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Two promoter-specific host factors interact with adjacent sequences in an EIA-inducible adenovirus promoter

(adenovirus type 5 EIIA early promoter/gel shift assays/transcription factors/EIA activation)

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ABSTRACT **We previously reported the identification of a host factor (EIIA-EF) specific for an upstream transcriptional control sequence (−82 to −66) of the EIA-inducible adenovirus EIIA early promoter. The levels of this factor remained unchanged after virus infection of human cells. Another study also identified a factor (EIIF) specific for this same promoter, but the activity of this second factor was shown to increase severalfold after virus infection. We now show that these dramatically different results, both based on gel shift assays on the same promoter, may be explained by variations in protocol details and actually identify two distinct factors. When synthetic DNA copolymers [poly(dI)·poly(dC) or poly(dI-dC)·poly(dI-dC)] are used as competitors in gel shift assays, a factor specific for DNA sequences between −82 and −66 can be identified, whereas when natural eukaryotic DNAs (salmon sperm or calf thymus) are used as competitors a different factor specific for DNA sequences between −69 and −33 can be identified. We have mapped the DNA–protein contact residues for the EIIF by analyzing a series of linker scan mutants in gel shift assays and methylation interference experiments. The EIIA-EF and EIIF bind to two distinct but adjacent sequences. Competition experiments indicate that these two activities are due to two different factors. Consistent with the earlier reports, the levels of one (EIIA-EF) do not change after virus infection of human cells, whereas the levels of the other (EIIF) are increased severalfold.**

Eukaryotic RNA polymerase II promoters contain a complex array of cis-acting genetic elements that regulate basal, induced, and repressed transcription rates. These cis-acting regulatory sequences interact with a variety of general as well as gene-specific transcription factors. The interaction of the cis regulatory elements of promoters with the trans-acting promoter-specific DNA-binding proteins is important in control of tissue-specific, hormone-induced, viral-induced, and growth-related gene expression.

Adenovirus (Ad) provides a useful model system to study the control of eukaryotic gene expression. In human cells infected with Ad type 2 or 5, a set of five early viral promoters are coordinately expressed (1). Efficient transcription of these early viral promoters and of several cellular promoters is dependent on the 32-kDa phosphoprotein encoded by the viral pre-early EIA gene. Recent negative results, including failure to detect either DNA-binding properties for the EIA protein (2) or sequence elements in the EIA responsive promoters recognized by the EIA gene product, have raised the possibility that the transcriptional activation by EIA may be mediated by promoter-specific host factors (for review, see ref. 3). Viral infection of human cells may modify or increase the synthesis of host transcription factors. Alternatively, the transcription factors that are sequestered with the host promoters may upon infection be diverted to viral promoters by an as yet unidentified mechanism.

Utilizing gel shift assays (4–6), we previously demonstrated that the uninfected HeLa cells contained a protein factor specific for the upstream transcriptional control element (between −82 and −66) of the EIA responsive Ad EIIA early (E) promoter (7). The levels of this factor did not change as a result of virus infection. However, Kovesdi *et al.* (8), using experimental conditions similar to ours, identified a factor that not only showed different sequence specificity but whose level of activity increased dramatically upon virus infection. We have now performed experiments to resolve the inconsistencies of these two reports. We found that when synthetic DNA copolymers are used as competitors in gel shift assays a factor specific for the DNA sequence located between −82 and −66 is identified (EIIA-EF). However, when the synthetic DNAs are replaced with higher eukaryotic DNAs in these same assays, EIIA-EF is not detected. Instead, another factor (EIIF; we have retained the original name of this factor; see ref. 8) specific for adjacent DNA sequences between −69 and −33 is identified. Consistent with earlier reports (7, 8), the levels of only this second factor, EIIF, increase considerably during viral infections.

