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Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter

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We have previously shown that E1A-mediated induction of the adenovirus E2 transcription unit likely involves the post-translational activation of a previously limiting cellular factor termed E2F. However, this factor is not involved in E1A induction of several other viral genes, including the E4 gene, since it does not bind to the promoters of these genes. We have undertaken an analysis of proteins which bind to the E4 promoter in an attempt to define the basis for E1A control of this gene. Gel retardation binding assays revealed a large number of interactions with the E4 promoter consistent with the fact that at least 180 nucleotides of sequence are required for full promoter activity. The analysis was simplified by employing small probes as well as by using partially fractionated extracts. By so doing, we have identified at least seven discrete factor interactions involving the E4 promoter. Multiple interactions, as defined by discrete gel complexes, were identified with a site previously shown to be critical for promoter activity as well as E1A control. We find that one of these factors, termed E4F, is increased at least 10-fold in extracts prepared from Ad5 infected cells and that the increase requires the E1A gene. Furthermore, the activation is maximal by 3 h post-infection, consistent with the kinetics of activation of E4 transcription. Competition binding assays demonstrated that the E4F factor was E4 specific and did not interact with any other E1A inducible promoter. We therefore conclude that the induced E4F factor is likely responsible for the E1A-induced transcription of E4, thereby suggesting that E1A control must involve an activation of multiple promoter specific binding proteins.

Key words: adenovirus/transcription/E1A/genes/inducible factor

Introduction

The control of transcription initiation is mediated through the interaction of proteins with specific DNA sequences in the promoter and enhancer of a transcription unit. Thus, the identification of such proteins, an elucidation of the control of the interaction as well as the mechanism whereby such interactions stimulate transcription is of great importance to an understanding of the various phenomenon which alter gene activity. A system which has proved valuable for elucidating details of transcription control is the set of adenovirus genes stimulated by the viral E1A gene product (Nevins, 1987). Six viral transcription units and at least three cellular transcription units are activated through the action of E1A. We have shown previously that for the E2 gene, the induction of transcription likely involves an E1A-mediated activation of a cellular transcription factor (Kovesdi *et al.*, 1986a,b). This factor, termed E2F, is present in uninfected cells but the concentration of the active factor is low and apparently limits transcription. As a result of the action of E1A, there is a large increase in the level of active E2F, as measured by DNA binding, which in several different contexts closely correlates with the activation of E2 transcription (Reichel *et al.*, 1987; Kovesdi *et al.*, 1987; Reichel *et al.*, submitted). Furthermore, the activation of E2F does not require new protein synthesis (Reichel *et al.*, submitted) and we therefore conclude that E1A likely mediates an alteration of a pre-existing pool of factor to convert it to an active state, capable of DNA binding and thus stimulation of transcription.

Although E2F very likely is responsible for the E1A-mediated stimulation of E2 transcription and possibly E1A transcription as well, since it binds to two sites in the E1A enhancer (Kovesdi *et al.*, 1987), it cannot be involved in the stimulation of E1B, E3, E4 or ML. Given this finding, one may ask what is responsible for the E1A-mediated stimulation of these promoters and how is coordinate control effected? To approach these questions, we have undertaken an analysis of proteins binding to the E4 promoter and, in particular, a search for proteins that might mediate induction of transcription by E1A. Our criteria for a protein involved in E1A control is the same as that used for the analysis of the E2 gene. A factor must bind to sequences within the promoter that are critical for transcription and the factor must fluctuate with respect to fluctuation of transcription of the gene. This latter point is particularly important when the assays are merely DNA binding, since to demonstrate that a protein interacts with a specific sequence *in vitro* does not mean that it does so *in vivo*. However, if in addition to this specific binding there is also a correlation between the presence of the protein in cell extracts and transcription of the gene inside the cell, then the case becomes considerably stronger for the involvement of the factor in mediating transcription.

With these considerations in mind, we have assayed for proteins interacting with the adenovirus E4 promoter. Sequences in the E4 promoter which are critical for transcription and apparently for E1A control have been mapped. Furthermore, the previously identified E2F factor does not bind to the E4 promoter suggesting the involvement of an additional factor in E1A control. We find numerous interactions of proteins with the E4 promoter but of particular interest is one factor that binds to a sequence already identified as crucial for the regulation of this promoter (Lee and Green, 1987). We find that the level of the factor, as measured by DNA binding, increases significantly as a function of E1A.

Results

Multiple cellular factors interact with the E4 promoter

To assay for porteins binding to the E4 promoter, we have made use of the gel retardation assay as described previously (Fried and Crothers, 1981; Garner and Revzin, 1981; Kovesdi *et al.*,

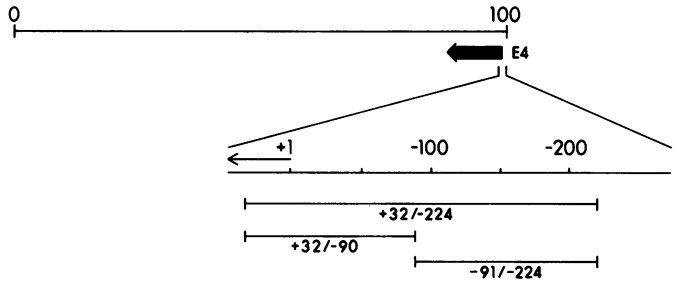

Fig. 1. Schematic diagram of the E4 promoter and derivation of probes utilized for binding assays.

1986a). A probe was prepared (Figure 1) that contained E4 promoter sequences from −224 to +32. This includes all of the E4 sequences previously shown to be critical for transcription (Gilardi and Perricaudet, 1984). When the probe was incubated with extracts of adenovirus infected HeLa cells, a number of interactions were evident as indicated by a complex, unresolved gel-shift pattern (data not shown). In fact, under these conditions it was impossible to resolve any discrete complexes.

In an attempt to clarify the picture, we have taken two approaches. First, we have made use of smaller probes for the binding assays allowing us to focus on specific regions of the promoter. Second, we have performed a simple fractionation of the extracts, by heparin-agarose chromatography, to separate various DNA binding proteins and thus simplify the analysis of the interactions. An example of such an assay is shown in Figure 2. When the entire E4 promoter probe is employed for the assays it is evident that discrete interactions are now visible as a result of the heparin−agarose fractionation. However, it is also evident that the complexity of the probe still largely precludes the analysis of these interactions. It is also clear from this analysis that we could not detect any significant differences in the profiles obtained with extracts of uninfected HeLa cells and adenovirus-infected HeLa cells.

