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Role of E2F Transcription Factor in E1A-Mediated *trans* Activation of Cellular Genes

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Adenovirus E1A-dependent *trans* activation of the adenovirus E2 gene involves the activation of the cellular transcription factor E2F. E2F binding sites have also been identified in the 5′-flanking region of a number of cellular genes, raising the possibility that such genes are targets for E1A *trans* activation. We now demonstrate that two genes that possess E2F recognition sites, N-*myc* and *DHFR*, are stimulated by E1A, dependent on the E2F sites. We also find that although there are multiple E2F sites in these promoters, a single intact E2F binding site is sufficient for E1A-mediated induction, although not to the full wild-type level. These results thus demonstrate that a variety of cellular genes that possess E2F binding sites are subject to E1A *trans* activation. Moreover, since the products of most of these genes are likely critical for cellular proliferation, there are obvious consequences of this *trans* activation for cellular phenotype.

Viral systems have proved to be invaluable tools in the study of complex cellular events. The DNA viruses that infect the nuclei of eukaryotic cells make use of the host cell transcriptional machinery, and thus the lessons learned from the study of these viruses have been informative for general considerations of eukaryotic transcription control mechanisms (10, 18). For instance, it is clear that most of the viral transcription units employ promoters and enhancers that utilize cellular transcription factors in a manner similar to that of their cellular counterparts. An added advantage of the viral systems is the fact that many viruses encode regulatory proteins that control the activity of some of these cellular transcription factors. These so-called *trans*-activating genes are responsible for the efficient stimulation of viral transcription during the lytic replication cycle. Perhaps the best studied of these viral regulatory genes is the E1A gene of adenovirus. The E1A gene products are not DNA binding proteins (5), and thus it is presumed that the action of E1A to stimulate transcription must be indirect. Indeed, several reports have shown that this E1A-dependent activation process involves the targeting of cellular transcription factors that bind to the promoter regions of the early viral genes (reviewed in reference 17). In some cases, the DNA binding activity of the factor is stimulated, whereas in other cases, no change in binding is seen, suggesting a change in function.

DNA binding assays identified a factor termed E2F that bound to functionally important sequences of the viral E2 promoter and which was increased in abundance upon viral infection (12). The time course of E2F induction during an adenovirus infection is coincident with the stimulation of E2 transcription, indicating a role for E2F in the regulation process (21). Recent experiments have now shown that the adenovirus-mediated stimulation of E2F DNA binding activity is a multistep process. First, there is an E1A-dependent increase in the level of DNA binding activity of the E2F factor, not involving new synthesis of protein and possibly as a result of an induced phosphorylation (2, 20). Second, the interaction of E2F with the E2 promoter is further augmented by the induction of cooperative binding to the two E2F binding sites in the E2 promoter as a result of the interaction of a 19-kDa E4 gene product with E2F (7, 9, 16, 20). This cooperative binding, which is dependent on the precise arrangement of E2F binding sites in the E2 promoter, leads to the formation of a very stable DNA-protein complex. Finally, recent experiments demonstrated that the E2F factor can be found complexed to cellular proteins in many cell types and that the E4 protein cannot interact with E2F in such complexes. However, E1A can dissociate these complexes, releasing free E2F to which the E4 protein can then bind (1).

Our previous experiments have identified E2F binding sites in the 5′-flanking region of a number of cellular genes including c-*myc*, N-*myc*, and the dihydrofolate reductase (DHFR) gene (3, 8, 15). Functional assays have demonstrated the importance of the E2F sites in the c-*myc* promoter for E1A-induced expression (8, 14, 25). We now show that E1A can *trans* activate these other cellular genes, dependent on the E2F sites in these promoters, indicating that the presence of an E2F site can render a gene sensitive to activation by E1A. Furthermore, we demonstrate a high degree of conservation of a dyad E2F binding site within these promoters and suggest that this site represents the normal, cellular binding site for E2F.

