The EMBO Journal vol.8 no.13 pp.4239–4250, 1989

TPA can overcome the requirement for EIa and together act synergistically in stimulating expression of the adenovirus EIII promoter

Leonard Buckbinder, Vicente J.Miralles and Danny Reinberg

Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane, Piscataway, NJ 08854-5635, USA

Communicated by P.A.Sharp

We have examined the control of gene expression from the adenovirus early region III (Ad-EIII) promoter, which contains two previously defined elements, the AP1 and ATF sites. We found that the AP1 element is capable of mediating activation by the adenovirus immediate early (EIa) gene products. Consistent with studies demonstrating that the AP1 site mediates signal transduction in response to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) we have shown that TPA can activate Ad-EIII expression and overcome the requirement for EIa. Together TPA and EIa elicited a synergistic response in expression from the Ad-EIII promoter during both transient expression assays and viral infections. This synergistic effect required the AP1 element. An EIII promoter construct, in which sequences upstream of the TATA box had been replaced with four AP1 sites, was responsive to TPA and EIa and in combination promoted the synergistic effect. The analysis of specific factors involved in transcription from the Ad-EIII indicated that proteins recognizing the ATF and AP1 sites were important in expression from this promoter *in vitro*. Purification of protein factors that specifically stimulated EIII expression resulted in the isolation of a set of factors of the AP1 family. Affinity purified AP1 recognized and activated transcription through both the AP1 and ATF elements. In addition, a protein fraction was identified with DNA binding activity specific for the ATF element. This fraction was dependent on the ATF site for transcriptional activity.

Key words: adenovirus/early region III promoter/intermediate early gene products

Introduction

EIa, the immediate early gene of adenovirus, encodes two major products, p289 and p243, which differ only by an internal block of 46 amino acids. The large product was shown to activate transcription of the six viral early promoters as well as some cellular genes [for review see Berk (1986) and references therein]. Although the mechanisms of EIa activation remain unknown, some details have been uncovered. The EIa p289 protein does not interact with specific DNA sequences (Ferguson *et al.*, 1985; Chatterjee *et al.*, 1988) and, is believed to exert its effects through the modification of cellular transcription factors. Several observations support this model of activation: (i) the DNA binding activity of a factor E2F, which binds to EIa responsive elements in the adenovirus early II (EII) promoter (Kovesdi *et al.*, 1986) and to the EIa enhancer (Kovesdi *et al.*, 1987), was increased by EIa (Kovesdi *et al.*, 1986); (ii) EIa activation of the EII promoter did not require new protein synthesis (Reichel *et al.*, 1988); (iii) a peptide containing the 46 amino acids unique to the EIa p289 protein activated EII expression when micro-injected into HeLa cells in the presence of cycloheximide, an inhibitor of protein synthesis (Green *et al.*, 1988); and (iv) VAI, an adenovirus gene transcribed by RNA polymerase III was activated by EIa through the modification of the cellular transcription factor TFIIIC (Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985; Hoeffler *et al.*, 1988; Yoshinaga *et al.*, 1986). Promoter elements mediating the EIa response have been identified (Nevins, 1981; Berk, 1987; Jones *et al.*, 1988). One of these elements includes the sequence 5′-ACGTCA-3′ and occurs in most of the viral early promoters (EIa, EII, EIII and EIV) [for review see Jones *et al.* (1988) and references therein]. This identical sequence was found in some cellular genes whose expression is regulated by the levels of cAMP (Montminy *et al.*, 1986). This element, termed the cyclic AMP response element (CRE), was shown to be recognized by a 43 kd phosphoprotein, CREB, present in PC12 cells (Montminy and Bilezikjian, 1987). Factors with similar specificity were identified in HeLa cells (Lee *et al.*, 1987a; Lin and Green, 1988); however, it appears that HeLa cells contain a large group of proteins that can recognize the CRE. Hurst and Jones (1987) isolated a 43 kd protein, ATF (activating transcription factor), that recognizes an element similar to the CRE present in the adenovirus EIII promoter which has the sequence 5′-TCGTCA-3′. We isolated proteins of 65 and 72 kd, EivF, that specifically recognized the CRE elements present in the adenovirus EIV promoter (Cortes *et al.*, 1988). We also identified proteins that ranged in mol. wt from 31 to 45 kd that bound to the CRE (Cortes *et al.*, 1988; Merino *et al.*, 1989). Subsequently, Hai *et al.* (1988), isolated proteins of 43 and 47 kd that bound to the CRE present in the Ad-EIV promoter.

It has been suggested that the CRE is a regulatory element common to most of the adenovirus early promoters and thus, may serve to coordinately control early gene expression in response to EIa. A large number of factors interact with the CRE and it appears that at least one cellular factor, termed E4F, was regulated by EIa during infection (Raychaudhuri *et al.*, 1989). In order to gain further insight into the role of the different factors that recognize the CRE, we have anlayzed DNA sequence elements and protein factors required for transcription from the adenovirus EIII and EIV promoters. The adenovirus EIII promoter contains a binding site for ATF located at −55 (Hurst and Jones, 1987), and an additional element at −90 that is thought to serve as a recognition site for the transcription factor AP1 (Garcia *et al.*, 1987; Hurst and Jones, 1987). AP1 is similar or identical

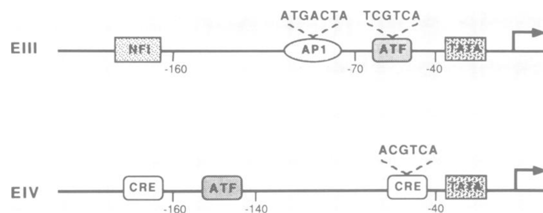

Fig. 1. Schematic representation of the adenovirus EIII and EIV promoters. The relative position of DNA elements that serve as recognition sites for specific DNA binding proteins are indicated.

to the product of the *jun* oncogene [for review see Vogt and Tjian (1988) and references therein], and has been implicated in the protein kinase C mediated cellular response to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Chiu *et al.*, 1987; Lee *et al.*, 1987b).

Studies presented here demonstrated that the DNA element centered at position −90 in the Ad-EIII promoter is a recognition site for AP1 and that this element is a target for EIa mediated induction. We demonstrated that during adenovirus infection the phorbol ester TPA was capable of overcoming the requirement for EIa in expression of the Ad-EIII promoter. TPA mediated expression of the Ad-EIII promoter was dependent on the AP1 element. Interestingly, TPA and EIa acted synergistically during viral infections to stimulate expression from the Ad-EIII promoter.

Results

Factors that regulate transcription from the adenovirus EIII and EIV promoters

A diagram of the Ad-EIII promoter showing sequences that serve as recognition sites for known specific DNA binding proteins is shown in Figure 1. These include an AP1 element (5′-ATGACTA-3′) centered at −85 (relative to the EIII-CAP site) and an ATF element (5′-TCGTCA-3′) centered at −55. The studies of Hurst and Jones (1987) analyzed factors that bound to the Ad-EIII promoter and using oligonucleotide competition assays, demonstrated that independent factors bound to the AP1 and ATF elements. Using DNA affinity chromatography with an oligonucleotide representing the DNA element centered at −55, they purified a factor of 43 kd, termed ATF. Hai *et al.* (1988) analyzed factors that interact with the adenovirus EIV promoter, which has an ATF recognition site and two related DNA elements known as CREs (5′-ACGTCA-3′) (see Figure 1). Using DNA affinity chromatography with the element centered at −50 in the Ad-EIII promoter, they purified factors of 43 and 47 kd also identified as ATF. The isolated proteins recognized the CRE and ATF elements and stimulated transcription from the Ad-EIII promoter *in vitro*. Our previous studies analyzing factors that specifically stimulated transcription from the Ad-EIII promoter resulted in the isolation of a mixture of proteins with mol. wts of 65 and 72 kd termed EivF. Both proteins bound independently to the CRE elements present in the Ad-EIV promoter and activated transcription; however, they did not bind to the ATF or AP1 DNA elements present in the Ad-EIII and Ad-EIV promoters (Cortes *et al.*, 1988; Merino *et al.*, 1989).

To assess the role of these related DNA elements in regulating transcription from the Ad-EIII and Ad-EIV