MATERIALS AND METHODS

Sources of HeLa cell suspension cultures, wild-type (wt), and *dl312* Ad5 variants, plasmids that contain wild-type Ad5 EIIA-E promoter, and the linker scan (LS) mutant derivatives were described in two of our previous reports (7, 9). Nuclear extracts from uninfected and virus-infected HeLa cells were prepared as described (10). Viral infection was carried out in the presence of cytosine arabinoside (25 μg/ml) for 7 hr. Protein concentrations were determined by the method of Bradford (11). DNA–protein complexes were resolved on polyacrylamide gels as reported (7) (see figure legends).

RESULTS

Recently, workers in numerous laboratories have used the mobility shift assays (gel shift assays) (4–6) to identify sequence-specific DNA binding proteins present in crude cell extracts. The interference of large amounts of nonspecific DNA-binding proteins in specific DNA–protein interactions was suppressed by including synthetic DNA copolymers (6) or bacterial DNAs (12). We have recently used this technique with poly(dI)·poly(dC) as the competitor DNA to identify a host factor specific for the EIA-inducible Ad5 EIIA early promoter. We probed the nuclear extracts from uninfected HeLa cells and from cells infected with wt Ad5 or *dl312* (a deletion mutant that lacks the EIA coding sequences) (13) with an end-labeled 166-base-pair (bp) DNA fragment (from

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Abbreviations: Ad, adenovirus; wt, wild type; LS, linker scan.
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*Hin*dIII/*Bgl* II site; see Fig. 1*C*), which contained the minimum promoter DNA sequences essential for both basal as well as EIA-induced transcription (9). Fig. 1*A* shows these results. In agreement with a previous publication (7), a factor complexed with the EIIA-E promoter DNA fragment is identified. Neither the levels nor the specificity of this factor as determined by methylation interference experiments (see below) changed when nuclear extracts were used from HeLa cells infected with wt Ad5 or *dl312* for 7 hr in the presence of cytosine arabinoside. To determine whether another competitor DNA would identify a different factor, we repeated these experiments with an alternating copolymer poly(dI-dC)·poly(dI-dC). No differences were found with regard to levels (Fig. 1*A*) or specificity (data for methylation interference experiments not shown).

When these experiments were repeated with higher eukaryotic DNAs as competitors, dramatically different results were obtained. Fig. 1*B* shows that when sonicated salmon or calf thymus DNAs are used as the competitors, the lanes corresponding to nuclear extracts prepared from infected cells showed two prominent bands (B-I and B-II). The radioactivity present in the slower migrating band (B-I) in the lane corresponding to infected cell extracts was present at levels severalfold higher than the corresponding bands appearing in lanes specific to nuclear extracts from uninfected cells or cells infected with *dl312* (Fig. 1*B*). Another band (B-II) with significant intensity appeared in all the lanes regardless of the type of nuclear extracts used. We believe that this band represents a nonspecific DNA–protein complex because (*i*) this complex was observed for every LS mutant of the EIIA-E promoter whether infected or uninfected cell extracts were used in gel shift assays (Fig. 2 *C* and *D*), and (*ii*) methylation interference experiments did not reveal any specific DNA protein contacts for this complex.

To determine which of the EIIA-E promoter sequences interact with the factors identified in the gel shift assays

