MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3806–3817
0270-7306/87/103806-12$02.00/0

Vol. 7, No. 10

The Abundance and In Vitro DNA Binding of Three Cellular Proteins Interacting with the Adenovirus EIIa Early Promoter Are Not Modified by the EIa Gene Products

P. JALINOT, B. DEVAUX, AND C. KÉDINGER*

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Faculté de Médecine, 67085 Strasbourg Cédex, France

Received 24 March 1987/Accepted 14 July 1987

Specific protein binding on the EIa-inducible adenovirus EIIa early (EIIaE) promoter was analyzed by the sensitive electrophoretic band-shift assay and by protection against DNase I digestion. Three factors were identified, and precise mapping of the cognate-binding sites revealed their correspondence to promoter elements essential for constitutive EIIaE transcription. One binds to the major upstream element located between −82 and −64 (with respect to the major EIIaE cap site), another appears to interact with sequences on either side of this region, and the last one binds to an element located further upstream. Comparison of the binding activities of the factors present in extracts from cells infected with wild-type adenovirus (adenovirus type 5) or with the EIa deletion mutant *dl*312 did not reveal striking differences. Not only were the general binding patterns indistinguishable, but the concentration of each of the identified factors as well as their affinity for the cognate-binding sites were unchanged. Our results suggest that the EIa-mediated activation of the EIIaE transcription complexes involves appropriate interactions between transcription factors, rather than their increased binding to DNA.

The understanding of the control mechanisms of gene pattern transcription during the development of eucaryotic organisms and in terminally differentiated cells is a major goal of molecular biology. At least a portion of this regulation appears to be achieved through the interaction of *trans*-acting proteins with *cis*-acting DNA promoter elements, resulting in positive or negative transcriptional effects (10, 47, and 50). Transcriptional activation of the adenovirus early genes by the viral immediate-early EIa gene products (44) provides an attractive model system for the study of the molecular mechanisms involved in eucaryotic gene induction. EIa-mediated stimulation is not restricted to adenoviral promoters since endogenous host genes such as the hsp70 heat shock (45) and β-tubulin genes (52) as well as other cellular or viral genes introduced into cells by transfection or infection (8, 16, 18) are also stimulated. Similarly, genes transcribed by RNA polymerase III are expressed at higher levels when cotransfected in the presence of the EIa transcription unit (2, 15).

With the exception of the adenovirus EIb promoter, in which the integrity of the TATA box is essential for the EIa-mediated induction (58), no discrete element could be delineated in the other adenovirus early-gene promoters which would be uniquely involved in the EIa-*trans*-activation process (3, 21, 35). This has been most thoroughly documented in the case of the adenovirus types 2 and 5 (Ad5) EIIa early (EIIaE) promoter, in which an extensive series of deletion and linker-scanning mutations (5, 11, 26, 31, 42, 60) have been analyzed in different transcription assays. No unique sequence element was identified that depressed EIa responsiveness without also decreasing constitutive promoter activity. These results strongly suggest that the stimulation of transcription induced by the EIa proteins involves the same host cell DNA-binding transcription factors as those used for uninduced transcription. The mechanism of EIa-mediated induction implies, therefore, that the EIa products do not act by binding directly to promoter sequences, but rather by interacting with or modifying preexisting host cell transcription factors. In the case of genes transcribed by RNA polymerase III, EIa *trans*-activation results in the increased accumulation of active transcription factor IIIC (24, 59). The mechanism of EIa-dependent induction of polymerase II-transcribed genes is still poorly understood. For the EIIaE transcription unit, Kovesdi et al. (32) have reported that the concentration or binding activity of a host cell transcription factor is increased in adenovirus-infected cells, while Siva Raman et al. (51) in a comparable study found no significant difference, although the factors identified in each study may be different.

Using both the electrophoretic band-shift assay and DNase I footprinting we characterized proteins which specifically bind to all the promoter elements previously shown to be critical for efficient and accurate transcription from the major EIIaE start site (+1). The binding activities of these proteins to the promoter were identical when assayed in extracts from HeLa cells infected with either wild-type Ad5 or with the EIa deletion mutant *dl*312. Our results suggest, therefore, that the EIa products do not stimulate EIIaE promoter activity by altering the capacity of these host cell transcription factors to bind to their corresponding promoter elements, but rather by inducing new protein-protein interactions.

MATERIALS AND METHODS

Crude cell extracts and partially purified protein fractions. HeLa cells were grown in Eagle minimal essential suspension culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 7% newborn calf serum, collected by centrifugation at a density of about 4×10^5 cells per ml, suspended at about 5×10^7 cells per ml in 50 mM Tris hydrochloride (pH 7.9)–10 mM α-thioglycerol–30% glycerol

* Corresponding author.

(final concentration), and frozen in liquid nitrogen. In some experiments the cells were infected at a multiplicity of infection of 100 PFU of virus per cell with either mutant *dl*312 (29) or wild-type Ad5 as described previously (57), in the presence of 10 mM hydroxyurea to prevent DNA replication. Cells were harvested 6 h postinfection and further processed or frozen as described above. The nuclear extracts were prepared as described by Dignam et al. (9) with the modifications introduced by Wildeman et al. (56). Nuclei were extracted at 0.42 M (NE0.42), 0.25 M (NE0.25), or 0.55 M (NE0.55) NaCl. Whole-cell extracts (WCE) were obtained by the procedure of Natarajan et al. (43), except that the final dialysis buffer (buffer A) contained 6 mM instead of 12.5 mM $MgCl_2$, resulting in type A WCE. Alternatively, extracts were prepared in which the ammonium sulfate precipitation step was omitted (type B WCE). In the latter case, the supernatant obtained after the 3-h high-speed centrifugation was frozen in liquid nitrogen, concentrated twofold by lyophilization (about 1 h), and dialyzed as above. Extracts prepared from fresh or frozen cells gave identical results (unpublished observation). Subsequent fractionation of the WCE by chromatography on heparin-Sepharose and DEAE columns has been previously described (41). Briefly, transcription factors present in the WCE were adsorbed on heparin-Sepharose and eluted with 0.6 M KCl, yielding the H0.6 fraction. These proteins were further fractionated on DEAE-cellulose by stepwise elution with buffers containing 0.15, 0.20, 0.25, and 1.00 M KCl, giving the DE0.15, DE0.20, DE0.25, and DE1.00 fractions, respectively. Protein concentrations were determined with the Pierce BCA reagent, following the instructions of the manufacturer.

Gel electrophoresis DNA binding assay. All DNA fragments (5′ end labeled with ^{32}P [36]) were purified by polyacrylamide gel electrophoresis and recovered by electroelution as described by Wildeman et al. (56). Unless indicated otherwise, 2 μg of protein from the WCE, nuclear extract, or H0.6 fraction or 0.4 μg from the DE fractions was used per assay. The optimum quantity of poly(dI-dC) (Pharmacia, Uppsala, Sweden) added to the binding reaction as nonspecific competitor DNA was determined empirically for each protein sample, but was generally 0.4 μg for WCE or NE and 0.8 μg for the H0.6 and DE fractions. Typically, about 5,000 cpm (~0.3 ng) of the labeled DNA fragments were mixed with the poly(dI)-poly(dC) in a buffer containing 20 mM Tris hydrochloride (pH 7.9), 70 mM KCl, 2 mM $MgCl_2$, 0.04 mM EDTA, 0.2 mM dithiothreitol, 12% (vol/vol) glycerol, and the protein fractions in a total volume of 10 μl. The mixtures were incubated for 25 min at 25°C and loaded directly onto 4.5% polyacrylamide gels (160 by 120 by 1.5 mm; acrylamide/bisacrylamide ratio, 80:1). Gels were always prerun for at least 1.5 h before sample loading and electrophoresed for about 2 h at 150 V and 18°C. Marker dyes (xylene cyanol and bromophenol blue), which destabilize the complexes, were omitted from the samples but run in parallel. The electrophoresis buffer (pH 7.5), which contained 6.7 mM Tris base, 3.3 mM sodium acetate, and 1 mM EDTA (53), was constantly recirculated. The gels were fixed for 10 min in 10% acetic acid–10% methanol and vacuum dried before autoradiography.

For quantitative purposes the retarded and free-running DNA bands were scanned on nonsaturated exposures of the gels. The corresponding amounts of DNA were deduced from standard curves constructed with the densities of known quantities of the same probe DNA, run along with the binding reactions. The quantification of specific binding factor and the apparent equilibrium constant of the binding reaction for each complex were determined (see Results) as described by Baker et al. (1).

Methylation interference experiments. The DNA probes, kinased at one end or the other, were methylated with dimethyl sulfate (38). About 2×10^5 cpm of the methylated probes were incubated with the WCE or DE0.15 fraction under the standard binding conditions and electrophoresed as described above, except that the gel was neither fixed nor dried before exposure. The DNA fragments were then electroeluted from the gel, phenol-chloroform extracted, and ethanol precipitated twice. Equal amounts of radioactivity of the different samples were treated with piperidine and analyzed on 8% polyacrylamide–8 M urea gels (38).

DNase I protection. The original DNase I footprinting assay (13) as modified by Boeuf et al. (5) was used.