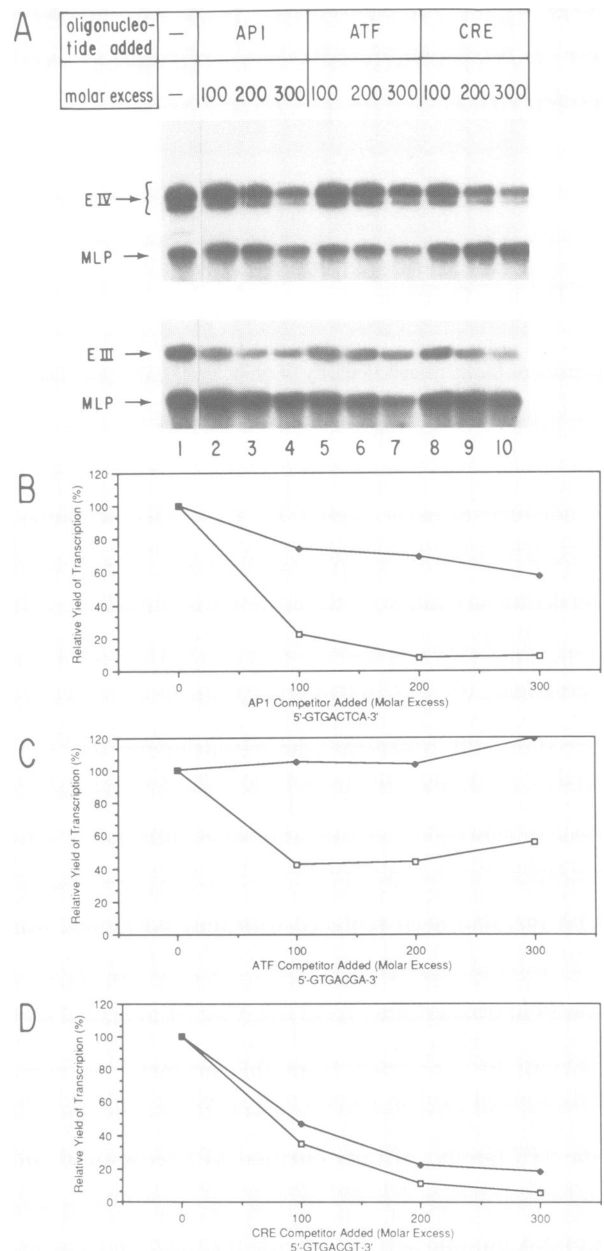

Fig. 2. Analysis of specific factors promoting transcription from the Ad-EIII and Ad-EIV promoters *in vitro*. **(A)** The effect of oligonucleotides containing consensus sequences that serve as recognition for different factors, as indicated at the top of the panel, were analyzed for transcription from the Ad-EIII and Ad-EIV promoters using a crude reconstituted system (see Materials and methods). A plasmid DNA containing the adenovirus MLP (2.0 μg/ml) was included in all the reactions as an internal control. The products of the reaction were analyzed by primer extension and separated by electrophoresis on polyacrylamide−urea gels. Arrows at the left of the autoradiogram indicate the position of specific transcription products. Two transcription start sites are used in the EIV promoter. **(B−D)** The effect of increasing oligonucleotide competitors on transcription from the Ad-EIII and Ad-EIV promoters is illustrated quantitatively in panels **B−D**. The radiolabeled band corresponding to transcripts initiated from the EIII, EIV and MLP presented in **panel A**, were excised and counted in a scintillation counter. The ratio of EIII/MLP (open boxes) or EIV/MLP (filled boxes) was calculated for each reaction. The effect of each competitor was determined by comparing the ratios of transcription in the competed reactions to that of the uncompeted case and is expressed as relative yield **(B−D)**.

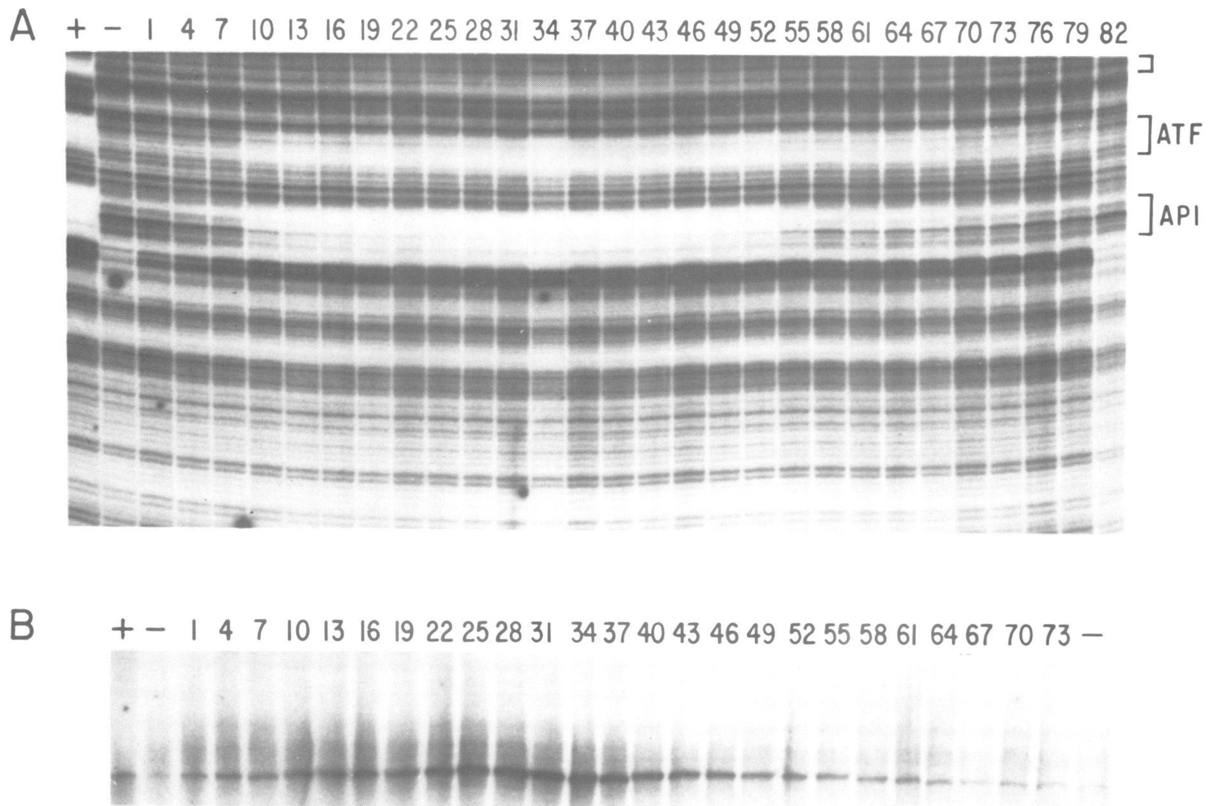

Fig. 3. Proteins recognizing both the AP1 and ATF DNA elements cofractionate with the EIII transcription stimulatory activity on gel filtration chromatography. (**A**) Aliquots (20 μl) of the indicated fractions were analyzed for their ability to bind the EIII promoter by DNase I protection. A DNA fragment (1 ng) containing the Ad-EIII promoter was 3′ end labeled using the *Eco*RI site located 238 nucleotides upstream from the CAP site. The pattern of digestion obtained in the absence of protein is indicated by the lane marked with the (−) symbol. The pattern of DNase I protection observed with an aliquot of the input material (DEAE−cellulose) is marked with the (+) symbol. The labeled brackets on the right indicate positions of the AP1 and ATF sites. The unlabeled bracket at the top indicates the position of an AP1-like site overlapping the TATA sequence. (**B**) Aliquots (8 μl) of the indicated fractions were analyzed for their ability to stimulate transcription from the EIII promoter using a run off assay as described in the Materials and methods. The DNA template (2.0 μg/ml) was pE3 CAT digested with *Hpa*I. The lanes marked by (−) symbols received 8 μl of sample buffer instead of protein fractions. The lane marked with a (+) symbol received an 8 μl sample of the input protein fraction. The low transcription activity of the input material is probably due to non-specific inhibitors that are sometimes found in this step.

promoters, increasing concentrations of oligonucleotides representing the AP1 (5′-TGACTCA-3′), ATF (5′-TCGTCA-3′) or CRE (5′-ACGTCA-3′) elements were added to transcription reactions that contained the Ad-EIII or Ad-EIV promoters. In addition, an internal control that lacks these sites, the Ad-MLP, was included. The ratio of transcription observed from the Ad-EIV and Ad-EIII promoters relative to the Ad-MLP in the presence of increasing concentrations of the oligonucleotides was compared with a control reaction carried out in the absence of the oligonucleotides (Figure 2A). This assay allowed us to systematically correct for non-specific competition by the added oligonucleotides. The addition of a 100-fold molar excess of oligonucleotides containing the AP1 recognition site resulted in a drastic inhibition of transcription from the Ad-EIII promoter (relative to the major late promoter); however, transcription from the Ad-EIV promoter was only slightly affected (Figure 2A, compare lanes 1 and 2). The addition of increasing concentrations of this oligonucleotide almost abolished transcription from the Ad-EIII promoter and had a modest effect in transcription from the Ad-EIV promoter (Figure 2A and B). A similar situation was observed when oligonucleotides containing the ATF recognition site were added to the reactions. The addition of 100-fold molar excess inhibited transcription from the Ad-EIII promoter by nearly 60%; however, transcription from the Ad-EIV promoter was not affected (Figure 2A, compare lanes 1 and 5 and C). A different situation was observed when oligonucleotides containing the CRE element were added to the reaction. In this case transcription from both promoters was severely affected (Figure 2A and D).

An interpretation consistent with these results is that transcription from the Ad-EIV promoter requires a factor(s) that specifically recognizes the CRE; this factor does not recognize the AP1 and/or ATF elements. However, transcription from the Ad-EIII promoter requires factors that recognize the ATF, AP1 and CRE elements. An alternative possibility is that only one or two factors regulate transcription from the Ad-EIII promoter and this factor(s) can bind to the three oligonucleotides used.