MATERIALS AND METHODS

Cells and virus. Vero and LMTK$^-$ cells were obtained from American Type Tissue Culture. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Plasmids and DNA fragments. Plasmid N-*myc*/RIBam/gem1 containing the transcriptional start site and 1,619 nucleotides of the mouse N-*myc* promoter was obtained from Jeff Friedman (Rockefeller University). The chloramphenicol acetyltransferase (CAT) gene from plasmid pCAT3M was cloned directly downstream of the N-*myc* promoter at the *Hin*dIII site. The full-length promoter was deleted to position −122 by digestion with *Nar*I, and double-stranded oligonucleotides containing wild-type E2F binding

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sites or mutant binding sites (TTTGGATCGAAACGCTTT GGACC) were ligated into the *Nar*I site to reconstitute nucleotides −123 to −142 (numbers are relative to the 5′ transcriptional start site [4]).

A *Xba*I-to-*Sma*I restriction fragment of 131 nucleotides was isolated and end labeled with [α-^{32}P]dATP with the Klenow fragment of DNA polymerase for use in the gel mobility shift analysis.

The DHFR-CAT wild-type and E2F double-mutant plasmids have been described previously (3). In this study, additional site-directed mutants were constructed which abolished either E2F site alone. The sequences of the mutagenic oligonucleotides are shown below (with the resulting sequence alteration underlined).

wild-type sequence:	5′-TGCAATTTCGCGCCAAACTTG-3′ 3′-ACGTTAAAGCGCGGTTTGAAC-5′
site 1 oligonucleotide:	5′-TGCAAAAACGCGCCAAACTTG-3′ 3′-ACGTTTTTGCGCGGTTTGAAC-5′
site 2 oligonucleotide:	5′-TGCAATTTCGCGCCTTTCTTG-3′ 3′-ACGTTAAAGCGCGGAAAGAAC-5′
site 1 + 2 oligonucleotide:	5′-TGCAATTTCGATCCAAACTTG-3′ 3′-ACGTTAAAGCTAGGTTTGAAC-5′

Probes for gel shift analysis were created by subcloning a *Fok*I-to-*Pst*I fragment from plasmid pDHFR-210CAT into BlueScript containing the CAT gene and isolating a *Xba*I fragment of 142 nucleotides. This fragment was end labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase.

Transfection of DNA and CAT assays. Vero or LMTK cells were split 1:4 in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum 8 h prior to transfection. Briefly, calcium phosphate precipitates (6) were formed containing 20 μg of DNA (1 to 5 μg of target DNA and 5 to 15 μg of the E1A-expressing plasmid pGE1A; plasmid pGEM2 was used as nonspecific carrier DNA). Cells were exposed to the DNA precipitates for 12 to 16 h and then washed with Dulbecco modified Eagle medium and placed in Dulbecco modified Eagle medium containing 10% fetal calf serum for 36 h before harvesting. CAT assays were performed exactly as described previously (6). The plasmid pBC12/RSV/SEAP (2 μg) was used as an internal control in each transfection (3a).

Mobility shift assays and off-rate analysis. DNA binding assays were performed as described by Hiebert et al. (8). The reaction mixtures contained 1 to 4 μg of protein and 0.1 ng of a DNA fragment ^{32}P end labeled with the Klenow fragment of DNA polymerase. The reaction mixture was incubated at room temperature for 30 min and analyzed on a 4% polyacrylamide gel containing 0.25× TBE at 4°C.

Off-rate analysis was performed as described by Raychaudhuri et al. (20). A standard DNA binding reaction mixture was prepared as above, and after a 30-min incubation at room temperature, a large excess (80 ng) of specific competitor was added, aliquots were removed at the various time points, and the DNA-protein complexes were analyzed immediately on a 4% polyacrylamide gel.

RESULTS

E2F binding sites in cellular promoters mediate E1A *trans* activation. A computer-assisted analysis of the GenBank data base has identified a number of cellular genes that contain possible binding sites for the E2F transcription factor, and direct binding assays have confirmed that each of these sites does indeed bind E2F (8, 15). Previous experiments have demonstrated that E2F is important for basal expression of both DHFR gene (3) and c-*myc* gene (19, 25) transcription and is involved in the E1A induction of the c-*myc* gene through the P2 promoter (8, 14, 25). We wished to extend these observations to determine the functional importance of E2F sites in other genes and thus the general relevance of E2F to activation of cellular transcription by E1A. Therefore, we used transient transfection assays to compare the wild-type promoters, containing all the signals necessary for efficient transcription, with promoters containing site-directed mutations which abolish E2F interactions.

