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Cooperative binding of two E2F molecules to an EIa-responsive promoter is triggered by the adenovirus EIa, but not by a cellular EIa-like activity

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The binding of the cellular E2F transcription factor to the central EIa-responsive element of the adenovirus EIIa early promoter (EIIaE) was compared in extracts of HeLa cells which had been infected with either wild-type adenovirus or the EIa-deficient mutant dl312. No quantitative differences in the E2F-binding activity were detected as a function of EIa gene expression. However, complexes formed by the E2F factor in the presence of EIa were qualitatively different from those formed on the same sequence element in the absence of EIa. Specifically, the formation of complexes containing two E2F molecules is favoured by EIa, probably through the induction of protein–protein interactions. Protein binding to EIIaE promoter in extracts from non-infected F9 embryonal carcinoma cells, prepared before and after *in vitro* differentiation of these cells was also analysed. The higher expression of EIIaE in undifferentiated cells, which was originally attributed to a cellular EIa-like function, may be correlated with the increased binding activity of a murine E2F-like protein which does not, however, result in the simultaneous occupation of both E2F sites on the EIIaE promoter, suggesting that the viral EIa and the presumptive cellular EIa-like functions *trans*-activate the EIIaE promoter through different pathways.

Key words: EIa *trans-* activation/DNA–protein interaction/protein–protein interaction/E2F/ATF/teratocarcinoma

Introduction

The understanding in molecular terms of the mechanisms underlying coordinate gene regulation in eukaryotic cells is a major goal of molecular biology. The control of viral and cellular promoters mainly involves the interaction of *trans*-acting proteins with specific DNA sequence elements, leading to positive or negative transcriptional effects. In particular, considerable amounts of information have been accumulated about the promoter structure and function of cellular genes which respond to exogenous stimuli, e.g. heat-shock, heavy metals, metabolites or hormones [for reviews see Guarante (1987), Maniatis *et al.* (1987), Evans (1988), Jones *et al.* (1988), Roesler *et al.* (1988) and Sorger and Pelham (1988)]. In each case transcriptional induction is mediated by the binding of activated transcription factors to specific recognition sequences.

Transcriptional activation of the adenovirus early genes by the viral immediate-early EIa gene products (Nevins, 1981; Shaw and Ziff, 1982) provides an alternative model system for the study of eukaryotic gene induction. The mechanism by which the EIa proteins mediate their effects appears, however, quite distinct in that it does not involve a unique promoter element. Extensive mutational analyses of the inducible viral promoters revealed that every sequence alteration which decreased EIa-responsiveness also reduced constitutive promoter activity (Berk, 1986). These observations suggested that the same host cell promoter-binding factors were used for both uninduced and EIa-induced transcription. Rather than binding themselves to a specific DNA sequence (Ko *et al.*, 1986), the EIa proteins may therefore interact with or modify some of these factors.

EIa-mediated *trans*-activation of the adenovirus EIIa early (EIIaE) promoter is mediated by a central EIa-responsive element, including both E2F-binding sites, together with elements located either further downstream or upstream (Zajchowski *et al.*, 1987). Previous DNA binding and protection experiments have indicated that the specific binding activities of the three distinct cellular proteins, ATF (formerly called EIIaE-EF or EIIaE-B), Cα and Cβ, were not affected by the EIa gene products in HeLa cells (Siva Raman *et al.*, 1986; Boeuf *et al.*, 1987; Jalinot *et al.*, 1987). On the other hand, Nevins and coworkers (Kovesdi *et al.*, 1986; Yee *et al.*, 1987) reported increased binding of the E2F protein to the EIIaE promoter, due to expression of the EIa products.

We show here that identical overall binding activities of the E2F protein are detected in the absence and presence of the viral EIa products. However, expression of EIa favours the simultaneous binding of E2F molecules to both of its recognition sites, suggesting that the EIa-mediated activation of this promoter is linked, at least in part, to the cooperative formation of E2F dimers on the EIIaE promoter.

We also comparatively examined protein binding on the EIIaE promoter, as a function of gene activity, in mouse embryonal carcinoma (EC) cells, before and after differentiation. In undifferentiated F9 EC cells early adenovirus promoters, including EIIaE, are indeed effectively expressed in the absence of viral EIa products, while after differentiation their expression becomes strictly dependent on the presence of viral EIa. This observation has led to the proposal that a cellular EIa-like activity specifically exists in undifferentiated F9 EC cells (Imperiale *et al.*, 1984). We recently found that the E2F-binding sites are also involved in this differential expression of EIIaE in F9 cells (H.Boeuf, P.Jansen-Durr and C.Kédinger, unpublished). Our present binding experiments reveal that the murine E2F homologue is present in undifferentiated as well as in differentiated F9 cells, but that the E2F-binding activity is reduced ~3-fold upon differentiation. In contrast to the viral EIa function, the endogenous EIa-like activity does not induce the formation of E2F dimers on the EIIaE promoter.