Transcription from the Ad-EIII promoter requires AP1

Using a functional transcription assay we attempted to purify from HeLa cell nuclear extracts the specific factor(s) required to drive transcription from the Ad-EIII promoter. The transcription stimulatory activity for the Ad-EIII promoter

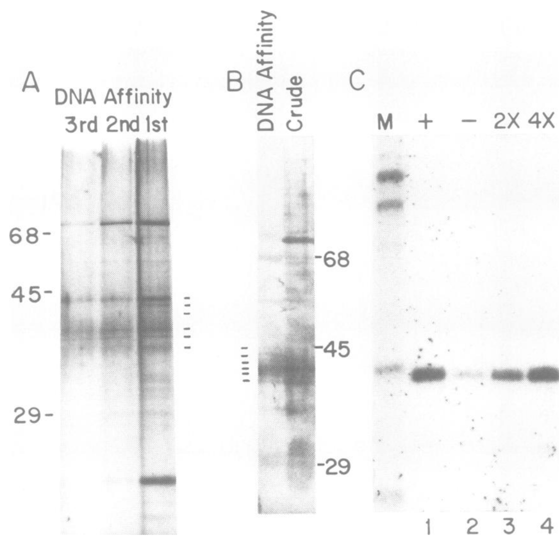

Fig. 4. Affinity purified AP1 is composed of a family of proteins that are immunologically related to c-jun and activate transcription from the EIII promoter *in vitro*. (**A**) Aliquots containing 20 footprint units (f.p.u.) (1 f.p.u. is defined as the amount of protein necessary to protect fully 0.6 nM of the AP1 site present in the Ad-EIII promoter) from three successive cycles of 'AP1' affinity chromatography, as indicated on the top of the panel, were analyzed for polypeptide composition by electrophoresis through a SDS−polyacrylamide gel (8%) followed by silver staining. The relative positions of protein mol. wt standards are indicated at the left. Marks at the right indicate the position of polypeptides that are enriched by affinity chromatography. (**B**) Protein fractions (20 f.p.u.) indicated by crude and DNA affinity (Sephacryl S-300 and third pass from the DNA affinity step respectively) were separated by electrophoresis through an SDS−polyacrylamide gel, the proteins transferred to nitrocellulose membrane and then incubated with antibodies directed against a jun fusion protein. The blots were washed and incubated with anti-rabbit IgG conjugated to alkaline phosphatase and developed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as previously described (Flores *et al.*, 1988). The positions of protein mol. wt standards are indicated at the right. Marks at the left indicate the positions of immunoreactive material that are enriched during AP1-DNA afffinity purification. (**C**) Aliquots of the protein pool derived after the third pass over the AP1 DNA affinity chromatography were assayed for their ability to stimulate transcription from the EIII promoter **Lanes 3** and **4** received 2 and 4 f.p.u. respectively. Reaction conditions were as described in Materials and methods and products were analyzed by primer extension. **Lane 1**, marked with the (+) symbol, contained 12 μl (10 f.p.u.) of the Sephacryl S-300 fraction. **Lane 2** received sample buffer and is marked by the (−) symbol. φx RF DNA digested with *Hae*III was used for mol. wt markers (**lane M**).

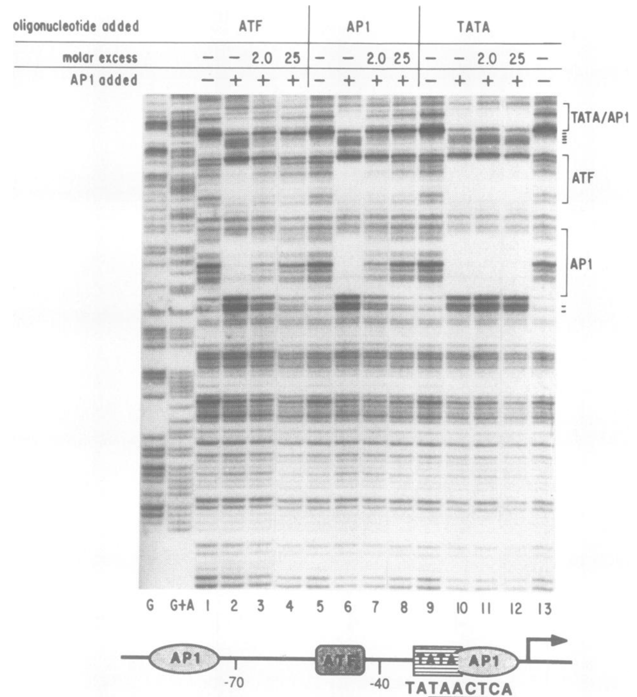

Fig. 5. Purified AP1 protein binds to the AP1, ATF and AP1/TATA sites in the Ad-EIII promoter. Aliquots (20 μl) of affinity purified AP1 were analyzed for their ability to bind to the Ad-EIII promoter. The pattern of DNase I cleavage obtained in the absence of added protein is indicated at the top of the figure by (−) symbols (**lanes 1, 5, 9** and **13**). The footprinting pattern obtained with AP1 in the absence of any added oligonucleotide competitor is shown in **lanes 2, 6** and **10**. The effect of oligonucleotides containing the recognition sites for ATF, AP1 and TFIID (TATA) are shown in **lanes 3** and **4**, **7** and **8** and **11** and **12**, respectively. Maxam and Gilbert DNA sequencing standards using G and G+A reactions are shown at the left. The brackets on the right indicate the different elements protected by the AP1 protein fraction. The diagram at the bottom of the figure summarizes the position of elements protected by AP1.

copurified with EivF over the first two chromatographic steps (data not shown, see Materials and methods). However, after chromatography over a gel filtration column in 1.0 M salt the two activities were partially separated (data not shown). The Ad-EIII transcription stimulatory activity coeluted with an activity capable of binding to both the AP1 and ATF elements (Figure 3, compare panels A and B). However, DNase I protection to the ATF element extended over fractions that were devoid of transcription activity (Figure 3 and see below). Transcriptionally active fractions were pooled and purified further by multiple passages over a DNA affinity column containing the AP1 recognition sequence. The third passage resulted in the isolation of several polypeptides in the mol. wt range 35−45 kd (Figure 4A). This is in agreement with studies which indicated that a family of proteins could bind to the AP1 recognition site (Angel *et al.*, 1988a; Bohmann *et al.*, 1988; Rauscher *et al.*, 1988). The polypeptides present in the DNA affinity purified fraction reacted with antibodies directed against a c-jun fusion protein (Angel *et al.*, 1988a) (gift of Dr M.Karin) which is similar or identical to AP1 (Vogt and Tjian, 1988) (compare Figure 4A, lane 3 with B). Affinity purified AP1 was transcriptionally active (Figure 4C, compare lane 2 with 3 and 4) and specific for the AP1/ATF recognition site; transcription from a DNA template lacking these sites was not affected by the addition of this protein fraction (see Figure 6A, lanes 14−18). Furthermore, the transcription stimulatory activity of the affinity purified AP1 protein fraction correlated with binding to the AP1 and ATF elements in the Ad-EIII promoter (Figure 5, lanes 2, 6 and 10). Interestingly, protection over the TATA sequence was also observed. In order to determine whether the binding to the three sites (AP1, ATF and TATA) was the result of multiple factors present in the affinity purified fraction, oligonucleotide competition experiments were carried out. The addition of increasing amounts (2- and 25-fold molar excess) of oligonucleotides containing the ATF (Figure 5, lanes 3 and 4) or AP1 (lanes 7 and 8) recognition sequence resulted in competition of the three areas protected from DNase I cleavage. However, the addition of oligonucleotides containing the TATA sequence had no effect (Figure 5, lanes

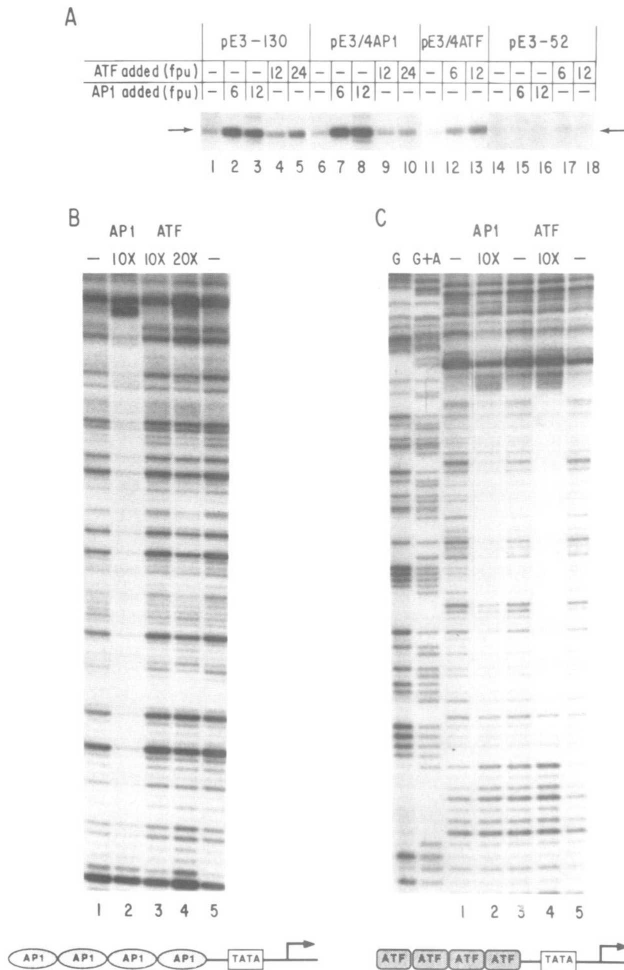

Fig. 6. Analysis of the DNA binding specificity and transcription stimulatory activity of the AP1 and ATF protein fractions. (**A**) The transcription stimulatory activity of the AP1 and ATF protein fractions was determined in a reconstituted transcription system (as described in Figure 4C), using four promoter constructs as indicated at the top of the figure. Plasmid DNA pE3-130 and pE3-52 contain EIII promoter sequences extending up to −130 and −52 respectively, and directed transcription of the CAT gene. pE3-130 contains the AP1 and ATF sites whereas pE3-52 contains a deletion of these two elements. The AP1 fraction used in this analysis was DNA affinity purified (Figure 4) and the ATF protein fraction was derived from the gel filtration step (fractions 58−67, see Figure 3). Transcription observed in the absence of added AP1 or ATF protein fractions is indicated by (−) symbols (**lanes 1, 6, 11** and **14**). Reactions receiving AP1 or ATF protein fractions are indicated at the top of the figure in the number of footprint units added (1 f.p.u. is defined as the amount of protein required to protect 0.6 nM of Ad-EIII 'AP1' or 'ATF' site, from DNase I cleavage). (**B**) The DNA binding specificity of the AP1 and ATF protein fractions was analyzed by DNase I protection using the pE3/4×AP1 promoter construct (diagrammed at the bottom of the figure). The promoter fragment was 3′ end labeled at the upstream *Eco*RI site (−126). The pattern of protection in the absence of added protein is indicated by (−) symbols (**lanes 1** and **5**). The addition of AP1 and ATF protein fractions is indicated at the top of the figure. 10 × AP1 or 10 × ATF is equivalent to 20 f.p.u. (**C**) The DNA binding specificity of the AP1 and ATF protein fractions was analyzed by DNase I footprinting using the pE3/4×ATF promoter construct (diagrammed at the bottom of the figure). Lanes containing Maxam and Gilbert DNA sequencing standards of the G and G+A reactions are indicated at the top of the figure.

