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Role of an adenovirus *E2* promoter binding factor in E1A-mediated coordinate gene control

(transcription control/trans-activation/promoter methylation)

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ABSTRACT **A product of the adenovirus gene *E1A* is responsible for the stimulation of transcription from six viral promoters as well as at least two cellular promoters. We have detected a HeLa cell factor, termed *E2* promoter binding factor (E2F), that appears to mediate the transcriptional stimulation of the viral *E2* promoter. Competition experiments revealed that E2F did not recognize and bind to the *E1B, E3, E4*, or major late promoter sequences. Furthermore, three additional promoters stimulated by E1A, heat shock protein 70, β-globin, and early simian virus 40, do not bind E2F. In contrast, the factor does recognize sequences in the *E1A* enhancer, and within the *E1A* enhancer are duplicated binding sites for E2F. Finally, a single E2F binding site from the *E1A* enhancer can confer increased transcription to a mouse β-globin promoter, dependent on the action of the *E1A* gene product. This stimulation requires binding of E2F since methylation of the binding site, which blocks binding *in vitro*, reduces transcription stimulation *in vivo*. We, therefore, conclude that E2F is likely to be responsible for the E1A-mediated stimulation of the *E1A* gene as well as the *E2* gene but is not involved in the activation of the other E1A-inducible promoters.**

The basis for coordinate control of transcription of a set of genes, in response to any given stimulus, is a crucial aspect of gene control in eukaryotic cells. In some cases, such control may be due to a single factor that recognizes multiple genes while in other cases multiple promoter-specific factors, controlled by a common regulatory stimulus, may be responsible. One system of coordinate gene control that is particularly amenable to study is the set of adenovirus early genes that are regulated by the product of the *E1A* gene (1–3). We have suggested (4) a mechanism for activation by E1A whereby there is enhanced binding of a cellular factor to critical promoter sequences. Indeed, we have identified a cellular factor, termed *E2* promoter binding factor (E2F), that appears to be responsible for the transcriptional stimulation of one of the viral genes (5). In this report, we have investigated the involvement of E2F in the coordinate control of the set of early genes.

MATERIALS AND METHODS

Cells and Virus. HeLa cells grown either in suspension in Joklik's modified minimal essential medium containing 5% (vol/vol) calf serum or in monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum were used. The growth and preparation of wild-type adenovirus type 5 (Ad5) and of the *E1A*-deletion mutant dl312 have been described (3, 6).

Nuclear Extracts. The preparation of nuclear extracts by the procedure of Dignam *et al.* (7) from adenovirus-infected HeLa cells was described (5).

Binding Assays. Binding assays were performed using an end-labeled E2 probe, depicted in Fig. 1, by procedures described (5).

***Hha* I Methylation.** Methylation with the *Hha* I methylase was carried out according to the instructions of the supplier (New England Biolabs).

Transfection Assays. HeLa cells were infected with 1000 particles per cell of Ad5 or dl312. After 8 hr, cells were transfected with 10 μg of β-globin-specific plasmids and 10 μg of pUC19 using calcium phosphate coprecipitation, and 4 hr later cells were glycerol-shocked (8, 9). The cells were maintained throughout in the presence of cytosine arabinonucleoside (25 μg/ml). Total RNA was prepared 48 hr after transfection by cesium chloride centrifugation (10) and analyzed for β-globin RNA using an SP6 RNA probe specific for the first two exons of β-globin (gift of R. Costa). Details of the procedures for hybridization of SP6 probes and analysis of protected fragments have been described (11).