Results

To gain insight into the process of the EIa-mediated activation of the EIIaE promoter, which is primarily dependent on the E2F-binding sites located between −70 and −30, we investigated the specific interaction of proteins to this region, in the absence or presence of the EIa gene products. As a first approach we undertook a comparative gel-shift analysis of extracts from HeLa cells which had been infected with the wild-type adenovirus-5 (wt) or EIa-deleted derivative, dl312 (dl).

E2F binding to a single binding site is not changed by EIa expression

The overall E2F-binding activities of wt- and dl-infected cell extracts were compared by incubating identical amounts of protein with a synthetic oligonucleotide probe (1 × E2F, see Figure 1A) comprising only one of the two adjacent E2F-recognition sites of the EIIaE promoter. One major retarded band (I) is obtained, with equal intensity in both dl and wt extracts (Figure 1B). The specificity of this complex was demonstrated by competition experiments. Preincubation of the extracts with a 100-fold molar excess of the E2F competitor oligonucleotide (see Figure 1A) completely prevents labelled complex formation (Figure 1B, lanes 6 and 13). By contrast, the same amount of an oligonucleotide altered in both E2F-binding sites (E2Fm, see Figure 1A) has virtually no effect (Figure 1B, lanes 7 and 14).

Besides the major complex I, two minor complexes involving E2F-specific binding are also observed with intensities varying from one extract to another. While the nature of the more slowly migrating complex is at present unknown, we suspect that the faster-migrating complex is most likely due to degradation of E2F.

When the E2F binding activity in wt and dl extracts was titrated by adding increasing amounts of labelled probe, the quantity of the retarded complex at any given DNA/protein ratio was indistinguishable in both extracts. A rough estimation of the apparent concentration of E2F could be deduced from such titration experiments (see Jalinot *et al.*, 1987), leading to values of $\sim 2 \times 10^{-8}$ M E2F in both extracts (i.e. 1000 molecules of active E2F per cell). Essentially the same results were obtained with extracts from uninfected HeLa cells (Figure 2C), indicating that similar amounts of E2F are present in uninfected and adenovirus-infected cells.

Having established that there is no detectable change in binding activity to a single E2F-binding site, we next investigated the interaction of E2F with DNA fragments spanning larger portions of the EIIaE promoter.

Different nucleoprotein complexes are detected by DNase I footprinting of the EIIaE promoter, in the absence and presence of EIa

DNase I footprinting experiments were performed with crude extracts from wt- or dl-infected cells on a EIIaE promoter fragment (P, see Figure 1A) spanning positions −87 to +62. To obtain detectable footprints, it was necessary to concentrate the standard crude extracts ~5-fold, to protein concentrations of at least 20 μg/μl. Under these conditions, a protection extending between −73 and −33, with a hypersensitive site at position −52, is detected with the wt extract (Figure 2A, lane 3). By contrast, only weak protections at positions −47 and −37 are observed with dl extracts. These results, indicating that DNase I-resistant complexes on the entire E2F-binding domain (−33 to −73 region) are formed with the wt extract only, are in apparent contradiction with those of the gel-shift experiments which revealed very similar E2F-binding activities in both extracts. To examine whether the E2F protein, present in cells which do not express the EIa products, is at all able to form DNase I-resistant complexes, we partially purified E2F from non-infected whole cell extracts (WCE, see Materials and methods) and assayed the footprinting activities of E2F-containing fractions. With the DEO.20 fraction, we detect a weak but significant protection over the −33 to −73 region. Since we have recently shown that the Cα protein which binds an element located at −110 to −120 [see Jalinot *et al.* (1987) and Figure 1A] has a weak affinity for the