11 and 12). In addition, the conentration of TATA oligonucleotide used in this analysis effectively competed for the binding of TFIID (the TATA binding protein) to the TATA motif present in the Ad-MLP (J.Carcamo and D.Reinberg, unpublished observations). This result indicates that the binding to these three sites was due to AP1. The observed protection to the TATA sequence was to an AP1 site that overlaps the TATA box (see underlined sequences in the diagram at the bottom of Figure 5). The significance of this sequence arrangement is currently unknown. However, it is interesting to mention that an AP1 recognition site also appears in the Ad-EIV promoter between nucleotides +6 to +13 (L.Buckbinder and D.Reinberg, unpublished observations). Thus, it is possible that these sites contribute to the regulation of adenovirus early gene expression.

During the purification of AP1, a protein fraction was identified that specificaly recognized the ATF site but failed to activate transcription from the Ad-EIII promoter (see Figure 3, fractions 58−67). In order to analyze this further, increasing amounts of the ATF protein fraction were added to transcription and footprinting reactions. In agreement with the results presented in Figure 3, the ATF protein fraction bound only to the ATF site (see Figure 3 and below) and stimulated, only slightly, transcription from the Ad-EIII promoter (Figure 6A, lanes 4 and 5). This protein fraction was capable of stimulating transcription, although to low levels, from an EIII promoter construct in which sequences upstream of −52 were replaced by two (data not shown) and four ATF sites (Figure 6, lanes 12 and 13). This stimulation was mediated via the ATF recognition site; a construct that replaced the ATF sites by four AP1 recognition sites was not affected by the ATF protein fraction (lanes 9 and 10) but was stimulated by the AP1 fraction (lanes 7 and 8). The stimulation by the AP1 protein fraction was specific for the AP1 or ATF recognition sites since the addition of affinity purified AP1 had no effect on transcription from a construct lacking these sites (Figure 6A, lanes 14−16). Consistent with the results presented in Figures 3 and 6A, the AP1 protein fraction was capable of binding to the AP1 and ATF sites (Figure 6B, lane 2, and C, lane 2 respectively). However, the ATF fraction bound to the ATF site but failed to bind to the AP1 recognition site (Figure 6C, lane 4, and B, lanes 3 and 4). These results demonstrated that transcription from the Ad-EIII promoter required AP1, which bound to both the AP1 and ATF recognition sites. A fraction that only bound to the ATF recognition site had a modest effect in transcription from the Ad-EIII promoter and did not affect the levels of AP1 protein required for optimal transcription from the Ad-EIII promoter (data not show).

The AP1 and ATF recognition sites can independently mediate the response to EIa

The expression of the EIII promoter is stimulated markedly by the EIa p289 protein *in vivo*. Previous studies have indicated that multiple elements in the Ad-EIII promoter mediated this response to EIa. Weeks and Jones (1985) indicated that sequences located between −105 and −82 (relative to the EIII-CAP site) were important in mediating basal and EIa induced expression. Furthermore, their studies demonstrated that a 148 bp DNA fragment derived from the Ad-EIII promoter, extending from nucleotides −230 to −82, was capable of conferring EIa inducibility when inserted 79 nucleotides upstream from the CAP site of the EIa insensitive thymidine kinase promoter (Weeks and Jones, 1983). Also, the studies of Leff *et al.* (1985) mapped another element

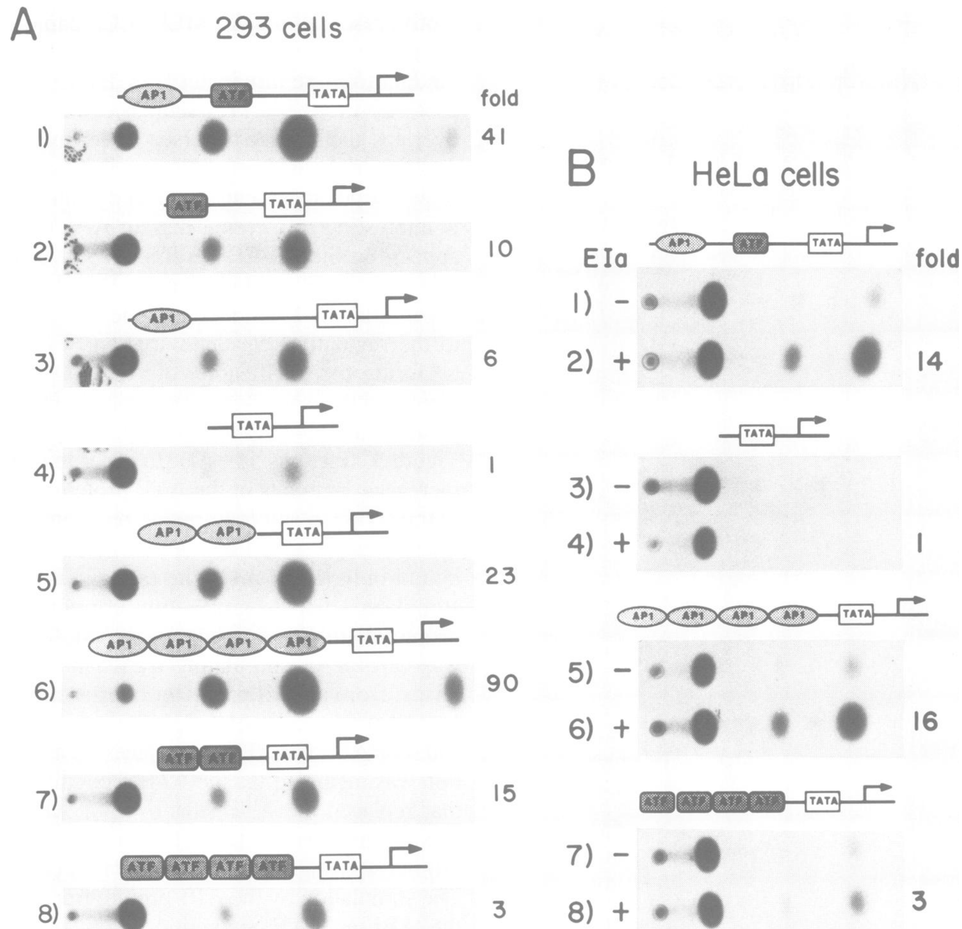

Fig. 7. Activity of different EIII promoter constructs in transfected 293 and HeLa cells. (**A**) EIII promoter constructs, diagrammed at the top of each panel, were transfected into 293 cells by the calcium phosphate coprecipitation method (Wigler *et al.*, 1977) as described in Materials and methods. Soluble extracts were prepared and CAT activity was analyzed by two independent methods. CAT activity was assayed using [^{14}C]chloramphenicol and acetylated products were resolved by TLC (Gorman *et al.*, 1982). Chromatographs were visualized by autoradiography and are shown in each panel. CAT activity was also determined quantitatively using [^{3}H]acetyl-coenzyme A and unlabeled chloramphenicol (Sleigh, 1986). These results (shown at the right of each panel) represent an average of three independent transfections and are expressed in the fold activity over the basal levels (obtained with a construct from which the DNA elements upstream of the TATA box were deleted, pE3-52). (**B**) EIII promoter constructs, diagrammed at the top of each panel, were transfected into HeLa cells (clone 2) by the calcium phosphate coprecipitation method, with or without a plasmid expressing the EIa gene products (pEIa) as indicated at the left of each panel. Analysis of CAT activity was as described in Figure 7A. Autoradiograms of the chromatographs are shown in each panel. Results of the quantitative analysis (shown at the right of each panel), represent an average of three independent transfections and are expressed in fold induction by EIa.

located between nucleotides −57 to −40 on the Ad-EIII promoter that was important for both EIa activated and basal levels of transcription. Based on these previous studies and results presented above, we further characterized EIII promoter elements *in vivo*. EIII promoter constructs containing deletions of the AP1 and/or ATF sites were used to direct expression of the bacterial chloramphenicol acetyltransferase gene (CAT), in transient expression assays in 293 cells (a cell line permissive for expression of promoters that are activated by EIa due to an integrated EIa gene). These results (Figure 7A), were in agreement with previous findings (Leff *et al.*, 1985; Weeks and Jones, 1985; Garcia *et al.*, 1987), indicating that the deletion of the AP1 (pE3-85, lane 2) or ATF (pE3Δ-82-52, lane 3) elements resulted in a reduction of the levels of expression; however, the promoter was still active. The deletions of both sites resulted in a construct (pE3-52, lane 4) that was almost inactive. Next, to analyze further whether the AP1 and/or ATF sites were capable of mediating EIa inducibility, chimeric plasmids containing two and four copies of the AP1 or ATF sites were constructed using an EIII promoter deletion in which all elements upstream of −52 were removed. All of these chimeric constructs resulted in CAT activity when transfected into 293 cells (Figure 7A). A plasmid containing two copies of the AP1 recognition site (pE3/2×AP1, lane 5) resulted in ~2-fold more activity than a similar construct containing two ATF sites (pE3/2×ATF, lane 7), but 2-fold less activity than an EIII promoter construct containing single AP1 and ATF sites pE3-130, lane 1). However, the placement of four copies of the AP1 recognition site upstream of the EIII-TATA sequence (pE3/4×AP1, lane 6), resulted in a construct that was ~2-fold more active than pE3-130. Interestingly, four copies of the ATF element yielded a construct capable of expressing the CAT gene; however, this construct was less active than one that contained two ATF sites (lane 8). In order to analyze directly whether these constructs were stimulated by EIa, the different plasmid DNAs were transfected into HeLa cells in the absence and presence of plasmid containing the EIa gene (pEIa) (gift of Dr L.Vales). While the overall level of activity in HeLa cells