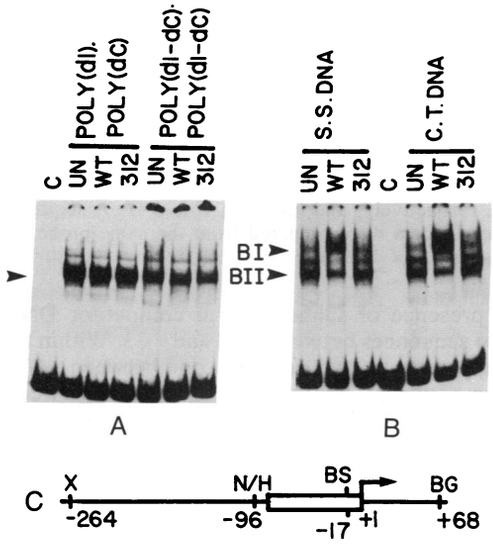

FIG. 1. Effect of different competitor DNAs on the detection of host factors specific for the EIIA-E promoter. Approximately 0.2 ng of the DNA fragment from *Bgl* II/*Hin*dIII (see *C*) was 3'-end-labeled and incubated with 10–12 μg of protein as described (7). The assay mixture contained 80 μg of the competitor DNAs per ml. (*A*) Assay with poly(dI)·poly(dC) or poly(dI-dC)·poly(dI-dC). (*B*) Assay with salmon sperm (S.S.) or calf thymus (C.T.) DNAs. (*C*) Physical map of the region of the plasmid that contains the EIIA-E promoter (7, 9). Lanes C, sample without protein. Arrowheads, DNA–protein complexes. B-I and B-II, specific and nonspecific DNA–protein complexes, respectively. X, *Xba* I; N, *Nar* I; H, *Hin*dIII; BS, *Bss*HII; BG, *Bgl* II.

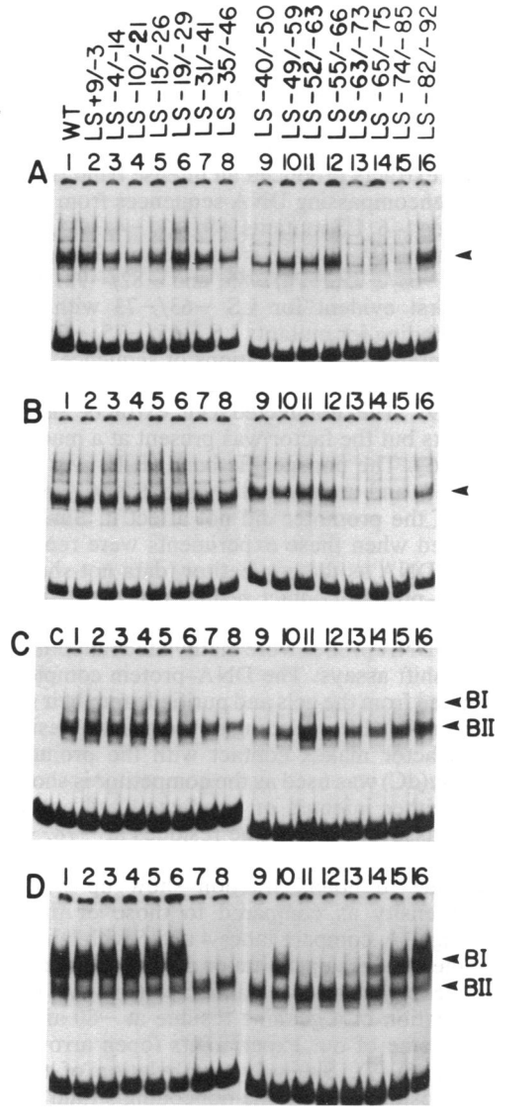

FIG. 2. Effect of LS mutations in the formation of DNA–protein complex in gel shift assays. The 166-bp DNA fragments from *Bgl* II/*Hin*dIII sites were used as probes. (*A*) Gel shift assays for LS mutants using uninfected nuclear extracts and poly(dI)·poly(dC) as the competitor. (*B*) Gel shift assays for LS mutants using nuclear extracts prepared from infected cells and poly(dI)·poly(dC) as the competitor. Arrowheads, DNA–protein complexes. (*C*) Gel shift assays for the LS mutants using uninfected HeLa cell nuclear extracts and salmon sperm DNA as the competitor. (*D*) Gel shift assays for the LS mutants with nuclear extracts prepared from infected cells and with salmon sperm DNA as the competitor. Specific and nonspecific DNA–protein complexes, B-I and B-II, respectively, are shown by arrows. The assay conditions are as described (7). Lane C, control sample without protein.