The analysis improved significantly, as shown in Figure 3A, when a smaller E4 probe, spanning the region from −91 to −224, was employed for the assay of heparin-agarose fractions. The pattern was complex but a number of discrete complexes were now evident, which were labeled a, b, c and d. Other bands were visible in the gel pattern but have not been pursued as they were either not well resolved or were not reproducibly observed. Once again, despite the appearance of distinct complexes there was no evidence of a difference in the gel patterns with fractionated extracts from Ad5-infected cells and uninfected cells.

The analysis of the heparin-agarose fractions using a probe spanning −90 to +32 is shown in Figure 3B. Distinct interactions with this probe were also clearly evident, as with the analysis with the −91 to −224 probe shown in Figure 3A. The level of several of the complexes (f and g) showed no differences between uninfected and infected extracts. However, in contrast to the previous analyses, there was a band identified by this procedure, labeled as e in the figure, which is greatly increased in the fractions from the Ad5-infected extract as compared to the same fractions from chromatography of uninfected HeLa cell extract. That this is a significant difference between the two extracts and not just random variation is suggested by the fact that the level of several other factors which are evident in this assay is equal in the two extracts. Furthermore, the difference in the level of this factor does not represent slight differences in fractionation as demonstrated in Figure 3C. Fractions 14−18,

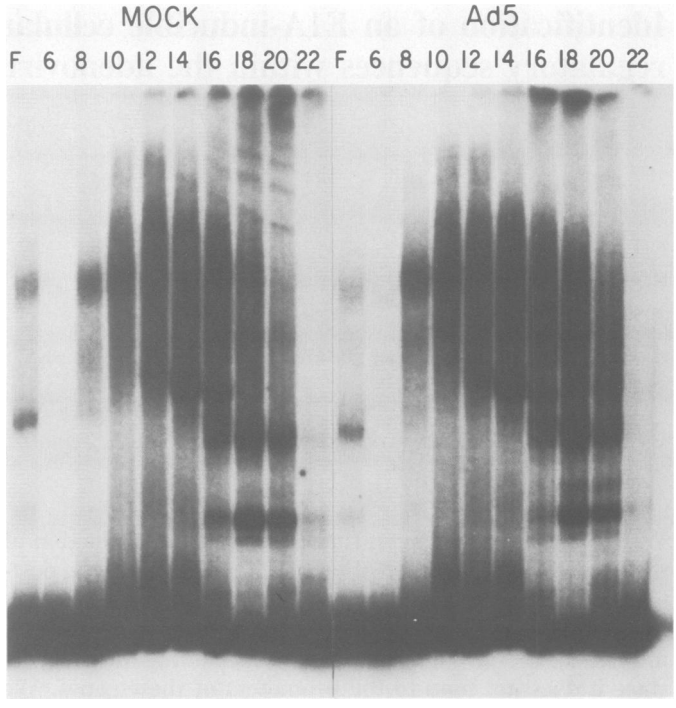

Fig. 2. Analysis of HeLa cell proteins that interact with E4 promoter sequences between positions +32 and −224. Mock infected or Ad5-infected HeLa whole cell extracts were prepared as described in Materials and methods. Extracts were fractionated by heparin-agarose chromatography and columns were eluted with a KCl gradient as described in Materials and methods. 10 μl of the flow-through (F) and 5 μl of the designated column fractions were used in gel retardation assays as described in Materials and methods. An *Eco*RI−*Hin*dIII fragment of plasmid pE4, labeled at the *Eco*RI site, was used as the probe.

encompassing the region of the peak activity along with adjacent fractions, were pooled and assayed for binding activity. It is clearly evident that the e complex is significantly higher in Ad5 extracts whereas the f and g complexes show no difference. We conclude that the factor responsible for generating the e complex, which we term E4F, is increased as a function of virus infection. Finally, once again we wish to point out that although there were several other complexes evident in this analysis, they have not all been pursued. Primarily, this is due to the fact that we have directed our attention to factors which might be subject to control or which were prominent and reproducible. By no means do we wish to suggest that we have identified all interaction with the E4 promoter.

Definition of sequences involved in complex formation

The results of Figure 3 suggest an interaction of several factors with sequences between −90 and +32 of the E4 promoter as well as sequences between −91 and −224. To pinpoint the binding sites, we have analyzed most of the interactions by methylation interference. A binding reaction was initiated employing partially methylated probe and chromatographic fractions derived from the experiment shown in Figure 3. Complexes and free probe were resolved in a native acrylamide gel, eluted from the gel, treated with piperidine to cleave at the methylated residues and then analyzed in an acrylamide-urea sequencing gel. As shown in Figure 4, distinct regions in the DNA making contact with the factors were evident. Analysis of the interactions of proteins with E4 sequences between −91 and −224 revealed areas of interaction at −151 (complex a), −175 (complex b), −102 (complex c) and −158 (complex d).

