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Purification and functional characterization of a cellular transcription factor that binds to an enhancer element within the adenovirus early EIIa promoter

(affinity chromatography/band-shift/gene regulation/HeLa cells/*in vitro* transcription)

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ABSTRACT **The adenovirus EIa-inducible early EIIa (EIIaE) promoter is comprised of several sequence elements essential for constitutive and induced expression. We report here the purification of the host-cell factor that interacts with the major upstream element of this promoter, extending between positions −90 and −70 with respect to the main EIIaE cap site and exhibiting enhancer properties. The purified factor, which corresponds to a 40- to 43-kDa polypeptide, specifically binds to its recognition site and stimulates EIIaE promoter activity when added to an *in vitro* transcription system, reconstituted from purified factors and RNA polymerase. The implication of this factor in the control of the other adenovirus early genes is discussed.**

Efficient transcription of the adenovirus early transcription units requires the presence of the viral pre-early EIa gene products (1, 2). The mechanism of this transactivation of the early transcription unit is still poorly understood. Extensive deletion and linker scanning mutational analysis (3–5) of the EIa-inducible EIIa early (EIIaE) promoter has indicated that EIa responsiveness involves the same promoter sequences as those required for uninduced expression, suggesting that the same host-cell transcription factors are required in each case. DNA binding studies have identified several of these factors, among which those recognizing sequence elements located between −90 and −70 [EIIAE-EF (6), EIIaE-B (7, 8), or EPF (9)] and between −70 and −30 [EIIF (9, 10) or EIIaE-A/Cα (8)]. Although the binding activity of the first of these factors is not affected by the EIa products (6, 8, 9), conflicting results have been reported as to the second one, showing that its binding to the EIIaE promoter is either unchanged (8, 11) or increased (9, 10, 12) in the presence of the EIa products.

Elucidation of the induction mechanism of this promoter will require the purification of these different DNA binding factors and the study of their interactions with each other, the DNA, and the EIa products. As a first step toward this goal we have characterized the EIIaE-B factor that interacts with an enhancer-like element (13) of the EIIaE promoter.

MATERIALS AND METHODS

HeLa whole cell extracts (WCE; ref. 14) were fractionated as outlined in Fig. 2 and as previously described (15, 16). Sequence-specific DNA affinity chromatography was carried out according to Kadonaga and Tjian (17). The double-stranded oligomer of the wild-type oligonucleotide (O.wt, Fig. 1*B*) was bound to CNBr-activated Sepharose 4B (Pharmacia) with a coupling efficiency of ≈50%, yielding 15–20 μg of DNA per ml of resin. The DNA binding activity of the chromatography fractions was analyzed by the electrophoretic band-shift assay (18) under the conditions described (8). Briefly, the various fractions were incubated with ≈5000 cpm of the 5′ end-labeled probe F or oligonucleotides (see Fig. 1) and carrier poly(dI·dC) as indicated in a final volume of 10 μl. The mixture was incubated for 25 min at 25°C before loading on a prerun 4.5% polyacrylamide gel (acrylamide/bisacrylamide, 80:1) and was electrophoresed for about 2 hr at 150 V and 18°C. The DNase I protection ("footprinting") assay (19), as modified by Boeuf *et al.* (7), was used (DNase I from Sigma had a specific activity of 1600 Kunitz units/mg of protein). Transcription assays were carried out as detailed in the legend to Fig. 7 and the specific transcripts were analyzed by quantitative S1 nuclease mapping (20) by using the coding strand of probe F, 5′ end-labeled at position +37.