shown in Fig. 1 *A* and *B* and to determine whether or not these sequences overlap, we examined the DNA–protein contact residues by analyzing a series of LS mutants of the promoter in gel shift assays and methylation interference experiments. Nuclear extracts from uninfected and virus-infected cells were probed with an end-labeled 166-bp DNA fragment from the LS mutants (*Bgl* II/*Hin*dIII sites; see Fig. 1*C*) in gel shift assays with poly(dI)·poly(dC) or salmon sperm DNA as competitors. The DNA–protein complexes resolved in polyacrylamide gels are shown in Fig. 2. In agreement with our previous report, factor binding was drastically reduced for LS mutants −63/−73, −65/−75, and −74/−85 (Fig. 2*A*, lanes 13–15) when poly(dI)·poly(dC) was used as the com-

petitor and unaffected for the remaining LS mutants. The phenotype of the LS mutants in this assay was essentially identical whether nuclear extracts came from infected or uninfected cells (Fig. 2 *A* and *B*).

When salmon sperm DNA was used as the competitor in gel shift assays, the results were dramatically different. Infected cell extracts produced an intense band (B-I) for the LS mutants encompassing DNA sequences from +9 to −29 (Fig. 2*D*, lanes 1–6, LS mutants +9/−3, −4/−14, −10/−21, −15/−26, and −19/−29) and from −65 to −92 (lanes 14–16; LS mutants −65/−75, −74/−85, and −82/−92. The factor binding is first evident for LS −63/−73 with a gradual increase in binding for mutants LS −65/−75, −74/−85, and −82/−92). Interestingly, mutations of sequence around position −50 do not affect the interaction of the factor (lane 10, LS −49/−59). The uninfected cell extracts also showed similar results but the factor was present at a much reduced level (Fig. 2*C*). The nonspecific band (B-II) was present in both infected and uninfected cell extracts, and the LS mutations of the promoter did not affect it. Similar results were obtained when these experiments were repeated with calf thymus DNA as the competitor (data not shown).

The DNA–protein contact residues of the factor binding were mapped by methylation interference experiments (7). End-labeled DNA probes were methylated randomly (14) and used in gel shift assays. The DNA–protein complexes were then extracted from the gels and purified, and their sequences were determined (14). Mapping of the guanine residues with which the factor makes contact with the promoter when poly(dI)·poly(dC) was used as the competitor is shown in Fig. 3*A*. When either infected or uninfected cell extracts were used in the assays, the guanine residues at −78, −75, −72, and −69 of the coding strand (shown by arrowheads) in lanes corresponding to bound fraction show up with a much reduced intensity as compared to those of the unbound fraction (Fig. 3*A*, compare lanes 4 and 6 with lanes 3 and 5). The guanine residues upstream or downstream of this region in the sequence ladders are unaffected (Fig. 3*A*, lanes 8 and 10). Methylation of a guanine residue at −80 affects factor binding in some of our experiments (open arrow, Fig. 3*A*, lanes 4 and 6) (7). Similarly, methylation of the guanine residue at position −73 in the noncoding strand also affects factor binding (data not shown). These results and the analysis of the LS mutants in gel shift assays confirm our earlier results and suggest that the EIIA-EF binds to a sequence between −82 and −66 of the EIIA-E promoter.

Using an identical strategy, we mapped the guanine residues within the promoter with which the factor makes contact when salmon sperm DNA was used as the competitor. Interaction of infected cell factor (complex B-I in Fig. 1*B*) with the EIIA-E promoter DNA fragment was affected when guanine residues at −63, −61, −45, −44, −43, −41, and −39 of the coding strand were methylated. In Fig. 3*B*, the guanine residues at these positions (shown by arrowheads) in lanes specific for infected cell extracts appear with a much reduced intensity from the bound fraction as compared to those of unbound fractions (Fig. 3*B*, compare lane 4 with lane 3). The factor from uninfected cells (B-I in Fig. 1*B*) also makes contact with these same guanine residues, as identical methylation interference pattern was obtained for the DNA–protein complexes isolated from uninfected cell extracts (Fig. 3*B*, compare lane 7 with lane 6). Similarly, methylation of guanine residues at −64, −62, −60, −42, and −40 of the noncoding strand interfered in factor binding whether the nuclear extracts came from infected or uninfected cells (Fig. 3*C*, compare lanes 2 and 5 with lanes 3 and 6). In contrast, the intensity of all the guanine residues in lanes corresponding to a nonspecific band (B-II in Fig. 1*B*) was essentially similar in both coding and noncoding strands (Fig. 3*B*, lanes 5 and 8; Fig. 3*C*, lane 4). Thus, we conclude that the factor detected