RESULTS

Identification of three different nucleoprotein complexes within the EIIaE promoter region. To detect specific binding of HeLa cell proteins to the promoter sequence of the adenovirus EIIaE transcription unit, we used the electrophoretic mobility shift assay. As similar results were obtained with DNA fragments extending from positions −280 or −169 to +37 with respect to the major start site, we used the shorter fragment in the following experiments (Fig. 1). In an attempt to optimize the release of the specific DNA-binding factors when preparing the extracts, a preliminary survey was performed with a standard WCE (WCE type A) and nuclear extracts prepared in the presence of 0.25, 0.42, or 0.55 M NaCl (NE0.25, NE0.42, and NE0.55, respectively;

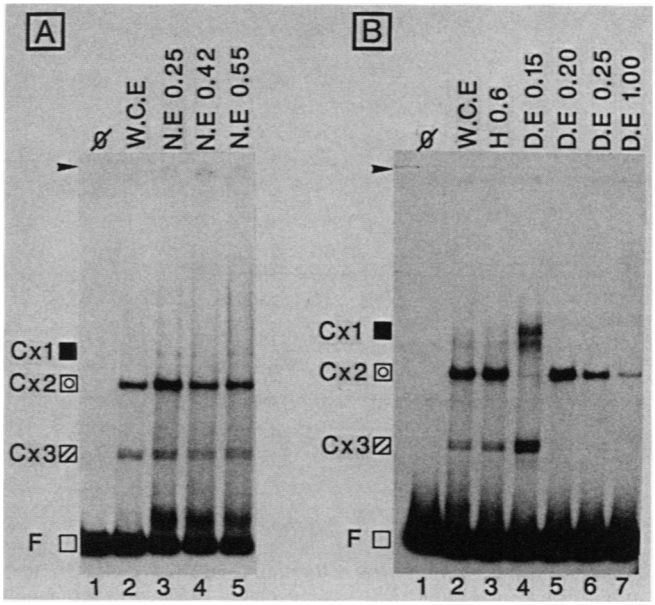

FIG. 1. Detection and fractionation of proteins that bind to the Ad2 EIIaE promoter region. (A) End-labeled fragment F (see Fig. 2B) was incubated without (Ø, lane 1) or with (lanes 2 to 5) the indicated extracts in standard gel electrophoresis DNA binding assays (see Materials and Methods). Unbound probe DNA is referred to as F (□), while retarded bands corresponding to the slowest-, intermediate-, and fastest-migrating complexes are referred to as Cx 1 (■), Cx 2 (⊡), and Cx 3 (▨), respectively. The arrowhead points to the top of the gel. (B) Binding assays with probe F in the absence (lane 1) or in the presence of WCE (lane 2) or chromatographic subfractions (see Materials and Methods) as indicated (lanes 3 to 7) were performed as described above.

see Materials and Methods). Equal quantities of protein from each extract yielded very similar band patterns in the retardation assay with the EIIaE fragment, revealing the presence, at roughly equivalent concentrations, of the same specific DNA-binding proteins in each extract (Fig. 1A).

A typical result of such an experiment is presented in Fig. 1. Three major sets of bands were found consistently. The complex with the lowest mobility (Cx 1) appears as a cluster of weak bands, while complex 2 (Cx 2) corresponds to a single strong band. Complex 3 (Cx 3), the faster-migrating band, appears as a doublet of intermediate intensity. For the sake of simplicity, the components responsible for this band shifting are called hereafter the Cx 1, 2, and 3 factors, respectively.

As a first attempt to separate and concentrate these factors, a WCE was fractionated according to a previously established chromatography procedure (40, 41). After adsorption of the extract on heparin-Sepharose, a majority of the loaded band-shifting activity was then eluted at 0.6 M KCl (H0.6 in Fig. 1B; compare lanes 2 and 3). Further chromatography of this fraction on DEAE-cellulose resulted in complete separation of factors Cx 1 and 3 from factor Cx 2. The Cx 1 and Cx 3 proteins were eluted together at 0.15 M KCl (Fig. 1B, DE0.15, lane 4), while the Cx 2-specific proteins were recovered essentially in the 0.20 M fraction (DE0.20, lane 5), with some residual activity eluting at still higher salt concentrations (DE0.25 and DE1.00, lanes 6 and 7). The specific activities of the factors which form Cx 1 and Cx 3 were significantly increased after DEAE chromatography (compare lanes 2 and 4).

To map the binding sites corresponding to these complexes, we performed DNA template competition experiments with a set of overlapping EIIaE fragments (Fig. 2).

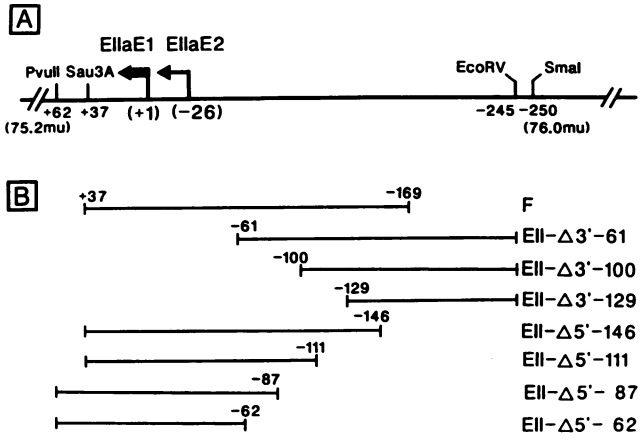

FIG. 2. Physical map of EIIaE promoter region and origin of the DNA fragments used in the binding experiments. The Ad2 region between the *Sma*I (76.0 map units [mu]) and the *Pvu*II (75.2 map units) sites is schematically represented (A), with the major (EIIaE1, +1) and minor (EIIaE2, −26) start sites and direction of transcription indicated by the arrows. The EIIaE promoter fragments used in the electrophoretic retardation experiments are shown in panel B. Fragment F corresponds to the *Bam*HI (−169)-*Sau*3A (+37) fragment of pBX−169 (11) subcloned into the *Bam*HI site of M13mp11 and reexcised by *Xba*I-*Sma*I. The Δ3' series of molecules were *Eco*RV-*Xba*I fragments prepared from the corresponding Mp9EII3' recombinants (60). The Δ5'−146 and Δ5'−111 fragments were generated as was fragment F after subcloning of the appropriate *Bam*HI-*Sau*3A segments. The Δ5'−87 and Δ5'−62 fragments were isolated from the pGD−87 and pGD−62 recombinants, respectively (28), by *Eco*RI-*Xho*I digestion.

DNA-protein complexes were formed with either the WCE (Fig. 3A and B) or the DE0.15 fraction (Fig. 3C and D) in the presence of a fixed amount of the labeled EIIaE fragment (F, Fig. 3) and increasing concentrations of the cold competitor fragments. In these experiments, either the WCE or DE0.15 fraction was used to preferentially reconstitute Cx 2 or Cx 1 and Cx 3, respectively.

Since both Δ3'−61 and Δ5'−146 EIIa fragments competed for the formation of all three complexes with the same efficiency as the fragment F itself, it was concluded that the binding sites for these complexes are located between positions −146 and −61. Similarly, because the Δ3'−61 fragment and all Δ5' fragments, except Δ5'−62, competed for the Cx 1 factor, the corresponding complex (Cx 1) must be positioned between −87 and −62. The region between −129 and −111 can then be assigned to Cx 2 since these positions correspond to the closest 3' and 5' boundaries of the noncompeting Δ3' and Δ5' fragments, respectively. Finally, the localization of Cx 3 between −146 and −129 is deduced from the observation that all Δ3' fragments and the Δ5'−146 fragment compete for its formation.

It is noteworthy that the formation of each of these complexes is essentially independent of the others. The formation of complex Cx 1 or 3 can indeed be competed separately from the two others with appropriate competitor DNA fragments (Fig. 3). Furthermore, Cx 2 can form in the absence of either Cx 1 or Cx 3 (Fig. 3A, lanes 11 to 13; Fig. 1B, lanes 5 to 7). These observations, together with the fact that the Cx 2 factor can be separated from the Cx 1 and 3 factors (Fig. 1B) and that the latter two factors can also be resolved by further chromatography on a sulfopropyl column (data not shown), strongly suggest that the proteins involved in each of these complexes are different.

Precise localization of the three binding sites by methylation interference studies. To refine the location of the nucleoprotein complexes on the EIIaE promoter and to determine the G residues in direct contact with the factor, we performed methylation interference experiments. Fragment F, 5' end labeled at either end, was submitted to random dimethyl sulfate treatment before specific complex formation with the crude WCE or the DE0.15 protein fraction. The bound fraction of the labeled probe (corresponding to Cx 1, 2, and 3) was then separated from the unbound molecules (F) by the gel retardation assay. The fragments were extracted from the gel and further processed to produce the G ladders on DNA sequencing gels (Fig. 4A and B). Those G residues whose methylation interfered with factor binding were revealed on the sequence ladders corresponding to the retarded fragment as weaker or absent bands. Taking the first unaffected G residues flanking the protected region as the upper limits of the binding sites, a maximal contact area for each complex on the EIIaE promoter could be delineated (see boxed sequences in Fig. 4C). The recognition sequences of these complexes are thus located within the regions between −82 and −64 for Cx 1, −127 and −112 for Cx 2, and −142 and −129 for Cx 3, in perfect agreement with the deletion mapping.

