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Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex

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The precise binding site in the adenovirus E2 promoter for the E1A-inducible factor E2F was determined. DNase footprinting revealed two distinct regions of protection which spanned sequences from −33 to −49 and from −53 to −71. Chemical modifications of DNA further delineated nucleotides involved in DNA−protein contacts in each binding region. The E2F binding sites are clearly distinct from the binding site for another E2 promoter binding factor, located at −68 to −80, previously described by SivaRaman *et al.* [(1986) *Proc. Natl. Acad. Sci. USA*, 83, 5914−5918]. As determined by DNase footprinting using crude nuclear extracts, both factors were present in extracts of Ad5-infected cells and were found to bind simultaneously to their respective sites on the promoter. In contrast, E2F was not evident in extracts of uninfected cells, whereas there was no difference in the −68 to −80 footprint as a function of the extract. Thus, although multiple factors interact with the E2 promoter, only the E2F factor is unique to the infected extract. The implications of the formation of a multi-factor promoter complex as a possible mechanism of transcriptional regulation are discussed.

Key words: E1A/*trans*-activation/E2F factor

Introduction

A complete understanding of the mechanisms underlying the control of gene expression requires an identification and detailed characterization of essential nucleic acid sequence elements and the protein factors which interact with those sequences. In the case of transcriptional regulation, much progress has been made in the elucidation of functionally important promoter elements and enhancer elements (for review, see Serfling *et al.*, 1985). In addition, several transcription factors have been identified that interact with critical sequences. In many cases they have been demonstrated to influence transcriptional activity, and several such factors have been purified to homogeneity (Briggs *et al.*, 1986; Chodosh *et al.*, 1986; Jones *et al.*, 1987).

The early genes of adenovirus represent an excellent system with which to study coordinate transcription regulation which is mediated by a single regulatory gene. The viral E1A gene product can stimulate the transcriptional activity of the five early promoters and also of several cellular genes (for review, see Nevins, 1986). The effects of E1A appear to be broad since many apparently unrelated genes are stimulated in transfection assays. The control by E1A extends beyond polymerase II transcription, since the adenovirus VA gene, a polymerase III transcription unit, can also be stimulated (Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985). Earlier experiments had suggested the involvement of cellular factors in viral transcription and E1A control (Nevins, 1981; Feldman *et al.*, 1982; Imperiale *et al.*, 1983, 1984) and recent evidence has supported this notion. In particular, a cellular factor was identified that interacted with the E2 promoter (Kovesdi *et al.*, 1986a). The level of this cellular factor, termed E2F, as measured by binding to specific E2 sequence, increased markedly upon viral infection and was dependent upon E1A, which is consistent with an *in vivo* analysis that suggested a role for E1A in the formation of stable promoter complexes (Kovesdi *et al.*, 1986b). Furthermore, binding of the factor to its recognition site could stimulate transcription of a linked heterologous promoter, but only when transfected in the presence of E1A protein (Kovesdi *et al.*, 1987). Thus, the interaction of E2F with its binding site and the E1A-mediated alteration of the factor appeared to be functionally important.

In addition to providing an attractive system with which to study transcription regulation, the action of the E1A gene products has several important biological consequences. The expression of E1A proteins leads to cell immortalization (Houwelling *et al.*, 1980) and, when expressed in conjunction with the E1B gene or with other oncogenes such as the *ras* gene, can lead to a transformed phenotype (Graham *et al.*, 1974. van der Eb *et al.*, 1977; Land *et al.*, 1983; Ruley, 1983). The role of E1A-mediated transcription regulation in these processes is unclear. Mutants exist which are defective in transformation but which can still activate viral transcription, suggesting that another function of E1A, perhaps the repression of enhancer-dependent transcription, is involved (Lillie *et al.*, 1986; Moran *et al.*, 1986). Particularly interesting with respect to the involvement of transcription activation in transformation is a correlation between control by E1A and differentiation of F9 teratocarcinoma cells. Undifferentiated F9 cells are rapidly growing, malignant tumor cells whereas differentiated F9 cells are non-tumorigenic (Martin, 1980; Strickland, 1981). Accompanying this change is the loss of a cellular E1A-like activity, which was first identified by its ability to functionally substitute for the viral E1A (Imperiale *et al.*, 1984). This phenotypic change in F9 cells is also paralleled by a loss of an E2F-factor activity (Reichel *et al.*, 1987). Thus, a correlation exists in the F9 system between the oncogenic state of the cell and the level of a specific promoter binding factor regulated by E1A.

Because the activity of E2F is involved in several aspects of viral and cellular regulation, it is important to understand the function of E2F in detail. Using a partially purified E2F fraction, we have delineated the precise binding site for E2F within the E2 promoter. We have also defined the interaction with the E2 promoter of an additional cellular factor, previously described by SivaRaman *et al.* (1986). We demonstrate that the sites of interaction of the two factors within the promoter are distinct and that the two factors can bind simultaneously. Previous experiments have indicated that an interaction could be detected *in vivo* on the E2 promoter in a wild-type infection (E1A+) but not in a dl312 infection (E1A−) (Kovesdi *et al.*, 1986b). Since the factor described by SivaRaman *et al.* (1986) appears to be essential for E2 transcription and is present at equal levels in

