'

bp) from the mouse V1 heavy chain promoter. The sequences of the two former oligonucleotides are shown in Fig. 4B, and the sequence of the latter is

Specific oligonucleotide probes synthesized to the primary and secondary binding sites on the major late (ML) and hsp 70 (HSP) promoters included the following sequences relative to the cap sites: ML(1), +5 to +43 (39 bp); ML(3), −164 to −119 (46 bp); HSP(2), −17 to +31 (48 bp); and HSP(1), −91 to −52 (40 bp). The sequences of specific oligonucleotides are shown in Fig. 4B.

DNase I protection assay. Site-specific protection of DNA fragments from partial DNase I digestion by bound proteins found in partially purified IE protein fractions was determined by a modification of the methods of Galas and Schmitz (12). A 15-ng sample of major late or hsp 70 promoter fragments was incubated with 4 μ g (protein) of IE protein-containing phosphocellulose fractions from wild-

performed essentially as previously described (1, 2). Prior to a 1-h transcription reaction, DEAE fractions from PrV-infected and mock-infected HeLa cells (8 μ g of protein) were incubated with oligonucleotides for 15 min at 30°C before addition to transcription reaction mixtures. Oligonucleotide concentrations were between 4- and 16-fold molar excess to promoter concentration in the transcription assays (template concentration, 2 μ g/ml). Analysis and quantitation of transcription products were done as described elsewhere (1, 2).

RESULTS

Multiple DNase I-protected sites on the major late and hsp 70 promoters are dependent on a functional IE protein. To further investigate the mechanism by which IE activates transcription, we examined the interaction of the IE protein with promoter sequences. DNase I protection experiments

FIG. 1. IE protein-dependent footprints on the major late promoter. DNase I-protected regions on the upper strand (A), 3' end labeled at the *Hind*III site, and on the lower strand (B), 3' end labeled at the *Xho*I site, of the major late promoter fragment (−260 to +192 relative to the cap site). Brackets denote the positions of the protected regions, and arrows indicate hypersensitive sites which are lost after heat treatment of the temperature-sensitive *ts*G IE protein. Also shown is the footprint of the upstream stimulatory factor (USF) which was present in all these fractions. The locations of the TATA box and cap site (+1) are also indicated. Prior to DNase I digestion, 15 ng of probe was incubated with 4 μ g (protein) of an IE protein-containing phosphocellulose fraction from wild-type (W)- or *ts*G (T)-infected HeLa cells or the corresponding fraction from mock-infected (M) HeLa cells. These fractions were heat treated for 20 min at 45°C prior to binding for the reactions as indicated. Identical digestion reactions in the absence of added fractions are shown (C), as well as Maxam-Gilbert sequencing G reactions (G). The positions, relative to the cap site, of the IE protein-dependent sites, ML(1 to 3), and the USF site for both strands are diagrammed at the bottom.

with IE-containing fractions, partially purified by chromatographic fractionation through DEAE-cellulose and phosphocellulose columns (2; Abmayr, Ph.D. thesis), revealed several protected sites on the major late promoter (Fig. 1). Of these, several sites (Fig. 1, sites 1 to 3) were protected only in IE protein-containing fractions from cells infected with wild-type or *ts*G pseudorabies viruses (Fig. 1A, lanes 2 and 3; Fig. 1B, lanes 1 and 2). The strongest protection was observed downstream of the cap site, while somewhat weaker protection was observed over the cap site and upstream. The differential affinity of these sites for the IE protein has been independently confirmed by Southwestern analysis (see Fig. 4 and below). In accordance with these results, the sites have been designated ML(1) (strong) ML(2), and ML(3) (weak) (Fig. 1). In addition, one other region of protection was found with both the IE protein-containing fraction and the corresponding fraction from mock-infected cells (Fig. 1A, lane 4; Fig. 1B, lane 3) and reflects binding of the cellular factor USF (27).

We have previously shown that the temperature-sensitive IE protein from the *ts*G mutant virus (14) is functionally

inactivated *in vitro* by mild heat treatment (1, 2). To test the dependence of protection at these sites on the presence of a functional IE protein, we used this temperature-sensitive IE protein. Prior to the binding assay, the wild-type, *ts*G, and mock-infected fractions were heat treated at 45°C for 20 min. As shown in Fig. 1A (lane 6) and 1B (lane 5), heat treatment of the *ts*G fraction abolished protection at these sites, such that the resulting digest resembled that obtained with the mock-infected fraction. In contrast, protection of these sites was unaffected by heat treatment of the wild-type protein (Fig. 1A, lane 5; Fig. 1B, lane 4).