Interaction of a factor recognizing the proximal EIIa promoter region with the distal Cx 2-binding site. In the experiments described above, probe F, which spans the entire EIIaE promoter (Fig. 2), allowed the identification of factor-binding sites only on the most upstream portion of this promoter. To screen more specifically for protein interactions with proximal elements, we used a shorter probe (EII-Δ5'−62, or F') lacking the three upstream binding sites. In the experiment shown in Fig. 5A (lane 1), a major shifted

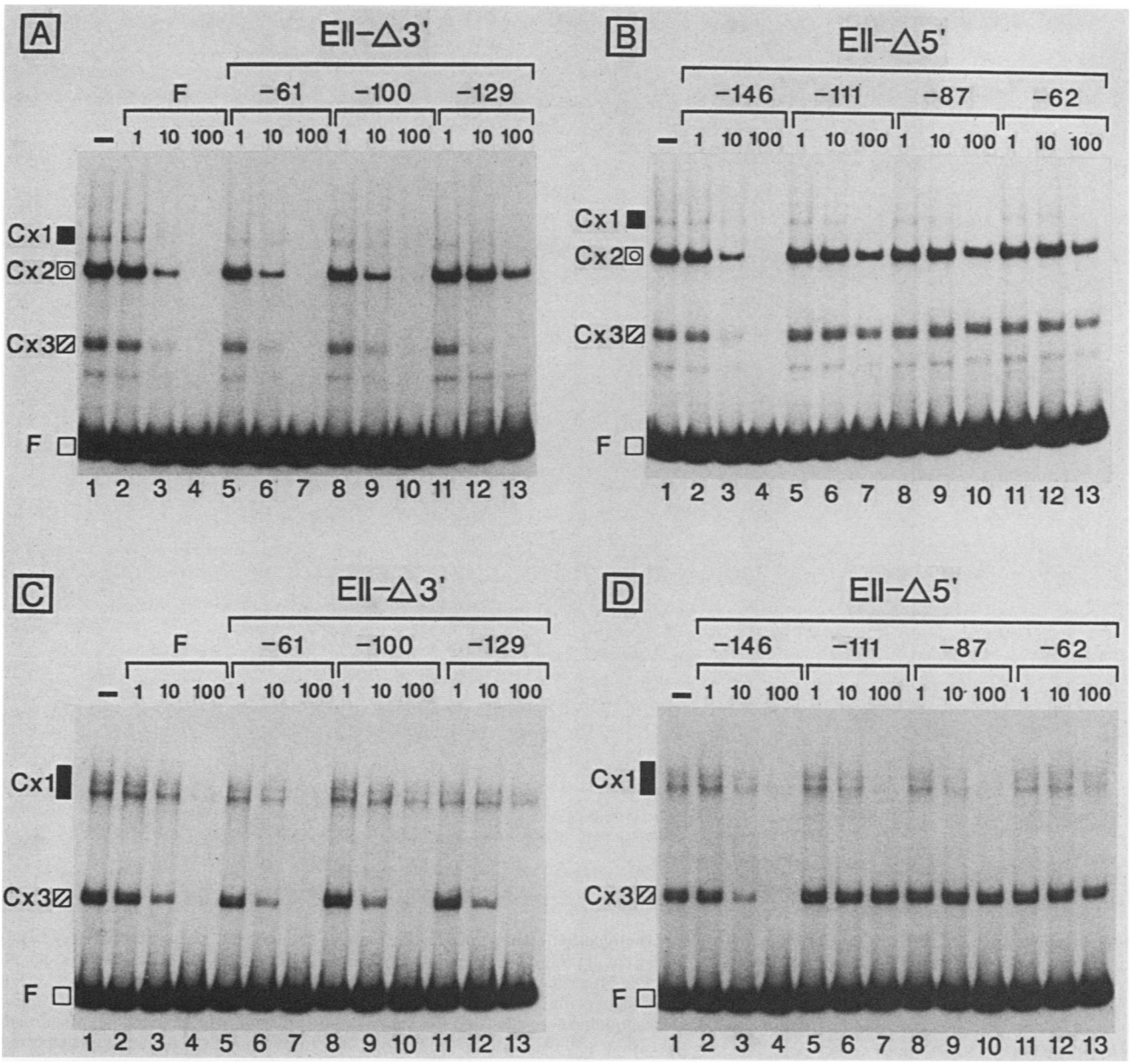

FIG. 3. Mapping of the protein-binding site on the EIIaE promoter by DNA competition experiments. Increasing amounts of the selected EIIaE unlabeled promoter fragments (Fig. 2B) were included as indicated in standard binding assays, in the presence of labeled fragment F and either WCE (A and B) or DE0.15 fraction (C and D). The competitor DNA was added at molar ratios of 1, 10, and 100 with respect to the amount of labeled probe present. The effects on specific complex formation of the addition of 3′ (A and C)- and 5′-(B and D)-deleted fragments were compared with that of the addition of the unlabeled probe F itself (lanes 2 to 4 in panels A and C), and to the pattern obtained in the absence of any EIIaE competitor DNA (lanes 1).

band (Cx 4) appeared after incubation of the truncated probe with the WCE. Methylation interference mapping of this Cx 4-binding site (Fig. 5B) pointed to a 5-nucleotide motif on the noncoding strand (between −44 and −40; Fig. 5C). Interactions with the coding strand involved the residues at −42 and −40; however, the boundaries (especially the 5′ limit) are less well defined on this strand owing to the absence of G residues. It is striking that the nucleotide sequence of the minimal binding site thus revealed for Cx 4 around position −42 (5′-GGCGC-3′) is identical to the sequence corresponding to Cx 2 around position −117. That this latter sequence element can also bind the Cx 4 factor was confirmed by competition experiments. While EII-Δ3′−129 (Fig. 2) was a very poor competitor for Cx 4 (Fig. 5A, lanes 5 to 7), a fragment comprising the Cx 2-binding site (EII-Δ3′−100; Fig. 2) competed efficiently (Fig. 5A, lanes 2 to 4). These findings strongly support the possibility that the same or a closely related factor is involved in the formation of both Cx 2 and Cx 4 complexes. The binding activity of the Cx 4 factor, however, appeared much weaker than that of the Cx 2 factor, since higher protein concentrations were required for the Cx 4 complex to be visualized. Furthermore, while the Cx 2-binding site readily competed for the binding of factor Cx 4 (Fig. 5A, lanes 2 to 4), the reverse was not true (Fig. 3B, lanes 5 to 7). If both complexes consist of the same factor, the proximal binding site is recognized with a much

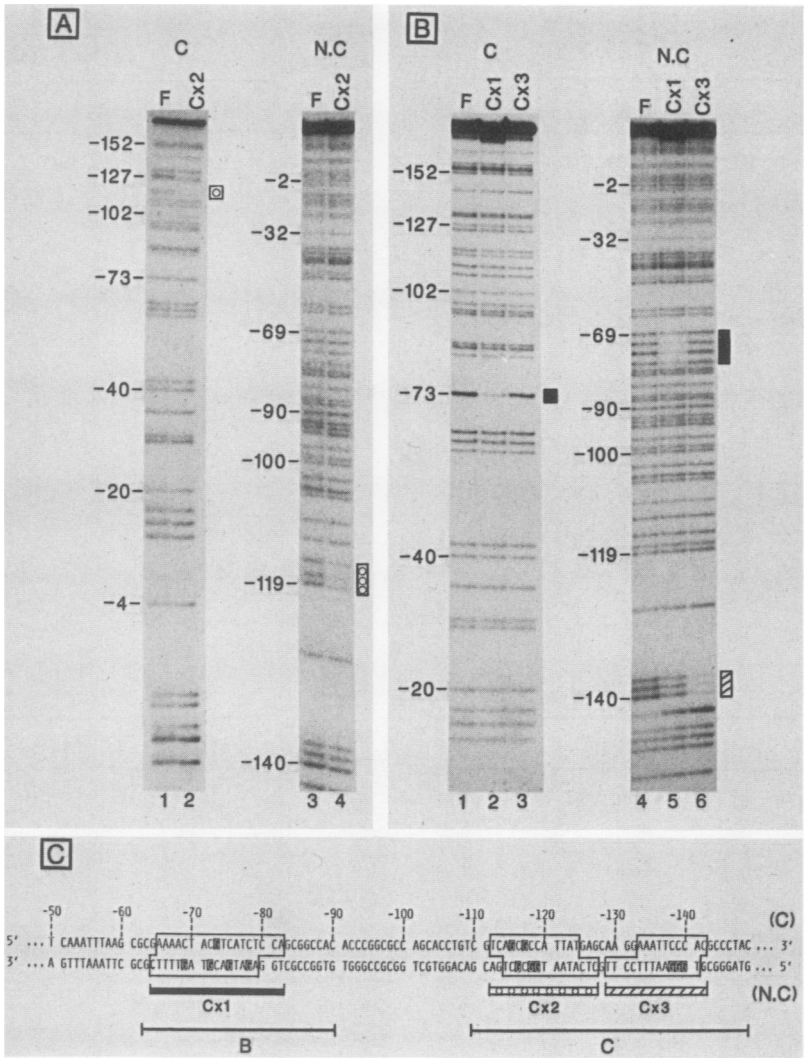

FIG. 4. Fine mapping of Cx 1, 2, and 3 binding sites by methylation interference. DNA binding assays were performed under standard conditions, except that probe F (5′ end labeled either on the coding [C] or the noncoding [N.C] strand) was treated with dimethyl sulfate before incubation with the WCE (A) or the DE0.15 fraction (B). After electrophoresis, the DNA present in each band (F, Cx 1, 2, and 3, Fig. 1) was extracted, treated with piperidine, and analyzed on sequencing gels (see Materials and Methods). Numbers on the left of the G sequence ladders of probe F denote the positions of the corresponding G residues on the EIIaE promoter, with respect to the major start site (+1). The boxes (■, Cx 1; ⊡, Cx 2; ▨ Cx 3) span those G residues whose methylation prevents the formation of the respective complexes. The sequence of the relevant portion of the EIIaE promoter is provided in panel C. The G residues which are involved in the formation of specific nucleoprotein complexes are shaded, and the maximal interaction sequence is framed. The boxes below the sequence delimit the binding area for each complex with their corresponding symbols. The bars marked B and C span the sequences involved in constitutive EIIaE1 promoter function (4, 60).

lower affinity. Divergence between the sequences which flank the conserved motif may account for their unequal binding capacities. This is further supported by the observation that the bound fraction of probe F′ is hypermethylated on the G residue (position −45) just next to the conserved sequence of Cx 4 (Fig. 5B, lane 4), indicating that modification of that residue facilitates complex formation.