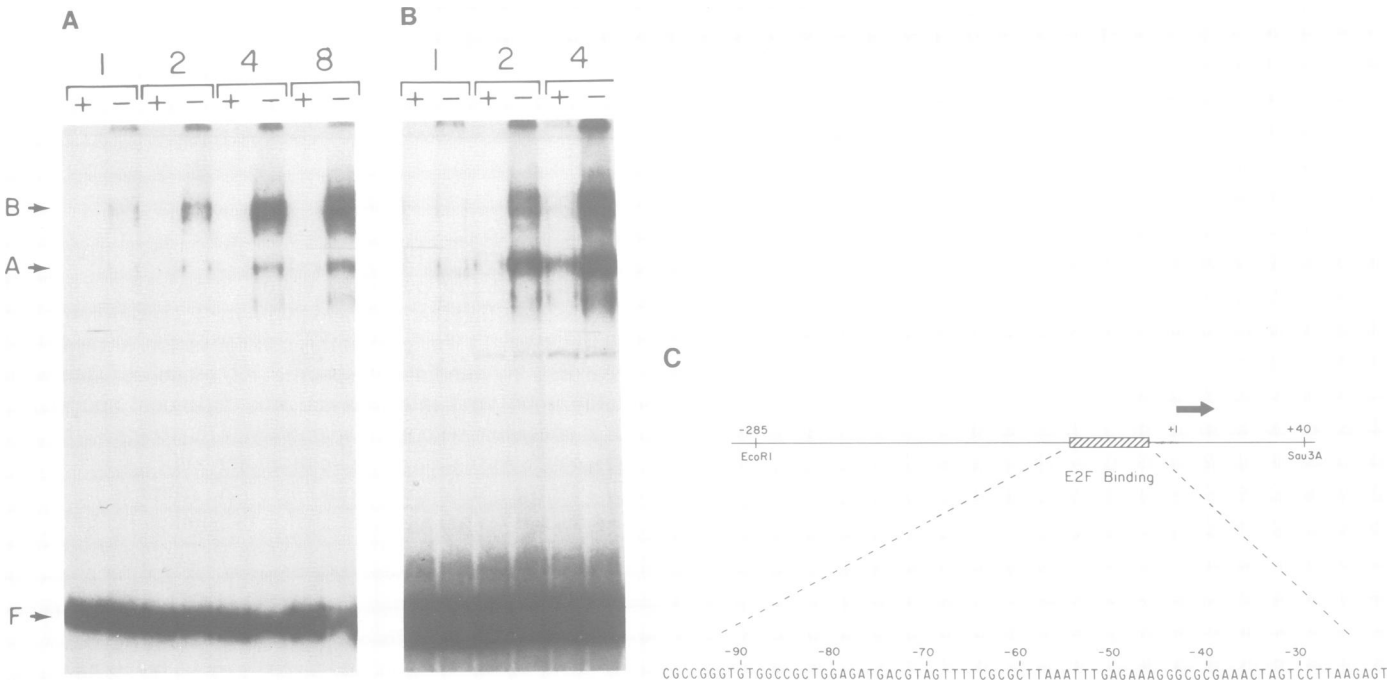

Fig. 1. Binding of E2F to the E2 promoter. **(A)** Effect of E2F concentration on formation of complexes. Gel retardation assay for binding of partially purified E2F to the E2 probe. As indicated above each pair of lanes, 1, 2, 4 or 8 μl of partially purified E2F (Mono Q fraction, 1.25 mg/ml) were added to 0.05 ng of labeled probe, in a 12.5 μl reaction volume (see Materials and methods). Lanes labeled − or + indicate the presence or absence of specific competitor (0.25 μg of plasmid containing sequences from −21 to −98). **(B)** Effect of DNA concentration of the formation of complexes. Assays were conducted as described in **(A)** but with 10-fold higher amount of probe DNA. **(C)** Derivation and sequence of the E2 probe employed in binding assays.

extracts of infected and uninfected cells whereas the E2F factor increases as a function of E1A, we suggest that the increase in E2F triggers the formation of a stable, functional complex.

Results

Definition of E2F binding sites

A precise localization of the factor binding sites on the E2 promoter is critical to the elucidation of the mechanism of transcriptional stimulation, particularly with respect to the involvement of multiple factors in the transcription of this gene. Specifically, in addition to the E2F factor, an additional factor appears to interact with sequences slightly upstream of the E2F site (SivaRaman *et al.*, 1986). Since there was considerable overlap in the apparent binding sites, we have attempted to define precisely the site of interaction of E2F. A partially purified fraction of E2F (see Materials and methods) was used for binding assays and the fraction was carefully titrated in order to obtain the maximal specific binding to the E2 promoter. The partially purified fraction is enriched in E2F-binding activity over binding activities which are not specific to the E2 promoter. Unless otherwise noted, all binding studies were performed with a probe which contains sequence from −21 to −98 of the E2 promoter. As shown in Figure 1A, the partially purified E2F fraction gave rise to two specific DNA−protein complexes, which are denoted as A and B. As is apparent in the adjacent lanes, both complexes could be specifically competed with DNA containing the E2 promoter. At the lowest protein concentration, the ratio of the two complexes was nearly 1:1 while at the highest protein concentration there was approximately five times more of the B complex than the more rapidly migrating A complex. Furthermore, as shown in Figure 1B, an increase in probe concentration resulted in a shift in the ratio of the A and B complex in favor of the A complex. These results demonstrate that the formation of the two complexes depends upon the relative concentration of DNA and factor and that the two states may differ in the number of E2F molecules per promoter fragment.

We next examined E2F binding using DNase footprinting analysis. The E2 promoter probe, labeled at either the 5′ end or the 3′ end, and partially purified E2F fraction were incubated under identical conditions to those in lane 8 of Figure 1. After binding, the mixtures were treated with DNase, and then analyzed by gel electrophoresis in a non-denaturing acrylamide gel. Bands corresponding to complex A and complex B were excised from the gel, and then were analyzed in a denaturing sequencing gel. As is shown in Figure 2, the footprint from the complex B displayed two clear areas of protection: one from −33 to −49, denoted site I, and a second from −53 to −71 denoted site II. The region between the two sites was accessible to cleavage by DNase. No other regions of protection were evident using the partially purified E2F fraction, and similar information was obtained with either 5′ or 3′ labeled probes. Thus, it appears that the interactions which result in complex B are due to the binding of E2F to two distinct sites in the E2 promoter. An examination of the sequence within these two domains of protection revealed a perfectly repeated octamer of TTTCGCGC. Additional evidence (presented below) indicated that at least a portion of this octamer sequence is the binding site for E2F.