We have also examined DNase I protection patterns on the human hsp 70 promoter in the presence of IE protein-containing fractions (Fig. 2A and B). Two DNase I-protected regions were evident on this promoter. The most strongly protected region was upstream, HSP(1), while a region of somewhat weaker protection was detected downstream, HSP(2) (Fig. 2). Furthermore, protection at both of these sites was lost upon heat treatment of the *ts*G but not of the wild-type fraction, indicating dependence on a functional IE protein (compare lanes 5 and 6 in Fig. 2A and 2B).

FIG. 2. IE protein-dependent footprints on the hsp 70 promoter. DNase I-protected sites on the upper strand (A), 3' end labeled at the *SalI* site, and on the lower strand (B), 3' end labeled at the *NcoI* site, of the hsp 70 promoter fragment (−264 to +112 relative to the cap site). Reactions contained wild-type IE protein (W), tsG IE protein (T), or the corresponding mock-infected cell fractions that had been incubated either at 4 or 45°C as described in the legend to Fig. 1. All symbols indicated are as defined in the legend to Fig. 1. The positions, relative to the cap site (+1), of IE protein-dependent footprints, HSP(1) and HSP(2), are diagrammed at the bottom.

IE protein bound directly to major late and hsp 70 promoter fragments. Our previous functional studies have shown that IE protein can facilitate the promoter interactions of the cellular factor TFIID (2). While the IE protein-containing fractions tested in the experiments shown in Fig. 1 and 2 did not contain TFIID activity or convey DNase I protection of the TATA box, it is possible that the IE protein-dependent binding observed was due to unidentified cellular proteins which cofractionated with IE protein. Alternatively, the observed binding might reflect the direct promoter interactions of the IE protein.

To investigate these possibilities, we tested direct binding of promoter fragments to the IE protein by Southwestern analysis. Southwestern blots (Fig. 3) illustrate the binding of major late (Fig. 3A) and hsp 70 (Fig. 3B) promoter fragments to electrophoretically separated proteins from extracts prepared from wild-type virus or mock-infected cells. Each promoter fragment bound a 180-kilodalton (kDa) protein from infected-cell extracts (lanes 2) which was not present in mock-infected cell extracts (lanes 1). Subsequent Western analysis of the same nitrocellulose strips confirmed that this protein was in fact the IE protein. The antibody binding revealed the presence of the 180-kDa IE protein only in the infected-cell extracts (lanes 4) at the exact position of the promoter-binding activity. We have repeated this experiment with other promoter constructs and oligonucleotide probes (see below), and in all cases the only detected DNA-binding activity specific to infected-cell extracts coincided with the IE protein as revealed by Western analysis.

FIG. 3. Southwestern and Western analysis of the major late promoter fragment (A), hsp 70 promoter fragment (B), and antibody binding to the PrV IE protein. A 75-μg sample of wild-type PrV-infected (I; lanes 2 and 4) and an 80-μg sample of mock-infected (M; lanes 1 and 3) HeLa cell nuclear extract proteins were electrophoretically separated on sodium dodecyl sulfate-acrylamide gels and blotted to nitrocellulose in each panel as described in Materials and Methods. Binding of the promoter fragments (DNA) to the 180-kDa (kd) IE protein (only present in the infected-cell extracts) is illustrated by the Southwestern blots of the major late (A) and hsp 70 (B) promoter fragments. The same strips were subsequently tested by Western analysis (Ab) as described in Materials and Methods to confirm that the probe binding was coincident with the location of the IE protein.

FIG. 4. Southwestern analysis of site-specific oligonucleotide probe binding to the IE protein. (A) Proteins from mock-infected (M) and wild-type PrV-infected (I) HeLa cell extracts were separated and transferred to nitrocellulose as described in the legend to Fig. 3. Nitrocellulose strips cut from the blot were probed under the same conditions with equal labeled oligonucleotide probes at identical specific activities and concentrations as described in Materials and Methods. The specific oligonucleotide probes used contained major late or hsp 70 promoter sequences that were protected in the footprinting analysis (see Materials and Methods) and included the sites designated MLP(1), MLP(3), HSP(2), and HSP(1). Nonspecific oligonucleotides containing a mutated octamer sequence (m-octa) or the adenovirus VA1 gene B-block sequence (B-Block) were also tested for binding sizes are shown in kilodaltons (kd). (B) Sequences of the oligonucleotide (OLIGO.) probes and the relative extent of binding as determined by densitometry of autoradiograms. The DNase I-protected regions on these sequences (from Fig. 1 and 2) are indicated by solid lines above or below the protected nucleotides.