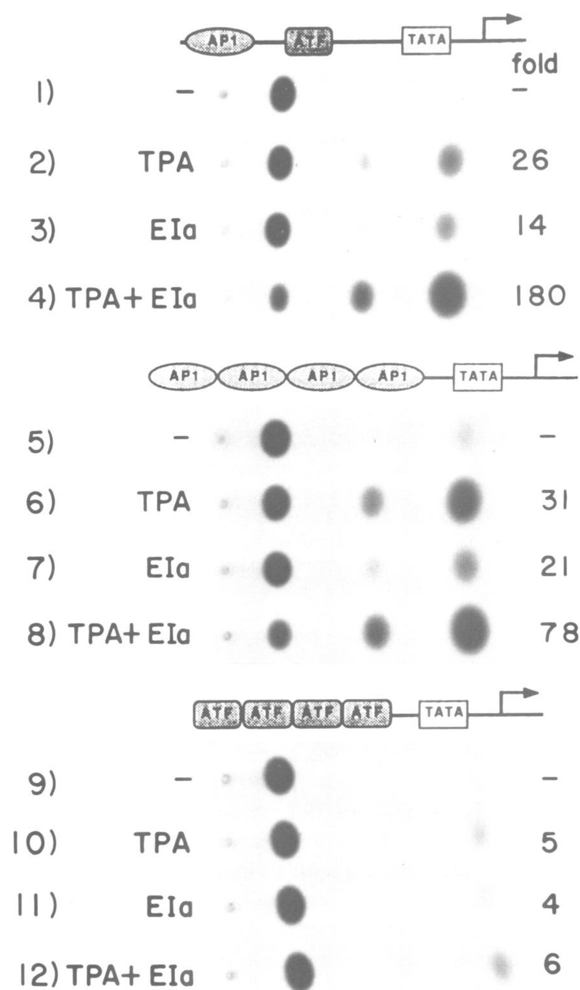

Fig. 8. The effect of EIa and TPA on the expression of the EIII promoter in transfected HeLa cells. EIII promoter constructs, diagrammed at the top of each panel, were transfected into HeLa cells by the calcium phosphate coprecipitation method with or without a plasmid expressing the EIa gene products as indicated at the left of each panel. After 16 h media were changed and transfected cells were either mock treated (DMSO alone) or treated with TPA, as indicated at the left of each panel. After an additional 24 h cells were harvested and assayed for CAT activity as described in Figure 7. Autoradiograms of the chromatographs are shown in each panel. Results of the quantitative analysis are given at the right of each panel and are expressed in fold induction by EIa and/or TPA. These results represent an average of three independent experiments.

was lower than that observed in 293 cells, the expression of the EIII constructs was stimulated by EIa (Figure 7B). The level of EIa activation observed in HeLa cells is probably underestimated due to the limitations inherent in cotransfection experiments. The fold-stimulation of a construct containing four AP1 recognition sites was comparable to the activity observed with the control template, a plasmid DNA containing EIII promoter sequences up to −130 (Figure 7, compare lanes 6 and 2 respectively). Interestingly, a plasmid that contains four ATF sites was stimulated by EIa only 3-fold (Figure 7, compare lanes 7 and 8). A plasmid that contained two ATF sites (pE3/2×ATF) was stimulated to the same extent (data not shown). These constructs (pE3/2×ATF and pE3/4×ATF) were transcriptionally active *in vitro* and dependent on a protein fraction that recognized the ATF element (Figure 6 and data not shown). Thus, the low activity observed with this construct in transfected 293 and in HeLa cells containing pEIa, may indicate that the ATF site is a weak element in mediating the EIa response.

EIa and TPA act synergistically to activate transcription from the Ad-EIII promoter in transfected HeLa cells

In the previous section we indicated that the DNA element centered at position −85 in the Ad-EIII promoter directly participated in mediating the response to EIa. Furthermore, we demonstrated that this element was recognized by factors of the AP1 family. The AP1 recognition site, which is also known as TPA responsive element (TRE) (Angel *et al.*, 1987), can also mediate the transcriptional response to the phorbol ester TPA (Angel *et al.*, 1987; Lee *et al.*, 1987b). Thus, we investigated the effect of TPA in transcription from the Ad-EIII promoter. A plasmid DNA construct containing the EIII promoter (pE3-130) directing expression of the bacterial chloramphenicol acetyltransferase gene was transfected into HeLa cells. The transfected cells were treated with TPA and the levels of CAT activity were compared with those obtained when the cells were cotransfected with pEIa. Expression from the Ad-EIII promoter was stimulated 14-fold by EIa (Figure 8, compare lanes 1 and 3). When the transfected cells were treated with TPA the expression from the Ad-EIII promoter was stimulated 26-fold (Figure 8, compare lanes 1 and 2). This result indicated that TPA was capable of overcoming the requirement for EIa. Interestingly, when cells cotransfected with the EIII promoter construct and pEIa were treated with TPA, the stimulation observed was 180-fold (Figure 8, lane 4). This stimulation could not be explained by an additive effect rather, it suggested that EIa and TPA acted synergistically in stimulating transcription from the Ad-EIII promoter. Stimulation of expression from the Ad-EIII promoter by TPA was also observed when the cells were treated with cycloheximide, an inhibitor of protein synthesis (data not shown, see below), and indicates that this induction probably proceeds through the modification of pre-existing cellular factors.

In order to determine which DNA elements were responsible for mediating the synergistic effect, chimeric constructs containing EIII promoter sequences up to −52 and multiple AP1 or ATF sites were analyzed. As previously indicated, a construct containing four AP1 sites responded to EIa with levels of expression similar to those observed with the wild type EIII construct pE3-130 (see Figure 8, lane 7). as expected, this construct was also stimulated by TPA (Figure 8, lane 6). Interestingly, while the level of expression in response to either EIa or TPA was similar or slightly higher than those observed with the wild type EIII construct, the level of expression observed in response to both EIa and TPA was approximately one-half that of the wild type EIII construct (Figure 8, compare lane 8 with 4 respectively). Chimeric constructs containing two (data not shown) or four copies of the ATF site (Figure 8, lanes 9−12) were stimulated slightly by EIa (lane 11) and TPA (lane 10) and no synergistic effect was observed (lane 12).

These results suggested that the AP1 recognition site is required for the synergistic response between EIa and TPA. The ATF recognition site was unable to mediate this response alone. Interestingly, one or two copies of the AP1 recogni-

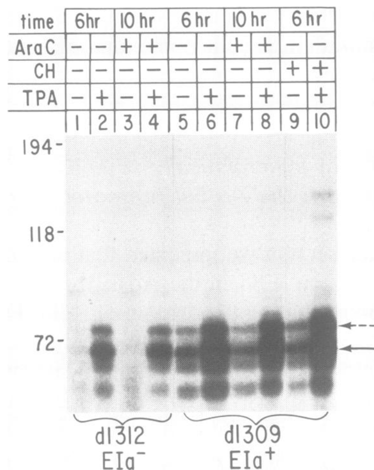

Fig. 9. TPA activates expression of the EIII promoter during adenovirus infection. HeLa cells (clone 2) were infected with adenovirus dl312 (**lanes 1–4**) or dl309 (**lanes 5–10**) as indicated at the bottom of the figure. After virus adsorption (see Materials and methods) cells were mock treated (DMSO alone) or treated with TPA as indicated at the top of each panel. Infections were allowed to proceed for 6 or 10 h (4.5 or 8.5 h of treatment respectively) at which time cells were harvested and cytoplasmic RNA prepared. Infections allowed to proceed for 10 h were maintained in the early phase of the infection by the addition of Ara C (50 μg/ml) as indicated at the top of the panel. Some experiments received the protein synthesis inhibitor cycloheximide (CH, 10 μg/ml) 15 min prior to TPA addition as indicated (**lanes 9** and **10**). RNA initiated at the EIII CAP site was analyzed from each infection by RNase T2 protection as described in Materials and methods. Arrows indicate the expected positions of protected EIII products. The position of molecular size standards are indicated at the left in nucleotides.

tion site were unable to mediate the synergistic effect (data not shown); however, a single AP1 site in combination with an ATF site (the pE3-130 promoter construct) was capable of mediating the synergistic response, generating levels of gene expression higher than those observed with a construct containing four AP1 sites. Thus, we postulate that the AP1 and ATF recognition sites cooperate in this response.