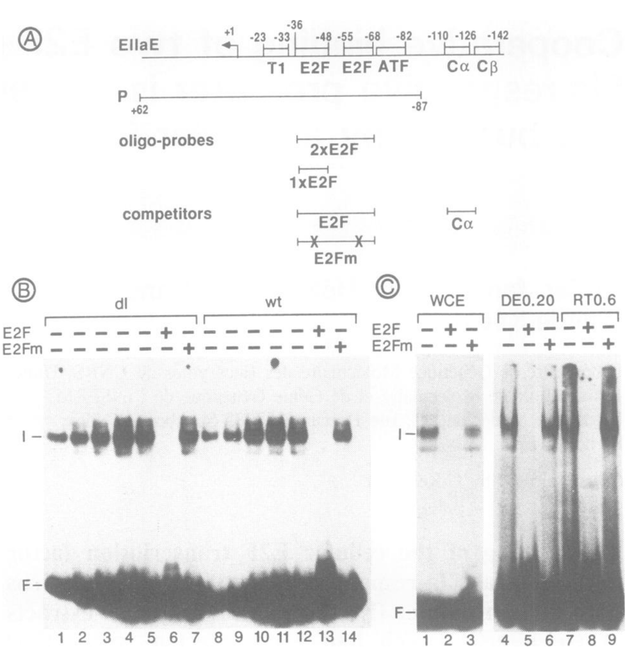

Fig. 1. E2F-binding activity on a single E2F-binding site. (**A**) The structure of the EIIaE promoter region is depicted, with the positions (relative to the major start site, +1) of the critical promoter elements and cognate factors [derived from Jalinot *et al.* (1987) and Jones *et al.* (1988)]. The promoter fragment P used in footprint experiments comprises EIIaE sequences between −87 and +62, flanked by linker DNA sequences (not shown), depending on its origin (see Materials and methods). Oligo-probes used in gel-shift experiments are double-stranded synthetic oligonucleotides spanning one (1 × E2F, −31 ACTAGTTTCGCGCGCTTTCT −50) or two E2F (2 × E2F, −35 GTTTCGCGCCCTTTCTCAAATTTAAGCGCGAAAA −68) binding sites. Oligonucleotides used as specific competitors in the binding reactions span positions −36 to −68 (E2F, identical to 2 × E2F), and −106 to −126 (Cα) respectively. An oligonucleotide (E2Fm, −35 GTTTACTCAGATAACTCAAATTTAAGTACTAGAA −68) with sequences altered (underlined) at both E2F-binding site (×) was used as non-specific competitor. (**B**) Gel-shift experiments were carried out as described in Materials and methods, using the 5′ end labelled 1 × E2F oligonucleotide, 3 μg of either dl-infected (dl) or wt-infected (wt) cell extracts in the presence of 1 μg poly(dA.dT). The amount of labelled probe was ~0.1 ng (**lanes 1** and **8**), 0.2 ng (**lanes 2** and **9**), 0.4 ng (**lanes, 3, 5–7, 10** and **12–14**) and 0.8 ng (**lanes 4** and **11**). Where indicated (+) the extracts were preincubated with 20 ng of the unlabelled E2F or E2Fm competitor oligonucleotides. F refers to unbound probe. I refers to the major E2F-specific complex. (**C**) Gel-shift experiments were performed with 3 μg of WCE from uninfected cells, 2 μl of the DEO.20 fraction or 2 μl of the RTO.60 fraction. Fractions were preincubated with a 100-fold molar excess of the E2F or the E2Fm oligonucleotide where indicated (+).

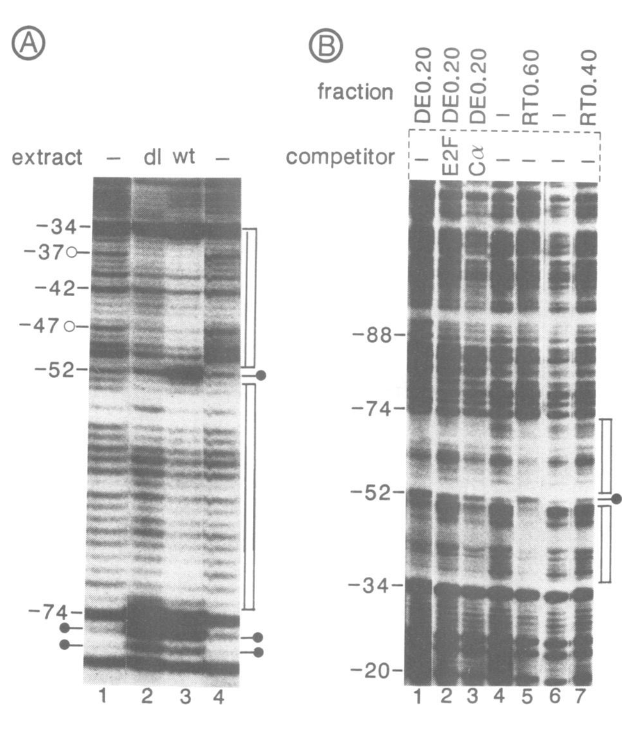

Fig. 2. Comparative DNase I footprinting of crude extracts and partially purified E2F fractions on the EIIaE promoter. (**A**) The *Eco*RI−*Pvu*II promoter fragment P (see Materials and methods), 3′ end labelled at the *Eco*RI site (transcribed strand) was incubated with 80 μg of either dl or wt extract and processed for DNase I protection analysis as described in Materials and methods. The results are shown in lanes 2 and 3 together with the digestion pattern of the naked probe (**lanes 1** and **4**). Protected regions are spanned by open boxes, single protected nucleotides are marked by open circles, hypersensitive sites are denoted by closed circles. Markings on the left and right correspond to the pattern obtained with dl and wt extracts respectively. Coordinates are given with respect to the major EIIaE start site. (**B**) Four microlitres of a 30-fold concentrated aliquot of the DEO.20 fraction (**lanes 1−3**) or 4 μl of a 5-fold concentrated aliquot of the RTO.60 (**lane 5**) or RTO.40 (**lane 7**) fractions (see Materials and methods and Figure 1C) were incubated with the *Eco*RI−*Hin*dIII promoter fragment P, 5′ end labelled at the *Hin*dIII site (transcribed strand). In **lanes 2** and **3** the DEO.20 fraction was preincubated with 20 ng of E2F or Cα competitor oligonucleotides (see Figure 1A) respectively. **Lanes 4** and **6** correspond to naked probe analysis. The reactions were processed and the results presented as in panel (A). Markings on the right refer to protection seen in **lanes 1, 3** and **5**.