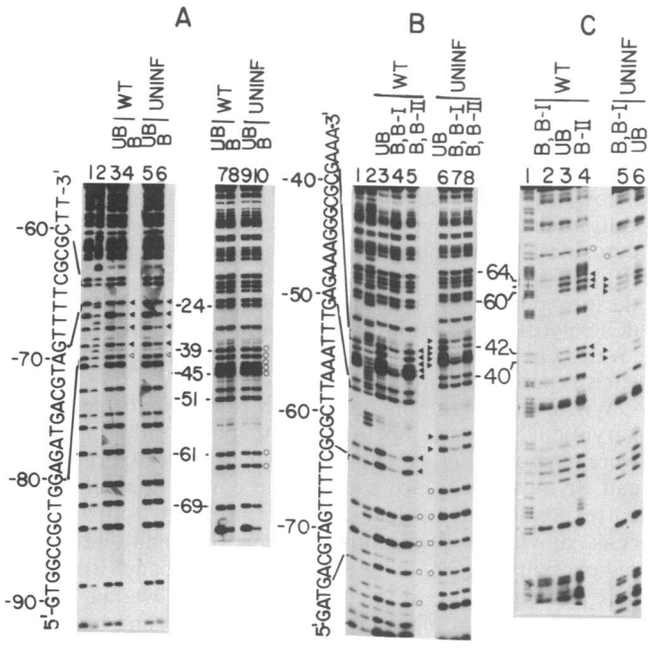

FIG. 3. Methylation interference assays. (*A*) Effect of methylation of coding strand sequences on EIIA-EF binding. A 166-bp DNA fragment from the *Hin*dIII/*Bgl* II site was 5′-end-labeled (coding strand) at the *Hin*dIII site and was methylated by dimethyl sulfate (14). The probe was then incubated with the nuclear extracts prepared from infected (lanes 3, 4, 7, and 8) or uninfected (lanes 5, 6, 9, and 10) cells. The bound (B) and unbound (UB) fragments were extracted from the gel and processed further to generate sequence ladders. Arrowheads, guanine residues that interact with the EIIA-EF. Circles, guanine residues that interact with EIIF. Methylation of the guanine residue at −80 shown by an open arrowhead affects factor binding in some experiments (7). (*B*) Effect of methylation of coding strand sequences on EIIF binding. A strategy identical to that in *A* was used. B-I and B-II, specific and nonspecific DNA–protein complexes, respectively. Arrowheads, guanine residues that interact with EIIF. Circles, guanine residues that interact with EIIA-EF. Lanes 3–5, extracts from virus-infected cells; lanes 6–8, extracts from uninfected cells. (*C*) Effect of methylation of noncoding strand sequences on EIIF binding. A 5′-end-labeled 166-bp DNA fragment (*Bgl* II/*Hin*dIII site; labeled at *Bgl* II site) was used as a probe. Lanes 2–4, extracts from virus-infected cells; lanes 5 and 6, extracts from uninfected cells. Lanes 1 and 2 in *A* and *B* are G and G+A sequence ladders, respectively, and lane 1 in *C* is a G+A sequence ladder. Sequence ladders were prepared from the same probe used in gel shift assays.

in the presence of salmon sperm competitor DNA (EIIF) binds to sequences between −69 and −33. Within this region there appear to be two domains, one between −47 and −33 and one between −69 and −54. Both domains contain the sequences 5′ TTTCGCGC 3′ at −67 to −60 on the coding strand and at −43 to −36 in the noncoding strand (see Fig. 5). This same sequence is also repeated twice in the EIA promoter, at −296 and at −234. The amount or the activity of this factor is low in uninfected cells and increases severalfold after virus infection.