TPA can overcome the EIa requirement for the expression of the EIII gene in adenovirus infected HeLa cells

In the previous section we showed that TPA was capable of replacing EIa in activation of the EIII promoter and, that TPA and EIa acted synergistically in promoting transcription from this promoter using transient expression assays in HeLa cells. Next, we analyzed whether TPA has a similar effect in the expression of the EIII promoter during a viral infection. HeLa cells were infected with either a virus containing a deletion of the EIa gene, dl312 (Jones and Shenk, 1979a), which cannot produce EIa proteins or the parental virus, dl309, (Jones and Shenk, 1979b) which produces wild type EIa proteins. The infected cells were exposed to TPA and the infection allowed to proceed for a total of 6 or 10 h (for details see legend to Figure 9). Cells infected for 10 h were maintained in the early phase of infection by inhibiting DNA replication with AraC; this was verified by analyzing the expression of the Ad-IX promoter which is not expressed until after the onset of DNA replication (Crossland and Raskas, 1983). RNA was isolated and transcripts initiated at the Ad-EIII CAP site were analyzed by hybridization to an antisense probe that spanned

Table I. Quantitation of TPA effects on EIII expression

	c.p.m.		fold	
	6 h	10 h	6 h	10 h
(–)	197	250	1.0	1.0
TPA	1407	1501	7.1	6.0
EIa	1220	1059	6.2	4.2
TPA + EIa	8921	8363	45.3	33.0
			Synergism 3.4	Synergism 3.2

The radiolabeled bands corresponding to transcripts initiated at the EIII CAP site in Figure 9, were excised from the dried gel and counted in a liquid scintillation counter. Analysis for infections of 6 and 10 h are given in columns 1 and 2 respectively, and are expressed in counts per minute above background (determined by excising an equivalent part of the dried gel where sample was omitted). The fold of induction by TPA, EIa or TPA + EIa was calculated for 6 and 10 h of infection and is given in columns 3 and 4 respectively. Synergism was quantitated by dividing the fold induction of the TPA + EIa sample by the sum of individual treatments as indicated in the Webster dictionary (i.e. for 6 h, 45.3/6.2 + 7.1 = 3.4).

the EIII-CAP site followed by digestion with RNase T2. As expected, transcription from the Ad-EIII promoter was not observed in cells infected with a virus that failed to produce the EIa proteins (Figure 9, lanes 1 and 3). However, when the dl312 infected cells were treated with TPA, the EIII promoter was expressed compare lanes 2 and 4 with 1 and 3 respectively). The level of TPA-dependent expression of the EIII promoter was similar or slightly higher than that observed in cells infected with a virus that expresses the EIa gene (dl309) (Figure 9, compare lanes 2 and 4 with 5 and 7 respectively; also see Table I). Therefore, TPA was capable of inducing the expression of the EIII gene during adenovirus infection to levels similar to those observed with EIa. We next analyzed the joint effects of TPA and EIa during a viral infection. HeLa cells infected with adenovirus dl309 were treated with TPA and then analyzed as above. After 6 h of infection, the overall induction of Ad-EIII expression in response to both, TPA and EIa was ~45-fold (Figure 9, compare lanes 6 and 1; also see Table I). Individually, EIa and TPA stimulated the expression of this promoter ~6- and 7-fold respectively (see Table I). Thus, TPA and EIa acted synergistically in stimulating the expression of the Ad-EIII promoter during adenovirus infection. This synergism did not require protein synthesis; approximately the same level of stimulation was observed in the presence of cycloheximide (Figure 9, compare lanes 9 and 10). Because the cells were treated with TPA for a longer period of time in the transient expression analysis (24 h during transfection versus 4 h in infection), we investigated whether TPA would have a greater effect if the infections were allowed to proceed for a longer period of time. The levels of EIII transcripts were not changed significantly after 10 h of infection (Figure 9, compare lanes 1 and 2 with 3 and 4, and 5 and 6 with 7 and 8 respectively; also see Table I) and indicated that the full effect of TPA was realized within 4 h after treatment.

Discussion

The studies described here demonstrated that the AP1 recognition site, which is known to be recognized by a family of proteins including the protooncogene c-*jun*, mediates the

majority of the response to EIa. Furthermore we demonstrated that the phorbol ester TPA, which is known to activate the protein kinase C mediated signal transduction pathway, can overcome the requirement for EIa and together EIa and TPA act synergistically in stimulating expression from the Ad-EIII promoter.

Protein factors involved in transcription from the EIII promoter

We have previously shown that a 65−72 kd factor, EivF, purified from uninfected HeLa cells activated transcription *in vitro* through the CRE elements present in the Ad-EIV promoter. Since the Ad-EIII promoter was sensitive to competition by the CRE we analyzed whether purified EivF could activate this promoter in a reconstituted transcription system. Surprisingly, we found that EivF had no effect on transcription from the Ad-EIII promoter, and determined that EivF did not recognize any EIII promoter elements (Merino *et al.*, 1989). We purified specific factors activating EIII transcription *in vitro* and found that proteins recognizing the consensus AP1 element were required. The affinity purified AP1 protein fraction was composed of a number of polypeptides that range in mol. wt from 35 to 45 kd. This is consistent with results obtained by others (Angel *et al.*, 1987). In addition, a number of polypeptides in the affinity purified preparation reacted with antibodies directed against the c-*jun* oncogene (Angel *et al.*, 1988a), shown previously to recognize the AP1 element alone or in conjunction with the product of the *fos* oncogne (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988; Gentz *et al.*, 1989; Turner and Tjian, 1989).

The affinity purified AP1 fraction was able to recognize the AP1 and ATF sites present in the Ad-EIII promoter. However, the gel filtration chromatographic step of the AP1 purification revealed fractions that bound only to the EIII-ATF site. Hurst and Jones (1987), demonstrated using HeLa cell nuclear extracts that factors interacting with the AP1 site were competed for by both the AP1 and ATF oligonucleotides, while factors interacting with the ATF site were poorly competed by the AP1 oligonucleotide. They also purified factors that recognized the ATF site (5′-TCGTCA-3′) and found the activity in a polypeptide of 43 kd. It was suggested that this factor did not interact with any other Ad-EIII promoter elements. Based on sequence specificity it is likely that the DNA binding activity present in our ATF preparation is the same as the one purified by these authors. Furthermore, the ATF fraction described in these studies stimulated transcription from a template that contained multiple ATF recognition sites. It is interesting to note that Hai *et al.* (1988) purified factors present in HeLa cells that recognize the CRE (5′-ACGTCA-3′). Using this element for DNA affinity chromatography, these authors purified what they also identified as ATF and AP1 factors; each composed of two polypeptides of 43 and 47 kd. Both the AP1 and ATF protein fractions that we have isolated bind efficiently to the CRE (Merino *et al.*, 1989), and thus the binding specificity and polypeptide composition of the DNA affinity purified AP1 appears to be equivalent to the activity contained in their CRE purified factors.

Our results demonstrated that factors recognizing the consensus AP1 site also protect an AP1 like site (5′-TATAACTCA-3′) that overlaps the EIII TATA box. Since it is known that the EIII promoter is down regulated late during infection (Binger and Flint, 1984), which may be due to high levels of EIa (Zajchowski *et al.*, 1988), this repression could be through activation of AP1, which at high concentraton may displace the TATA binding factor and turn off EIa expression. It is also known that EIa can repress enhancer driven expression, as in the simian virus 40 (SV40) early promoter (Velcich and Ziff, 1985) and this effect was localized to the 12S gene product (Lillie *et al.*, 1986). The AP1 site, an enhancer element present in the SV40 early promoter, is also found in the EIII promoter and may be a target of repression by the EIa 12S gene product. We have investigated this possibility by examining the ability of the EIa 12S gene product to repress TPA induced expression of the EIII promoter during transient expression and adenovirus infection in HeLa cells. During transient expression, the TPA induced expression of the EIII promoter in the presence of the 12S gene product (Stephens and Harlow, 1987) was reduced ~70% (L.Buckbinder and D.Reinberg, unpublished observations). However, when the same experiments were performed using viral constructs that only express the large or small EIa proteins [pm 975, Montell *et al.* (1982) and dl500, Montell *et al.* (1984) respectively] we found that the virus encoding only the 12S product did not repress TPA mediated expression of the EIII promoter. The differences observed between transient expression and adenovirus infections are currently unknown. However, it is possible that in a viral background with only the 12S products, other viral products, e.g. those encoded by the EIb gene, can have an effect that suppresses the repression by the 12S product (White *et al.*, 1988; Vales and Darnell, 1989). This remains to be further analyzed.

EIa activation of the EIII promoter; a link to protein phosphorylation

The AP1 and ATF sites proved to be critical for expression of the EIII promoter *in vitro*. Thus, we analyzed the contribution of these elements for EIII expression *in vivo*. In agreement with previous studies (Leff *et al.*, 1985; Weeks and Jones, 1985; Garcia *et al.*, 1987), we found that promoter sequences located upstream of the AP1 site (−130) contribute only slightly to expression. However, the deletion of sequences encompassing either the AP1 or ATF sites have a pronounced effect on expression. We demonstrated that individually the AP1 and ATF elements are capable of mediating the response to EIa; however, our results indicated that the AP1 site is a much better activator of EIa induced expression. Furthermore, we showed that the requirement for EIa was overcome by TPA in expression of the EIII promoter and that this activation was through the AP1 site. This is consistent with studies demonstrating that the AP1 site mediates transcriptional induction in response to TPA (Angel *et al.*, 1987; Lee *et al.*, 1987b).

Interestingly, expression from the adenovirus EII, EIII and EIV promoters can be induced by cAMP (Engel *et al.*, 1988; Leza and Hearing, 1988; Sassone-Corsi, 1988). This induction appears to proceed through the CRE present in the Ad-EII and Ad-EIV promoters. Elements mediating the cAMP response in the EIII promoter are yet to be defined but are likely to include the AP1 and/or ATF site(s). It is important to stress that even though the ATF and CRE elements differ by only one nucleotide and others have suggested that these sites are homologous, our studies suggest that they are functionally different. Previous studies indicated

that two copies of the CRE inserted immediately upstream of the CAP site in the Ad-EIV promoter were potent activators in response to EIa (Miralles *et al.*, 1989). In contrast, our results indicated that two or four copies of the ATF element appear to be at best, weak activators of the EIa response. In light of these differences, it is premature to say if the ATF element is homologous to the CRE.