Additional proteins in the range of 116 kDa also appeared to bind to these fragments. These proteins bound nonspecifically to all of the fragments and oligonucleotides examined and were present in both infected and mock-infected extracts (Fig. 3 and 4). We therefore did not pursue the nature of these proteins further.

IE protein bound with differential affinities to sequences corresponding to DNase I-protected sites. The above results indicate that the IE protein was able to bind to promoter fragments and suggested that the IE protein-dependent DNase I-protected sites were due to the direct binding of the IE protein. To gain more direct evidence for the sequence specificity of IE binding, we tested the binding of IE to double-stranded oligonucleotide probes which corresponded to the sequences contained in the protected sites. The results of a Southwestern analysis performed with the specific as well as nonspecific oligonucleotides are shown in Fig. 4A. All binding reactions were matched for both oligonucleotide concentration and specific activity to allow direct comparison of the results. As shown, all of the oligonucleotides corresponding to DNase I-protected sites bound to the IE protein. In contrast, two nonspecific oligonucleotides (one containing a mutant octamer sequence and one containing the B-block of the adenovirus VA1 gene) did not display any detectable binding.

Consistent with the results of the footprint analysis described above (Fig. 1 and 2), IE apparently bound to the specific oligonucleotides with different affinities (Fig. 4). The strongest binding observed was to the oligonucleotides which corresponded to the protected sites ML(1) and HSP(1) as designated in Fig. 1 and 2, respectively. Substantially weaker binding was observed to the two other site-specific oligonucleotides tested, ML(3) and HSP(2). The relative binding of IE to these oligonucleotides correlated with the extent of DNase I protection of the corresponding sites on promoter fragments when IE concentrations were titrated. The ML(1) site was protected at lower IE protein concentrations than was ML(3), and the HSP(1) site was protected at lower IE protein concentrations than was HSP(2) (data not shown). These data confirm the site-specific binding of the IE protein to the major late and hsp 70 promoter

fragments previously shown and further illustrate that both low- and high-affinity binding sites exist on each of these promoters.

The sequences of these oligonucleotide probes are shown in Fig. 4B. The solid lines above and below each sequence indicate the protected bases. The largest homology between the two high-affinity sites, ML(1) and HSP(1), is the sequence CCNCATCG. However, we have also detected in the adenovirus EIII promoter a strong protected site (-10 to +13 relative to the cap site) which does not contain this sequence (data not shown). Alternatively, the sequence GCGAGG is present in both the ML(1) site and the strong EIII site. However, only four of these bases (CGAG) are present in the HSP(1) site. In addition, partial homologies to this sequence are present in the ML(3) site (GCGAG) and the HSP(2) site (GAGG). Thus, while small homologies are observed within these binding sites, it seems unlikely that these homologies alone can account for the sequence specificity and the differing affinities of IE protein binding to these regions. Although we cannot eliminate the possibility that the differences in sequence specificity and affinity are actually due to the presence of several forms of the IE protein, we believe that this is unlikely. In contrast to the 175-kDa herpes simplex virus ICP4 protein, of which several forms have been identified (23), no higher-molecular-weight species of the PrV IE protein have been observed by similar methods of analysis (this paper).

IE protein-dependent transcription was preferentially inhibited by oligonucleotides complementary to binding sites. To investigate the functional relevance of site-specific binding of the IE protein, we tested the effect of competitor binding sites on IE-dependent transcription from the major late promoter (Fig. 5). As shown previously (1, 2), the addition of IE protein-containing fractions to transcription reactions performed at low template concentrations stimulated the level of transcription obtained (compare lanes 1 and 2). However, when the IE protein-containing fractions were incubated with oligonucleotides corresponding to the high-affinity IE protein-binding sites ML(1) and HSP(1), the subsequent level of transcription obtained was decreased in a dose-responsive manner (lanes 3 to 6 and 7 to 10). At a

FIG. 5. IE protein binding to site-specific oligonucleotides (oligo) preferentially inhibited transcription which is dependent on the IE protein. A 8- μ g sample of a DEAE-cellulose IE fraction was incubated with oligonucleotide probes prior to its addition to an in vitro transcription reaction (lanes 3 to 22). Oligonucleotide concentrations are indicated as ratios of molar excess to promoters in the subsequent transcription assay. The oligonucleotides tested are as described in the legend to Fig. 4, except a longer octamer-sequence-containing probe (h-octa; 43 bp) was also tested. Lanes 1 and 2 show the products of identical transcription reactions without oligonucleotides but with the IE protein-containing fraction (lane 2) or the corresponding fraction from mock-infected cells (lane 1).