Plasmids. The pE2 plasmid has been described (12). The pE1b plasmid is an *Hpa* I/*Hin*dIII fragment of adenovirus type 2 (4.4 to 8.0 map units) in pBR322. The pE3 plasmid (gift of D.-W. Huang) is an *Eco*RI/*Sst* I fragment of adenovirus type 2 (76.0–76.8 map units) in pUC12. The pE4 plasmid (gift of P. Fischer, Columbia Univ.) contains Ad5 sequences from 93.5 to 100 map units in pBR322. pML is a *Pst* I/*Hin*dIII fragment of adenovirus type 2 (14.0–17.0 map units) in pGEM1. pβ-glo contains mouse globin sequences from position −1221 to position +482 (relative to the cap site at +1) in pUC13. pSV40 is the pSV2-neo vector (13). The pE1A plasmid contains Ad5 sequences from the left terminus of the genome (0 map units) to the *Xba* I site at 3.8 map units, and the pE1A(−188) plasmid has been described (14).

The E1A-Enh plasmid contains Ad5 sequences from the *Hpa* II site at position 188 to the *Sac* II site at position 353 inserted in pUC13. The Enh-A is an *Hpa* II/*Bal* I (position 188 to position 270) insert; Enh-B is a *Bal* I/*Sac* II (position 270 to position 353) insert; Enh-C is a *Hin*PI/*Hin*PI (position 216 to position 335) insert; Enh-D is a *Hin*PI/*Fnu*DII (position 216 to position 279) insert; and Enh-E is a *Fnu*DII/*Hin*PI (position 279 to position 335) insert.

RESULTS

Competition Assays for E2F Binding. To determine if E2F recognizes any of the other early adenovirus promoters that are stimulated by E1A, we have utilized DNA clones of each of the promoters as competitors for binding of E2F to the *E2* promoter. The probe used for binding is shown in Fig. 1*A* along with the sequence of the binding site. As shown in Fig. 1*C*, an excess of the *E2* promoter effectively eliminates binding of E2F to the labeled probe as assayed by the gel-shift technique (15, 16). In contrast, an excess of the *E1B, E3, E4*,

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Abbreviations: Ad5, adenovirus type 5; E2F, *E2* promoter binding factor.

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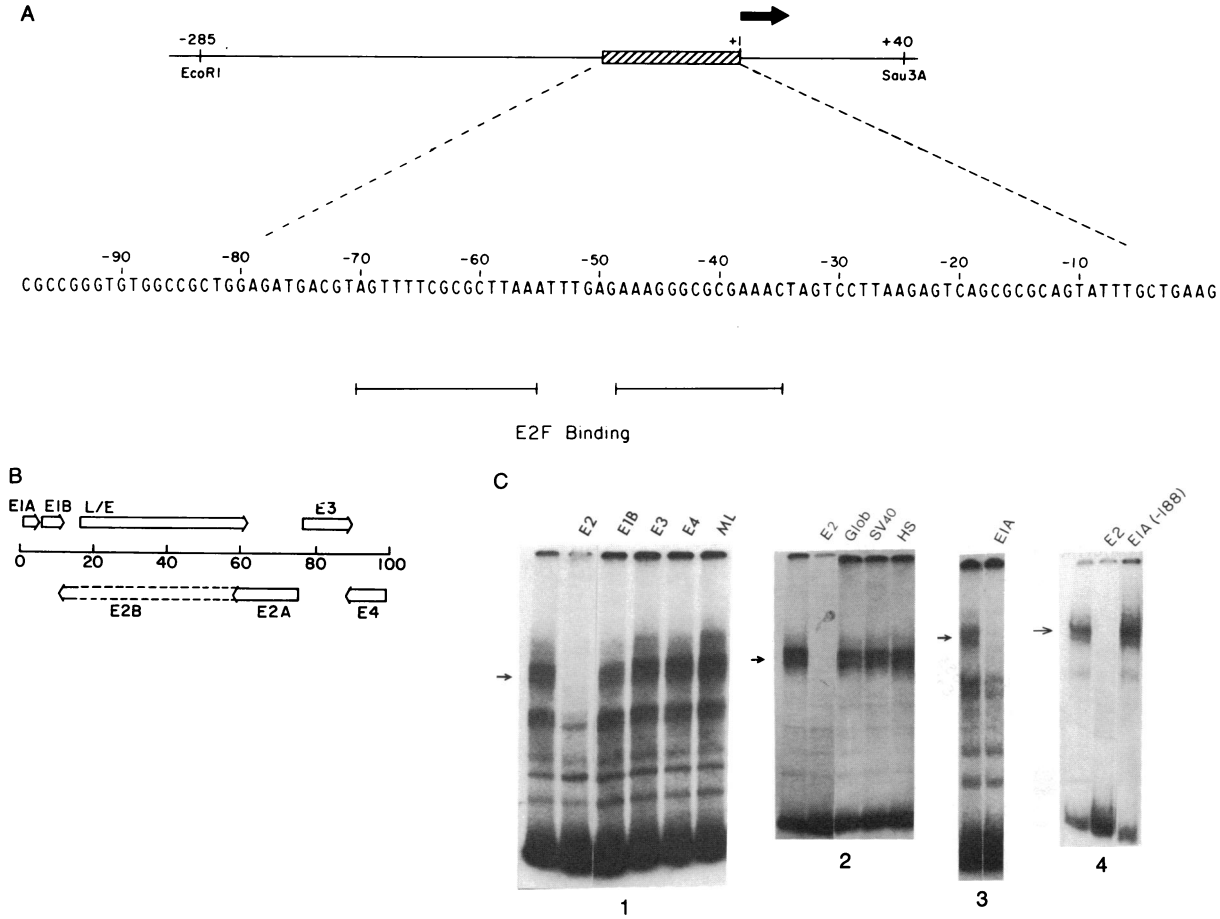