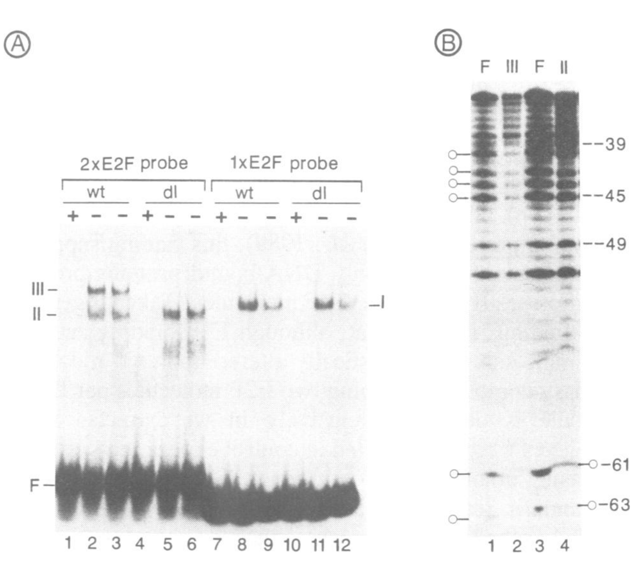

Fig. 3. Characterization of the E2F-specific complexes. (**A**) The 5′ end labelled 2 × E2F (**lanes 1−6**) or 1 × E2F (**lanes 7−12**) oligonucleotides were used in standard gel-shift assays, with 3 μg of either wt or dl extracts, in the presence of 1.2 μg (**lanes 3, 6, 9** and **12**) or 0.6 μg (the other lanes) of poly(dA.dT) as non-specific competitor. Where indicated (+), 20 ng of unlabelled E2F competitor oligonucleotide (see Figure 1A) were added to the binding reaction. I, II and III refer to specific nucleoprotein complexes discussed in the text. F denotes the unbound probe. (**B**) Complexes corresponding to bands II and III in panel (A) (**lane 2**) and unbound probe (F) were excised from a preparative retardation gel run with a DMS-treated 2 × E2F probe 5′ end labelled on the non-transcribed strand. After purification the corresponding DNA was cleaved at both methylated A and G residues and analysed as described in Materials and methods. Residues whose methylation interfered with complex formation are marked by open circles, on the left for complex III and on the right for complex II. Coordinates are given with respect to the EIIaE major start site. The same result (not shown) was obtained when band II was excised from the gel corresponding to lane 5 in panel (A).

−30 to −70 element as well (unpublished data) it was important to show that the observed footprint obtained with concentrated fractions is not due to binding of Cα to the low-affinity binding site. Competition for the footprint with the E2F-binding site, but not an oligonucleotide containing the Cα-binding site, reveals that E2F binding indeed produces the specific protection. This result was confirmed by the finding that the RTO.6 fraction, which no longer contains the Cα protein (not shown), gives rise to a very clear E2F-specific footprint, indistinguishable from that obtained with the crude extract from wt-infected cells. We conclude therefore that the failure to detect E2F-specific DNase I protections on fragment P in the absence of the EIa products is indeed not due to an intrinsic inability of E2F to bind to its recognition sites, but to a lower resistance of these complexes against DNase I digestion. Since the promoter fragment used for the footprinting experiments contains two adjacent E2F-binding sites, one reason for the conflicting results might be that EIa does not change the interaction of E2F with a single binding site, but stabilizes the complex formed on the promoter by two separate E2F proteins.