16-fold molar excess (relative to the promoter concentration), the level of transcription was reduced 4.0-fold by the ML(1) oligonucleotide and 3.6-fold by the HSP(1) oligonucleotide (compare lanes 6 and 10 with lane 2). In contrast, nonspecific oligonucleotides did not reduce the levels of transcription when present at these concentrations (lanes 11 to 14, 15 to 18 and 19 to 22). In fact, addition of these oligonucleotides actually increased transcription. At a 16-fold molar excess of the nonspecific oligonucleotides, the levels of transcription observed were increased by 1.38-, 1.35-, and 1.17-fold relative to the control, for the m-octa, h-octa, and B-block oligonucleotides, respectively. Under conditions in which IE protein was not required for transcription, i.e., when nonspecific DNA was added (2), the addition of oligonucleotide probes inhibited transcription approximately 2-fold when present at a 16-fold molar excess. However, this inhibition was observed for all these oligonucleotides and was not specific to those containing IE protein-binding sites (data not shown). Thus, oligonucleotides which contain IE protein-binding sites preferentially inhibit transcription which is dependent on the action of the IE protein.

DISCUSSION

In this study, we have demonstrated site-specific binding of the PrV IE protein to double-stranded DNA of class II gene promoters. Our results correlate well with previous studies on the herpes simplex virus ICP4 protein, which also binds to DNA in a site-specific fashion (10, 23). As with ICP4, binding of the PrV IE protein did not appear to require a strong consensus sequence. Moreover, our results demonstrate that all sequences were not recognized equally and that some sites did have a higher affinity than others. In addition, we have shown that binding of the temperature-sensitive IE protein was eliminated by the same mild heat treatment which eliminates its transcriptional activating function both in vivo and in vitro (1-3, 14). Furthermore, oligonucleotides corresponding to high-affinity IE protein-binding sites inhibited transcription which is dependent on the IE protein.

Functional relevance of site-specific promoter binding of IE proteins. Our results demonstrate that a transcriptionally

active IE protein is required for site-specific promoter binding. The relevance of DNA binding to function is also suggested by our observation that ML(1) or HSP(1) oligonucleotides preferentially inhibited transcriptional stimulation by the IE protein (Fig. 5). While these observations suggest that the promoter-binding and *trans*-activating functions of the IE protein are related, they do not prove that binding is actually required for transcriptional stimulation. For example, one alternative interpretation is that DNA binding simply inactivates a catalytic function of the IE protein such that the oligonucleotide-bound IE protein is unable to function. In this case, however, one might expect that IE protein bound to promoter sequences would be similarly inactivated, leaving no free IE protein to stimulate in vitro transcription. Thus, in vitro stimulation would actually have been prevented under the transcription conditions that we have used. Since we have reproducibly observed stimulation, even at low concentrations of IE protein, we believe that this interpretation is unlikely.

Our results also demonstrate that the IE protein bound with differing affinities to multiple sites, of very degenerate sequence homology, on both the hsp 70 and major late promoters. The multiplicity of binding sites may account for the observation that, other than the TATA box, no specific site in the major late promoter is required for IE protein-mediated stimulation of transcription (2) since deletion of individual sites would not eliminate IE protein binding to the promoter.

In contrast to the observations reported here, Chlan et al. (7) have shown that wild-type and temperature-sensitive IE proteins bind nonspecifically to single-stranded DNA-Sepharose at both 0°C and 40°C (the nonpermissive temperature). Together with our results, these observations suggest that while the inactivated temperature-sensitive IE protein can apparently nonspecifically bind nucleic acids, it differs from a functional IE protein in its ability to bind double-stranded DNA in a site-specific manner.

In addition to having the ability to stimulate transcription from a variety of cellular and viral promoters, ICP4 and the IE proteins of pseudorabies and cytomegalovirus play a role in negative regulation of their own transcripts (14, 26, 32).