If the two binding activities detected in the gel shift assays are due to two different proteins, then a DNA fragment that contains a recognition sequence for one factor should not compete with detection of the other factor in gel shift assays. We previously showed that among the several early adenoviral promoters, both the EIV and the EIA promoters compete efficiently with the EIIA-E promoter for the EIIA-EF binding in gel shift assays (7). This is likely due to the DNA sequence elements they contain that are completely or partially homologous to the EIIA-EF binding site (5′ TGACGTA 3′). Similarly, DNA sequences homologous to the

EIIF-binding sites of the EIIA-E promoter are repeated twice in the EIA promoter sequences (see above). Therefore, we tested synthetic double-stranded oligonucleotides that contain either the EIIA-EF or the EIIF recognition sequences and the LS mutant −74/−85, which has an intact EIIF binding sequence and a mutated EIIA-EF binding site (Fig. 2 *A* and *B*) for their ability to compete for their respective factors (see Fig. 4 for nucleotide sequences of these oligonucleotides and legend for details). As shown in Fig. 4, mutant LS −74/−85 competes efficiently for the EIIF binding (Fig. 4*B*, lanes 8 and 9), whereas even at 50-fold molar excess, it fails to compete for the EIIA-EF (Fig. 4*A*, lanes 3 and 4). Similarly, an oligonucleotide that contains the EIIA-EF-binding site competes efficiently for the EIIA-EF (Fig. 4*C*, lanes 11–13) but fails to compete for the EIIF (Fig. 4*D*, lanes 21–23). Two oligonucleotides that contain DNA sequences from the EIA region and that are homologous to the EIIF binding sites (Fig. 4, EIA-I and EIA-II) compete efficiently for the EIIF (Fig. 4*D*, lanes 24–29) but are unable to compete for EIIA-EF even at high concentrations (Fig. 4*C*, lanes 14–19). These results suggest that the two different activities detected in the gel shift assays are most likely due to two different proteins.

DISCUSSION

Although purification and biochemical characterization of the molecules responsible for the two activities we have detected are required to prove that the EIIA-EF and EIIF are two separate proteins, the following preliminary observations suggest that this is so: (*i*) the two factors bind to sequences that are different and for the most part nonoverlapping; (*ii*) DNA fragments or oligonucleotides that contain factor-binding sequences for EIIA-EF or EIIF can compete efficiently only for the cognate factors; and (*iii*) the relative abundance of only one of the factors is strikingly different in infected and uninfected cells.

The observation that the type of competitor DNAs used in gel shift assays can make an enormous difference when detecting promoter-specific host factor for the EIIA-E promoter is intriguing. At this point, one can only speculate as to why this is so. Perhaps salmon sperm or calf thymus DNAs may contain sequences similar to the EIIA-EF binding sites and when present in very large amounts be able to compete out the EIIA-EF in gel shift assays. Similarly, recognition sequences of the EIIF involve alternating G·C pairs. The synthetic DNA copolymers may mimic these sequences and thus bind to the EIIF, allowing the detection of EIIA-EF.

We believe that the factor detected in our gel shift assays with salmon sperm DNA as competitor is identical to the EIIF reported by Kovesdi *et al.* (8). Our findings that this factor binds to sequences between −69 and −33 and that it is increased severalfold after viral infection is consistent with their observations. F9 teratocarcinoma cells were found to contain this factor, which correlated with their ability to transcribe the EIIA-E promoter in the absence of EIA (15, 16). Upon differentiation, activity of this factor was lost in F9 cells with concomitant loss of their ability to transcribe the EIIA-E promoter (15, 16). This observation, combined with the findings that this factor binds in a sequence-specific manner to the EIIA early promoter (results presented in this paper and ref. 8), suggests that EIIF is required for transcription of the EIIA-E promoter. The evidence that EIIA-EF is also required for EIIA-E transcription rests on the binding data presented in this and a previous paper (7) and its binding site is vital to expression of the promoter in transfection assays (9, 17). Some LS mutants that overlap the EIIF binding sites and were expected to show decreased transcription failed to show a clear phenotype in transfection assays (9). However, Zajchowski *et al.* (17) have shown that a LS mutant of the EIIA-E promoter around −40 transcribed with a much reduced efficiency. To resolve these inconsistencies, we are presently reintroducing the LS mutants into the virus itself to study their phenotype in the more natural context of viral chromosome.