Also shown in Figure 2 is the footprint derived from complex A. This footprint is similar to that of complex B but is incomplete, indicating only partial protection. Specifically, a densitometric scan of the footprint revealed ~50% protection over both sites I and II as compared with the free, unprotected DNA. Given the observation that complex A is of apparent lower mol. wt than complex B (Figure 1) and that complex A forms at lower factor concentration, complex A is likely to be the result of one E2F

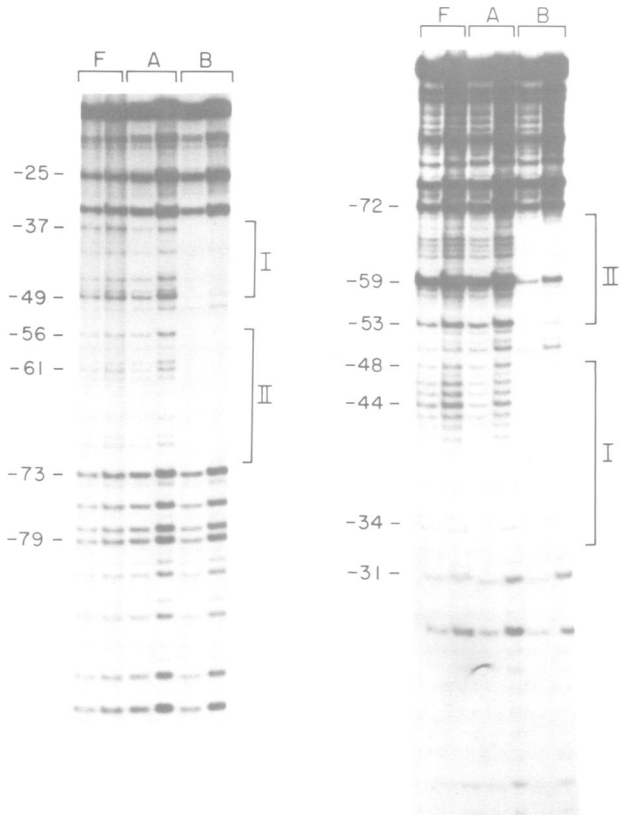

Fig. 2. DNase footprint analysis of E2F binding. The E2 probe was end labeled, incubated in a binding reaction with partially purified E2F, digested with DNase and then fractionated on an acrylamide gel. The binding conditions were identical to lane 8 of Figure 1. Bands corresponding to complex A and B as well as free DNA were eluted and the DNA was then analyzed in a 12% acrylamide−urea sequencing gel. Two different amounts of each sample were analyzed. The panel on the left shows an analysis of the coding strand and the panel on the right shows the non-coding strand. Positions of bands relative to the E2 transcription initiation site at +1, as determined from a parallel run of a sequencing cleavage ladder, are indicated. F, free DNA; A, complex A; B, complex B.

molecule bound to the promoter, with no preference for site I or site II. In contrast, complex B likely represents E2F molecules bound to both sites. If the binding of E2F to the E2 promoter was an ordered reaction in that one site must be occupied prior to the other site, then the expected result for the complex A footprint would be clear protection of one of the sites but not the other. If, however, E2F could initially interact equivalently with either site, then the expected result when a single factor was bound would be a 50% footprint for both sites, which was the observed result.

In addition to the DNase footprint analysis, protection from methylation was used to define accurately the E2F binding site. An analysis of G residues within the E2 promoter that are protected by E2F from methylation by dimethylsulfate (DMS) is shown in Figure 3. On each strand, there are four G residues clearly protected from DMS methylation (indicated by filled circles) and one which is only partially protected (open circle). Furthermore, on one strand there was an apparent hypermethylation of one G residue (indicated by the triangle).

The E2F binding site was also analyzed by the use of site-specific methylation. The *Hha*I methylase recognizes the sequence GCGC and methylates the middle C residue on each strand (Smith, 1979). Within the −21 to −98 E2 promoter sequence

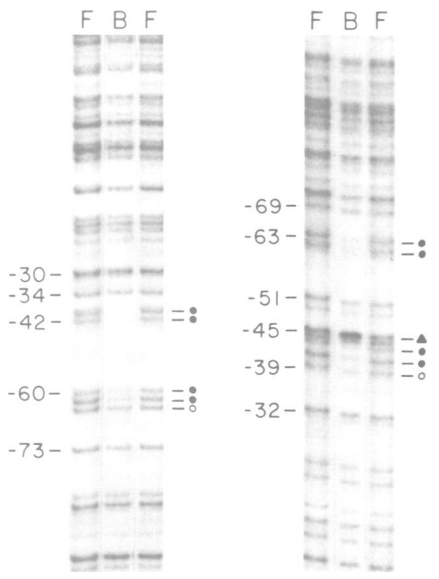

Fig. 3. DMS protection footprint analysis of E2F binding. End-labeled E2 probe was incubated with nuclear extracts from Ad5-infected cells and the complex was treated with DMS as described in Materials and methods. The complex was then fractionated in an acrylamide gel and bands corresponding to complex B and free DNA were isolated. The DNA was purified, treated with piperidine and then analyzed in a 5% acrylamide−urea sequencing gel. Analysis of the coding strand is shown in the panel on the left and the non-coding strand is the panel on the right. F, free DNA; B, bound DNA. Bands protected from methylation are indicated by the filled circles; partial protection is indicated by the open circles; and a hypermethylation site is indicated by the filled triangle.