Gel-shift assays with a probe comprising two E2F-binding sites reveal EIa-dependent, cooperative binding of E2F

To test the hypothesis that EIa favours the simultaneous binding of two E2F molecules to the EIIaE promoter, comparative gel-shift assays were performed with oligonucleotides comprising either a single E2F-binding site (1 × E2F) or both E2F sites (2 × E2F, see Figure 1A) in their natural orientation. The results of this experiment are striking: while as expected from Figure 1 identical complexes are formed with wt extracts and dl extracts (band I) with the 1 × E2F oligonucleotide (Figure 3A, lanes 7−12), a slower-migrating complex (complex III) is uniquely detected with wt extracts (lanes 1−3), in addition to another complex (complex II) obtained with both extracts on the 2 × E2F probe (lanes 1−6). Since in this case two distinct E2F-binding sites are present, complex III could correspond to DNA molecules simultaneously bound by two E2F molecules. This interpretation was confirmed by DMS-interference. In complex III the G residues at positions −41, −43, −44, −45, −61 and −63 (on the non-transcribed strand) are specifically undermethylated, indicating that both E2F-binding sites are occupied (Figure 3B, lane 2). In complex II the G residues at −61 and −63 are protected, indicating predominant occupancy of the distal (relative to the start site) E2F-binding site in this complex (lane 4). When we analysed the proteins present in complexes II and III by a preparative gel-shift assay followed by UV crosslinking (Cereghini *et al.*, 1988), we obtained DNA−protein adducts of an apparent mol. wt of 70 kd from both complexes, indicating that a protein of ~55 kd is present in each case (not shown). Since it has recently been reported that E2F is a 54 kd protein (Yee *et al.*, 1989), this finding supports the conclusion that the only DNA-bound proteins present in complexes II and III are E2F molecules. Taken together, these findings indicate that, although E2F-binding activity to a single site is not drastically altered upon wt infection, a ternary complex containing two E2F molecules per DNA molecule is detected exclusively in wt extracts. Such complexes were not detected in control experiments, where increasing amounts of protein from dl extracts were used in standard retardation assays with a fixed amount of labelled probe (data not shown). Since gel-shift experiments are performed under conditions of large probe excess, essentially bimolecular protein−DNA complexes should be revealed in the absence of protein−protein interactions. Furthermore, control experiments (not shown) indicated that both complexes II and III were competed with very similar efficiencies by oligonucleotides comprising either the distal or the proximal E2F-binding site alone. This observation rules out the formal possibility that, in wt extracts, E2F exhibits a higher intrinsic affinity than in dl extracts for its distal binding site. It is likely therefore that the formation of the ternary complexes observed on the 2 × E2F oligonucleotide with wt extracts is favoured by interactions between the E2F molecules, probably due to an EIa-dependent modification of E2F, leading to their cooperative binding to the promoter.

The additional faster-migrating complexes seen in Figure 3A (lanes 1−6) may correspond to degradation products of E2F since they are specifically competed by the E2F oligonucleotide.

The EIa-like activity in undifferentiated F9 embryonal carcinoma cells does not favour E2F dimerization on the EIIaE promoter

To gain insight into the mechanism underlying the modulation of EIIaE expression by the EC cell-specific EIa-like activity, we analysed binding of factors to the E2F sites in extracts from either F9 EC cells or EC cells that had been induced to differentiate by retinoic acid and cAMP [F9(RA+cA)]. Gel-shift assays using the oligo probe 1 × E2F (Figure 4A, lanes 1−10) revealed one major E2F-specific complex (A). Titration of the E2F-specific activity with increasing amounts of labelled probe revealed that, depending on the particular extract, 3- to 5-fold more E2F-binding activity is detected in extracts from undifferentiated F9 cells, as compared to their differentiated derivatives. In some experiments (not shown), the E2F-specific complex obtained with extracts from differentiated cells migrates slightly faster than that obtained with extracts from undifferentiated F9 cells. Since this is not reproducibly observed with all extract preparations, we believe that this difference most likely reflects partial E2F degradation, rather than two different forms of the factor. On the other hand, it is not excluded that the preferential lability of E2F in extracts from differentiated cells may be due to a particular modification of the protein induced during the differentiation process. This has not been further investigated.