Based on the results presented here and those published earlier (7–9, 17), the EIIA-E promoter appears to contain multiple promoter domains (Fig. 5): (*i*) a region between −29

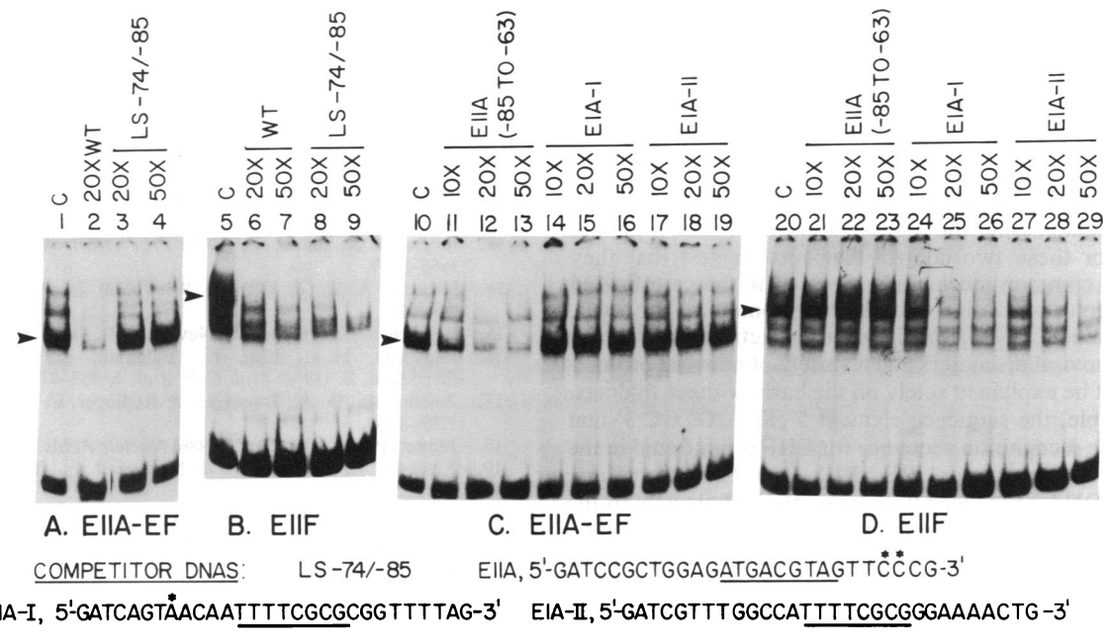

FIG. 4. Effect of different competitor DNAs on EIIA-EF and EIIF binding with the EIIA-E promoter. The oligonucleotides for both strands were hybridized to form duplex structures and used in gel shift assays. The details of the DNA fragments or oligonucleotides are as follows: LS −74/−85, a DNA fragment from the *Bss*HII/*Hin*dIII site. EIIA, from −85 to −63 (nucleotides shown by asterisks are substituted nucleotides to prevent possible interaction with EIIF). EIA-I, from −296 to −272 (the nucleotide shown by an asterisk is a substituted nucleotide to avoid the possible binding of the EIIA-EF). EIA-II, from −234 to −217. The numbers above the lanes (10×, 20×, 50×) indicate the molar excess of the unlabeled competitor DNAs. Lanes C, control samples, which did not receive any competitor DNA.