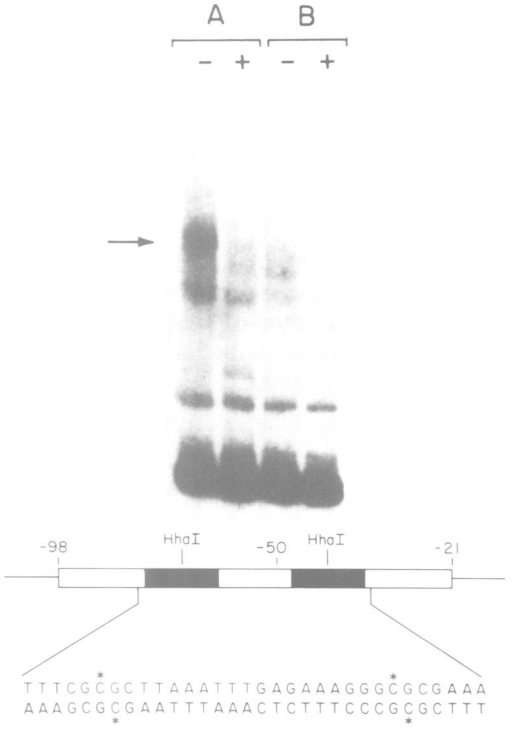

Fig. 4. Effect of methylation on the binding of E2F. The E2 probe was methylated with *Hha*I methylase as described in Materials and methods. The sites for methylation are depicted in the sequence shown at the bottom of the figure. Unmethylated **(A)** and methylated DNA **(B)** were then assayed for E2F binding in nuclear extracts from Ad5-infected cells by a gel retardation assay. Lanes marked − and + refer to the absence or presence respectively of specific competitor DNA.

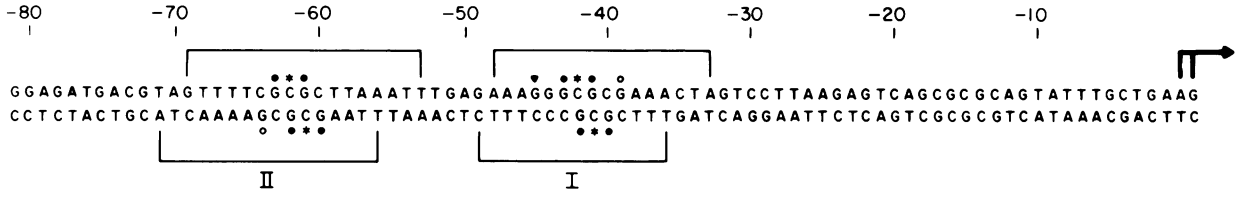

Fig. 5. Summary of E2F binding to the E2 promoter. The sequence of the E2 promoter is shown. The sites of protection from DNase cleavage are indicated by the brackets. Sites of protection from methylation by DMS are indicated by the solid circles and partial protection to DMS is indicated by the open circles. A site hypermethylated by DMS is indicated by the solid triangle. *Hha*I methylation sites are indicated by the asterisks.

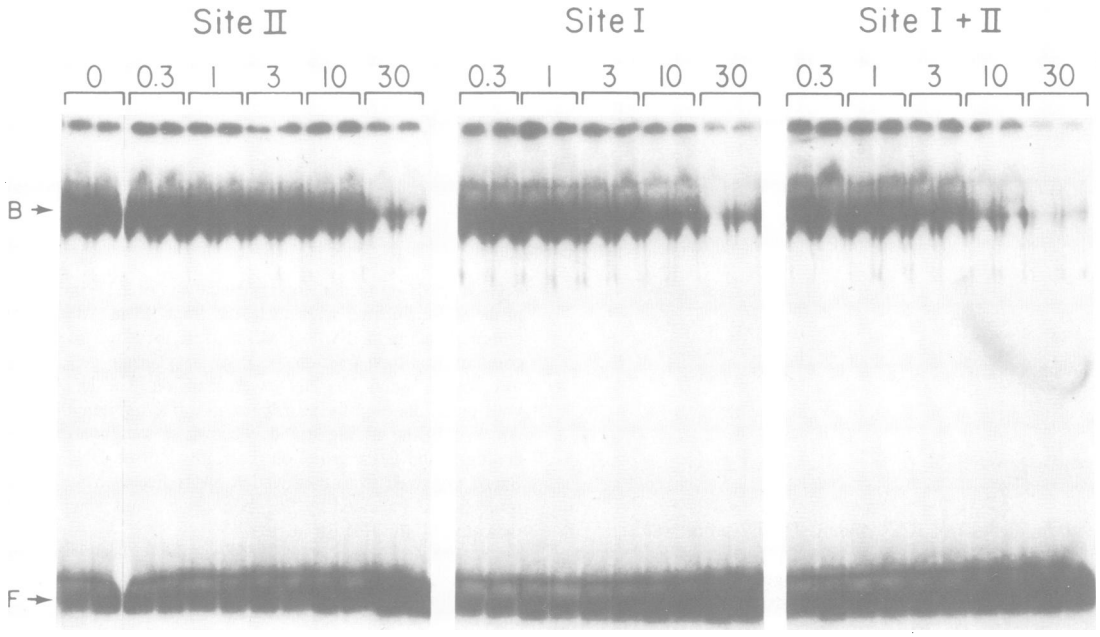

Fig. 6. Competition assays for interaction of E2F with E2 promoter binding sites I, II and I + II. Binding of E2F was assayed as described in Figure 1. Specific competitor DNAs were prepared as described in Materials and methods. Each binding assay was carried out in duplicate (each pair of lanes) and included the indicated competitor. The molar concentration of binding sites in the competitor relative to the concentration of binding sites in the probe is indicated above the lanes.

there are two *Hha*I recognition sites, each within the expected E2F binding sites (see Figure 4). The binding of E2F to both methylated and unmethylated probes was examined by a gel retardation assay. Methylation of the probe was ~90% complete as judged by digestion with *Hha*I endonuclease and, as shown in Figure 4, methylation of the probe resulted in the near total inhibition of E2F binding to the promoter. Thus, these C residues are clearly involved in the recognition of the binding sites by the factor.