RESULTS

Purification of the EIIaE-B Factor. The purification of the EIIaE-B factor (hereafter called EIIaE-B) was followed by the electrophoretic band-shift assay (8) using the probe F, which spans the EIIaE region between −111 and +37 (see Fig. 1*A*). Under the experimental conditions used here the EIIaE-B factor binds most readily to this probe fragment, leading to the formation of the specific DNA–protein complex initially named Cx1 (8). Throughout the purification Cx1 appears as a double band, each one corresponding to the interaction of a protein with the same DNA sequence (P.J., unpublished observation). The reason for this heterogeneity has not been further investigated. The purification scheme is outlined in Fig. 2, in which the salt elution steps that have been used for each type of chromatography are indicated. The fractions containing the majority of the EIIaE-B binding activity, as revealed by band-shift analysis (Fig. 3 and ref. 8), corresponded to the heparin-Ultrogel/0.6 M KCl (H0.6), the DEAE/0.15 M KCl (DE0.15), the sulfopropyl/0.18 M KCl (SP0.18), and the red-trisacryl/1 M KCl (RTI.00) eluates.

The apparent molecular mass of EIIaE-B was determined by glycerol density gradient centrifugation of the RT1.00 fraction. Identical results were obtained whether the gradients were run at 100 (not shown) or 300 mM KCl (Fig. 4). The gradient fractions were assayed for Cx1 formation (Fig. 4), and the specific binding activity was found to sediment between marker proteins of 30 and 43 kDa, suggesting an apparent molecular mass between 35 and 40 kDa for EIIaE-B.

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Abbreviations: WCE, whole cell extracts; O.wt, wild-type oligonucleotide; O.mut, mutated oligonucleotide.

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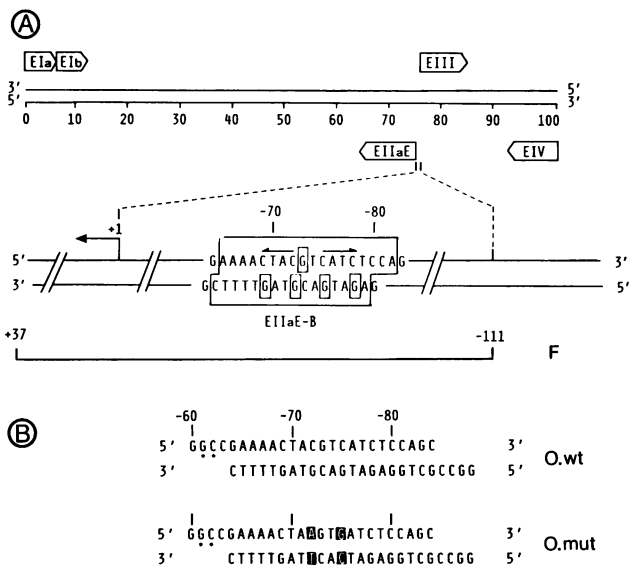

FIG. 1. Organization of the adenovirus early transcription units and nucleotide sequence of the EIIaE-B binding site. (*A*) Genomic map of the adenovirus showing the viral early transcription units with their respective orientation. The promoter region of the EIIaE unit is schematically represented below, and the binding site of EIIaE-B (framed sequence element), as defined by dimethyl sulfate interference experiments (8), is shown with the essential guanines boxed. The divergent arrows correspond to a symmetrical motif discussed in the text. The major cap site (+1) and direction of transcription are indicated by the arrow pointing to the left. Probe F, used for the electrophoretic band-shift assay and S1 nuclease mapping, extends from position −111 to +37, with respect to this cap site (8). (*B*) Sequences of double-stranded oligonucleotides encoding the wild-type (O.wt) or mutated (O.mut) binding site of EIIaE-B. The mutations (shaded nucleotides) alter the central portion of the binding site by changing the C·G pairs at positions −72 and −75 into A·T and G·C pairs, respectively. In both molecules the nucleotides at −61 and −62 (dotted) were permutated with respect to the natural sequence to generate complementary ends.