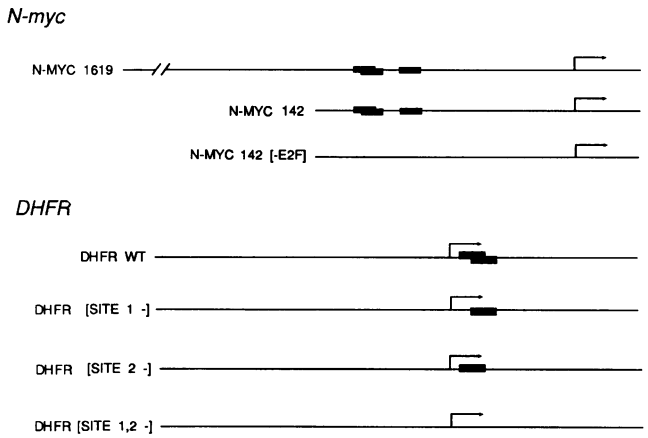


FIG. 1. Schematic representation of the N-*myc* and DHFR wild-type and mutant promoters. (A) Construction of the N-*myc* promoter mutations. The full-length N-*myc* promoter is shown schematically at the top. An N-*myc* promoter deleted to position −122 was constructed by isolating the small *Nar*I-to-*Bam*HI restriction fragment and reintroducing this fragment back into the parent vector after addition of a *Bgl*II linker at the *Nar*I site. The N-*myc* 142 and 142 (-E2F) promoters were reconstructed from N-*myc*-122 by synthesizing double-stranded oligonucleotides encoding nucleotides −123 to −142 containing either wild-type or mutated E2F binding sites. Boxes depict E2F binding sites. Arrows indicate the first transcriptional start site. (B) The DHFR promoter (top) containing all signals necessary for full expression (23) was used for oligonucleotide-directed site-specific mutagenesis of the first (DHFR [site 1]), the second (DHFR [site 2]), or both (DHFR [site 1,2]) E2F binding sites. WT, wild type.

We began our analysis by comparing an N-*myc* promoter extending to −1619 relative to the cap site with promoters deleted to −142 and containing either wild-type or mutant E2F sites (Fig. 1). The N-*myc* promoter contains a single E2F site at −120 to −127 and two overlapping E2F sites at −142 to −131. The constructs were tested by cotransfection with an E1A-expressing plasmid into LMTK$^-$ cells. Transfection of the E1A-expressing plasmid pGE1A with the −1619 N-*myc*/CAT construct resulted in an approximately fivefold stimulation of CAT activity over that seen in cells transfected with the target plasmid alone (Fig. 2A). This level of induction or a slightly higher level was also achieved with the N-*myc* promoter deletion mutant that retained only 142 bp of upstream sequence that includes the E2F binding sites. However, a −142 promoter containing site-directed mutations that abolished all three possible E2F binding sites was not responsive to E1A. These results therefore demonstrate that the N-*myc* promoter is indeed responsive to E1A