FIG. 1. Competition assays for binding of E2F to other E1A-inducible promoters. (*A*) DNA sequence in the *E2* promoter containing the binding site for E2F (5). (*B*) Schematic diagram of the early adenovirus transcription units that are subject to E1A control. (*C*) Binding reactions were performed using nuclear extract from adenovirus-infected HeLa cells (5) and the E2 probe as depicted in *A*. The first lane in each panel (labeled 1, 2, 3, or 4) is binding in the absence of specific competitor DNA. Competing DNAs were present at a 50-fold molar excess to that of the probe. In each case, the plasmids representing the indicated promoter elements contained sequences known to be critical for E1A-induced expression of the gene. The E1A competitors contain Ad5 sequences from the left end of the genome (0 map units) to 3.8 map units [the E1A poly(A) site is at 4.5 map units] (20). The E1A(−188) plasmid contains the entire E1A transcription unit but is deleted of sequences upstream from nucleotide −188.

and major late promoters had little or no effect on the binding (Fig. 1*C1*). In addition, three other promoters known to be stimulated by E1A, heat shock protein 70 (17, 18), β-globin (19), and early simian virus 40 (19), were tested. None of these promoters would compete for binding of E2F (Fig. 1*C2*). Thus it is clear that E2F does not bind to these promoters, and E1A stimulation of these other promoters cannot proceed through this factor. We conclude that additional factors must be used by these promoters, and if *E2* activation is an example, then the level or activity of some of these factors must be regulated by E1A.

E2F Binds to the *E1A* Enhancer. In contrast to the above results, we did find that a plasmid containing most of the *E1A* transcription unit (0–3.8 map units) effectively eliminated binding of E2F to the *E2* promoter (Fig. 1*C3*). The E2F binding site must be within 310 nucleotides of the left end of the viral genome since the E1A(−188) plasmid does not contain *E1A*-specific sequence upstream of nucleotide 310 (nucleotide −188 relative to the *E1A* initiation site) and does not compete for binding (Fig. 1*C4*). Additional competition assays employing *E1A* upstream sequences (positions 0–454 and 0–353) (Fig. 2*A*) indicated that the factor did indeed bind in the upstream region (Fig. 2*C*). Within this region are the sequences defined by two groups as the *E1A* enhancer (21, 22). The fragment termed Enh contains the enhancer element as defined by Hearing and Shenk (21), and the Enh-A and Enh-B fragments are the two halves of the element (Fig. 2*A*). Another subclone, Enh-C represents an internal region of the enhancer, and the clones Enh-D and Enh-E are the two halves of the Enh-C DNA. When each of these DNAs were used in competition assays for binding of E2F to the *E2* promoter, the results shown in Fig. 2*C* were obtained. Clearly, the *E1A* enhancer element competes effectively indicating that a binding site for E2F lies within this region. In addition, each half (Enh-A and Enh-B) of the enhancer element was an effective competitor suggesting that the E2F binding site is duplicated in the Enh fragment. Also effective as a competitor was the fragment Enh-C. However, the two halves of this fragment, Enh-D and Enh-E, did not compete.