Factor titration experiments (8) have been used to estimate the purification and recovery of EIIaE-B. A constant, nonsaturating amount of proteins from WCE or RT1.00 was incubated with increasing concentrations of the labeled probe F and, after electrophoretic separation and quantitation of the bound (Cx1) and unbound (F) probe molecules, the fraction of bound DNA ([Cx1]/[F]) was represented as a function of the actual amount of retarded DNA ([Cx1]) (data not shown). On these Scatchard plots the concentration of active EIIaE-B protein present in the binding reactions is given by the [Cx1] value corresponding to the crossing-over point of the straight line extrapolated to the abscissa. A concentration of ≈0.1 nM was found for the EIIaE-B factor in the binding reaction by using 1 μg of WCE in a 10-μl assay (as an example, see data in ref. 8). Taking a molecular mass of 40 kDa for EIIaE-B, this factor then represents ≈0.004% of the WCE total protein content. When the same titration experiment was applied to the RT1.00 fraction, it was found that EIIaE-B constitutes ≈5% of the protein content of this fraction, indicating an increase of EIIaE-B specific activity of >1000-fold between the WCE and RT1.00 fractions. Incidentally, the EIIaE-B concentrations found for the WCE indicate that there are ≈20,000 molecules of EIIaE-B factor per cell (1 μg of WCE protein corresponding in this particular experiment to about 3×10^4 cells).

EIIaE-B Specific Binding Activity Is Elicited by a Single Polypeptide of ≈40 kDa. As revealed by $NaDodSO_4$/PAGE of various active fractions (Fig. 5*A*), only three prominent bands (a, b, and c) are retained in the 30- to 43-kDa range of