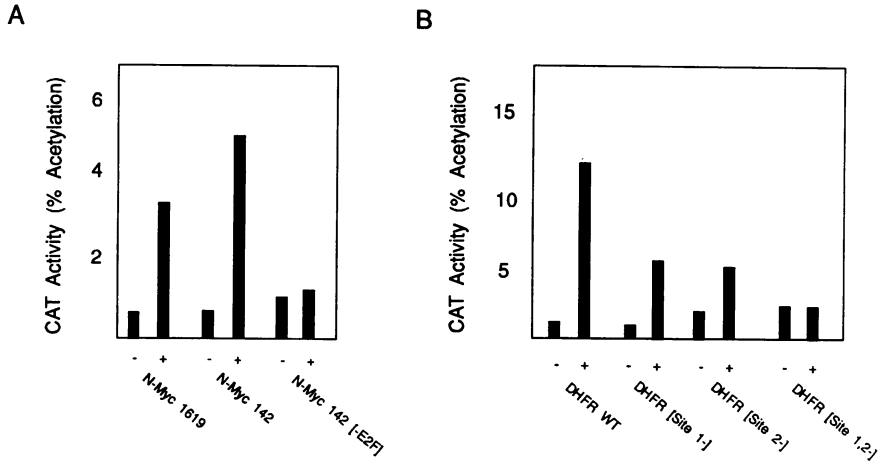


FIG. 2. Activation of the N-*myc* and DHFR promoters by E1A. (A) LMTK$^-$ cells were cotransfected with 2 μg of the N-*myc*-CAT construct and 0 (−) or 10 μg (+) of the E1A-expressing plasmid pGE1A. The N-*myc* construct used is indicated below each group of lanes. Extracts were prepared and assayed for CAT activity as described in Materials and Methods. (B) The wild-type (WT) and DHFR mutant promoters were linked to the CAT reporter gene and cotransfected with the E1A-expressing plasmid pGE1A. Extracts were prepared 36 h after transfection and assayed for CAT activity as described in Materials and Methods. Each transfection contained an internal control, as described in Materials and Methods, to correct for transfection efficiencies.

and that the E2F binding sites are required for this activation.

The DHFR promoter is also efficiently *trans* activated by adenovirus E1A as shown in Fig. 2B. Recent experiments have indicated that sequences within 147 bp of the DHFR major transcription start site contain all the signals necessary for efficient transcription (23). This minimal promoter has been demonstrated to contain two overlapping E2F binding sites, similar to those found at −142 to −131 in the N-*myc* promoter (Fig. 1), located at positions +3 to +13 relative to the major transcription start site (Fig. 2A). To determine the importance of these sites for E1A *trans* activation, we used cotransfection assays to test DHFR promoters that were truncated to position −147 and which contained either wild-type or mutant E2F binding sites (Fig. 2B). Cotransfection of the −147 DHFR promoter with the E1A-expressing plasmid resulted in a 5- to 10-fold stimulation of transcription from the DHFR promoter. Mutation of both of the E2F binding sites virtually abolished *trans* activation by E1A. Thus, as was the case for the N-*myc* promoter, the E2F binding sites are critical for *trans* activation of the DHFR promoter by E1A. From these results, as well as previous experiments (8, 25), we conclude that the c-*myc*, N-*myc*, and DHFR promoters are all responsive to E1A *trans* activation and that this response is dependent on E2F binding.

A single E2F recognition site is sufficient for E1A *trans* activation. An inspection of the E2F binding sites within the E1A-regulated genes revealed a common motif differing from that found in the adenovirus type 5 E2 promoter. The DHFR, N-*myc*, and c-*myc* promoters each contain a motif consisting of two E2F binding sites which, while oriented in the same manner as in the E2 promoter, overlap by 4 bp, forming a near-perfect dyad repeat (Fig. 3). In contrast, although the E2F sites in the E2 promoter also are a dyad repeat, the centers of the elements in the E2 promoter are separated by 25 bp or slightly more than two helical turns. The functional significance of a nucleotide sequence is often suggested by evolutionary conservation, and such an analysis of these E2F recognition sites is summarized in Fig. 3. Clearly, the dyad symmetry of the overlapping E2F recognition site motif is highly conserved through evolution. This is particularly striking in the DHFR locus, where this sequence is absolutely conserved among human, hamster, and mouse genes. In fact, this element shows greater conservation than the DHFR coding sequence. Interestingly, this motif is also present in the adenovirus type 5 E1A enhancer, and a comparison of the adenovirus E1A enhancer sequence in a variety of viral serotypes reveals strong conservation. As the E1A gene must be expressed immediately upon infection, it is perhaps not surprising that it contains transcription factor binding motifs similar to that found in cellular promoters. One might presume that the E1A gene must function as a cellular promoter without the benefit of alterations induced by the E1A gene products or other early viral genes such as E4.

Although the E2F binding sites in four independent promoters conserve this motif, this arrangement is not critical for the E1A *trans* activation event. We mutated either half of the element or the entire element in the context of the DHFR promoter (Fig. 1). The constructs were transfected into LMTK$^-$ cells with or without pE1A. As shown in Fig. 2B, mutation of one-half of the element, regardless of which half was mutated, did not eliminate E1A *trans* activation. Mutation of the entire element, however, abolished the response to E1A. The data shown in Fig. 2B are representative of multiple experiments performed in both LMTK$^-$ and Vero cell types and have been corrected for minor fluctuations in transfection efficiency. We therefore conclude that although the overlapping sites are highly conserved, only one-half of this motif, representing a single E2F recognition site, is required for a response to E1A, consistent with previous results (13). It does appear, however, that optimal activity requires the full site, since the extent of *trans* activation was reduced with each of the half-site mutants, particularly the site 2 mutant. This is also consistent with other experiments in which the constitutive basal activity of the single-site mutants has been lower than that of the wild-type DHFR promoter, depending on the growth state of the cells at the time of transfection (26).