Binding of factors Cx 1, 2, and 3 to promoter sequences of other adenovirus early genes. To determine whether the nuclear factors which, in this band shift assay, bind to the EIIaE promoter also interact with other adenovirus early genes, we performed additional competition experiments. Increasing amounts of DNA fragments spanning the promoter region of each of the other early transcription units (EIa, EIb, EIII, and EIV) were added to standard binding assays. Either the WCE (Fig. 6A) or the DE0.15 fraction (Fig. 6B) was used to examine their effect on Cx 2 or Cx 1 and Cx 3 formation, respectively.

Under these conditions, both EIa and EIV promoter sequences strongly competed for the Cx 1, 2, and 3 factors (Fig. 6, lanes 2 to 4 and 11 to 13). The EIb fragment efficiently competed for Cx 2, but did not significantly affect Cx 1 and 3 formation (Fig. 6, lanes 5 to 7), while the EIII fragment did not impair Cx 2 or Cx 3 formation, but moderately competed for Cx 1 (Fig. 6, lanes 9 and 10). However, this latter effect seems specific since it was not observed when equivalent concentrations of random pBR fragments were used (data not shown). These results suggest, therefore, that the promoter regions of both EIa and EIV transcription units contain binding sites for Cx 1, 2, and

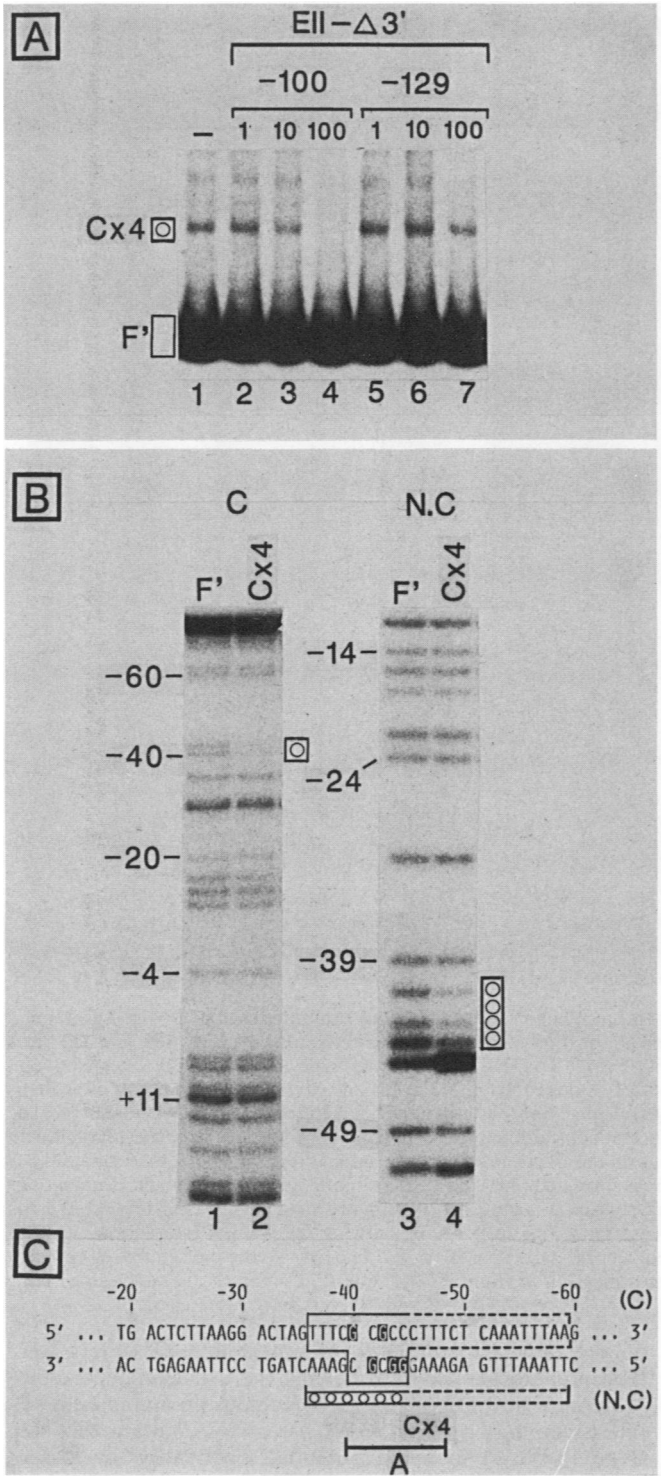

FIG. 5. Mapping of the protein-binding site in the EIIaE proximal promoter region. The EII-Δ5'−62 DNA fragment (Fig. 2B) was used as the labeled probe (F') with 6 μg of type A WCE and 1.2 μg of poly(dI-dC) in the binding reaction (A), either alone (lane 1) or in the presence of 1, 10, or 100 molar excess of cold EII-Δ3'−100 (lanes 2 to 4) or EII-Δ3'−129 (lanes 5 to 7) competitor DNA. Cx 4 refers to the major retarded band observed in this assay. Methylation interference mapping of the Cx 4 binding site is shown (B) on both coding (C) and noncoding (N.C) strands. The G residues involved in Cx 4 complex formation are shaded on the corresponding nucleotide sequence of the EIIaE promoter (panel C), where the framed sequences represent the maximal interaction site. The dashed lines extending to the next unaffected 3' G residue on the coding strand delimit a less probable binding region. The boxes with the circles (⊡) span the regions corresponding to Cx 4 in each panel. The bar marked A delimits a sequence element involved in constitutive promoter activity (60).

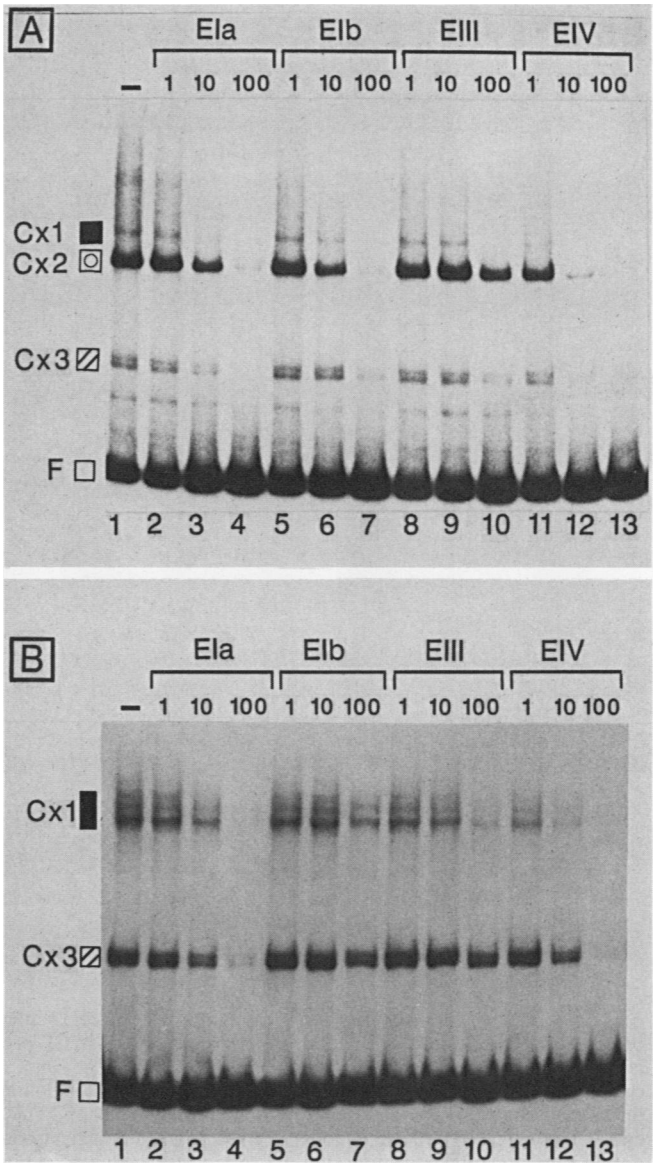

FIG. 6. DNA binding competition experiments with adenovirus heterologous promoter fragments. Factors involved in specific complex formation on fragment F were titrated in WCE (A) or DE0.15 (B) by increasing amounts of competitor DNA fragments, as described in legend to Fig. 3. The following Ad2 promoter fragments were used: EIa, between nucleotides 0 (position −497) and 451 (−47); EIb, between nucleotides 1569 (−130) and 2045 (+347); EIII, between nucleotides 27372 (−237) and 27634 (+26); EIV, between nucleotides 35360 (+250) and 35937 (−327). The values in parentheses refer to the positions of the cleavage sites with respect to the start site of the corresponding transcription unit; the other values give the coordinates of the sites on the Ad2 genome. Symbols are as described in the legend to Fig. 1.