A summary of all of the binding data is shown in Figure 5. DNase protection defines the two distinct regions indicated by the brackets that includes sequences between −33 and −71. Chemical modifications further define bases which participate in the binding of E2F to the promoter. The common element in both sites is the sequence TTTCGCGC. Furthermore, the analyses summarized in Figure 5 place the C2F binding sites within the region between −33 and −71 of the E2 promoter and clearly indicate that there is little overlap between the E2F binding sites and the binding site for the factor detected by SivaRaman *et al.* (1986). Although these authors described the binding site as extending from −66 to −82, this was based in part on inferences from binding to linker scanning mutants. Precise localization was only achieved with methylation interference assays where these borders of protection were between −69 and −80. DNase protection assays described below define a binding site between −68 and −80.

Quantitative competition analysis of binding sites

The data in Figures 1 and 2 indicate that the two regions of E2F protection may be independent binding sites. To establish this, we have carried out competition assays for binding using as competitors DNA fragments encompassing site I, site II and site I + II. To measure the affinity of single- and double-site binding interactions, gel retardation analysis under quantitative conditions was used. As outlined by Chodosh *et al.* (1986) the concentration of the specific complex is directly proportional to the concentration of factor when the probe is in excess. Under the conditions in the assay, the intensity of the band obtained in a gel retardation assay should be directly proportional to the concentration of the E2F DNA complex.

As shown in Figure 6, site I alone and site II alone could compete for E2F binding indicating that these represent independent binding sites. However, the affinity for binding to the single site fragments was less than that observed for the binding to the double site fragement. As determined by densitometric scanning, the molar amount of binding site required to give 50% competition was 3-fold or 4-fold more for site I and site II respectively than that required when the two sites were on the same DNA frag-

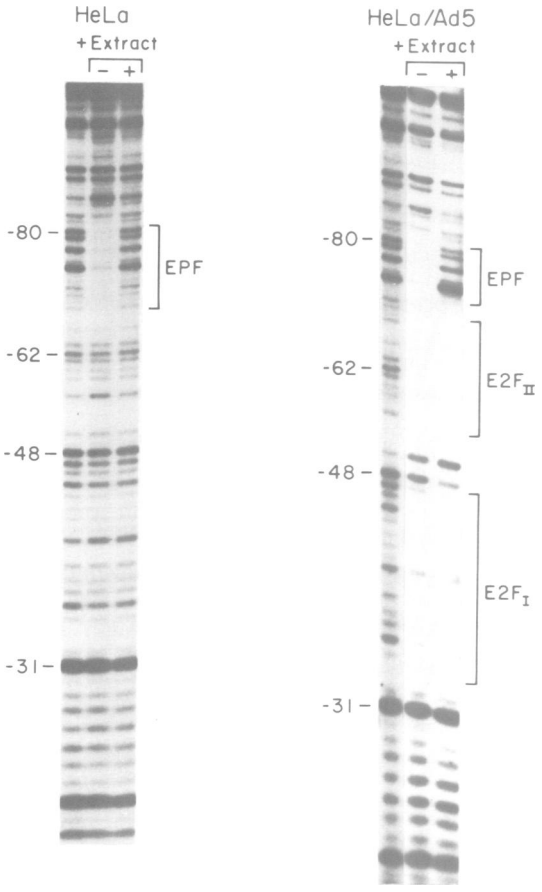

Fig. 7. Direct footprint analysis of E2 binding factors in nuclear extracts. The E2 promoter probe (−21 to −98) was incubated with extract from uninfected HeLa cells (left panel) and Ad5-infected HeLa cells (right panel). The binding reactions were performed in the presence of poly(dI)·poly(dC) (100 μg/ml) as nonspecific competitor. After 30 min at room temperature, the binding reactions were treated with DNase (2 min at room temperature), the DNA was phenol extracted and analyzed in 8% polyacrylamide−urea gels. The left lane in each panel shows a reaction performed in the absence of nuclear extract. The two right lanes in each panel represent experiments done in the presence of nuclear extract. Lanes marked (−) refer to the absence and (+) to the presence of a fragment which comprises the EPF binding site.

ment (site I + site II). For instance, a 10-fold molar excess of site I or site II competitor over the probe resulted in 2-fold reduction whereas the 10-fold excess of site I + site II resulted in 6-fold reduction. Threfore, the binding of E2F appears to be of maximal affinity when both sites are present, suggesting that there is cooperativity in the binding reaction.

Multiple factors interact with the E2 promoter

As described in the preceding section, two distinct binding factors interact with the E2 promoter. The E2F factor interacts with two distinct sites between −33 and −71. In addition, a recent report described a factor in nuclear extracts that interacts with E2 sequences immediately upstream of this region (SivaRaman *et al.*, 1986). To establish firmly that these two activities represent distinct binding activities, the E2 DNA interactions for both factors were explored by direct DNase footprinting using extracts prepared from infected and uninfected cells. For simplicity and clarity, we have referred to the factor initially described by SivaRaman *et al.* (1986) as EPF, denoting early promoter factor, as it appears to interact with at least three of the early viral promoters (E1A, E2 and E4).