Transcription activation by E1A does not require coopera-

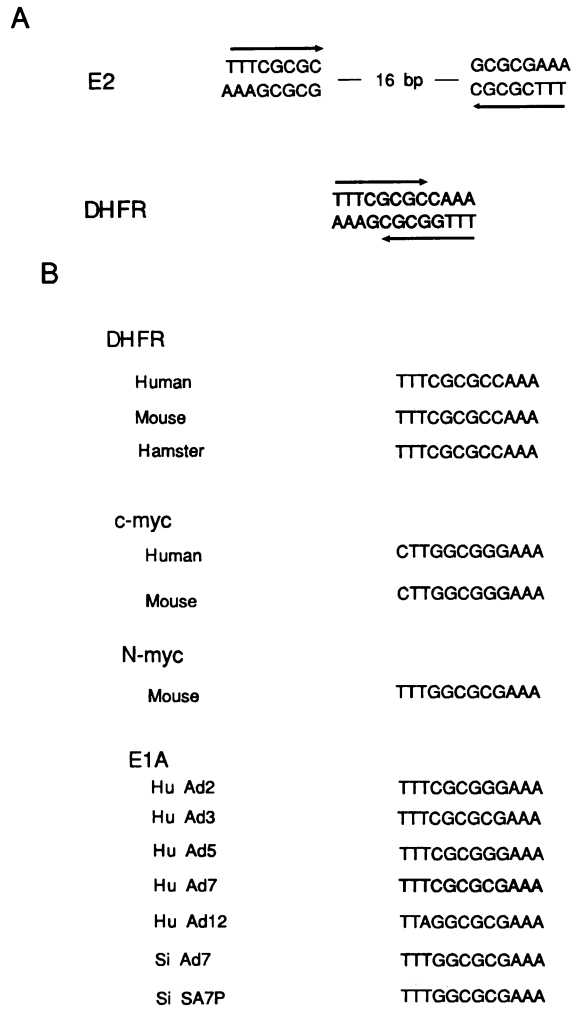


FIG. 3. Conserved E2F binding site motif. (A) E2F binding sites in the adenovirus type 5 E2 promoter and DHFR promoter. Arrows indicate the relative 5'-to-3' orientation of the binding site. (B) Conservation of the dyad E2F binding site present in cellular genes and the viral E1A gene. Hu, human; Si, simian; Ad2, adenovirus type 2; SA7P, simian adenovirus type 7P.

tive E2F binding. The results presented in Fig. 2 demonstrate that an E2F site in the DHFR promoter is required for E1A-mediated *trans* activation. Previous studies with the adenovirus E2 promoter have shown that the normal activation involves an E1A-dependent stimulation of E2F binding activity as well as an induced cooperativity of E2F binding dependent on the product of the early E4 gene (7, 20). Since this is dependent on E4, as well as the precise arrangement of sites in the E2 promoter, it would seem unlikely that cooperative binding of E2F contributes to activation of the DHFR gene by E1A. Indeed, comparison of E2F binding to a DHFR promoter containing both sites in the dyad motif versus a promoter containing mutations in the first site, the second site, or both E2F sites indicated that only a single complex could form on the promoter, at least in vitro, and that there was no indication of cooperativity (Fig. 4A). Furthermore, using extracts containing the E4 protein also caused no alteration in the pattern of DNA-protein complexes, suggesting that the E4 protein also could not induce a cooperative interaction.