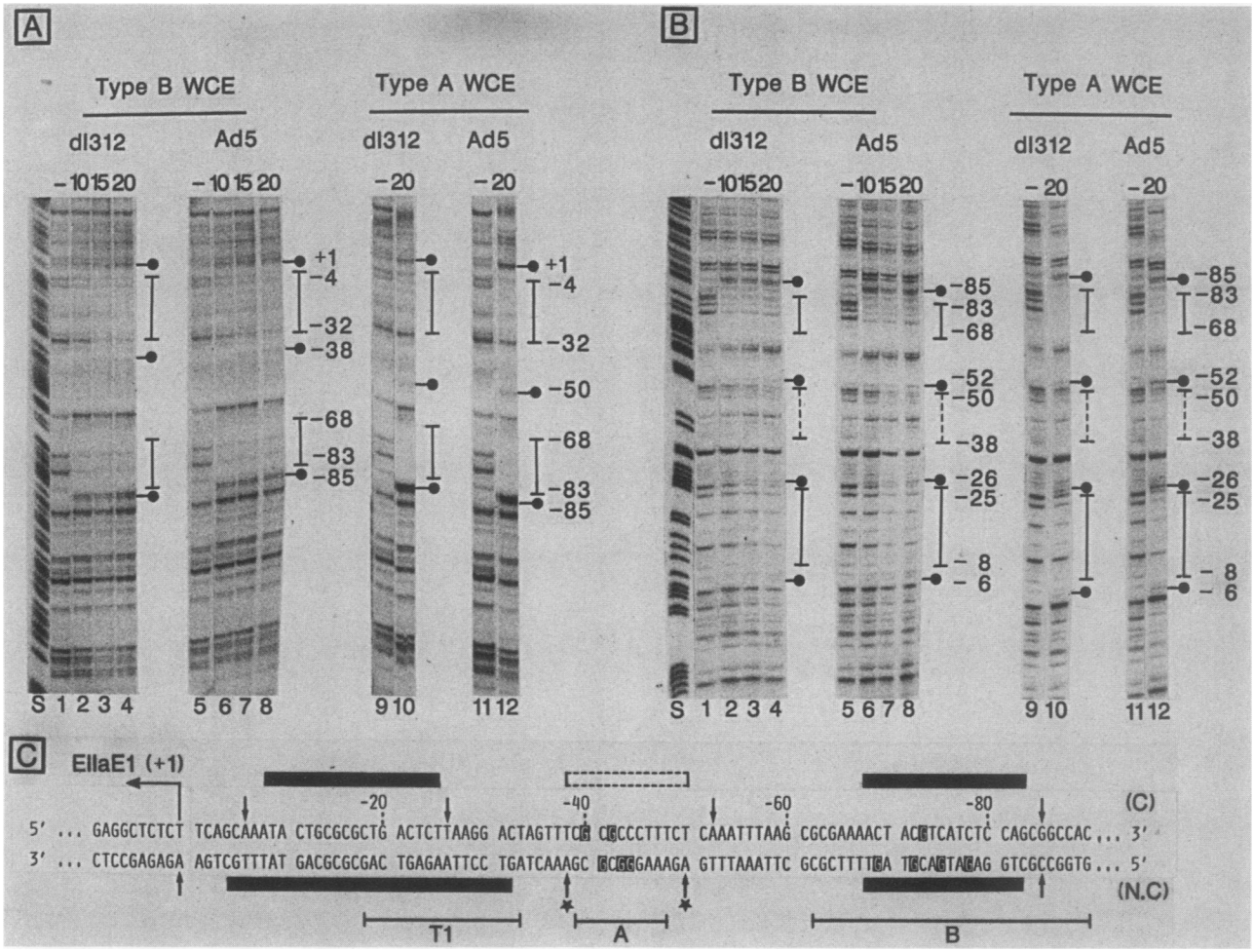

FIG. 7. DNase I comparative footprinting of Ad5- and *dl*312-infected HeLa cell extracts on the EIIaE promoter region. DNase I protection provided by type A (lanes 9 to 12) or type B (lanes 1 to 8) WCE prepared 6 h postinfection from HeLa cells infected with Ad5 or *dl*312 virus is shown on the noncoding (A) and coding (B) strand of the *Sma*I (−250)-*Pvu*II (+62) EIIaE fragment. Type A and B WCEs, respectively, correspond to extracts precipitated or not precipitated by ammonium sulfate as described in Materials and Methods. The amount of proteins (in micrograms) used in each assay is indicated on the top. The DNase I digestion pattern of the naked probe is shown under −. The corresponding G+A sequence ladder is given in lanes S. The strongest protections are marked with solid bars, weaker ones are marked with dotted bars, and hypersensitive sites are shown with heavy dots. Positions on the EIIaE promoter are indicated with respect to the major start site (+1). The nucleotide sequence of the relevant EIIaE promoter region is provided (C), with the horizontal arrow indicating the major start site (EIIaE1, +1) and direction of transcription. G residues involved in Cx 1 and 4 complex formation are shaded. DNase I-protected areas are spanned by closed or dashed boxes next to the corresponding DNA strand, the dashed box showing the weaker interaction. Vertical arrows point to DNase I-hypersensitive sites (stars refer to sites observed in either type A or B WCE). The bars below the sequence represent the promoter elements (T1, A, and B) essential for efficient constitutive transcription from EIIaE1 (60). C, Coding; N.C, noncoding.

3 factors, while EIb and EIII sequences are recognized only by Cx 2 and Cx 1 or related factors, respectively.

Abundance and binding activity of Cx 1, 2, and 3 factors in cell extracts are not affected by EIa gene products. The experiments presented above were all performed with extracts or fractions derived from uninfected HeLa cells. Since the EIIaE transcriptional unit, like the other early genes of adenovirus, is subjected to transcriptional activation by the EIa gene products, it was of interest to examine the binding properties of extracts prepared from adenovirus-infected cells. The patterns of specific nucleoprotein complexes formed on the EIIaE promoter with extracts from HeLa cells infected with either *dl*312, a mutant adenovirus lacking the EIa region, or the parental wild-type adenovirus (Ad5) were compared. The cells were harvested 6 h postinfection, a time at which EIIaE gene transcription is maximal with Ad5, but undetectable with *dl*312 (data not shown). Extracts were then prepared either by following the usual procedure (see Materials and Methods), which includes an ammonium sulfate precipitation (type A WCE), or by omitting this step (type B WCE) to avoid potential inactivation or loss of unprecipitable components.

A comparison of *dl*312- and Ad5-infected cell extracts was first undertaken by DNase I footprinting experiments. The protection pattern conferred to the EIIaE promoter fragment by EIa-lacking (Fig. 7A and B, lanes 1 to 4, 9, and 10) and EIa-containing (lanes 5 to 8, 11, and 12) extracts appeared essentially indistinguishable, regardless of the type of WCE extract used (type A or B). Detailed analysis of the digestion patterns, summarized in Fig. 7C, revealed a strong protection on both strands between −83 and −68. A weaker protection observed only on the coding strand (Fig. 7B)