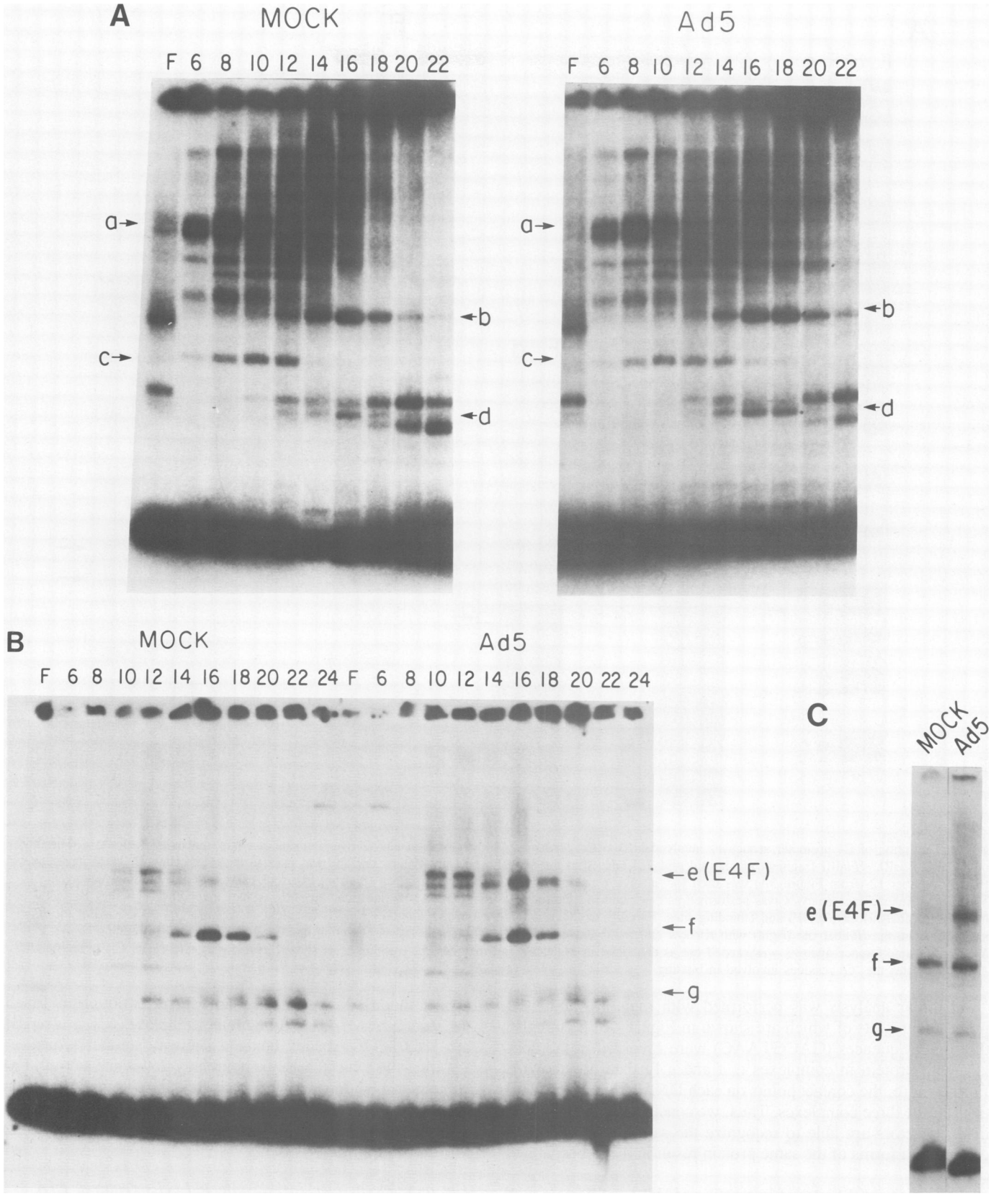

Fig. 3. Analysis of HeLa cell proteins that interact with subregions of the E4 promoter. **A.** Analysis of HeLa cell proteins binding to E4 promoter sequence between protein −91 and −224. Details of the procedures are the same as in legend for Figure 2 except that an *Eco*RI−*Hin*dIII fragment of plasmid pE4-224, labeled at the *Eco*RI site, was used as probe. Specific and reproducible interactions are indicated by the arrows and labeled as a, b, c, d. **B.** Analysis of HeLa cell proteins binding to E4 promotor sequences between position +32 and −90. In this case the *Bam*HI−*Hin*dIII fragment of plasmid pE4-90, labeled at the *Hin*dIII site, was used as probe. Arrows indicate specific interactions which are designated as e, f, g. **C.** Heparin-agarose fractions 14 to 18, as obtained in panel B, were pooled. 5 μl of pooled fraction from both mock infected and Ad5-infected extracts were assyed for E4F activity (complex e) using the +32/−90 DNA probe.

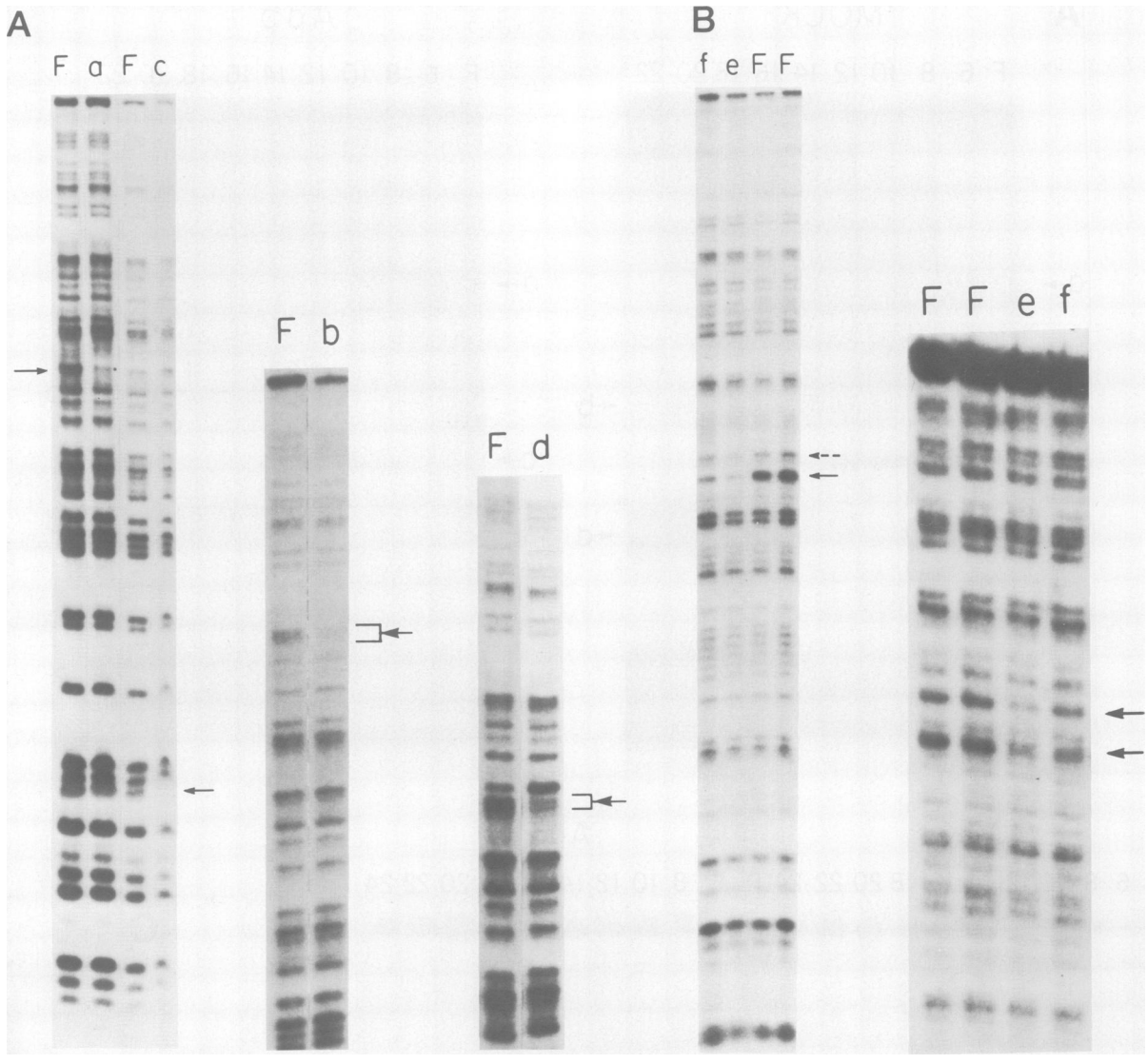

Fig. 4. Definition of E4 promoter sequences involved in complex formation. **A**. Methylation interference analysis of shifted bands a, b, c and d. Partially methylated *Eco*RI−*Hin*dIII fragment of plasmid pE4-224 labeled at the *Hin*dIII site with Klenow enzyme was used in these experiments. Heparin-agarose fraction number 12 (from Figure 3A) was used for 'a' and 'c'. For 'b' and 'd', fraction number 18 (from Figure 3A) was used in binding reactions. Methylation interference analysis was performed as described in Materials and methods. Arrow indicates the 'G' residues involved in binding. **B**. Methylation interference analysis on e (E4F) and f binding sites. Partially methylated DNA probes, a *Bam*HI−*Hin*dIII fragment of plasmid pE4-90 labeled at the *Bam*HI site (left) or at the *Hin*dIII site (right) with Klenow enzyme, were used along with heparin-agarose fraction number 16 (Figure 3B) in binding assays. Methylation interference analysis was carried out as described in Materials and methods. **F**: free DNA, **e**: shifted band for e (E4F) and '**f**': shifted band f. Arrow indicates the 'G' residues involved in binding.