An examination of the sequence of the *E1A* enhancer element provides a rational explanation for the binding assays (Fig. 2*B*). It can be seen that there are two sequence elements in the *E1A* enhancer [TTTCGCG(C/G)] that are close approximations of the binding site in the *E2* promoter. As long as one of the sequences was left intact, competition was efficient. In the case of the Enh-C clone, only one copy of the sequence remains, and this was still an effective competing DNA. The cleavage to produce the Enh-D and E DNAs cut through this sequence thereby destroying the potential binding site. This would strongly suggest that the *E2*-like TTTCGCG(C/G) sequence was the binding site.

Definition of the E2F Binding Site in the *E1A* Enhancer. That these *E2* homologous sequences are indeed the binding sites is substantiated by the results shown in Fig. 3. The A element

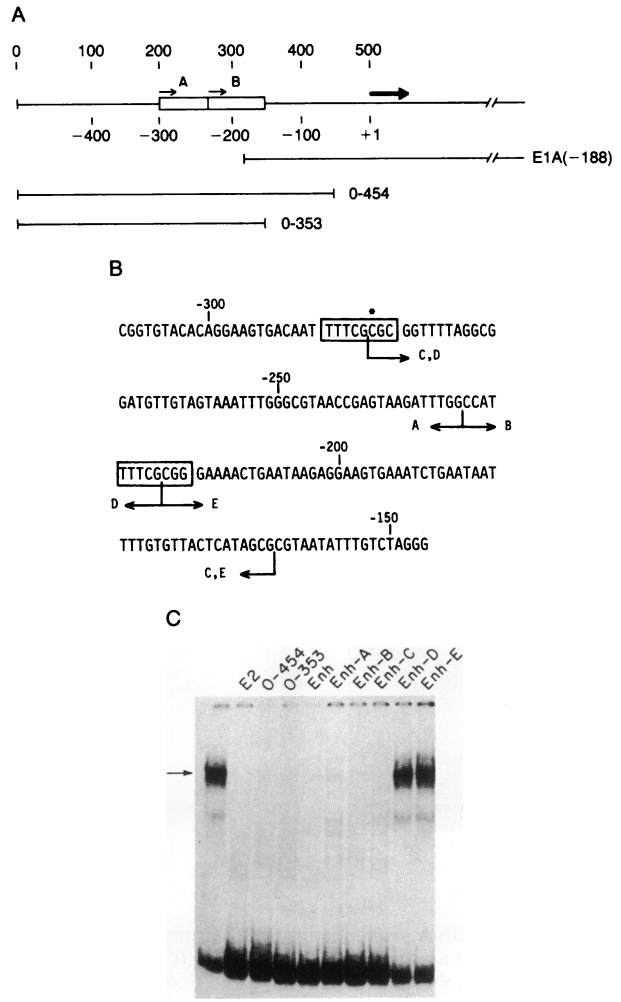

FIG. 2. *E1A* sequences that bind the E2F factor. (*A*) Schematic diagram of the upstream region of the *E1A* transcription unit. The enhancer as defined in ref. 21 is denoted by the open box. Various subclones of the *E1A*-upstream sequences are shown below. (*B*) Sequences of the *E1A* enhancer. Depicted is the adenovirus sequence upstream of the *E1A* transcription initiation site from nucleotides −310 to −46 [nucleotides 189–353 of the Ad5 genome (20)]. The two sequences homologous to the E2F binding site in the *E2* promoter are indicated by the boxes. The site for *Hha* I methylation in the first sequence is depicted by an asterisk (*). Finally, the boundaries of the various Enh subclones are indicated by arrows. (*C*) Competition assays for binding of E2F to the *E1A*-upstream sequences as defined with the *E1A* enhancer subclones.