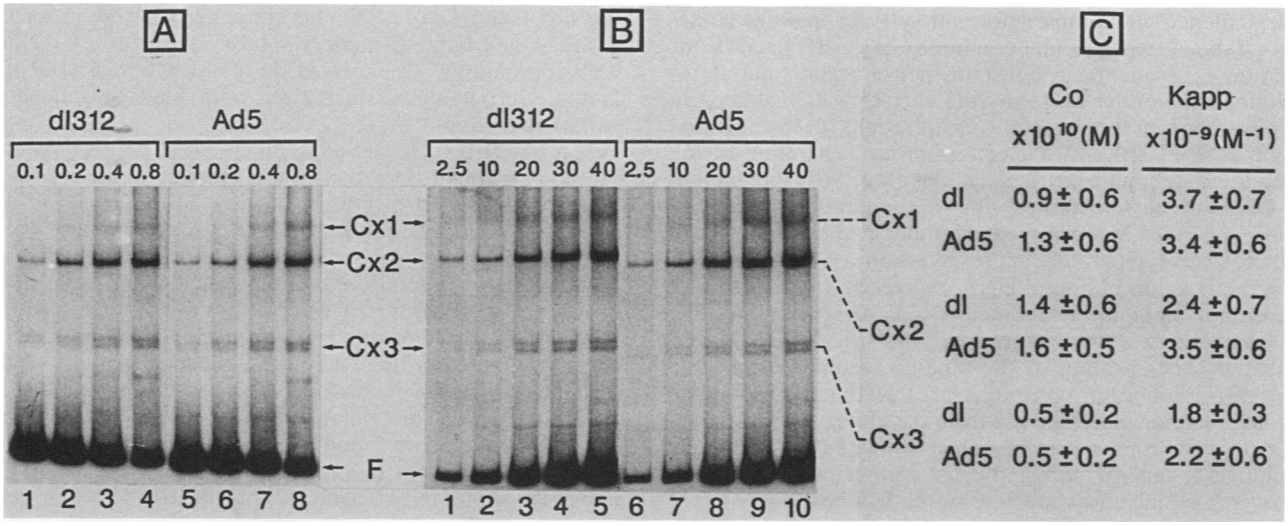

FIG. 8. Effect of EIa gene products on concentration and binding activity of the factors involved in Cx 1, 2, and 3 complexes. A probe saturation experiment (A) was performed by adding increasing amounts of protein (0.1 to 0.8 μg as indicated) from either *dl*312 (lanes 1 to 4)- or Ad5 (lanes 5 to 8)-infected cell extracts (type A WCE) to a fixed amount (5,000 cpm) of ^{32}P-labeled probe F (Fig. 2B). The binding and retardation analyses were as described in Materials and Methods except that the amount of poly(dI-dC) was adjusted with respect to the amount of total protein used. Panel B shows binding reactions containing increasing amounts of probe F (2.5×10^3 to 40×10^3 cpm, as indicated) which were done in the presence of 1 μg of type A WCE prepared from *dl*312 (lanes 1 to 5)- or Ad5 (lanes 6 to 10)-infected cells. The amount of bound and unbound probe DNA was determined for each sample (see Materials and Methods), and the data from this and an identical, but separate, experiment were analyzed (C) as described by Baker et al. (1). Co corresponds to the molar concentration of each factor in the binding reaction, as loaded on the gel, assuming a mole-to-mole relation of factor to probe.

occurred between −50 and −38. Clear interactions also appeared further downstream, between −25 and −8 on the coding strand (Fig. 7B) and between −32 and −4 on the opposite strand (Fig. 7A). This latter protection appeared slightly stronger with type A Ad5-infected cell extract (compare lanes 12 and 10, Fig. 7A and B). However this difference was not found reproducibly and, as it was never observed with type B extracts, may be related to variations in the ammonium sulfate precipitation step. Each of these binding areas is flanked with DNase-hypersensitive sites. An additional site was also often observed around position −60 on both strands. As previously noted (5), there appears to be a close correspondence between the localization of these footprints and the sequence elements essential for constitutive EIIaE promoter function: the pseudo TATA-box T1 and the upstream elements A and B (Fig. 7C). The hypersensitivity at −60 may correspond to an interaction with the pseudo TATA box T2, located between −60 and −52, and corresponding to an essential promoter element for transcription from the minor start site at −26 (5, 60). Furthermore, a striking correlation existed between these DNase I protections and the methylation interference mapping of the gel-retarded complexes. These results strongly suggest that the factors visualized by the two upper footprints (−83 to −68 and −50 to −38) correspond to the Cx 1 and Cx 4 factors, respectively.

Surprisingly, no clear protection over the most-upstream EIIaE promoter element (element C), comprising the Cx 2- and Cx 3-binding sites (Fig. 4C), could be visualized with any of the WCE preparations, while nuclear extracts or partially purified fractions generate strong footprints on element C (5). This discrepancy may reflect a lower concentration of the relevant DNA-binding factors in the WCE and/or the presence of additional proteins in this extract which interfere with the specific binding in the protection assay. On the other hand, this assay reveals specific protein binding to the T1 region (5) (Fig. 7). However, under various experimental conditions, we did not succeed in visualizing such an interaction with the electrophoretic band-shift assay. It is likely that the T1 factor is stabilized by association with the upstream-binding factors on the EIIaE promoter in the protection experiments and that such cooperative interactions are not visualized in the retardation assay, which essentially reveals single protein-DNA interactions.

As a second approach, we compared the gel retardation profiles generated on the EIIaE promoter region by type A WCEs prepared from *dl*312-and Ad5-infected cells. Under standard conditions, the patterns of retarded bands produced by the two extracts (Fig. 8A) were undistinguishable, and in addition, identical results were obtained when type B extracts were used (data not shown). A recent report has demonstrated that the dissociation rates of nucleoprotein complexes are not altered by the migration through a nondenaturing polyacrylamide gel and are identical to the solution values (48). Therefore, we used the gel retardation assay to examine whether the concentrations or affinities or both of the Cx 1, 2, and 3 factors were altered in the presence of the EIa products. Since the complexes were fully formed after a 5-min incubation at 25°C and were stable for several hours (data not shown), the reaction mixtures were systematically incubated at 15 min before electrophoresis to ensure binding equilibrium. Analysis of probe saturation curves, obtained for each complex by adding increasing amounts of extracts from *dl*312- or Ad5-infected cells to a fixed amount of labeled probe (Fig. 8A), revealed that the complex formations followed a first-order reaction; indeed, plotting the logarithm of the fraction of probe retarded for each complex (Cx 1 to 3) as a function of the logarithm of the amount of total protein present in the reaction mixture yielded straight lines with, in each case, a slope value close to 1.0 (data not shown). This observation rules out any cooperativity in the binding of the three factors (49), in agreement with their

independence in complex formation (see above). Under these defined experimental conditions, we performed factor titration experiments. A constant, nonsaturating amount of proteins from either *dl*312 or Ad5 extract was incubated in the presence of increasing concentrations of the labeled probe F (Fig. 2B). After electrophoretic separation of the bound and unbound DNA molecules (Fig. 8B), the amount of DNA in each retarded and free-migrating band was determined (see Materials and Methods). The data for each complex were separately represented on a Scatchard plot, with the fraction of bound DNA expressed as a function of the actual amount of retarded DNA. In each case, the experimental values were then fitted to a straight line (1), and both the concentration of the binding factor (Co) and the apparent equilibrium constant (K_{app}) of the corresponding binding reaction were derived from the plots. As determined (Fig. 8C) by comparing the values obtained with extracts from *dl*312- and Ad5-infected cells, neither of these parameters was significantly affected by the EIa products.

To compare the binding activity of the Cx 4 factor in *dl*312- and Ad5-infected cell extracts, we repeated the same analysis (Fig. 9) using increasing amounts of the EII-Δ5′−62 probe (Fig. 2B). Although a precise quantitation of the data was hampered by the weakness of the retarded signal (Cx 4), no striking difference appeared between the two extracts. This strongly suggests that neither the concentration nor the affinity of the Cx 4 factor is affected by the EIa products.

DISCUSSION

Interaction of EIIaE binding factors with promoter elements. Using the sensitive electrophoretic band-shift assay, we identified four types of nucleoprotein complex (Cx 1, 2, 3, and 4) assembling on the Ad2 EIIaE promoter region. Precise mapping of the protein-binding sites revealed a tight correlation with sequence elements previously shown to participate in the promoter activity. These results strongly suggest that the cognate DNA-binding proteins correspond to transcription factors. By both in vivo (4, 60) and in vitro (5, 11) mutational analyses of the EIIaE transcription unit, we have previously delineated distinct promoter elements involved in efficient transcription from the major EIIaE1 start site. These elements are positioned as follows: the pseudo TATA box element T1 between −33 and −18; the upstream element A between −48 and −39; the upstream element B between −90 and −68; and the weaker upstream element C between −146 and −111. The cellular factors which interact with elements A and B, respectively generating Cx 4 and Cx 1, will therefore be referred to as the EIIaE-A and EIIaE-B factors. Those involved in Cx 2 and Cx 3 formation will be termed the EIIaE-Cα and EIIaE-Cβ factors, respectively, since they both bind to sequences within element C.

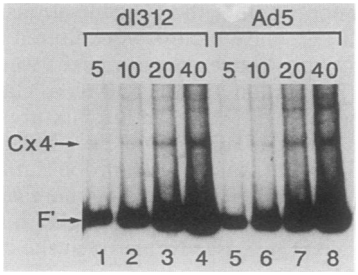

FIG. 9. Effect of EIa gene products on the binding of the factor involved in the Cx 4 complex. Increasing amounts (5×10^3 to 40×10^3 cpm, as indicated) of probe F′ (EII-Δ5′−62; Fig. 2B) were incubated in the presence of 6 μg of *dl*312 (lanes 1 to 4)- or Ad5 (lanes 5 to 8)-infected cell extracts (type A WCE), and formation of specific complexes was examined by the retardation assay (Materials and Methods).

The EIIaE-B factor binds to an essential EIIaE upstream promoter element. Deletion of this element alone results in dramatic decreases of both in vivo (~50-fold) and in vitro (~10-fold) promoter efficiency (5, 60). An enhancer activity has been shown to be associated with this promoter element (28). Sequence alterations within this element abolish or drastically weaken protein interactions with this region (5). All together, these results indicate that EIIaE-B is a critical transcription factor involved in the enhancer activity of the promoter. A similar factor has previously been identified by Siva Raman et al. in a comparable study (51). In agreement with their results, we found that the binding of the EIIaE-B factor to the B element is most efficiently competed by EIa and EIV promoter sequences, suggesting that the same factor is used by all three promoters. This conclusion is further supported by the observation that these promoters share enhancer properties (17, 20, 22, 26). Sequence homologies between these functionally related elements in the EIa, EIIaE, and EIV promoters have already been stressed (6, 17, 20, 28, 55). If the EIIaE-B factor plays a central role in the enhancer activity of these elements, it is unlikely, however, that it represents an ubiquitous general enhancer factor. Indeed, DNA fragments making up the simian virus 40 72-base-pair repeat (51) or the immunoglobulin heavy-chain gene enhancer (unpublished observation) clearly fail to bind this factor.