A similar analysis was performed with the −90 and +32 probe, the results of which are shown in Figure 4B. Strikingly, the same sequences (GACGTAAC) were involved in the contact with protein in complexes e, f and g (g data not shown). Furthermore, the contact points on each DNA strand were identical for the three complexes. Thus, we conclude that either three distinct factors are able to recognize the same DNA sequence or modified forms of the same protein result in the gel-separated complexes.

A summary of the sites of interaction of HeLa cell factors with the E4 promoter is depicted in Figure 5. Five distinct regions of protein binding to the promoter were observed from the methylation interference assays. Given the distinct sites of interaction and the fact that they derived from distinct gel complexes, we conclude that these are different factors interacting with these sites. The interaction at the −46/−53 site has been observed with three complexes (e, f, g). Whether these are different factors or modified forms of the same factor is not clear. Finally, the sequence at −46/−53 involving complexes e, f, g is repeated at −163/−170 (shaded boxes). There is another sequence at −140/−146 which has some homology to this site as well. From competition binding assays described below, as well as the work of others (Lee and Green, 1987), it would appear that the upstream site or sites can bind the same factor that recognizes the −46/−53 site.

The increase in E4F in Ad5-infected cells requires E1A

From the result depicted in Figure 3B and C we conclude that the factor generating complex e, which we designate as E4F, is substantially increased in extracts of adenovirus-infected cells. This result, together with the fact that the E4F binding site is critical for E4 transcription and, in particular, the E1A-induced E4 transcription (Gilardi and Perricaudet, 1984; Lee and Green, 1987), suggests that the increase in the factor is responsible for

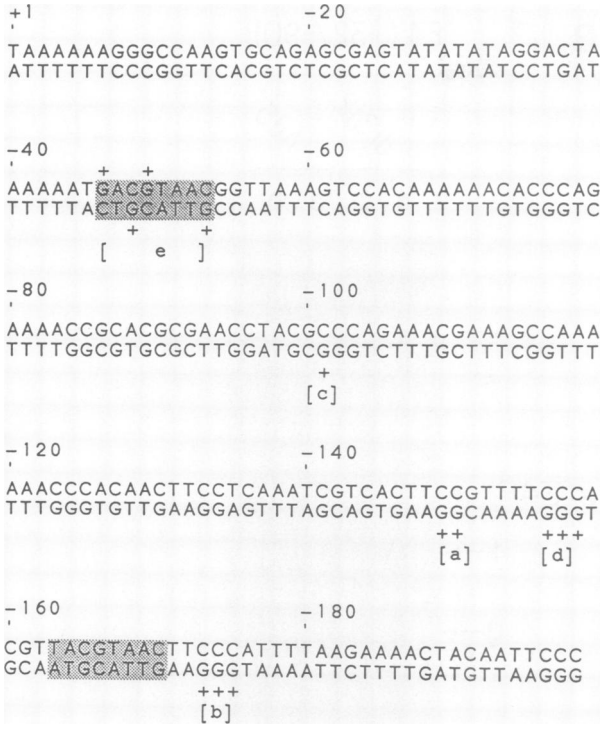

Fig. 5. Sequence of the E4 promoter and depiction of sites of interaction of E4 promoter binding proteins. The residues involved in protein binding, as defined by methylation interference, are denoted by the '+' symbols. The gel complexes which give rise to the protections are indicated in the brackets. The conserved sequence to which the E4F factor binds is shaded.

the stimulation of E4 transcription by E1A. Thus we should expect to find a correlation between the presence of the factor and transcription of the E4 gene. As depicted in Figure 6 this is indeed the case in two separate circumstances. First, analysis of the kinetics of induction of the E4F factor during a WT infection reveals a rapid increase within 3 h of infection and then a maintenance of this level through 4.5 h of infection (Figure 6). Thus, the level of E4F closely corresponds to transcription of the E4 gene, at least through the activation process (Nevins *et al.*, 1979; Nevins, 1981). The decline in E4 transcription that normally occurs is the result of the action of the 72-kd E2A gene product (Nevins and Winkler, 1980; Handa *et al.*, 1983) whose action is presumably dominant. In addition, the kinetics of induction of E4F are clearly different from that of E2F, the factor likely responsible for the activation of E2 transcription. The level of E2F in extracts does not begin to rise until 4–5 h post-infection and reaches a peak at 6–7 h (Reichel *et al.*, submitted), again coincident with E2 transcription (Nevins *et al.*, 1979; Nevins, 1981) as is the case for the E4F factor and E4 transcription.

Secondly, the increase in E4F requires the E1A gene since no increase was observed in cells infected with the E1A mutant dl312 (Figure 6B). It is therefore evident from these two analyses that there is a good correlation between the level of the E4F factor in cell free extracts and the transcription of the E4 gene in virus-infected cells. This observation, together with the finding that an E4F binding site can confer E1A stimulation to a linked promoter (Lee and Green, 1987), strongly suggests that the E4F factor mediates E1A stimulation of E4 transcription.

The E4F factor is E4-specific

The binding site for E4F, as identified by methylation interference (Figure 4) includes the sequence GACGTAAC between positions −46 and −53. An almost identical copy of this sequence, TACGTAAC, is found at position −163 to −170 in the E4 promoter and a similar but not identical sequence is located at −140 to −146. Although we did not detect the E4F factor binding to the −91 to −224 probe in the initial assay shown in Figure 3A, it is clear from assays of Figure 7A that E4F must bind to these sequences since the −91/−224 fragment competes for E4F binding as efficiently as does the homologous binding site (+32/−90). The g complex is also efficiently competed whereas the f complex is only inefficiently competed, even by the homologous fragment, the reason for which is not clear (Figure 7A). We presume that the interaction of E4F with the −91/−224 fragment was not evident in the analysis shown in Figure 3A due to the complexity of the pattern in that region of the gel and/or because of interference due to the binding of other proteins.