We found that together EIa and TPA acted synergistically in promoting EIII expression during a viral infection as well as in a transient expression assay. We found that this synergistic response could be potentiated through one type of element, the AP1 site. We can speculate that EIa and TPA act synergistically in EIII expression by causing the phosphorylation of different sites in a single factor (i.e. *jun*); or, since *jun* and *fos* hetrodimers constitute at least one AP1 DNA binding species, that EIa and TPA induce the phosphorylation of separate subunits. Although only one type of element is sufficient for the synergistic response (the AP1 site) multiple copies are still required, and thus EIa and TPA may operate through distinct factors utilizing the same DNA element. This possibility is interesting, for our studies indicate that one copy of each the AP1 and ATF elements (pE3-130) promotes the synergistic response but two copies of either site alone do not. It is interesting to note that a similar situation was shown for the adenovirus EIV promoter, where EIa and cAMP were shown to act synergistically in activating its expression (Engel *et al.*, 1988). The EIII and EIV promoters share a common element, the ATF site, but also possess unique sites, the AP1 and CRE sites respectively. Thus, it will be interesting to compare the regulation of the EIII and EIV promoters in response to combinations of EIa, TPA and cAMP.

The data accumulated thus far indicate that EIa is able to activate transcription through elements utilized in both the TPA (this paper) and the cAMP responses (Engel *et al.*, 1988; Leza and Hearing, 1988; Sassone-Corsi, 1988) and strongly suggest a link between EIa activation and protein phosphorylation. Recent evidence suggests that EIa induction may proceed through the activation or synthesis of a kinase that modifies specific transcription factors (Reichel *et al.*, 1988; Raychaudhuri *et al.*, 1989; Merino *et al.*, 1989). This idea is supported by the following observations: (i) the DNA binding activity of factors that recognize the CRE, AP1 and ATF elements are regulated by their phosphorylation states (Yamamoto *et al.*, 1988; Raychaudhuri *et al.*, 1989; Merino *et al.*, in preparation); (ii) factors recognizing the AP1 consensus sequence are phosphorylated *in vivo* (Merino *et al.*, unpublished observations); and (iii) the *jun* oncogene is induced by post-translational modification in response to TPA and serum (Angel *et al.*, 1988b; Lamph *et al.*, 1988; Ryder and Nathans, 1988). The activation of transcription factors by phosphorylation appears to be a mechanism used by EIa in activation of early gene expression. However, the kinase(s) and pathway(s) utilized in this response remain unknown.

Materials and methods

Plasmid DNA and oligonucleotides

The Ad-EIII promoter construct; pE3 CAT and the deletion mutations −130, Δ82-52, −85, −52 have been described previously (Weeks and Jones, 1985, and Garcia *et al.*, 1987 respectively). The sequence of oligonucleotides were as follows: CRE, 5′-GATCCGTGACGTGATCGATGTGACGTAG-3′ and its complement 5′-GATCCTACGTCACATCGATCACGTCACG-3′; ATF, 5′-GATCCCCGTGACGAG-3′ and its complement 5′-GATCCTCGTCACGGG-3′; AP1, 5′-GATCCGTGACTCAATCGATGTGACTCAAG-3′ and its complement 5′-GATCCTTGAGTCACATCGATTGAGTCACG-3′; MLP-TATA, 5′-GATCCGGCTATAAAAGG-3′ and its complement 5′-GATCCCTTTTATAGCCG-3′; NF1, 5′-GATCCGAATTCTGCTAGCTCTGGCTTTGGGCCAAGAGCCGCGAGCTCG-3′ and its complement 5′-GATCCGAGCTCGCGGCTCTTGGCCCAAAGCCAGACCTAGCAGAATTCG-3′.

Oligonucleotides used for competition in transcription and footprinting assays were phosphorylated with T4 kinase and ligated with T4 DNA ligase as described for the preparation of DNA affinity columns (Kadonaga and Tjian, 1986).

Plasmids pE3/2×AP1 and pE3/4×AP1 were constructed by inserting one and two copies respectively of the AP1 oligonucleotide (each oligonucleotide contained two copies of the AP1 recognition site) into the unique *Sma*I site located at position −52 (relative to the EIII CAP site) of plasmid pE3-52. Plasmids pE3/2×ATF and pE3/4×ATF were similarly constructed by inserting two and four copies of the ATF oligonucleotide into the *Sma*I site of plasmid pE3-52. The nucleotide sequence of the different DNA constructs was determined by DNA sequencing using Sequenase, as described by the manufacturer, USB. The SP6 vector used for RNA analysis of the EIII gene (pSP E3) was constructed by cloning the 375 bp *Eco*RI fragment (containing promoter and CAT coding sequences) from pE3-52 into the *Eco*RI site of pSP18 (BRL). Proper orientation producing EIII antisense message was determined by restriction analysis.

Transcription reactions in vitro

Transcription reactions *in vitro* were as previously described (Cortes *et al.*, 1988), except in Figure 1 in which crude phosphocellulose fractions (phosphocellulose 0.1 M fraction 12 μg, 0.5 M fraction 6 μg and 1.0 M fraction 1.5 μg), served as the source of transcription factors (Reinberg and Roeder, 1987). The general transcription factors IIA, IIB, IIE/IIF, IID and HeLa RNA polymerase II were purified as previously described (Reinberg and Roeder, 1987; Reinberg *et al.*, 1987; Flores *et al.*, 1988). Reactions analyzed in run-off assays contained plasmid DNA pE3 CAT digested with restriction endonuclease *Hpa*I. Transcripts initiated at the EIII CAP site generated RNA molecules of 1550 nucleotides. Reactions analyzed by primer extension were modified as follows: RF template replaced linear DNAs, reaction mixtures were scaled up by 3-fold and reactions were terminated by the addition of an equal volume of stop solution (10 μg/ml sonicated salmon sperm DNA, 10 mM EDTA, 300 mM Na acetate, 0.1% SDS). The primer used consisted of 20 nucleotides of CAT coding sequence (5′-CTCCATTTTAGCTTCCTTAG-3′).

Purification of AP1

AP1 was purified from HeLa cell nuclear extracts (10^{11} cells), prepared as previously described (Dignam *et al.*, 1983). AP1 copurified with EivF and the general transcription factor IIB during the first two fractionation steps, which included chromatography on phosphocellulose and DEAE−cellulose (Reinberg and Roeder, 1987; Cortes *et al.*, 1988). The third chromatographic step was performed using gel filtration on a Sephacryl S-300 column; where sample preparation and chromatographic conditions have been described (Cortes *et al.*, 1988). Every third fraction (1 ml) from this column was dialyzed for 2 h against 4 l of buffer C [20 mM Tris−HCl, pH 7.9 (4°C), 20% (v/v) glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride (PMSF)] containing 100 mM KCl. Aliquots of each dialyzed fraction were then assayed for DNase I protection using a labeled EIII promoter DNA fragment as well as for transcription stimulatory activity as described above. Transcriptionally active fractions were pooled and typically contained 5−8 mg of protein. DNA affinity chromatography using a resin containing multiple AP1 recognition sites was performed with a fraction of the gel filtration pool (0.5−1.0 mg of total protein), using conditions previously described (Lee *et al.*, 1987b), except that chromatography was performed at 22°C. Non-specific competitor DNAs used in the DNA affinity chromatography were composed of 6 μg/ml poly[d(I−C)], 6 μg/ml poly(dA) poly(dT) and 5 μg/ml NF-I oligonucleotide. Competitor DNAs were reduced by one-half for the second pass and one-quarter for the third pass respectively. For reapplication, active fractions were pooled (1−2 ml), concentrated by spin filtraton (Millipore, Ultrafree-MC), and diluted to 100 mM KCl with buffer Z [25 mM HEPES−NaOH, pH 7.8, 12.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20% (v/v) gycerol, 0.05% (v/v) Nonidet P-40].

AP1 affinity column

Oligonucleotides for the AP1 affinity column were prepared essentially as described by Kadonaga and Tjian (1986). The ligated oligonucleotides were

coupled to CNBr activated sepharose 4B (Pharmacia) as described (Hurst and Jones, 1987). The coupling efficiency was ~60% giving an oligonucleotide concentration of ~60 μg/ml of resin. Storage conditions and regeneration of the column were according to Kadonaga and Tjian (1986).

Cell culture and virus stocks

HeLa cells used for nuclear extract preparation and purification of transcription factors were harvested during logarithmic growth ($7-10 \times 10^5$ cells/ml) from Joclick's modified medium (Hazelton) supplemented with 5% calf serum. HeLa and 293 cells used in transfections and infections were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% heat inactivated fetal calf serum (FCS) (Hazelton). Viral stocks of the parental wild type adenovirus dl309 and EIa deletion mutant Dl312 (Jones and Shenk, 1979a and Jones and Shenk, 1979b respectively) were grown in monolayers of HeLa S3 and EIa complementing 293 cells respectively.

DNA transfections and CAT assays

HeLa (clone 2) and 293 cells were grown in 10 cm tissue culture dishes and selected at 50% confluence for transfection. Only HeLa cells that were passaged between four and 10 times were used for transfection experiments. Transfection of DNA into cells was performed by the calcium phosphate coprecipitation method (Wigler *et al.*, 1978), using 10 μg of total DNA (5 μg of plasmid DNA containing the Ad-EIII promoter, 2 μg of plasmid DNA containing the Ad-EIa gene or 2 μg of calf thymus DNA and 3 μg of plasmid DNA containing the β-globin promoter). After 24 h, the medium was changed and cells were treated with 100 ng/ml TPA (Sigma) in 100% dimethylsulfoxide (DMSO) or DMSO alone. Twenty four hours later, the cells were collected by centrifugation, washed with ice cold phosphate buffered saline, and resuspended in 0.25 M Tris−HCl buffer pH 7.5. Soluble extract was prepared and assayed quantitatively for CAT activity using [^{3}H]acetyl-coenzyme A and chloramphenicol as described previously (Sleigh, 1986). These results were confirmed by visualizing CAT activity on TLC plates using [^{14}C]chloramphenicol and acetyl-coenzyme A essentially as described (Gorman *et al.*, 1982). Transfections were repeated at least three times with at least two different preparations of each plasmid DNA.