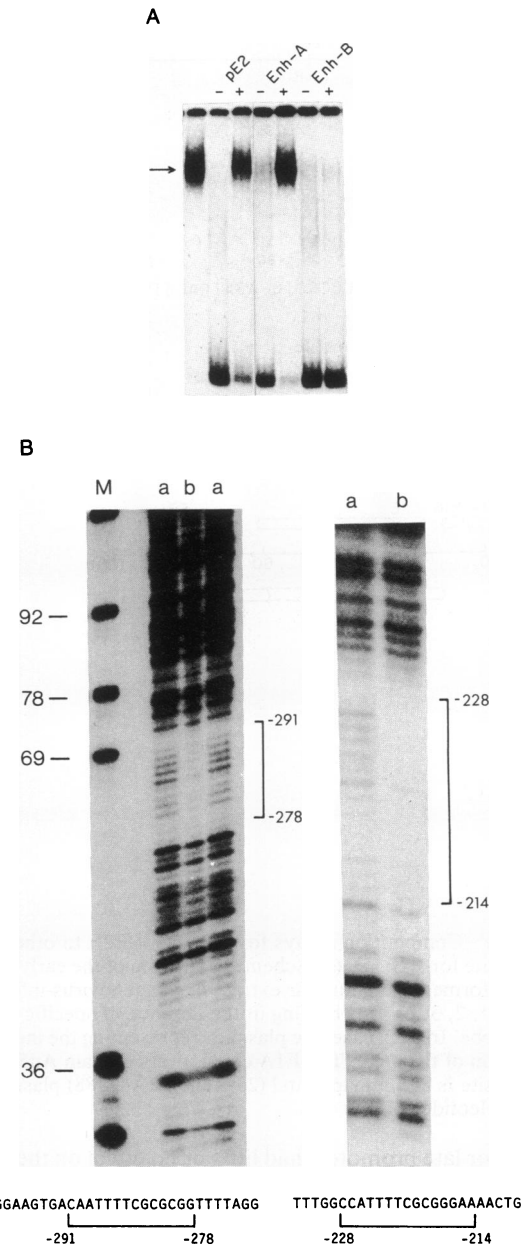

FIG. 3. Delineation of E2F binding sites within the *E1A* enhancer. (*A*) Methylation inhibits the binding of E2F to the *E1A* enhancer. The pE2 plasmid and the Enh-A and Enh-B subclones were treated with the *Hha* I methylase. Methylated (+) or unmethylated (−) plasmid was used as competitor for E2F binding to the *E2* promoter probe. (*B*) (*Upper*) DNase footprint analysis of E2F binding. The Enh-A and Enh-B fragments were end-labeled and used for binding. After DNase digestion, the complexes were separated from free DNA by gel electrophoresis. Bound DNA and free DNA were eluted from the gel, purified, and analyzed in an acrylamide/urea sequencing gel. M, marker; a, free DNA; b, bound DNA. (*Lower*) Sequence at each site of protection.

contains the sequence GCGC, which is a substrate for the *Hha* I methylase (23), while the B element contains a GCGG sequence, which is not a substrate for the *Hha* methylase. Methylation of the A DNA abolished its ability to compete for E2F binding, but treatment of the B DNA with the methylase had no effect on its ability to bind. Finally, we have delineated the binding sites by a "footprinting" experiment (24). The Enh-A and the Enh-B fragments were end-labeled and used for a binding reaction followed by DNase treatment. The bound complexes were separated from free DNA by gel electrophoresis, and the DNA was eluted and analyzed in a sequencing gel. As shown in Fig. 3*B*, there is a region of protection in each fragment that coincides with the predicted binding sequence. We thus conclude that E2F indeed binds to a duplicated site in the *E1A* enhancer similar in sequence to the binding sites in the *E2* promoter.