Conditions were optimized for the detection of both EPF and E2F binding activities. Of most importance was the nature of the non-specific competitor DNA. Our initial assays for E2F binding and the assays presented here have used sonicated salmon sperm DNA as the competitor. Using crude nuclear extracts in the presence of salmon sperm DNA, the only factor which binds to the E2 promoter is E2F (Kovesdi *et al.*, 1986a). The EPF factor is completely excluded. However, if poly(dI)·poly(dC) is substituted for salmon sperm DNA, the EPF factor is now readily detected in gel retardation assays (SivaRaman *et al.*, 1986). The E2F factor can also bind in the presence of poly(dI)·poly(dC) but apparently the EPF factor is more abundant in extracts than the E2F factor. Thus, the complexes detected in the presence of poly(dI)·poly(dC) and assayed by gel retardation are due to binding of EPF.

As shown in Figure 7, when uninfected extracts are used in the presence of poly(dI)·poly(dC) as competitor, the region from −68 to −80 was specifically protected, in agreement with the binding site previously described by SivaRaman *et al.* (1986). In addition, this is the only region of protection which was observed with uninfected extracts. In contrast, when extracts from

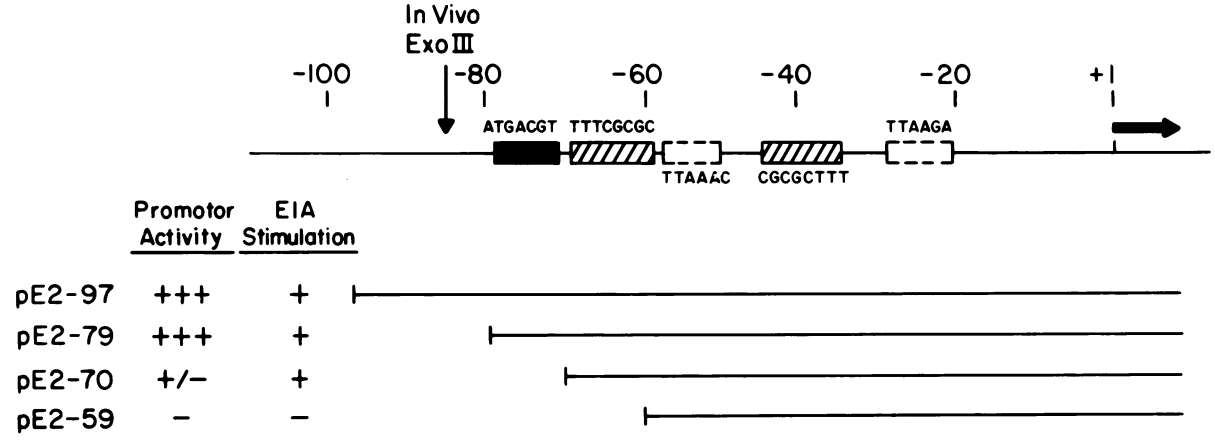

Fig. 8. Schematic representation of factor binding sites on the E2 promoter. Shown at the top is the location of the binding site for the EPF factor (solid box) and the two E2F binding sites (hatched boxes). Also shown are locations of potential factor binding sites (broken boxes), which are predicted by mutagenesis data (see Discussion). Shown below are endpoints of 5′ deletion mutants in relation to factor binding sites. The *in vivo* activity of the mutants (Imperiale *et al.*, 1984) is summarized.

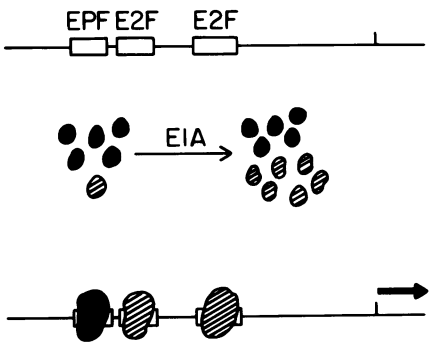

Fig. 9. Model for the control of E2 transcription by E1A. The two E2F binding sites and the single EPF binding site in the E2 promoter are depicted. In the absence of E1A (e.g. dl312 infection), the E2 promoter is unoccupied (Kovesdi *et al.*, 1986b) and transcriptional activity is low. In the presence of E1A (e.g. wild-type infection), the promoter is occupied by factors and transcription proceeds efficiently. The level of EPF (solid figures) does not change as a function of E1A whereas the level of E2F (hatched figures) increases markedly.

Ad5-infected cells were used, the region from −68 to −80 was still protected, but an additional region of protection from −33 up to this site, which coincides with the two E2F binding sites, is now apparent. In addition, a fragment specific for the sequence between −70 and −80 (see Materials and methods) completely eliminates binding of the EPF factor but had no effect on the binding of E2F (+ lane). Thus, both E2F and EPF can bind simultaneously on the same promoter. Furthermore, it is clear from this analysis that the extracts from uninfected and infected cells differ not by the presence of the EPF factor but by the presence of the E2F factor.

Discussion

We have presented a detailed analysis of the binding of HeLa cell factors to the adenovirus E2 promoter and a summary of the data is shown in Figure 8. As demonstrated by a variety of analyses, there are two distinct binding sites for the E2F factor within the E2 promoter, from −33 to −49 and from −53 to −71. Binding can clearly occur independently to each site but the binding of E2F is of maximal affinity when both binding sites are present. Previous experiments have demonstrated that E2F can also bind to the E1A enhancer (Kovesdi *et al.*, 1987). Similar to the interaction with the E2 promoter, there are duplicated binding sites for E2F in the E1A enhancer and binding can occur to each site independent of the other (Kovesdi *et al.*, 1987). In contrast to the inverted orientation of the sites in the E2 promoter, the binding sites in the E1A enhancer are in the same orientation and are separated by 55 nucleotides. The functional significance of these different arrangements of E2F binding sites, if any, is not clear.