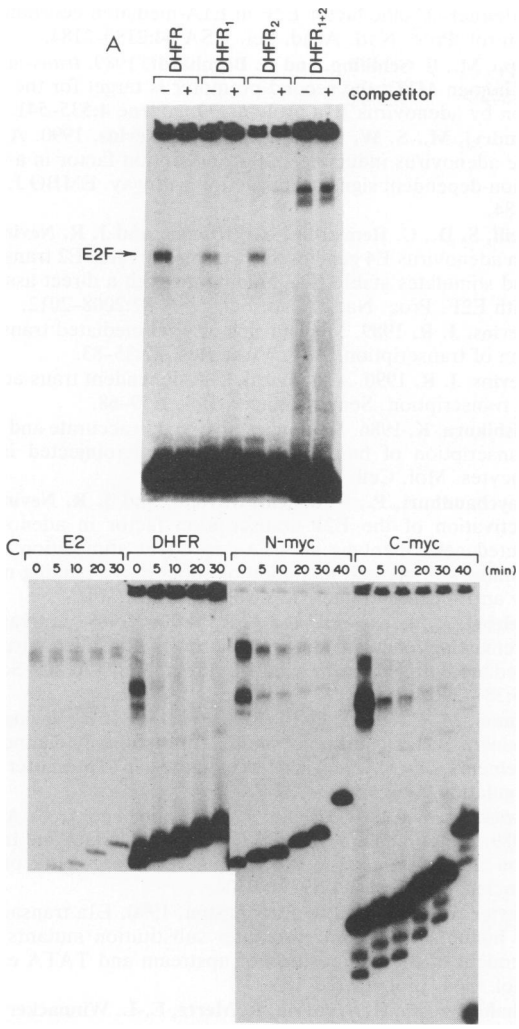
The lack of a cooperative interaction between two factors is also shown by a dissociation rate analysis depicted in Fig. 4C. The E2F DNA complex was formed with the promoters containing the dyad E2F site, and after equilibrium was reached, an excess of cold DNA was added and the stability of the complex was measured. In contrast to the cooperative binding of the E2F-E4 complex on the E2 promoter that results in the formation of a very stable DNA-protein complex (7, 20), the interaction of E2F with the DHFR promoter, the N-*myc* promoter, or the c-*myc* promoter was unstable as indicated by the rapid dissociation rate. The half-life of the complex was less than 5 min, independent of the source of E2F. We therefore conclude that although there are two potential E2F sites in the DHFR promoter, only a single E2F complex is formed, at least as measured in in vitro binding assays.

DISCUSSION

These experiments illustrate several important points. First, transcription from the N-*myc* and DHFR promoters can be stimulated by the adenovirus E1A gene product. Second, these promoters contain sequences that interact with the E2F transcription factor, and these sequences are required for the E1A-mediated *trans* activation of these promoters. Finally, the identification of E2F binding sites in multiple cellular promoters has led to the recognition of a cellular E2F binding site which is composed of two overlapping half-sites. Although the conservation suggests a functional importance, only a single E2F site (a half-site) is required for E1A *trans* activation. We suspect that the majority of the E1A effect through E2F is the result of a stimulation of E2F DNA binding activity, dependent on E1A, that may involve phosphorylation of the factor (2, 20). This results in an increased concentration of active factor that can bind to DNA. Recent experiments also demonstrate that E2F can be found in complexes with cellular proteins and that E1A can dissociate E2F from these complexes (1). If these complexes are inhibitory for transcription, then this action of E1A may also contribute to the *trans* activation of an E2F-dependent promoter.

Whether E1A can *trans* activate every gene that may possess an E2F binding site is not clear, but the fact that each gene thus far assayed does respond in this way indicates that this may be a general phenomenon. Although our assays have utilized transient transfections to measure the effects of E1A and the role of the E2F elements in this control, our previous experiments have shown that the endogenous c-*myc* gene is stimulated by adenovirus infection (8). Moreover, other experiments have shown that amplified, endogenous DHFR genes were stimulated by adenovirus infection (30). Subsequent experiments by these investigators suggested that the activation was posttranscriptional since transcription rates appeared to be unchanged (29). However, since the transcription rates measured in these experiments were quite low, such a conclusion must be considered tenuous. Our findings concerning the role of the E2F transcription factor in the E1A-mediated activation of the DHFR gene would suggest that at least one component of this activation involves control of transcription initiation.