Binding of E2F to the *E1A* Enhancer Site Increases Transcription. Although it was clear from the results above that E2F binds to the *E1A* enhancer, it was not clear that this binding occurs *in vivo* or is of any consequence to *E1A* promoter activity. We have inserted the Enh-A fragment into a plasmid containing the β-globin promoter as shown in Fig. 4*A* to assay the ability of E2F to stimulate transcription *in vivo*. We have investigated whether the presence of this *E1A* fragment stimulated transcription from the β-globin promoter, and if so did this require binding of E2F. To assess the requirement for E2F binding, we have taken advantage of the *Hha* I methylation site in the E2F binding site of the Enh-A

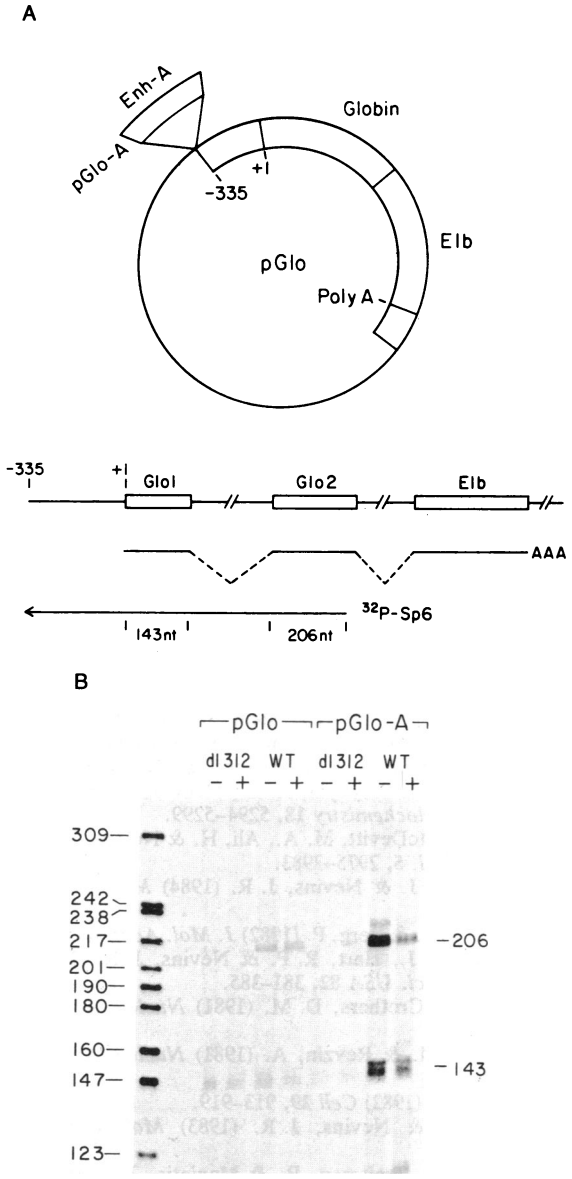

FIG. 4. Assay for functional role of E2F binding to the *E1A* enhancer. (*A*) Schematic diagram of the pGlo and pGlo-A plasmids used to assay for Enh-A function. A chimeric plasmid containing the first two exons of the mouse β-globin gene fused to the Ad5 *E1B* region from map units 9.6 to 15.5 (25) was modified by discarding the globin sequences upstream of the *Hin*dIII site at nucleotide −335. Into this clone (pGlo) was ligated the Ad5 Enh-A fragment, in the same orientation relative to β-globin as relative to the *E1A* promoter, producing the plasmid pGlo-A. The RNA produced from either plasmid contains the first two exons of globin spliced to the 3′ exon of *E1B*. The SP6 transcript used for RNA assays initiates within the second globin exon. (*B*) SP6 assays of β-globin-initiated transcripts in transfection assays. RNA from HeLa cells transfected with methylated (+) or unmethylated (−) plasmid and infected with dl312 or wild-type Ad5 was measured for SP6 assay.

fragment. By methylating the plasmid with the *Hha* I methylase, we can prevent the binding of E2F to the Enh-A element and thus relate DNA binding to transcriptional activity of the plasmid; in essence, a site-directed mutagenesis.