The binding data presented here also firmly establish that E2F is distinct from the promoter binding factor EPF which was initially described by SivaRaman *et al.* (1986). Furthermore, it is clear from the results shown in Figure 7 that the two factors can occupy the respective binding sites simultaneously. Based on promoter mutagenesis data, both factors are essential for promoter activity and therefore we surmise that the functional complex involves each of the factors. As depicted schematically in Figure 8, promoter deletions that remove the EPF binding site severely reduce promoter activity and linker scanning mutants that disrupt this site impair the function of the promoter (Murthy *et al.*, 1985). A deletion which eliminates the E2F site II essentially abolished promoter activity. Interestingly, the −70 deletion, which eliminates the EPF site but retains both E2F sites, is still weakly transcribed and retains E1A inducibility (Imperiale *et al.*, 1985). There are certain inconsistencies, in particular a linker scanning mutation at −55 to −66 in the E2 promoter that disrupts the E2F site II but which apparently does not impair promoter activity (Murthy *et al.*, 1985). However, the E2F binding site is duplicated in the E2 promoter and if one site is sufficient for promoter function in the presence of the EPF site, then only the mutation of both sites would abolish transcriptional activity.

Indeed, it appears that interaction of E2F with a single E2F binding site can be functional, although the presence of two sites may increase transcription efficiency. A single E2F binding element was found to confer increased transcription to a heterologous promoter (β globin) but only in the presence of E2F protein (Kovesdi *et al.*, 1987). Importantly, when E2F binding was blocked by the methylation of the E2F site, the E1A-inducible transcription was also inhibited. Therefore, the binding of E2F to a promoter element is functionally important, yielding an increased rate of transcription, but only as a function of the E1A protein. Given this result and the fact that the level of the E2F factor as measured by binding increases markedly as a function of E1A, we suggest that the stimulation of the E2 promoter by the action of E1A is mediated through the E2F factor. In view of this and because the EPF factor is present at equal levels in extracts of infected and uninfected cells, we conclude that EPF is an essential factor for full transcription of the E2 gene but is not likely to be involved in the regulation by E1A. The EPF factor may be a more general transcription factor since it is utilized by several promoters. Competition experiments indicate EPF binds to the E1A and E4 promoters as well as to the E2 promoter (SivaRaman *et al.*, 1986) and EPF also appears to interact with at least one cellular promoter element (T.Fisch, unpublished data).

In vivo exonuclease III protection experiments, which utilized nuclei from Ad5-infected cells, indicated a complex on the E2 promoter with a 5′ boundary at −85 (Kovesdi *et al.*, 1986b). These data are consistent with the simultaneous binding of the two E2F factors and of EPF which was detected in the *in vitro* binding experiments (see Figure 7). In sharp contrast, this complex was absent on the E2 promoter in nuclei of dl312 (E1A−) infected cells. This observation suggests that the activation of E2 transcription as a function of E1A may involve the formation of a stable promoter complex, a possibility previously suggested by Gaynor and Berk (1983). In view of this, it is interesting that the EPF factor is apparently present at equal levels in uninfected and in infected cells. Evidently, the presence of the EPF protein in the cell is not sufficient to ensure its interaction with the promoter. As depicted in Figure 9, we suggest that the formation of a functional complex of factors on the E2 promoter, including EPF and two E2F molecules, can only take place when the level of E2F rises as a result of E1A. The apparent dependence of EPF binding on the presence of E2F certainly cannot be absolute since the EPF factor can bind to the promoter independent of the binding of E2F as shown in Figure 7. However, within the cell there may be an influence of E2F on binding of EPF.

Finally, although two factors have been identified that interact with the E2 promoter, it is possible that additional factors may be components of an active transcription complex. In particular, based on promoter mutagenesis data (Murthy *et al.*, 1985; Zajchowski *et al.*, 1985), there is likely to be a factor which interacts with a TATA-like sequence between −28 and −22 although such a factor has not yet been detected in binding assays using

crude extracts. In addition, a linker scanning mutant that disrupts a CAAT-like sequence between the two E2F binding sites exhibits a phenotype of increased transcription (Murthy *et al.*, 1985; Zajchowski *et al.*, 1985). Certainly a final understanding of the mechanisms by which these factors interact to stimulate transcription of this promoter will require the isolation of all factors and a reconstitution of the active complex.

These observations on the interactions of proteins with the E2 promoter may be relevant to the general case of functional protein interactions with promoters and enhancers. The interaction of multiple factors with transcriptional regulatory elements appears to be a common occurrence. Possibly the best studied example is the regulatory region of the SV40 early gene. There are five binding sites for the Sp1 factor in the promoter region (Dynan and Tjian, 1983) and there are as many as four sites for protein binding to the enhancer (Wildeman *et al.*, 1986), at least three of which appear to involve distinct proteins (R.Tjian, personal communication). Furthermore, each of the interactions appear to be of functional significance (Zenke *et al.*, 1986). A requirement of multiple factors for transcription complex formation would increase specificity of the interactions and ensure that a particular gene would be efficiently expressed only in appropriate instances. However, if it were necessary to regulate each of the factors to achieve the desired result, the required complexity would overwhelm the genetic capabilities of the cell. An efficient mechanism of regulation might be the tight control of only one of the factors in a complex formation. For instance, transcription of the immunoglobulin heavy chain locus in a lymphocyte is paralleled by the presence of proteins bound to the enhancer (Church *et al.*, 1985; Ephrussi *et al.*, 1985). These interactions are not found on the enhancer in a fibroblast where the gene is not transcribed. However, it appears that most of the proteins that interact with the heavy chain enhancer may not be lymphocyte specific but rather are present in most cell types (Peterson *et al.*, 1986; Sen *et al.*, 1986a,b; Singh *et al.*, 1986). Thus, as with the EPF factor which is present in dl312-infected cells but apparently does not bind to the promoter (Kovesdi *et al.*, 1986b), these Ig enhancer binding proteins are present in a fibroblast but do not interact with the enhancer. In contrast, a lymphocyte-specific factor, which recognies the octamer sequence that is common to the Ig enhancer and promoter, has been identified (Landolfi *et al.*, 1986; Staudt *et al.*, 1986). Lipopolysaccharide treatment of lymphocytes, which stimulates heavy chain transcription, also increases the level of the octamer binding protein. We might speculate that the involvement of this factor in heavy chain transcription could be similar to the role of E2F in adenovirus E2 gene transcription.