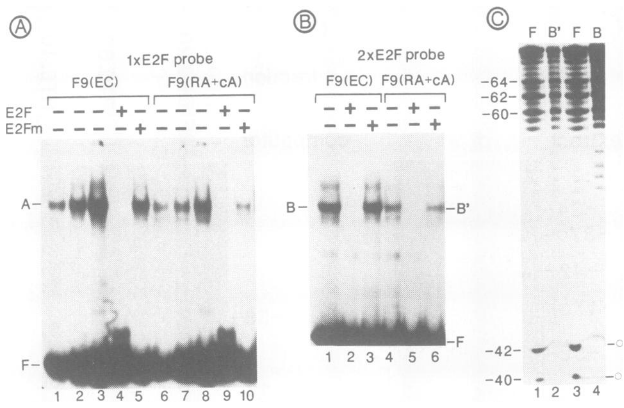

Fig. 4. Comparative analysis of specific nucleoprotein complexes formed on the E2F-specific oligonucleotides with extracts from undifferentiated and differentiated F9 cells. (**A**) The 5′ end labelled 1 × E2F (0.3 ng in **lanes 1** and **6**, 0.6 ng in **lanes 2, 4, 5, 7, 9** and **10** or 1.2 ng in **lanes 3** and **8**) oligonucleotide was incubated in standard gel-shift reactions with 3 μg of F9 EC (**lanes 1−5**) or F9(RA+cA) (**lanes 6−10**) cell extracts, 1 μg poly(dA.dT), without (−) or with (+) 20 ng of the E2F or E2Fm competitor oligonucleotides. 'A' refers to E2F-specific complexes. (**B**) The 5′ end labelled 2 × E2F oligonucleotide (~0.6 ng) was incubated under standard gel-shift conditions as in panel (A). (**C**) Complexes corresponding to bands B and B′ in panel (B) (**lanes 1** and **3** respectively) and unbound probe (F) were excised from a preparative band-shift assay run with a DMS-treated 2 × E2F probe. After purification, the corresponding DNA sequences were cleaved at methylated G residues and analysed as described in Materials and methods. Open circles denote residues whose methylation interferes with formation of complexes B and B′. Coordinates are given relative to the promoter major start site.

Essentially the same result was obtained with the 2 × E2F oligo probe (Figure 4B, lanes 1−6). One major retarded band (B, B′) is produced by extracts from undifferentiated or differentiated F9 cells, with 3- to 5-fold higher intensities in the presence of EC cell extracts (cf. lanes 1−3 and 4−6). That this band, like band A in Figure 4A, corresponds to an E2F-specific complex is demonstrated by its disruption only by a competitor oligonucleotide with intact E2F-binding sites. DMS-interference analysis of the complexes, formed on the 1 × E2F and 2 × E2F oligonucleotides respectively, indicates that only one, mainly the proximal E2F site, is occupied, as judged from the protection on positions −40 and −42, in each case (Figure 4C). A slower-migrating, E2F-specific complex is also detected with the 2 × E2F probe in both extracts. It is most likely, however, that this minor complex does not correspond to the binding of two E2F molecules, since similar amounts of a low-mobility complex are also reproducibly observed with the 1 × E2F probe (in Figure 4A, see in particular lanes 3 and 8). Importantly, roughly the same proportion of a slower-migrating complex is detected with extracts prepared from differentiated F9 cells (Figure 4B, seen in lanes 4 and 6 after longer exposure), indicating that the appearance of this variant E2F is not linked to the undifferentiated phenotype. Finally, DMS-interference analysis revealed that, also in these complexes, only one binding site is occupied as in the major complexes (not shown). Although the nature of these marginal complexes is presently unknown, they may be due to a variant form of the murine E2F protein.

Altogether these results indicate that, whereas E2F-binding activity is readily detectable in both cell types, differentiation of F9 cells is accompanied by a significant reduction of E2F-binding activity. In contrast to the viral EIa-dependent alteration of E2F, the cellular EIa-like activity does not promote the cooperative binding of two E2F molecules on the EIIaE promoter.

Discussion

The DNA-binding properties of a factor, termed E2F, which interacts with the critical EIa-responsive element of the adenovirus EIIaE promoter has been examined. We show that the factor is present in uninfected HeLa cells and that no change in its overall binding activity occurs in the presence of the viral EIa gene products. Our results indicate, however, that it undergoes an EIa-dependent modification which leads to the cooperative formation of stable complexes between two E2F molecules and their binding sites on the EIIaE promoter *in vitro*. We also show that a murine protein (the E2F-like protein) that binds to the E2F recognition sites is present in undifferentiated embryonal carcinoma cells. Interestingly, in these cells, where the EIIaE promoter is active, no ternary E2F–DNA complexes are detected. Upon differentiation of these cells, we detect a reduction of the E2F-binding activity, concomitant with the down-regulation of the EIIaE promoter. These results indicate that in F9 cells, unlike in HeLa cells, the active state of the EIIaE promoter is not correlated to the formation of ternary E2F–DNA complexes *in vitro*, but is associated with a higher E2F-binding activity. This suggests that activation of the EIIaE promoter by viral EIa may involve another pathway than activation by the endogenous EIa-like function.

In contrast to the findings of Kovesdi *et al.* (1986), the E2F protein is readily detected by gel-shift assays in extracts from dl-infected and uninfected HeLa cells. In fact, no change in E2F-binding activity occurs when the cells are infected with wt virus. Our results indicate, however, that E2F from wt-infected cells is modified to give rise to specific ternary complexes comprising one DNA molecule together with two E2F molecules. This conclusion is based on the following observations: (i) ternary complexes were formed on the 2 × E2F oligo probe only with wt extracts; and (ii) footprints spanning the two E2F-binding sites with strong hypersensitivity of the nucleotides in between, were generated only by wt extracts. This latter finding is in agreement with the EIa-dependent increase of E2F binding to the EIIaE promoter which has been repeatedly reported by others (Reichel *et al.*, 1988; Yee *et al.*, 1987), using either DNase I or exonuclease III protection techniques on an authentic promoter fragment comprising two E2F sites. On the other hand, we have no explanation for the failure of these authors to detect E2F binding in the absence of EIa by gel retardation assays. It is possible, however, that under their experimental conditions mainly complexes containing two E2F molecules are visualized, while monomeric E2F complexes would escape detection. The observed differences in E2F binding are not caused by an increased DNA-binding activity of the protein to its cognate site in the presence of EIa; rather, the specific nucleoprotein complex is strengthened by EIa-dependent interactions between adjacent E2F molecules. The cooperativity of E2F binding is most clearly attested by the requirement of both E2F sites (Figure 3) and by the higher stability of complex III, compared with complex II, when challenged by increasing amounts of unlabelled E2F oligonucleotides (not shown).