Methylated or unmethylated DNA was transfected into HeLa cells that were infected either with dl312 (E1A$^-$) or Ad5 (E1A$^+$). RNA was then prepared and assayed for β-globin-specific transcripts using an SP6 probe, as shown in Fig. 4*A*, and the results are shown in Fig. 4*B*. Transfection of the pGlo plasmid into HeLa cells infected with dl312 produced a barely detectable level of β-globin-directed transcripts. Transfection of the same plasmid into cells infected with Ad5 yielded a slightly increased level of β-globin-initiated transcripts (≈3-fold), consistent with the observation that E1A can stimulate the β-globin promoter (19). This was clearly seen with the 206-nucleotide protected fragment. Furthermore, methylation of the pGlo plasmid had no effect on its activity. Thus, although there are a number of methylation sites in the plasmid, methylation at these sites apparently does not influence β-globin promoter activity. Transfection of the pGlo-A plasmid into HeLa cells infected with dl312 produced an equal level of transcripts compared to that without the *E1A* element. Thus, there is no evidence of an effect due to this *E1A* element in the absence of E1A protein. Strikingly, however, transfection of this plasmid into cells infected with Ad5 produced a high level of β-globin transcripts, ≈8-fold higher than that without the enhancer. Therefore, this E1A-A element can confer an increased transcription to the β-globin promoter but only in the wild-type infected cells and thus we presume it is a function of *E1A*. This result is fully consistent with the fact that E2F, which binds to this *E1A* element, increases markedly in cells infected with Ad5 but not in cells infected with dl312 (5).

Comparison of the activity of the methylated pGlo-A plasmid to the unmethylated pGlo-A suggested that E2F does indeed mediate this increase in transcription. Methylation of the E1A-A element significantly reduced the ability of the plasmid to be stimulated in *trans* by E1A. Although methylation did not completely abolish the effect of the E1A-A element, nevertheless there was a reduction by a factor of 4 to 5 in β-globin transcripts from the plasmid with the methylated element as compared to the unmethylated element resulting in a level of expression only slightly higher than the plasmid lacking the E1A-A element. Thus, the binding of E2F to the *E1A* element appears to be critical for the increased transcription in the presence of the E1A protein. This observation, along with the fact that E1A mediates an increase in the level of E2F as measured by binding activity (5), strongly argues for a role of this factor in E1A-mediated transcription stimulation.

DISCUSSION

Our work has established (5) that E2F binds to duplicated sequences in the *E2* promoter. These duplicated sequences are in fact two binding sites for E2F (A. Yee, R.R., I.K., and J.R.N., unpublished data) consistent with the findings presented here concerning the binding sites for E2F in the *E1A* enhancer. Although it has not been shown directly, we suggest that E2F is in part responsible for *E2* transcription and critically required for E1A stimulation of *E2* transcription. E2F binds to sequences in the *E2* promoter that have been shown to be essential for transcription and stimulation by E1A (14, 26). There is one discrepancy with this conclusion since a linker-scanning mutant localized to nucleotides −66/−55 (relative to the *E2* initiation site at +1) disrupts the distal E2F binding site but does not affect promoter activity (27). However, it must be kept in mind that there are two E2F sites in the *E2* promoter and elimination of one, without affecting other elements of the promoter, might not impair activity. Certainly it appears that a single E2F binding site from the *E1A* enhancer can mediate an effect (Fig. 4). In addition, the level of active E2F increases markedly after infection with adenovirus, and this increase depends on a functional *E1A* gene (5). Thus, we conclude that the increase in this factor is largely responsible for the stimulation of the *E2* promoter by E1A. Furthermore, a change in the level of E2F during F9 teratocarcinoma cell differentiation correlates with the ability of these cells to support *E2* transcription (28).