A slightly divergent sequence GACGTAGT, is present in the E2 promoter at position −74 to −67. This E2 promoter sequence is recognized by a factor which is found at equal levels in extracts of infected and uninfected cells (SivaRaman *et al.*, 1986; Yee *et al.*, 1987). Competition assays revealed no binding of the E4F factor to the E2 promoter (Figure 7A). Furthermore, using an oligonucleotide containing either the E4 sequence or the E2 sequence for a binding assay demonstrated that the binding activity in the heparin fractions containing E4F recognized the E4 sequence specifically and did not form a complex with the E2 sequence (Figure 7B). Thus, we conclude that distinct factors recognize the upstream site of the E2 promoter and the regulatory sites of the E4 promoter. In order to determine if E4F could recognize any other E1A inducible promoter, competition experiments were carried out using a large excess of unlabelled promoter DNAs from E1B, E3, ML and Hsp70 genes. As shown in Figure 7C none of these promoters competed for E4F binding. Although there does appear to be competition for the g complex by the other promoters, this is largely the result of the low level of the signal in this particular sample rendering the quantitation inaccurate. Therefore, we conclude that the E4F factor is specific for the E4 promoter and is probably not responsible for E1A regulation of the remaining early viral promoters, including E1B, E3 and ML.

Discussion

The activation of the early genes of adenovirus represents a system of coordinate gene control whereby a single regulatory gene product, the 289 aa E1A protein, stimulates the activity of six viral promoters (Nevins, 1987). Furthermore, in addition to the viral promoters, the activity of several cellular promoters is influenced by E1A. This phenomenon presents two basic questions, the answers to which will be of considerable importance to mammalian cell gene control. First, what is the molecular basis for the activation of any given promoter by E1A and, second, how is coordinate control of a variety of promoters achieved? As a result of the experiments reported here, as well as our previous experiments, we can now begin to address these questions. We have shown in previous experiments that the E1A-mediated activation of the E2 promoter involves a stimulation of the formation of stable promoter–protein complexes within the infected cell (Kovesdi *et al.*, 1986b). Analysis of extracts for E2 binding proteins revealed a factor, termed E2F, that appeared to be responsible for formation of the stable complex (Kovesdi *et al.*, 1986a; Yee *et al.*, 1987). In the absence of E1A, the E2F factor appears to be limiting for the E2 promoter, but as a result of E1A action, the level of active E2F rises dramatically. Furthermore, the level of E2F in various extracts correlated

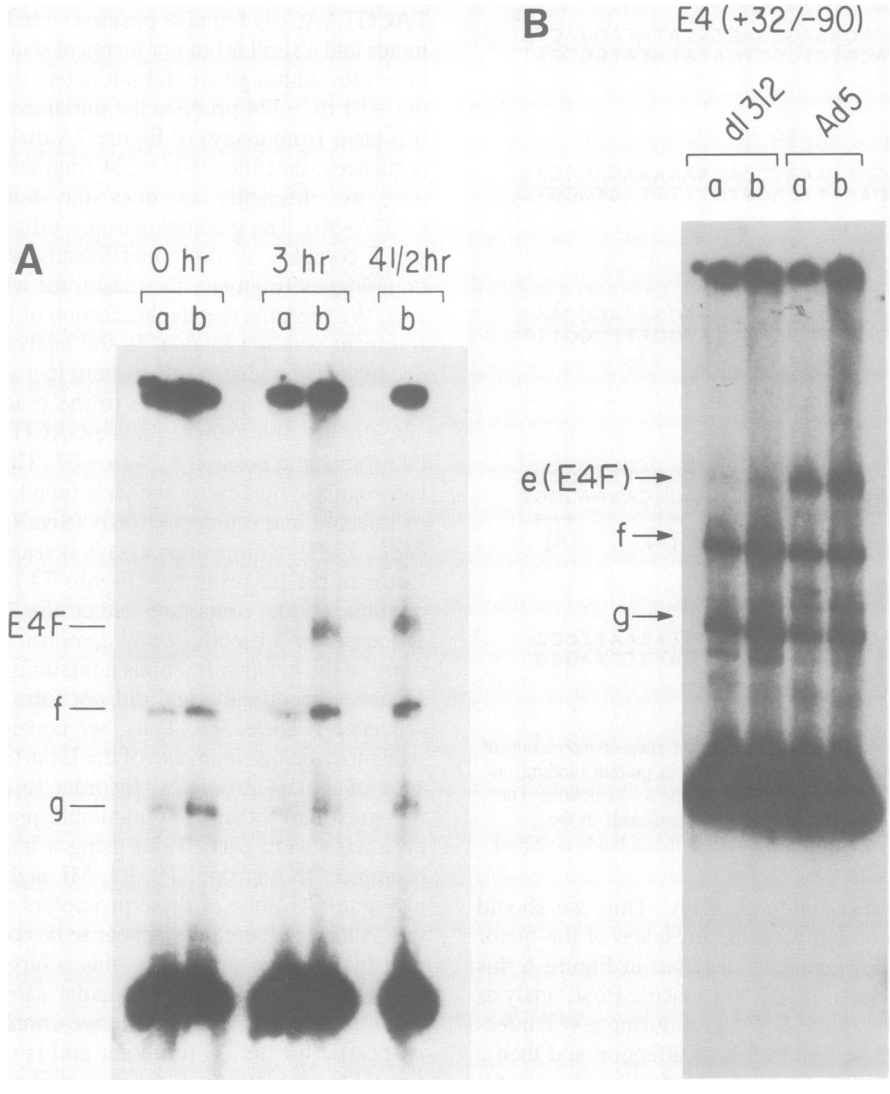

Fig. 6. Characterization of E4F induction. **A.** Time course of induction of E4F. HeLa whole cell extracts were prepared at 0 h, 3 h and 4.5 h post-infection from cells infected with wild-type Ad5, as described in Materials and methods. 15 mg of each of the extracts were fractionated stepwise by heparin-agarose chromatography as described in Materials and methods. 3 μl (**a**) and 7 μl (**b**) of 0.4 M heparin-agarose eluates were assayed for E4F activity by gel retardation assay using the +32/−90 probe. **B.** Induction of E4F requires E1A gene products. Whole cell extracts were prepared from cells infected with either dl312 or WT Ad5. Extracts were fractionated stepwise by heparin-agarose chromatography as described in Materials and methods. 3 μl (**a**) and 7 μl (**b**) of 0.4 M eluates were analyzed for E4F activity using the E4 (+32/−90) probe.

with the transcription of the E2 gene within the cell (Reichel *et al.*, 1987; Kovesdi *et al.*, 1987; Reichel *et al.*, submitted). Based on the results presented here, we suggest that the E4F factor plays an analogous role for E4 transcription. We find that the level of E4F, again as measured by DNA binding, rises substantially as a function of E1A from a low level in extracts of uninfected or dl312-infected cells to much higher levels in extracts of Ad5-infected cells. In addition, previous experiments have shown that the upstream binding site(s) homologous to the E4F site are critical for E1A-induced transcription and can confer E1A stimulation to a linked promoter (Gilardi and Perricaudet, 1984; Lee and Green, 1987). Based on our competition data and the fact that the −43/−54 site can functionally substitute for the upstream sites (Lee and Green, 1987) we conclude that the E4F binding site is indeed crucial for E1A control of E4 transcription and that E4F mediates this process. Finally, a number of other proteins were found to interact with the E4 promoter, but none of these were altered as a function of E1A. Thus, these proteins may be important for E4 transcription but are unlikely to be the limiting factors involved in regulation.