Our observation that complexes of identical mobilities were formed on the 1 × E2F probe, with both dl and wt extracts, clearly indicates that the E2F dimerization does not occur before binding to DNA, but only in the presence of both E2F-binding sites. It is tempting to speculate that EIa induces a modification of the E2F protein to promote dimer formation, as has been described for the CREB protein from rat brain, which undergoes dimerization on the cognate binding site only after phosphorylation (Yamamoto *et al.*, 1988). Interestingly, it has recently been shown that protein phosphorylation is involved in the EIa-dependent activation of the cellular transcription factor TFIIIC (Hoeffler *et al.*, 1988).

Transient expression studies with EIIaE promoter mutants carried out by several different laboratories (summarized in Zajchowski *et al.*, 1987) have revealed that EIa *trans*-activation of the EIIaE promoter requires essentially the −30 to −70 element. Since the modification of E2F described in this study represents the only detectable change of a promoter binding protein in extracts of cells where the EIIaE promoter is efficiently transcribed, we suggest that this alteration should at least in part account for the promoter activation. In contradiction with the present results, it has been concluded from a study on the EIa-responsiveness of the EIa promoter itself that a single E2F-binding site could confer EIa-dependent increased activity to a heterologous promoter (Kovesdi *et al.*, 1987). Close examination of the sequence of the EIa-promoter fragment used in the latter study reveals, however, clear homology not only to several E2F-binding sites, but also to an ATF-recognition sequence, further supporting the notion that several distinct promoter elements contribute to EIa-responsiveness.

It has been reported that down-regulation of the EIIaE promoter in differentiated F9 EC cells is accompanied by a decrease of protein binding to the E2F sites in extracts of these cells (La Thangue and Rigby, 1987; Reichel *et al.*, 1987). Whereas we detect a moderate but significant reduction in E2F-binding activity we have no explanation for the failure of Reichel *et al.* (1987) to detect any E2F-binding activity in differentiated F9 cell extracts. In this respect it is important to note that the differentiated phenotype of the F9(RA+cA) cells used in the present study, as well as the concomitant down-regulation of EIIaE-promoter activity, have been unambiguously established (Boeuf *et al.*, unpublished).

On the other hand, unlike in Ad-infected HeLa cells, no ternary E2F-specific complexes are formed on the 2 × E2F oligonucleotide with extracts from either undifferentiated or differentiated cells. Since in HeLa as well as in F9 cells the −30 to −70 element is critical for promoter activation (Boeuf *et al.*, unpublished), these findings suggest that the processes leading to activation of EIIaE by the viral EIa or the endogenous EIa-like functions are not the same.

Materials and methods

Cells and virus

HeLa cells, grown in suspension in Eagle minimal essential medium supplemented with 7% calf serum, were infected with adenovirus-5 (wt) or its EIa-defective dl312 derivative (dl) at 10 p.f.u./cell, and harvested 6 h post-infection, as described (Jalinot *et al.*, 1987). Mouse F9 embryonal carcinoma (EC) stem cells were grown on plates in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. F9 EC cells were induced to differentiate as described (Hogan *et al.*, 1986), by treatment with 0.1 μM retinoic acid and 1 mM dibutyryl cyclic AMP for 5 days [F9(RA+cA)].

Crude cell extracts and partially purified fractions

Whole cell extracts were prepared from uninfected, dl- or wt-infected HeLa cells or from F9 EC or F9(RA + cA) cells as described (Jansen-Durr *et al.*, 1988). Uninfected HeLa extracts were fractionated as previously described (Moncollin *et al.*, 1986; Zheng *et al.*, 1987; Jalinot *et al.*, 1988) by successive chromatography over hepatin−Ultrogel, DEAE−5PW and Red-Trisacryl. The E2F-binding activity, monitored by gel-shift assays (see Figure 1C), was eluted at 0.6 M KCl from the heparin column, at 0.2 M KCl from the DEAE column (fraction DEO.20) and at 0.6 M KCl from the Red-Trisacryl column (fraction RTO.60). On this latter column, E2F was separated from the Cα protein [see Jalinot *et al.* (1987) and Figure 1A], which eluted at 0.4 M KCl (fraction RTO.40) as determined by gel-shift analysis, using an appropriate probe (not shown).