These results also underscore the fact that E1A-mediated activation of transcription can be specific and can require a distinct promoter element, in this case an E2F binding site. Other reports have suggested that E1A *trans* activation was nonspecific, often showing no evidence for requirement of a



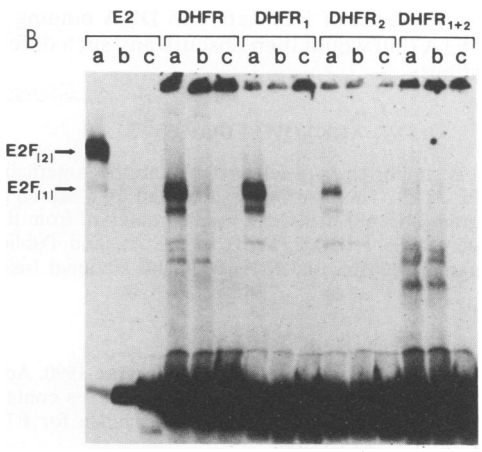


FIG. 4. Interaction of E2F with the DHFR promoter. (A) Restriction fragments of the DHFR promoter containing the dyad E2F binding sites or mutations in the first site ($DHFR_1$), the second site ($DHFR_2$), or both sites ($DHFR_{1+2}$) were 3′ end labeled and used to measure E2F DNA binding in DNA mobility shift assays with HeLa cell nuclear extract as the source of E2F. −, assay with no competitor added; +, assay containing 10 ng of an E2 promoter fragment containing E2F binding sites. (B) Mobility shift analysis with partially purified E2F from adenovirus type 5-infected HeLa cells. DHFR probes are the same as in panel A. E2, E2 promoter fragment containing both E2F binding sites. Lanes a, shift assay with no competitor; lanes b, shift assay containing E2 promoter fragments as a competitor; lanes c, shift assay containing no E2F. (C) Off-rate analysis of E2F binding to the DHFR promoter. The E2F-DNA complex was allowed to form for 30 min on ^{32}P-end-labeled DNA fragments from the E2 promoter or the DHFR promoter before a large excess of cold competitor DNA was added. Samples were removed at the times indicated and immediately loaded onto a native 4% polyacrylamide gel.

specific promoter element, leading to the interpretation that general transcription factors may be targeted (11, 24, 27). A more likely explanation is that some promoters may contain multiple E1A-responsive elements such that the elimination of any one is not sufficient to prevent *trans* activation. Furthermore, it is also clear that a TATA factor can be targeted by E1A (22, 28), and in many cases, this is likely responsible for the general activation that is observed. The results we present here clearly show that a unique site can be critical for E1A *trans* activation.

Our analyses also suggest that the E2F binding sites in these cellular promoters are intrinsically different from those present in the adenovirus E2 promoter. The E2 promoter contains two E2F sites that together form a dyad repeat separated by 25 bp. This arrangement is critical for the cooperative binding of the E2F-E4 protein complex since any alteration in the spacing or orientation of the recognition sites prevents stable complex formation (7, 20). As far as we are aware, this precise arrangement is found only in the E2 promoter. This unique arrangement of the E2F sites in the E2 promoter is critical for the stable binding of the E2F-E4 complex (7, 20). In contrast, the E2F recognition element found in the DHFR promoter consists of an overlapping motif that places the two E2F binding sites on opposite sides of the DNA helix. Interestingly, this motif is also conserved in the E1A enhancer of various serotypes of adenovirus. Of the six serotypes available in the DNA sequence library (GenBank), five contain a perfect match of the dyad binding site (adenovirus types 2, 3, 4, 5, and 7), while adenovirus type 12 contains a 10 of 12 bp match. The high degree of conservation of this overlapping motif in both cellular and viral regulatory elements strongly suggests that it is of functional significance.

Although it is possible that the arrangement of E2F sites in the DHFR promoter is for the same purpose as the arrangement of sites in the E2 promoter, to allow cooperative binding of a yet-to-be-identified E2F protein complex, we found no evidence for such specificity. We believe that there is likely another explanation for the conservation of this element. A common theme of DNA recognition is the binding of protein dimers to a recognition site with dyad symmetry, each subunit of the dimer making contact with one half-site of the recognition sequence. Although there is no evidence to support the notion that E2F binds as a dimer, there is also no evidence against it. We suggest that this is the normal mode of E2F binding and that the motif found in the DHFR promoter is the full site optimal for binding. In this context, the full site is likely not found in the E2 promoter because of the altered binding properties induced by the E4 protein. The E2F protein can bind a half-site, as present in the E2 promoter or in the DHFR mutants, but presumably with somewhat reduced affinity. Indeed, recent assays suggest a slightly increased half-life on the full site compared with that on a half-site (26). We imagine that the

increase in the level of E2F active in DNA binding brought about by E1A (20) would then obscure any such difference in binding affinity.

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