Based on the results of recent experiments, Lee and Green (1987) came to a rather different conclusion than our own. These authors reported the identification of a cellular factor termed E4F1 that recognized the same sequences to which E4F binds but which did not change upon infection. Given the absence of a change of DNA binding, they suggested two possible mechanisms by which E4F1 could be involved in E1A control of E4 transcription. One would involve negative control whereby the action of E1A eliminated a repressor thus allowing E4F1 to act. Alternatively, they suggested a role for E1A in redistributing cellular factors, in this case releasing E4F1 from other sites making it available for E4. Thus, the total amount of the factor would not change but rather the amount of available factor would increase. Our data provide an alternative view and suggest that, although E4F1 may bind to the E4 promoter, it does not necessarily do so inside the cell since there are other factors which also can bind to the same sequences. The differences between our results and those of Lee and Green raise two issues. First, why have

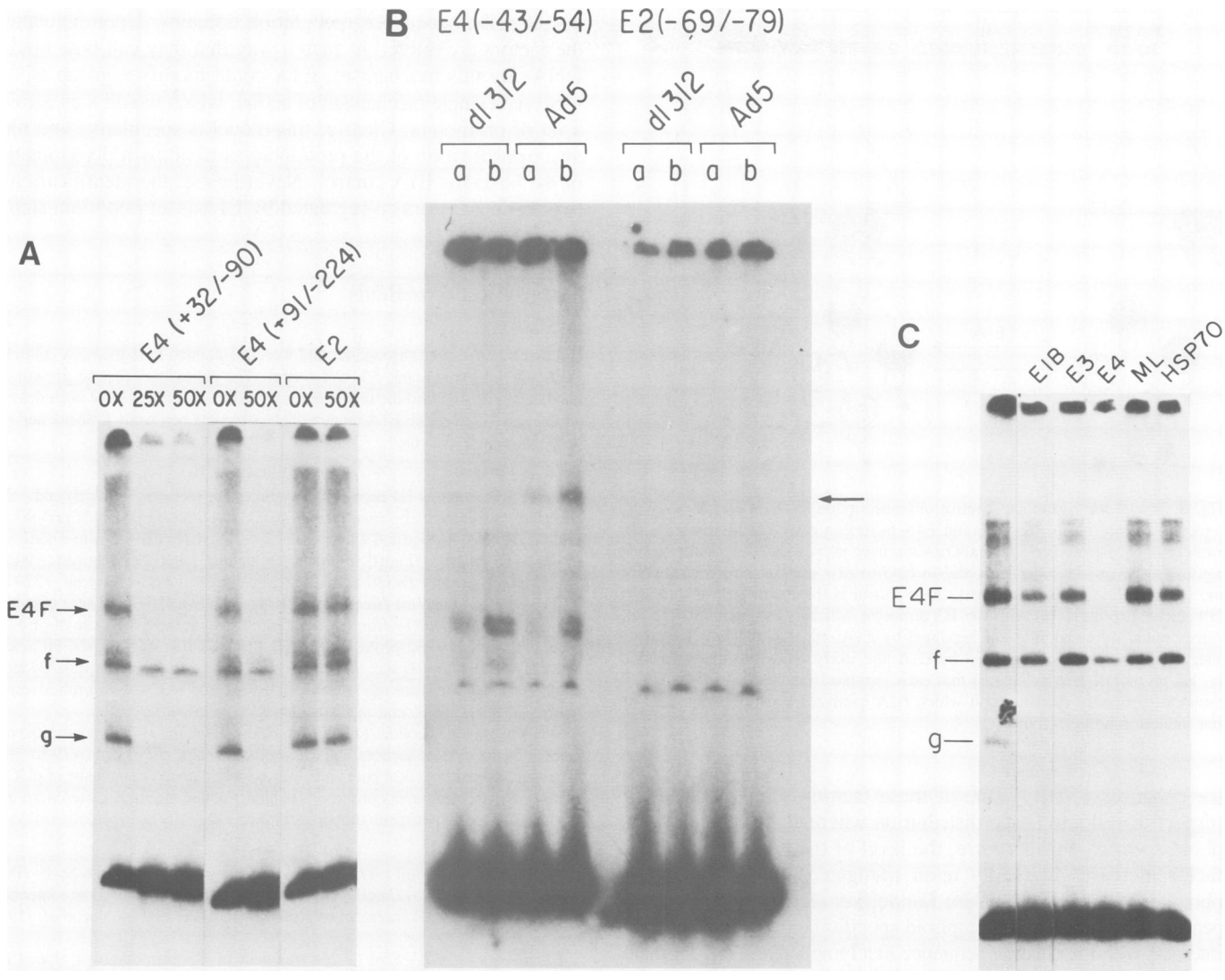

Fig. 7. Binding of E4F is specific to the E4 promoter. **A**. Competition assay for E4F interaction with E4 and E2 promoters. 7 μl of 0.4 M heparin-agarose fraction of Ad5-infected HeLa cell extract and end labeled E4 (+32/−90) probe was used to assay for E4F binding. Preparation of competitor DNA is described in Materials and methods. 25- or 50-fold molar excess of the competitor DNA, as indicated in the figure, were included in the binding assays. **B**. Assays for binding to the related E4 and E2 sites. E4 (−43/−54) is an oligonucleotide of the E4F binding site (E4 promoter sequence from position −43 to −54) with flanking sequences from the pUC19 polylinker region. E2 (−69/−79) is an oligonucleotide containing the binding site (SivaRaman *et al.*, 1986; Yee *et al.*, 1987) in E2 promoter (sequences from position −69 to −79) with flanking sequences from pUC19 polylinker regions. Preparation of probe E4 (−43/−54) and E2 (−69/−79) is described in Materials and methods. Extracts for binding were those described in Figure 6B. **C**. E4F is specific for the E4 promoter. 5 μl of pooled heparin-agarose fraction, as described in the legend to Figure 2B, were used in E4F binding assays along with unlabeled competitor DNA as indicated in the figure. Preparation of competitor DNA is described in Materials and methods. 100 ng of each competitor DNA was used in this experiment.

we detected a factor apparently not observed in the assays of Lee and Green? Two possibilities are likely explanations. Their analyses involved direct footprint assays in which an abundant factor would likely preclude the assay of a less abundant factor. Indeed, we were only able to visualize the E4F factor after fractionation of extracts and by employing small probes for binding. In addition we have utilized whole cell extracts rather than nuclear extracts as used by Lee and Green. In fact, we have not detected the E4F factor in nuclear extracts suggesting that it is not efficiently extracted under the standard conditions.