Element A, which binds factor EIIaE-A, constitutes another sequence component essential for efficient EIIaE promoter activity. Deletion of this element or its alteration by linker substitution reduced by 5- to 10-fold specific EIIaE in vivo and in vitro transcription (5, 60). Binding of the EIIaE-A factor appears to be strongly dependent on the sequence 5′-GGCGC-3′ (starting at position −44 on the noncoding strand), as indicated by methylation interference experiments.

Element C appears as a weak EIIaE upstream promoter element, since deletion of all sequences upstream of position −97 (27) or to −111 (4) reduced promoter activity only about twofold as measured in transfection experiments. Within this region, the EIIaE-Cα factor binds to a sequence making up the 5′-GGCGC-3′ motif which is also recognized by the EIIaE-A factor, in element A. This observation, together with the ability of a DNA fragment comprising element C to compete for the binding to element A, suggests that these sites bind in fact the same protein. An additional 5′-GGCGC-3′ motif located between the EIIaE-Cα and EIIaE-B binding sites (at position −100; Fig. 4C) may account for a weak protection against DNase I which has been detected over this region with partially purified WCE fractions (5). Whether this element, as well as similar G+C-rich elements located further downstream (at −64 and −18), also bind factor EIIa-Cα/A is presently unknown. Multiple binding sites for given factors have previously been reported (39, 54), and their role in cooperative binding to DNA has been suggested. It may be relevant in this respect that both EIIaE-A and -Cα binding sites are on the same side of the double helix since they are separated by a integral number of helical turns (seven turns, assuming 10.5 base pairs per turn). It is likely that the role of the stronger Cα binding site might have been underestimated in the in vivo transcription systems (transient expression of transfected DNA), in which

high copy numbers of template molecules are generally taken up by the cells. Under these conditions, the relative importance of the multiple binding sites may indeed be reduced (46).

The close proximity of the EIIaE-Cβ binding site to that of the EIIaE-Cα factor suggests the existence of interactions between the two factors, although, as pointed out in the Results, each complex can form independently of the other. Clearly, additional information is required to understand the functional relationship between the A, Cα, and Cβ components.

The ability of the EIa and EIV promoters to compete for the binding of both EIIaE-Cα and -Cβ factors to element C suggests that these factors are also implicated in the activity of these early viral genes. It will be of interest to identify their binding sites on these promoters and to see whether they are juxtaposed as well. The observation by Kruczek and Doerfler (34) that DNA methylation at the *Hha*I sites (5'-GCGC-3') in the EIa upstream region decreases its promoter activity supports the possibility that the EIa counterparts of the G+C-rich EIIaE-A/Cα binding sites, which encode *Hha*I sites, correspond to important promoter elements.

No apparent effect of EIa gene product on EIIaE factor binding. The EIa products, which do not bind to DNA by themselves (as suggested by Ferguson et al. [12]), could mediate gene induction by two general mechanisms: (i) the relief of a negative regulatory factor bound to the noninduced promoter or (ii) the activation of positive transcription effectors, or both. In the case of the EIIaE promoter, no mutations have been found that abolish EIa responsiveness without also impairing constitutive promoter activity. This observation eliminates the possibility that a repression-derepression mechanism is involved in the EIIaE promoter modulation. The other alternative could imply, as in the case of genes transcribed by RNA polymerase III (24, 59), an increase in the concentration or affinity or both of a preexisting transcription factor(s). Our results show, however, that the nucleoprotein complexes generated on the EIIaE promoter with extracts from *dl*312 (i.e., EIa depleted)- and Ad5 (i.e., EIa containing)-infected cells were indistinguishable by both footprint and band-shift pattern analysis. This indicates that the factors detected in these extracts have identical binding activities. The possibility remained, however, that the higher affinity of these factors could be fortuitously counterbalanced by their weaker release into the cell extracts, as a consequence of their stronger interaction with the cellular or viral DNA. Measurements of the concentrations and apparent binding equilibrium constants of factors EIIaE-A (Cx 4 in Fig. 9), EIIaE-B, -Cα, and -Cβ (respectively, Cx 1, 2, and 3 in Fig. 8) in both extracts rule out this possibility. Whether this is also true for the factor(s) which equally binds the T1 region in both extracts, as suggested by the footprints, remains to be established. These results are in agreement with and further extend those of Siva Raman et al. (51) who found, using the same band-shift assay, that the net concentration of the EIIaE-B factor was the same in *dl*312- and Ad5-infected cell extracts and that it did not change during the course of EIa protein accumulation. However, no information about other EIIaE binding factors was provided by these authors.

In contradiction with these results and ours are those of Kovesdi et al. (32, 33) who observed binding to the EIIaE region between −74 and −33 (thus including element A and part of B) only in the presence of the EIa products. Although we cannot exclude the possibility that some differences in the technical procedures [such as using salmon sperm DNA instead of poly(dI-dC) as the nonspecific competitor] may explain part of this discrepancy, we have recently found (B. Devaux, G. Albrecht, and C. Kédinger, submitted for publication), in agreement with the present results, that the same viral genomic DNA footprints on regions A, B, and C were produced in *dl*312- or Ad5-infected cell nuclei under conditions in which EIIaE transcription is detected only on the wild-type genome.

It appears, therefore, that the EIa-mediated stimulation is most likely not achieved through a stronger binding of preexisting factors to the EIIaE promoter sequences, but rather by more efficient interactions between these factors. Studies with chimeric promoter constructions have recently shown that only specific combinations of EIIaE promoter elements confer EIa responsiveness to a heterologous, uninducible promoter (D. Zajchowski et al., Gene, in press). It is also worth recalling that sequences flanking element B have been implicated in the EIa dependence of the enhancer activity of this minimal element (28). The G+C-rich motifs present at positions −119 and −100 on one side of element B and at positions −64 and −44 on the other may play crucial functions in this induction mechanism. A possible model for this induction would be that in the absence of the EIa products, an interaction between the factors binding to these regions results in the formation of a DNA loop which would impede appropriate contacts among these specific factors and RNA polymerase. Upon induction, the EIa products themselves or an as yet unidentified protein-binding factor would favor the formation of active transcription complexes (14) by preventing the establishment of such a structure. A similar model has previously been proposed for the regulation of the bacterial arabinose operon (37). On the other hand, there are notable examples supporting the view that DNA-binding transcriptional activators may exert their effect through direct contacts with neighboring proteins, without involving increased DNA-binding efficiency. Mutants of bacteriophage λ or P22 repressor proteins bind to their repressor-binding sites as well as their wild-type counterparts do, but fail to stimulate transcription from the adjacent PRM promoter (19, 23). Detailed mutational analysis of the yeast activator proteins GAL4 (7, 30) and GCN4 (25) demonstrated, as for the phage proteins, that DNA binding and transcription activation are separable functions, encoded by distinct domains of the factors. These studies, which showed that DNA binding of the proteins per se was not responsible for the activation, stress the predominant role of protein-protein interactions in the induction mechanism. Purification and fine characterization of the factors implicated in the process of EIIaE promoter activation will be required to verify whether these observations also apply to this promoter.

ACKNOWLEDGMENTS

We are very grateful to G. Albrecht for critical reading of the manuscript. We also thank H. Boeuf for helpful advice for the footprinting and, together with D. Zajchowski and J. M. Egly, for generous gifts of material. Special thanks go to C. Hauss and the cell culture group for technical assistance, to C. Kister, C. Werlé, B. Boulay, and to the entire secretarial staff for all aspects of preparing this manuscript.

This work was supported by the CNRS (P.J., ATP 6984), the Ministère de l'Industrie et de la Recherche (84V0803), the Association pour le Développement de la Recherche sur le Cancer, and the Ligue Française contre le Cancer (B.D.).