Adenovirus infections and RNA analysis

HeLa clone 2 cells were grown in 15 cm plates to near confluence and infected at an m.o.i. of 20 for each virus used. Virus was diluted to 1.5 ml with serum free DMEM and allowed to adsorb to the cells for 1 h at which time 25 ml of DMEM containing 2% FCS was added. Cytosine arabinoside (Ara C) was added to 50 μg/ml at a time of infection, feeding and every 6 h thereafter. Cells were harvested and lysed as described previously (Nevins, 1981).

Equal amounts of total cellular RNA (5−10 μg) from each infection were used for RNase T2 anlaysis. SP6 RNA probe was synthesized using 1 μg of pSP E3. Conditions of probe synthesis and hybridization were as described (Melton *et al.*, 1984), except that the temperature for hybridization was 58°C. Digestion with RNase T2 was as described (Vales and Darnell, 1989).

Acknowledgements

We wish to thank Dr Lynne Vales for helpful discussion and reading the manuscript. We also thank Drs K.J.Marians and F.H.Mermelstein for reading the manuscript, Dr R.Gaynor for providing us with plasmid DNA containing different deletions of the Ad-EIII promoter and Drs M.Karin, T.Bos and P.Vogt for providing the antibodies against the c-jun protein. The expertise of Dr Nai-Sheng Lin in preparing the nuclear extracts and Mr D.Wong for assistance in preparing plasmid DNAs is acknowledged. We also thank the members of the laboratory for active discussions. This work was supported by grants from the National Institute of Health, Grant GM 37120, the National Science Foundation, Grant DMB 88-19342, the NJ Commission on Cancer Research Grants 687-035 and 688-026 and a grant from the Foundation of the University of Medicine and Dentistry of New Jersey, L.B. was a recipient of a fellowship from Kirin Beer Company. V.J.M. was a recipient of a fellowship from NATO−Spain. D.R. was a recipient of an American Cancer Society Junior Faculty Award (JFRA-205).

References

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729−739.
Angel,P., Allegretto,E.A., Okino,T.S., Hattori,K., Boyle,W.J., Hunter,T. and Karin,M. (1988a) *Nature*, **332**, 166−171.
Angel,P., Hattori,K., Smeal,T. and Karin,M. (1988b) *Cell*, **55**, 875−885.
Berk,A.J. (1986) *Annu. Rev. Genet.*, **20**, 45−79.
Binger,M.H. and Flint,S.J. (1984) *Virology*, **136**, 387−403.
Bohmann,D., Bos,T.J., Admon,A., Nishimura,T., Vogt,P.K. and Tjian,R. (1988) *Science*, **238**, 1386−1392.
Chatterjee,P.K., Bruner,M., Flint,S.J. and Harter,M.L. (1988) *EMBO J.*, **7**, 835−841.
Chiu,R., Imagawa,M., Imbra,R.J., Bockoven,J.R. and Karin,M. (1987) *Nature*, **329**, 648−651.
Cortes,P., Buckbinder,L., Leza,M.A., Rak,N., Hearing,P., Merino,A. and Reinberg,D. (1988) *Genes Dev.*, **2**, 975−990.
Crossland,L.D. and Raskas,H.J. (1983) *J. Virol.* **46**, 737−748.
Dignam,D.J., Lebovitz,R.M. and Roder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1485.
Engel,D.A., Hardy,S. and Shenk,T. (1988) *Genes Dev.*, **2**, 1517−1528.
Ferguson,B., Krippl,B., Adrisani,O., Jones,N.C., Westphal,H. and Rosenberg,M. (1985) *Mol. Cell. Biol.*, **5**, 2653−2661.
Flores,O., Maldonado,E., Burton,Z., Greenblatt,J. and Reinberg,D. (1988) *J.Biol. Chem.*, **263**, 10812−10816.
Garcia,J., Wu,F. and Gaynor,R. (1987) *Nucleic Acids Res.*, **15**, 8367−8385.
Gaynor,R.B., Feldman,L.T. and Berk,A.J. (1985) *Science*, **230**, 447−450.
Gentz,R., Rausher,F.J.,III, Abate,C. and Curran,T. (1989) *Science*, **243**, 1695−1699.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044−1051.
Green,M., Lowenstein,P.M., Pusztai,R. and Symington,J.S. (1988) *Cell*, **53**, 921−926.
Hai,T., Liu,F., Allegretto,E.A., Karin,M. and Green,M.R. (1988) *Genes Dev.*, **2**, 1216−1226.
Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E. and Leder,P. (1988) *Cell*, **55**, 917−924.
Heoffler,W.K. and Roeder,R.G. (1985) *Cell*, **41**, 955−963.
Hoeffler,W.K., Kovelman,R. and Roeder,R.G. (1988) *Cell*, **53**, 907−920.
Hurst,H.C. and Jones,N.C. (1987) *Genes Dev.*, **1**, 1132−1146.
Jones,N.C. and Shenk,T. (1979a) *Proc. Natl. Acad. Sci. USA*, **76**, 3665−3669.
Jones,N.C. and Shenk,T. (1979b) *Cell*, **17**, 683−689.
Jones,N.C., Rigby,P.W.J. and Ziff,E.B. (1988) *Genes Dev.*, **2**, 267−281.
Kadonaga,J.T. and Tjian,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5889−5893.
Kouzarides,T. and Ziff,E. (1988) *Nature*, **336**, 646−651.
Kovesdi,I., Reichel,R. and Nevins,J.R. (1986) *Cell*, **45**, 219−228.
Kovesdi,I., Reichel,R. and Nevins,J.R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2180−2184.
Lamph,W.W., Wamsley,P., Sassone-Corsi,P. and Verma,I.M. (1988) *Nature*, **334**, 629−633.
Lee,K.A.W. and Green,M.R. (1987) *EMBO J.*, **6**, 1345−1353.
Lee,K.A.W., Hai,T., SivaRaman,L., Thimmappaya,B., Hurst,H.C., Jones,N.C. and Green,M.R. (1987a) *Proc. Natl. Acad. Sci. USA*, **84**, 8355−8359.
Lee,W., Mitchell,P. and Tjian,R. (1987b) *Cell*, **48**, 741−752.
Leff,T., Elkiam,R. and Sassone-Corsi,P. (1985) *Nucleic Acids Res.*, **13**, 1209−1221.
Leza,M.A. and Hearing,P. (1988) *J.Virol.*, **62**, 3003−3013.
Lillie,J.W., Green,M. and Green,M.R. (1986) *Cell*, **46**, 1043−1051.
Lin,Y.-S. and Green,M.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3396−3400.
Melton,D.A., Krug,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035−7036.
Merino,A., Buckbinder,L., Mermelstein,F.H. and Reinberg,D. (1989) *J. Biol. Chem.*, in press.
Miralles,V.J, Cortes,P., Stone,N. and Reinberg,D. (1989) *J. Biol. Chem.*, **264**, 10763−10772.
Montell,C., Courtois,G., Eng,C. and Berk,,A.J. (1984) *Cell*, **36**, 951−961.
Montell,C., Fisher,E.F., Caruthers,M.H. and Berk,A.J. (1982) *Nature*, **295**, 380−384.
Montminy,M.R., Sevarino,K.A., Wagner,J.A., Mandel,G. and Goodman,H.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6682−6686.
Montminy,M.R. and Bilezikjian,L.M. (1987) *Nature*, **328**, 175−178.
Nevins,J.R. (1981) *Cell*, **26**, 213−220.
Rauscher,F.J.,III, Cohen,D.R., Curan,T., Bos,T.J., Vogt,P.K., Bohmann,D., Tjian,R. and Franza,B.R.,Jr (1988) *Science*, **240**, 1010−1016.
Raychaudhuri,P., Bagchi,S. and Nevins,J.R. (1989) *Genes Dev.*, **3**, 620−627.
Reichel,R., Kovesdi,I. and Nevins,J.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 387−390.

Reinberg,D. and Roeder,R.G. (1987) *J. Biol. Chem.*, **262**, 3310–3321.
Reinberg,D., Horikoshi,M. and Roeder,R.G. (1987) *J. Biol. Chem.*, **262**, 3322–3330.
Ryder,K. and Nathans,D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8464–8467.
Sassone-Corsi,P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7192–7196.
Sassone-Corsi,P., Ransone,L.J., Lamph,W.W. and Verma,I.M. (1988) *Nature*, **336**, 692–695.
Sleigh,M.J. (1986) *Anal. Biochem.*, **156**, 251–256.
Stephens,C. and Harlow,E. (1987) *EMBO J.*, **6**, 2027–2035.
Turner,R. and Tjian,R. (1989) *Science*, **243**, 1689–1694.
Vales,L.D. and Darnell,J.E.,Jr (1989) *Genes Dev.*, **3**, 49–59.
Velcich,A. and Ziff,E. (1985) *Cell*, **40**, 705–716.
Vogt,P.K. and Tjian,R. (1988) *Oncogene*, **3**, 3–7.
Weeks,D.L. and Jones,N.C. (1983) *Mol. Cell. Biol.*, **3**, 1222–1234.
Weeks,D.L. and Jones,N.C. (1985) *Nucleic Acids Res.*, **13**, 5389–5402.
White,E., Denton,A. and Stillman,B. (1988) *J. Virol.*, **62**, 3445–3454.
Wigler,M.S., Silverstein,S., Lee,L.S., Pellicer,A., Cheng,Y.C. and Axel,R. (1977) *Cell*, **11**, 223–232.
Yamamoto,K.K., Gonzales,G.A., Biggs,W.H.,III and Montminy,M.R. (1988) *Nature*, **334**, 494–498.
Yoshinaga,S., Dean,N., Han,M. and Berk,A.J. (1986) *EMBO J.*, **5**, 343–354.
Zajchowski,D.A., Jalinot,P. and Kedinger,C. (1988) *J. Virol.*, **62**, 1762–1767.

Received on July 5, 1989; accepted on September 18, 1989