Second, given this complexity, how can one decide which is the authentic interaction? We suggest that if DNA binding is the only criteria then it is not possible to say that a given factor that binds *in vitro* actually does so *in vivo*. In fact, even the demonstration that a binding activity is a transcription factor that is able to stimulate the promoter *in vitro*, still does not mean that the factor functions in this way *in vivo*. Other information must support this suggestion. In our view, a correlation between the presence of a factor (or relative levels) in a cell free extract and the rate of transcription of the gene to which the factor binds provides compelling evidence that the factor is involved in the transcription of the gene. Furthermore, if this correlation exists for more than one predicted circumstance, as is the case here for E4F (increased as a function of E1A and the kinetics of activation coincide with transcription), then the probability that the protein is involved becomes significantly greater. Such is the case for E4F as is true for the E2 binding protein E2F. A quite similar circumstance exists for the enhancer of the immunoglobulin heavy chain transcription unit. Two factors have been found to bind to the octamer sequence which is known to be a critical sequence

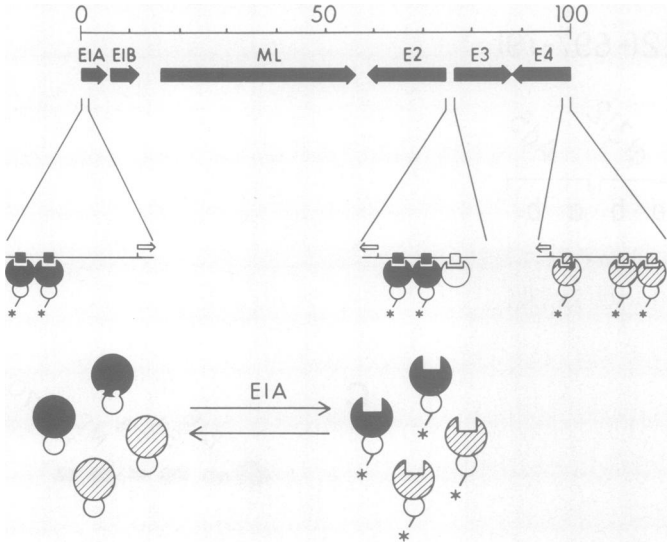

Fig. 8. Model for coordinate control of transcription by E1A. Schematic depiction of the role of the E2F and E4F factors in early adenovirus transcription control. Previous experiments have shown the E2F factor (solid figures) to be regulated by E1A and to bind to two sites in the E2 promoter and two sites in the E1A enhancer. Also shown is the interaction of the EPF factor (open figure) with the E2 promoter which is essential for E2 transcription but apparently not regulated by E1A. Finally, the E4F factor (hatched figures) is also regulated by E1A and likely binds to three sites in the E4 promoter. It is postulated that each regulated factor possesses a common regulatory domain upon which E1A catalyzes a modification (*) that induces binding activity.

for enhancer activity. One of these factors is ubiquitous with respect to cell and tissue distribution whereas the other is lymphoid specific. Furthermore, the level of the lymphoid specific factor increases markedly upon mitogen stimulation of lymphocytes (Staudt *et al.*, 1986; Landolfi *et al.*, 1986). It thus appears likely that it is the lymphoid specific factor which actually interacts with the octamer sequence in a lymphocyte. The ultimate proof that a given factor interacts with a sequence within the cell and is responsible for the stimulation of transcription of the gene is a genetic approach that demonstrates that the presence of the factor is essential for transcription. Experimentally, this is approachable in yeast (gene disruption), but in mammalian cells this becomes a difficult task. Short of this, a correlation between the level of a factor in extracts and transcription of the gene provides compelling evidence for a link between the two.

If these factors are mediating E1A control of the E4 promoter (E4F) and the E2 promoter and the E1A promoter (E2F), then how are they coordinately controlled? In the case of E2F we have recently shown that the increase in level of active E2F does not require new protein synthesis and we thus conclude that E1A likely mediates a modification of a pre-existing pool of inactive factor (Reichel *et al.*, submitted). We do not know if the same is true for E4F because the experiment is complicated by the very rapid activation of E4F. That is, cycloheximide must be added after sufficient E1A protein is made but before there is any significant rise in E4F. The timing of this experiment has proved difficult for E4F since the level is already rising by 1.5 h post-infection whereas E2F did not begin to increase until 4–5 h. However, assuming that the increase in E4F is due to a modification of a pre-existing factor, a likely possibility given the rapid activation as well as analogy to E2F, we would speculate that coordinate control could be achieved in a manner as depicted in Figure 8. We suggest that E2F and E4F might possess common regulatory domains, that are the site of action of E1A, but distinct DNA sequence recognition domains. Upon activation, the factors are altered in such a way that they can now bind to DNA. By this mechanism, E1A could modify a group of factors, via a single mechanism of action, which would then activate a set of promoters. Clearly, this model is speculative and must await purification of the protein so as to allow a determination of the basis for E1A control. Nevertheless, the identification of two distinct factors co-regulated by E1A is an important step in this direction.

Materials and methods

Cells and virus

HeLa cells were used throughout and were maintained in suspension culture in MEM (Joklik) containing 5% calf serum. The procedures for the growth and purification of Ad5 and the methods for infection of cultures have been described (Nevins, 1980). The E1A mutant dl312 was obtained from T.Shenk and was propagated in 293 cells.

DNA and probes

Plasmid pE4 contains E4 promoter sequences betweeen +32 (*Taq*I) and −224 (*Hph*I) cloned into *Acc*I−*Sma*I sites of pUC13. To obtain plasmids pE4-90 and pE4-224, pE4 was digested either with *Fnu*dII and *Hin*dIII or with *Fnu*dII and *Eco*RI. The *Fnu*dII−*Hin*dIII fragment (containing sequences between −90 and +32) and the *Eco*RI−*Fnu*dII fragment (containing sequences between −224 and −91) were cloned into the *Hinc*II−*Hin*dIII sites and *Hinc*II−*Eco*RI sites of pUC13 respectively. An *Eco*RI−*Hin*dIII fragment labeled at the *Eco*RI site of pE4 was used as the −224/+32 probe. A *Bam*HI−*Hin*dIII fragment of pE4-90 labeled at the *Hin*dIII or *Bam*HI site, and an *Eco*RI−*Hin*dIII fragment of pE4-224 labeled at an *Eco*RI site were used as the −90/+32 and −224/−91 probes respectively. DNAs were 3′ end labeled with Klenow enzyme.