LITERATURE CITED

1. **Baker, R. E., O. Gabrielsen, and B. D. Hall.** 1986. Effects of tRNA Tyr point mutations on the binding of yeast RNA polymerase III transcription factor C. J. Biol. Chem. **261:** 5275–5282.
2. **Berger, S. L., and W. R. Folk.** 1985. Differential activation of RNA polymerase III-transcribed genes by the polyomavirus enhancer and the adenovirus EIa gene products. Nucleic Acids Res. **13:**1413–1428.
3. **Berk, A. J.** 1986. Adenovirus promoters and EIa transactivation. Annu. Rev. Genet. **20:**45–79.
4. **Boeuf, H., D. A. Zajchowski, P. Jalinot, C. Goding, B. Devaux, C. Hauss, and C. Kédinger.** 1986. Intricate sequence elements involved in constitutive and trans-activated expression from the adenovirus early E2A promoter region, p. 203–215. *In* M. Botchen, T. Grodzicker, and P. A. Sharp (ed.), Cancer cells: DNA tumor viruses, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Boeuf, H., D. A. Zajchowski, T. Tamura, C. Hauss, and C. Kédinger.** 1987. Specific cellular proteins bind to critical promoter sequences of the adenovirus early EIIa promoter. Nucleic Acids Res. **15:**509–527.
6. **Bos, J. L., and H. C. Ten Wolde-Kraamwinkel.** 1983. The EIb promoter of Ad12 in mouse L-cells is activated by adenovirus region EIa. EMBO J. **2:**73–76.
7. **Brent, R., and M. Ptashne.** 1985. A eucaryotic transcriptional activator bearing the DNA specificity of a procaryotic repressor. Cell **43:**729–736.
8. **Chen, I. S. Y., A. J. Cann, N. P. Shah, and R. B. Gaynor.** 1985. Functional relationship between HTLV-II x and adenovirus EIa proteins in transcriptional activation. Science **230:**570–573.
9. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **14:**1475–1489.
10. **Dynan, W. S., and R. Tjian.** 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (London) **316:**774–778.
11. **Elkaim, R., C. Goding, and C. Kédinger.** 1983. The adenovirus-2 EIIa early gene promoter: sequences required for efficient *in vitro* and *in vivo* transcription. Nucleic Acids Res. **11:**7105–7117.
12. **Ferguson, B., B. Krippl, O. Andrisani, N. Jones, H. Westphal, and M. Rosenberg.** 1985. EIa 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind to DNA. Mol. Cell. Biol. **5:**2653–2661.
13. **Galas, D., and A. Schmitz.** 1978. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5:**3157–3170.
14. **Gaynor, R. B., and A. J. Berk.** 1983. Cis-acting induction of adenovirus transcription. Cell **33:**683–693.
15. **Gaynor, R. B., L. T. Feldman, and A. J. Berk.** 1985. Transcription of class III genes is activated by viral immediate early proteins. Science **230:**447–450.
16. **Gaynor, R. B., D. Hillman, and A. J. Berk.** 1984. Adenovirus early region 1A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. Proc. Natl. Acad. Sci. USA **81:**1193–1197.
17. **Gilardi, P., and M. Perricaudet.** 1984. The E4 transcriptional unit of Ad2: far upstream sequences are required for its transactivation by EIa. Nucleic Acids Res. **12:**7877–7888.
18. **Green, M. R., R. Treisman, and T. Maniatis.** 1983. Transcriptional activation of cloned human β-globin genes by viral immediate-early gene products. Cell **35:**137–148.
19. **Guarente, L., J. S. Nye, A. Hochschild, and M. Ptashne.** 1982. A mutant lambda repressor with a specific defect in its positive control function. Proc. Natl. Acad. Sci. USA **79:**2236–2240.
20. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1a transcriptional control region contains a duplicated enhancer element. Cell **33:**695–703.
21. **Hearing, P., and T. Shenk.** 1985. Sequence-independent autoregulation of the adenovirus type 5 EIa transcription unit. Mol. Cell. Biol. **5:**3214–3221.
22. **Hen, R., E. Borrelli, P. Sassone-Corsi, and P. Chambon.** 1983. An enhancer element is located 340 base pairs upstream from the adenovirus 2 EIa capsite. Nucleic Acids Res. **11:**8747–8760.
23. **Hochschild, A., N. Irwin, and M. Ptashne.** 1983. Repressor structure and the mechanism of positive control. Cell **32:** 319–325.
24. **Hoeffler, W., and R. G. Roeder.** 1985. Enhancement of RNA polymerase III transcription by the EIa gene product of adenovirus. Cell **41:**955–963.
25. **Hope, I. A., and K. Struhl.** 1986. Functional dissection of a eucaryotic transcriptional activator protein, GCN4 of yeast. Cell **46:**885–894.
26. **Imperiale, M. J., R. P. Hart, and J. R. Nevins.** 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and EIa-induced transcription. Proc. Natl. Acad. Sci. USA **82:**381–385.
27. **Imperiale, M. J., and J. R. Nevins.** 1984. Adenovirus 5 E2 transcription unit: an EIa-inducible promoter with an essential element that functions independently of position or orientation. Mol. Cell. Biol. **4:**875–882.
28. **Jalinot, P., and C. Kédinger.** 1986. Negative regulatory sequences in the EIa-inducible enhancer of the adenovirus-2 early EIIa promoter. Nucleic Acids Res. **14:**2651–2669.
29. **Jones, M., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17:**683–689.
30. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eucaryotic regulatory protein. Science **231:**699–704.
31. **Kingston, R. E., R. J. Kaufman, and P. A. Sharp.** 1984. Regulation of transcription of the adenovirus EII promoter by EIa gene products: absence of sequence specificity. Mol. Cell. Biol. **4:**1970–1977.
32. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in EIa trans-activation. Cell **45:**219–228.
33. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. EIa transcription induction: enhanced binding of factor to upstream promoter sequences. Science **231:**719–722.
34. **Kruczek, I., and W. Doerfler.** 1983. Expression of the chloramphenicol acetyl transferase gene in mammalian cells under the control of adenovirus type 12 promoters: effect of promoter methylation on gene expression. Proc. Natl. Acad. Sci. USA **80:**7586–7590.
35. **Leff, T., J. Corden, R. Elkaim, and P. Sassone-Corsi.** 1985. Transcriptional analysis of the adenovirus-5 EIII promoter: absence of sequence specificity for stimulation by EIa gene products. Nucleic Acids Res. **13:**1209–1221.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Martin, K., L. Huo, and R. F. Schleif.** 1986. The DNA loop model for Ara repression: Ara C protein occupies the proposed loop sites in vivo and repression-negative mutations lie in the same sites. Proc. Natl. Acad. Sci. USA **83:**3654–3658.
38. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
39. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46:**795–805.
40. **Miyamoto, N. G., V. Moncollin, J. M. Egly, and P. Chambon.** 1985. Specific interaction between a transcription factor and the upstream element of the adenovirus-2 major late promoter. EMBO J. **4:**3563–3570.
41. **Moncollin, V., N. G. Miyamoto, X. M. Zheng, and J. M. Egly.** 1986. Purification of a factor specific for the upstream element of the adenovirus-2 major late promoter. EMBO J. **5:**2577–2584.
42. **Murthy, S. C. S., G. P. Bhat, and B. Thimmapaya.** 1985. Adenovirus EIIa early promoter: transcriptional control elements and induction by the viral pre-early EIa gene, which appears to be independent. Proc. Natl. Acad. Sci. USA **82:**2230–2234.

43. **Natarajan, V., M. J. Madden, and N. P. Salzman.** 1984. Proximal and distal domains that control *in vitro* transcription of the adenovirus IVa2 gene. Proc. Natl. Acad. Sci. USA **81:** 6290–6294.
44. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1a gene product. Cell **26:**213–220.
45. **Nevins, J. R.** 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1a gene product. Cell **29:**913–919.
46. **Pelham, H.** 1985. Activation of heat-shock genes in eukaryotes. Trends Genet. **1:**31–35.
47. **Ptashne, M.** 1986. Gene regulation by proteins acting nearby and at a distance. Nature (London) **322:**697–701.
48. **Revzin, A., J. A. Ceglareck, and M. M. Garner.** 1986. Comparison of nucleic acid-protein interactions in solution and in polyacrylamide gels. anal. Biochem. **153:**172–177.
49. **Riggs, A. D., H. Suzuki, and S. Bourgeois.** 1970. Lac repressor-operator interaction. J. Mol. Biol. **48:**67–83.
50. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1:**224–230.
51. **Siva Raman, L., S. Subramanian, and B. Thimmapaya.** 1986. Identification of a factor in HeLa cells specific for an upstream transcriptional control sequence of an E1a-inducible adenovirus promoter and its relative abundance in infected and uninfected cells. Proc. Natl. Acad. Sci. USA **83:**5914–5918.
52. **Stein, R., and E. B. Ziff.** 1984. HeLa cells β tubulin gene transcription is stimulated by adenovirus 5 in parallel with early genes by an E1a-dependent mechanism. Mol. Cell. Biol. **4:**2792–2801.
53. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **37:**889–901.
54. **Topol, J., D. M. Ruden, and C. S. Parker.** 1985. Sequences required for *in vitro* transcriptional activation of a drosophila hsp70 gene. Cell **42:**527–537.
55. **Weeks, D. L., and N. Jones.** 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral genes. Mol. Cell. Biol. **3:**1222–1234.
56. **Wildeman, A. G., P. Sassone-Corsi, T. Grundström, M. Zenke, and P. Chambon.** 1984. Stimulation of *in vitro* transcription from the SV40 early promoter by the enhancer involves a specific trans-acting factor. EMBO J. **3:**3129–3133.
57. **Wilhelm, J., O. Brison, C. Kédinger, and P. Chambon.** 1976. Characterization of adenovirus type 2 transcriptional complexes isolated from infected HeLa cell nuclei. J. Virol. **19:**61–81.
58. **Wu, L., D. S. E. Rosser, M. C. Schmidt, and A. Berk.** 1987. A TATA box implicated in E1a transcriptional activation of a simple adenovirus 2 promoter. Nature (London) **326:**512–515.
59. **Yoshinaga, S., N. Dean, M. Han, and A. J. Berk.** 1986. Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1a-dependent increase in transcription factor IIIC concentration. EMBO J. **5:**343–354.
60. **Zajchowski, D. A., H. Boeuf, and C. Kédinger.** 1985. The adenovirus-2 early EIIa transcription unit possesses two overlapping promoters with different sequence requirements for E1a-dependent stimulation. EMBO J. **4:**1293–1300.