It does appear that E2F is not the only factor that is utilized by the *E2* promoter, based both on binding data and promoter mutagenesis. A site upstream of the distal E2F binding site has been defined by mutagenesis to be important for promoter activity (14, 27) and is recognized by a factor in HeLa nuclear extracts (ref. 29; A. Yee, R.R., I.K., and J.R.N., unpublished data). However, this factor does not fluctuate as a function of viral infection (ref. 29; A. Yee, R.R., I.K., and J.R.N., unpublished data) suggesting that the critical component with respect to regulation is E2F.

We have now demonstrated that E2F binds to the *E1A* enhancer as well as to the *E2* promoter, but does not bind to any of the other early adenovirus promoters. Two points of importance are evident from this result. First, if E2F does indeed mediate stimulation of the *E2* promoter by E1A, then it is clear that a single factor cannot be responsible for E1A stimulation of all of the viral promoters. That is, E2F cannot mediate stimulation of *E1B, E3, E4*, or the major late gene. Second, at least one factor is shared and utilized by more than one of the transcription regulatory regions. E2F does bind to the *E1A* enhancer, and, as indicated by the transfection results, the binding is likely to be important for activity. Analyses of the sequence requirements for *E1A* transcription (21, 30) are consistent with the conclusion that the E2F binding sites are critical for *E1A* transcription. Deletion of the entire enhancer sequence severely impaired *E1A* transcription whereas deletion of only half of the enhancer had little effect. Hearing and Shenk (21) pointed to a duplicated sequence in this element as the basis for this result. Although the sequence identified by Hearing and Shenk (21) is not the E2F binding site, the E2F binding site is also duplicated and its presence correlates with activity. This fact, and the results that we have presented here, provide evidence that the binding of E2F to the enhancer region is important for full *E1A* expression. In the absence of E1A protein, *E1A* transcription proceeds at a reduced level but well above that of *E2* (3). We would suggest that there might be two distinct phases of *E1A* transcription, as defined by the absence or presence of the E1A protein. The binding of other factors, besides E2F, to the *E1A* promoter might enable the initial activation to occur and for transcription to proceed at a significant level. Then, as E1A protein is produced, E2F increases in abundance, binds to the *E1A* enhancer, and further stimulates *E1A* transcription. In this case, binding of E2F to the *E1A* enhancer would not be an absolute requirement for *E1A* transcription but rather would just provide an additional boost.

Methylation of the E2F binding site afforded the equivalent of a site-specific mutation and has, therefore, allowed us to show that an alteration that adversely affects binding (methylation) also impairs function. This observation and the fact that the amount of active factor (as measured by binding) changes as a function of *E1A* strongly argues that E2F is responsible for stimulation of transcription. The methylation experiment has also allowed us to suggest, in specific terms, a role for methylation in gene control. It is well known that DNA methylation is correlated with decreased gene activity (31). Our experiment demonstrates a specific basis for this decrease, namely the inhibition of binding of a critical transcription factor.

Although we are far from an understanding of the overall basis for coordinate control of the early viral genes by *E1A*, we can speak to the role of E2F in transcription control. Since E2F is not used by the *E1B, E3*, and *E4* promoters, we presume that other factors must be involved in the regulation of these genes. How could a group of factors be coordinately regulated? Obviously, an answer to the question must await a detailed analysis of several of the factors. However, we might speculate that several distinct factors with different DNA sequence recognition domains might possess common regulatory domains. In this way, *E1A* could modify a group of factors that possessed common regulatory sites and that as a result were activated in their DNA binding capacity. These active factors could then turn on a group of promoters.

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