Materials and methods

Cells and virus

HeLa cells were grown in suspension in Joklik modified minimal essential medium (MEM) containing 5% calf serum. The growth and purification of Ad5 has been described previously (Nevins, 1981).

Gel retardation assay

The assays were performed essentially as described by Kovesdi *et al.* (1986b) with the following modifications. Buffer A containing 40 mM KCl was used in all reactions. In most of the assays, the reaction volume was 12.5 μl, and contained 0.1 ng of end-labeled E2 promoter probe (−21 to −98) and 1 μg sonicated salmon sperm DNA and E2F fraction. This ratio of specific to nonspecific DNA ensured maximal specific binding of E2F. Both the *Eco*RI and *Hin*dIII ends were labeled for gel shift assays. Binding was performed at room temperature for 30 min. The complexes were resolved from unbound DNA on a 4% polyacrylamide gel. The gel buffer was 1/4X TBE, and the gels were run at 4°C.

All quantitative assays were done under conditions of probe excess in which all factor was in the bound form. As outlined by Chodosh *et al.* (1986), these conditions allowed a direct correlation between the intensity of the band in the gel with the concentration of the E2F−DNA complex. In most cases, because two E2F bind per promoter, the concentration of E2F is twice the concentration of E2F−DNA complex.

Gel retardation assays in which a methylated probe was used were performed essentially as described above. The methylated probe was prepared by incubating the end-labeled −21 to −98 E2 promoter fraction with *Hha*I methylase and 80 μM S-adenosylmethionine. The methylated probe was purified by agarose gel electrophoresis.

Specific competitor DNA fragments were isolated by agarose gel electrophoresis. The boundaries of the site I and site II are −59 to +40 and −53 to −98 respectively. The site I + II fragment extends from −21 to −98.

Partial purification of E2F

HeLa cells at ~4 × 10^5 cells/ml were infected with Ad5 at a concentration of 2000 particles/cell by direct addition to the culture and were incubated for ~18 h in the presence of 25 μg/ml cytosine arabinoside. Nuclear extracts from 9 l of Ad5-infected HeLa cells were prepared by the method of Dignam *et al.* (1983). The crude extract (26 ml; 4 mg/ml) was loaded onto a 25-ml Heparin agarose column (Sigma), which was equilibrated in Buffer A [20 mM Hepes, pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol (w/v) and 0.02% sodium azide] and 0.1 M KCl. The column was then washed first with Buffer A containing 0.1 M KCl and then with Buffer A containing 0.25 M KCl. The E2F activity was eluted with an eight-column volume gradient ranging from 0.25 to 1.0 M KCl. The peak fractions eluted at ~0.45 M KCl. The active fractions were pooled and dialysed against Buffer B [50 mM Tris, pH 7.5, 0.2 mM EDTA, 1 mM EDTA, 10% glycerol (w/v) and 0.02% sodium azide] and 0.1 M KCl. The dialysed peak fractions were then concentrated ~6-fold by ultrafiltration (Amicon).

The Heparin agarose fraction (4 ml; 1.25 mg/ml) was then loaded onto a 1-ml f.p.l.c. Mono Q column (Pharmacia), which was equilibrated in Buffer B containing 0.1 M KCl. After extensive washing, E2F activity was eluted with a 20-column volume gradient ranging from 0.1 to 0.5 M KCl. The peak fraction was concentrated ~6-fold by ultrafiltration, and the KCl concentration was adjusted to 0.1 M.

Using quantitative conditions for the gel retardation assay, the estimated overall purification was 100-fold, with a 20% recovery of activity and a 0.2% recovery of protein. A typical yield of partially purified E2F was 1.2 ml at 0.2 mg/ml protein.

Footprinting analysis

DNase footprinting was performed essentially as described in Kovesdi *et al.* (1986b) with the following modifications. Because the partially purified fraction was in Buffer B which contained EDTA and EGTA, the concentration of $MgCl_2$ was adjusted to 1 mM prior to the addition of DNase to a concentration of 0.04 μg/ml. Digestion proceeded for 4 min at room temperature. The DNase-treated E2F−DNA complex was isolated by gel electrophoresis prior to analysis of the DNA on sequencing gels. Footprint data were obtained on both strands by using either *Eco* or *Hin*d end-labeled probes, and the position of the protected regions was determined by reference to a G + A Maxam−Gilbert ladder of the appropriate probe.

Methylation protection footprinting was performed by treating the binding reaction with DMS at a concentration of 2% (v/v). The reaction was terminated by the addition of 100 μl of a solution of 1.5 M sodium acetate and 1 M β-mercaptoethanol, pH 7.0, and the products were precipitated with ethanol. After cleavage with 1 M piperidine, the products were analyzed on a sequencing gel.

For the direct footprinting experiments in which both EPF and E2F binding were assayed (Figure 7), poly(dI)·poly(dC) was used instead of salmon sperm DNA. This modification of conditions allowed detection of both EPF and E2F in the infected extracts. After binding, DNase was added to the reaction at a concentration of 60 μg/ml. The reactions were terminated with addition of EDTA to 1 mM and SDS to 0.5% and extracted with phenol:chloroform. The reaction products were analyzed on a sequencing gel.

The −70 to −80 fragment, which was used for competition, was prepared from two complementary oligonucleotides: (GATC AGATGACGTAG) and (GATC CTACGTCATCT). Each oligonucleotide was purified by gel electrophoresis. The pair were annealed and then ligated to form multimers exactly as described by Kadonaga and Tjian (1986).

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