FIG. 5. Factor-binding sites for the EIIA-E promoter. Arrowheads, guanine residues that interact with the EIIF. Asterisks, guanine residues that interact with the EIIA-EF. The EIIF-binding region at −50 is interrupted to show that the sequences around this region are not involved in EIIF binding. A DNA sequence functionally analogous to the TATA box is shown as TATA. Bold arrow, transcription start site.

and −19, which is functionally analogous to the "TATA" box; (*ii*) a region between −69 and −33, which shows an imperfect palindromic structure and contains EIIF binding sites; and (*iii*) a distal control element between −82 and −66, which contains the EIIA-EF binding domain. Mutations around −50 do not affect the EIIF binding. It is interesting to note that the basal level of transcription of the LS mutant (−49/−59) that binds to the EIIF in this region increases severalfold in transfection assays (9). Whether this region contains a negative regulatory element is uncertain at present but such negative regulatory elements have been implicated in the regulation transcription of this promoter (18).

An *in vivo* exo III mapping study showed that a factor is bound to sequences up to approximately position −85 of the EIIA-E promoter in cells infected with wt Ad5 but not with *dl312* (19). It is tempting to speculate that this factor is EIIA-EF. If so, *in vitro* EIIA-EF and EIIF may bind to the promoter independently as we and others have shown (this report and refs. 7 and 8). One must hypothesize that *in vivo* EIIA-EF is unable to bind to the promoter by itself. Its interaction with the promoter may require EIIF, which may be limiting in uninfected cells or in cells infected with *dl312*.

Thus, it seems likely that activation of the EIIA-E promoter involves at least two host factors, EIIA-EF and EIIF. As suggested earlier (8), EIA may act to modify the activity of EIIF or to increase its synthesis, and the EIIF may then bind to the promoter by itself or in concert with EIIA-EF to form a stable transcription complex. If active binding of EIIA-EF to the promoter requires EIIF, it would imply that there may be protein–protein interactions during transcriptional activation. Close proximity of the binding sites for these two factors on the promoter suggests that this may be the case.

DNA sequences homologous to the EIIF binding site are repeated twice in the promoter sequences upstream of the EIA cap site (see above) as are sequences partly or completely homologous to the EIIA-EF. Such striking similarities between these two promoters in the organization of cis-acting control elements and the identification of two host factors specific for these two control elements suggest that they mediate a common mechanism involved in the coordinated transcriptional activation of these two promoters. However, the mechanism of EIA-mediated trans-activation of other early adenoviral promoters and a variety of nonviral promoters cannot be explained solely on the basis of these findings. For example, the sequence element 5′ TTTCGCGC 3′ that may be the recognition sequence for EIIF is not found in the EIA-activated promoter EIB, EIII, and EIV promoters, although DNA sequences fully or partly homologous to the EIIA-EF-binding site are present in EIII and EIV promoters. In addition, Ad infection of human cells results in an EIA-dependent increase of activity or concentration of TFIIIC resulting in increased transcription of RNA polymerase III genes (20, 21). Ad and pseudorabies viruses are presumed to utilize a common mechanism(s) for trans-activation of early Ad promoters (22). Cell-free extracts from pseudorabies virus-infected human cells stimulate transcription of Ad major late, EIII, EIV, and human histone 4 promoters *in vitro* as a result of an increase in the activity of transcription factors (23). Cell-free extracts prepared from Ad2-infected HeLa cells were also shown to stimulate transcription of Ad2 major late, EIII, and protein IX promoters *in vitro* (24). These results suggest that one or more general transcription factors may be involved in the *in vitro* activation of this variety of promoters. In transfection assays, activation of transcription of the β-globin gene by the viral EIA gene product requires only the TATA box element of the promoter (25). Finally, an *in vivo* mutational analysis of the Ad EIB promoter suggests that transcription factors specific for the TATA box region may be involved in the EIA-dependent stimulation of transcription (26). All of the above results raise the possibility that multiple pathways are involved in the transcriptional activation of genes by EIA.

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