While it is not yet known whether this control occurs by direct binding of the IE proteins to promoter sequences, the observed negative regulation has been shown to be very specific for IE gene promoters in both herpes simplex virus and cytomegalovirus (18, 26). Thus, it is unlikely that the binding observed in this study is related to negative autoregulation, since the binding sites described here are neither highly conserved nor specific to IE gene promoters.

Studies by Chatterjee et al. (6) suggest that the 289-amino-acid E1A protein may also bind directly to DNA, although this binding appears to be quite weak and displays no sequence preference. Interestingly, however, this form of the E1A protein has been shown to be the primary form responsible for transcriptional activation and contains a putative zinc finger domain characteristic of numerous DNA-binding proteins (for reviews, see references 15 and 24). Furthermore, a 49-amino-acid fragment of the E1A protein which contains this putative zinc finger is sufficient for transcriptional stimulation (21). At present, however, the ability of this fragment to bind to DNA has not been addressed.

Implications for mechanisms of *trans*-activation. Our previous studies have demonstrated that the pseudorabies IE protein does not activate transcription by increasing levels of transcription factors (1). Instead, IE protein stimulates *in vitro* transcription by facilitating the promoter interactions of the cellular factor TFIID (the TATA box-binding factor), a primary step in preinitiation complex formation (2). This conclusion is further supported by the *in vivo* observation that IE protein is not continuously required for reinitiation of transcription from previously induced promoters (4). Furthermore, *in vitro* functional analyses indicated that the rate of TFIID binding is enhanced in the presence of IE protein and demonstrated that TFIID is the only factor required during promoter activation (33). The present observations suggest that IE protein may stimulate TFIID binding via direct interactions at the promoter. Thus, through protein-DNA and/or protein-protein interactions, promoter-bound IE protein may increase the rate or stability of TFIID binding to the TATA/cap region. With regard to possible direct interactions between IE protein and TFIID, it is interesting that the strongest IE protein-binding site observed on the major late promoter ML(1) overlaps the downstream region also bound by TFIID (25, 31).

Studies of transcriptional activation of class III genes by E1A have demonstrated that the *in vivo* action of E1A results in a modified (more active) form of TFIIC (13). While direct demonstration of a TFIID modification by the action of either the PrV IE protein or E1A has not been achieved, Leong et al. (20) have suggested that the TFIID protein might also be activated by E1A. While it is not known whether factor modifications result from the action of the pseudorabies IE protein, such activities are not inconsistent with its promoter-binding properties. In fact the promoter-binding properties of IE protein could mediate factor modifications by concentrating IE protein at promoter sequences. However, it is important that inactivation of the *tsG* mutant IE protein *in vivo* abolishes activation of class II but not class III genes (3). This observation suggests that at least in the case of the PrV IE protein, activation of class II and class III genes may occur by multiple or distinct mechanisms.

In addition to the previously discussed role of the TATA-binding factor (TFIID) in E1A and IE protein-mediated stimulation, other cellular proteins have also been implicated as targets of E1A. These include the sequence-specific factor

ATF, which has a binding site common to many promoters (19), and E2F, which binds upstream of the adenovirus E2 promoter (16). Whether E1A alters the transcriptional activity of these proteins in solution or through interactions at the promoter and whether the PrV IE protein and ICP4 act similarly remain to be determined. However, the direct binding of IE protein to multiple sites on class II promoters may facilitate either the promoter binding or the modification (or both) of various transcription factors in addition to TFIID.

ACKNOWLEDGMENTS

We are grateful to Barbara Yoza, James Cromlish, and Lorenz Poellinger for useful advice and for contributing oligonucleotides. We thank Virginia Kozler for excellent assistance in the growth of cells and viruses.

This work was supported by Public Health Service grant CA-42567 from the National Cancer Institute to R.G.R. and general support from the Pew Trusts to The Rockefeller University. S.M.A. was supported by Public Health Service training grant T32 A107233 from the National Institutes of Health. J.L.W. was supported by Public Health Service fellowship GM11077 from the National Institutes of Health. M.H. was supported in part by a Japan Society for the Promotion of Science Fellowship for Research Abroad and in part by a Biomedical Research Fellowship of the Norman and Rosita Winston Foundation.

ADDENDUM

D. G. Tedder and L. I. Pizer (J. Virol. **62**:4661–4672, 1988) have recently provided direct evidence that ICP4 binding to specific sites in a herpes simplex virus gene contributes to transcription activation.

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