Probes for gel-shift and footprinting experiments

Promoter fragment P comprises EIIaE sequences between −87 and +62 with or without 10 or 30 bp of unrelated flanking sequences, depending on its origin. It was either an *Eco*RI (position equivalent to −100)−*Pvu*II (+62) fragment from pMTE87 (Boeuf *et al.*, 1986), a plasmid which contains EIIaE sequences between −87 and +719, or an *Eco*RI (position equivalent to −100)−*Hin*dIII (position equivalent to +94) fragment from the pUC-based derivative (pE87) of pMTE87, where the *Eco*RI−*Pvu*II fragment of pMTE87 was subcloned into the *Sma*I site of pUC19. This promoter fragment P was 5′ end labelled by incubating the *Eco*RI or *Hin*dIII linearized recombinants with 80 μCi [γ-^{32}P]ATP and 20 U T4 polynucleotide kinase for 1 h at 37°C. After incubation the recombinants were recut with *Pvu*II or *Eco*RI respectively, and the labelled P fragment was purified by electrophoresis on a non-denaturing 7% polyacrylamide gel.

The two strands of oligonucleotides spanning the proximal (1 × E2F, −31 to −50) or both (2 × E2F, −36 to −68) E2F-binding sites of the EIIaE promoter (see Figure 1) were chemically synthesized. Their sequence corresponded to the EIIaE wild-type sequence, except for 1 × E2F where the G at −44 was changed to C to eliminate potential homology with the Cα sequence (gel-shift competition experiments confirmed specificity of this oligonucleotide for E2F but not Cα protein binding). These oligonucleotides were end labelled by incubating 2 pmol of either the transcribed or non-transcribed strand with 40 μCi [γ-^{32}P]ATP and 10 U T4 polynucleotide kinase for 30 min at 37°C. After heating the reaction for 10 min at 68°C, 2 pmol of the complementary, unlabelled strand was annealed to the labelled strand by successive incubation of the mixture for 10 min at 68°C, 15 min at 37°C and 15 min at 25°C. The double-stranded oligonucleotide was then purified by electrophoresis on a non-denaturing 20% polyacrylamide gel.

Gel retardation assay

Gel-shift assays were performed essentially as described (Jalinot *et al.*, 1987). Briefly, protein fractions were incubated with poly(dA.dT) as nonspecific competitor for 3 min at 25°C. Then specific unlabelled oligonucleotides were added (at a molar excess of ~200-fold with respect to the labelled probe), where appropriate, and the incubation continued for 3 min. Finally, the 5′ end labelled probe (5000 c.p.m.) was added and the mixture, adjusted to 2 mM $MgCl_2$, 50 mM KCl in 10 μl final volume, was further incubated for 15 min at 25°C before loading on a 4.5% polyacrylamide gel premigrated 1 h at 180 V. Electrophoresis was carried out at the same voltage for 90 min at 18°C in 10 mM Tris-acetate, pH 7.5, 1 mM EDTA buffer. Gels were transferred onto Whatman DE81 paper and vacuum dried before autoradiography.

DMS-interference analysis

End-labelled probes were partially methylated by dimethylsulphate (DMS) as described (Jalinot *et al.*, 1987). Gel retardation experiments, scaled up to 20- to 50-fold, were performed with the DMS-treated probes. DNA−protein complexes were excised, the DNA was electroeluted and purified by phenol−chloroform extraction and ethanol precipitation. The methylated probe was then treated either for 30 min at 90°C with 1 M piperidine, to reveal methylated G residues (Siebenlist and Gilbert, 1980), or for 15 min at 90°C with 10 mM sodium phosphate pH 7.2, 1 mM EDTA, followed by the addition of sodium hydroxide to 0.1 M and further incubation for 30 min at 90°C, to reveal both methylated G and A residues (Cereghini *et al.*, 1988). The chemically cleaved DNA was precipitated and separated by electrophoresis on sequencing gels (Maxam and Gilbert, 1980).

DNase I footprinting experiments

About 0.5ng of the labelled probe fragment P was incubated for 15 min at 30°C with either whole cell extracts or particular chromatographic fractions in the presence of 200 ng poly(dA.dT). Where appropriate, fractions were further incubated with 20 ng of either E2F- or Cα-unlabelled oligonucleotides. After 10 min at 30°C, the mixture was digested for 5 min at 30°C with appropriate amounts of DNase I (Boeuf *et al.*, 1987). The DNase-resistant fragments were purified and separated on denaturing polyacrylamide gels. Positions of DNase I cleavage sites were determined by coelectrophoresis of G and G+A sequencing reactions of the same probe (Maxam and Gilbert, 1980).

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