Synthetic oligonucleotides (double-stranded) containing E4 promoter sequences from −54 to −43 sequences and E2 promoter sequences from −79 to −69, each with *Bam*HI linkers at both ends, were cloned into the *Bam*HI site of pUC19. *Eco*RI−*Hin*dIII fragments of these plasmids, labeled at *Hin*dIII sites were used as probe in the experiment shown in Figure 7B.

Competitor DNAs

E1B: *Hpa*I (−128)−*Sal*I (+72) promoter fragment was cloned into *Sac*I−*Hinc*II site of pUC19. *Eco*RI−*Hin*dIII fragment of this plasmid was used as competitor.

E2: *Eco*RI−*Hin*dIII fragment of plasmid pE2 described by Yee *et al.* (1987) was used.

E3: *Eco*RI (−237)−*Sca*I (+25) fragment of E3 promoter was cloned into pUC13 at *Eco*RI and *Sac*I sites. A *Rsa*I (−125) to *Bam*HI (in vector) fragment was subcloned into *Sma*I and *Bam*HI sites of the Bluescript plasmid. An *Eco*RI−*Sma*I fragment of this recombinant plasmid was used as E3 competitor DNA.

Hsp70: *Sac*I−*Sac*I fragment (−74 to +140) was cloned in pUC13 (kind gift of Dr C.Simon). An *Eco*RI−*Hin*dIII fragment of this plasmid were used as competitor.

E4: *Eco*RI−*Hin*dIII fragment of pE4 (containing E4 promoter sequence from −224 to +32). *Bam*HI−*Hin*dIII fragment of pE4-90 (containing E4 promoter sequence from −90 to +32) and *Eco*RI−*Hin*dIII fragment of pE4-224 (containing E4 promotor sequences from −224 to −91) were used as competitor DNAs.

ML: promoter sequences between *Xho*I (−280) and *Rsa*I (+45) restriction sites was cloned into *Sal*I−*Sma*I sites of pUC13. An *Eco*RI−*Hin*dIII fragment of this recombinant plasmid was used as competitor.

Preparation of whole cell extracts

Whole cell extracts were prepared from virus-infected or mock infected HeLa cells following a procedure described by Hoeffler and Roeder (1985) with certain modifications. HeLa cells were harvested by centrifugation at 1500 *g* for 15 min. Cell pellets were washed two times with 10 volumes of cold PBS. Cells were then resuspended in 1.5 packed cell volumes of hypotonic buffer (10 mM KCl, 10 mM Hepes pH 7.9, 1.5 mM MgCl, 0.5 mM DTT, 1 mM PMSF, 0.4 mM NaF and 0.4 mM sodium orthovanadate) and allowed to swell for 20 min on ice. Lysis was achieved with 35 strokes of a Dounce homogenizer. The cell lysate volume was measured and to this lysate was added 1.66 volumes of high salt buffer [1.6 M KCl, 20 mM Hepes, pH 7.0, 0.2 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.4 mM NaF, 0.4 mM sodium orthovanadate and 20% (v/v) glycerol]. The tubes containing this lysate were then placed on a tilt shaker for 30 min at 4°C and then centrifuged at 100 000 *g* for 60 min. The upper lipid layer was discarded and the clear supernatant was dialyzed against a large excess of buffer containing 50 mM KCl, 20 mM Hepes pH 7.0, 0.1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.4 mM NaF, 0.4 mM sodium orthovanadate and

10% glycerol for 90 min. The dialyzed material was centrifuged in Corex tubes at 9000 r.p.m. in SS34 rotor and the clear supernatant was stored at −70°C. From 1 l of spinner cell culture (6×10^5 cells/ml), 30 mg of protein at a concentration of 5 mg/ml was obtained. Ionic strength of the extract was equivalent to that of 0.15 M KCl.

Heparin-agarose fractionation of whole cell extracts

Heparin-agarose (Sigma) columns (1.5 ml) were equilibrated with buffer A [20 mM Hepes pH 7.9, 0.2 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF and 10% (v/v) glycerol] + 0.1 M KCl. Whole cell extracts (30 mg) were diluted 1.5-fold using buffer A minus KCl and slowly applied to the columns. The columns were then washed with 15 ml of buffer A + 0.1 M KCl. For gradient elution, 21 ml total volume of linear gradient from buffer A + 0.1 M KCl to buffer A + 0.7 M KCl were applied and 0.5 ml fractions were collected. E4F activity eluted from this column when ionic strength of this column buffer reached that of 0.32 M KCl. For step elution, columns were successively washed with 15 ml of buffer A + 0.25 M KCl and 15 ml of buffer A + 0.4 M KCl. 0.5 ml fractions were collected. The A_{280} peak fractions (fraction number 3−7) from 0.4 M wash were pooled and analyzed for E4F activity.

Gel retardation assay

The assays (35 μl total volume) were carried out in buffer containing 40−60 mM KCl, 20 mM Hepes pH 7.6, 1 mM $MgCl_2$ and 1 mM EGTA. The reaction mixes also contained 0.4−0.6 ng of 3′ end labeled DNA probes and 5 μg of poly(dIdC)−poly(dIdC) (Pharmacia). Reactions were initiated by adding heparin-agarose fractions and incubation was carried out at room temperature for 20 min. At the end of incubation 5 μl of 20% Ficoll solution was added to the reaction mixes and an aliquot of each reaction mixture was applied to 4% polyacrylamide gels. Electrophoresis was carried out as described by Yee *et al.* (1987).

Methylation interference analysis

Methylation interference analysis was performed essentially as described by Staudt *et al.* (1986) with the following modifications. DNA fragments were labeled at one of the 3′ ends using Klenow reaction. Dimethyl sulfate reaction was carried out for 5 min and this reaction was stopped by adding 1.5 M sodium acetate pH 7.0, 1.0 M 2-mercaptoethanol and 100 μg/ml poly(dIdC)−poly(dIdC). DNA was precipitated twice with ethanol, washed with 70% ethanol, dried and dissolved in the desired volume of TE. Five-fold scaled-up binding reactions with partially methylated DNA and heparin-agarose fractions were resolved by 4% polyacrylamide gel electrophoresis. Shifted bands (bound DNA) and free DNA were isolated by crushing the gels. The DNA was purified and heated with piperidine and then analyzed in 6% acrylamide−urea sequencing gels.

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