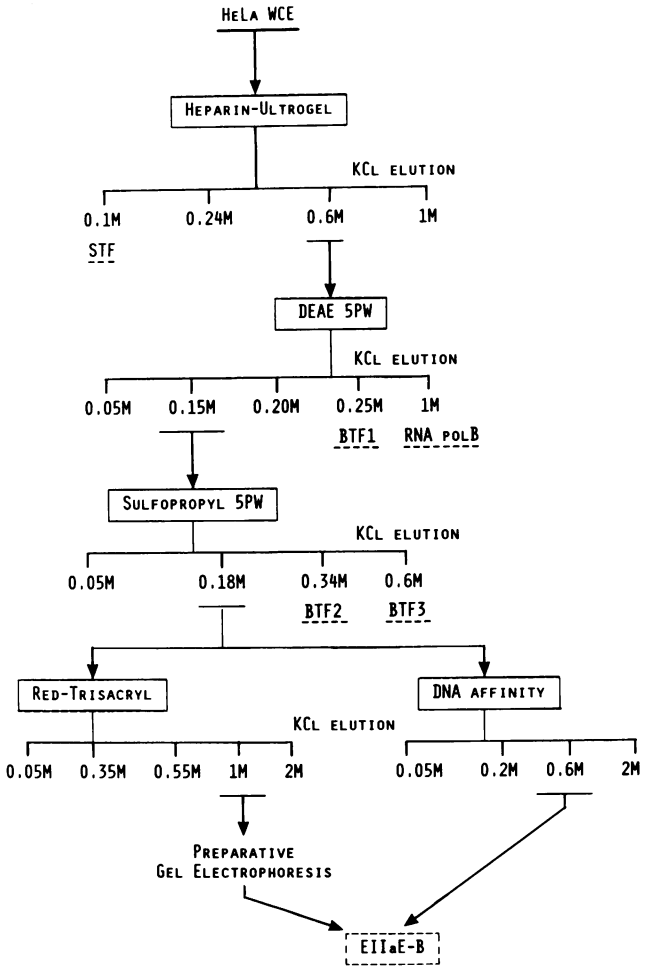

FIG. 2. Purification scheme of EIIaE-B. The relevant fractions (underlined) were chromatographed on the various columns (boxed) and step-eluted with KCl as described (15, 16). Typically, starting with 15×10^{10} HeLa cells (the equivalent of a 200-liter suspension culture), the total protein recovery was ≈10,000 mg in the WCE (6.8 mg/ml), 1100 mg in H0.6 (0.9 mg/ml), 135 mg in DE0.15 (0.6 mg/ml), 30 mg in SP0.18 (0.3 mg/ml), and 1.6 mg in RT1.00 (0.1 mg/ml). The general RNA polymerase B transcription factors (stippled) used in the reconstituted transcription assay are indicated (15, 16). The DNA-affinity column was saturated with dry milk proteins (5 mg/ml of gel) and successively washed with 2 M and 0.05 M KCl prior to adsorption of the sulfopropyl/0.18 M KCl eluate (SP0.18). Adsorption was performed in the presence of competitor poly(dI·dC) at a protein-to-competitor mass ratio of 2 in a buffer (21) containing 0.2 mM phenylmethylsulfonyl fluoride. Proteins were eluted with KCl in the same buffer but without poly(dI·dC).

the RT1.00 fraction (lane 2). To examine whether one of these bands exhibits the EIIaE-B binding activity, each band was excised, electroeluted, renatured, and tested for specific band-shift capacity (Fig. 5*B*). In this experiment double-stranded oligonucleotides encompassing the EIIaE-B binding site (Fig. 1*B*) were used as probes. Only band b led to the formation of a specific complex with the wild-type oligonucleotide (O.wt) element. No retarded band was observed with the control, mutated oligonucleotide (O.mut). This result indicates therefore that the 40-kDa protein present in band b (shown in Fig. 5*A*, lane 1, after electroelution, E.P.) corresponds to the EIIaE-B factor. From the gel in Fig. 5*A* (lane 2) it appears that band b corresponds to ≈5% of the total protein present in the RT1.00 fraction, in good agreement with the value derived from factor titration experiments (see above). It is noteworthy that the apparent molecular mass of band b is shifted to 43 kDa when run under nonreducing conditions (i.e., omitting 2-mercaptoethanol in

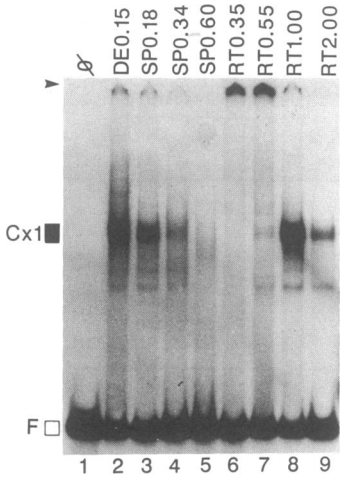

FIG. 3. Electrophoretic band-shift analysis of chromatography fractions. The various chromatography fractions shown in Fig. 2 [4 μg (lanes 2–5) or 2 μg (lanes 6–9)] were tested for EIIaE-B binding activity in the presence of labeled probe F, 0.8 μg (lane 2) or 0.2 μg (lanes 3–9) of poly(dI·dC), and 5 μg of bovine serum albumin (lanes 3–9). The positions of the specific complex (Cx1, ■) and the free probe (□) are indicated. Probe F alone migrated in lane 1 (Ø). The arrowhead points to the top of the gel.

the loading buffer and without boiling the sample; data not shown).

Purification of the EIIaE-B Factor by Affinity Chromatography. When fraction SP0.18 was further purified by affinity chromatography on a specific DNA-Sepharose column containing the polymerized specific oligonucleotide O.wt (Fig. 1*B*), the EIIaE-B binding activity was eluted at 0.6 M KCl [fraction A0.6(1)]. Analysis by $NaDodSO_4$/PAGE of the proteins recovered in the A0.6(1) fraction (Fig. 6*A*, lane 2) revealed a single major protein band migrating in the 43- to 30-kDa region and some proteins of higher molecular mass. After rechromatography of this fraction on the same affinity column, the major component of the eluate [A0.6(2)] was the 40-kDa protein (Fig. 6*A*, lane 3). The additional bands seen in the upper part of the gels (between 65 and 50 kDa, Fig. 5*A*, lane 1, and Fig. 6*B*, lanes 2 and 3) correspond to a silver staining artifact (26).

Fraction A0.6(1) was further tested for its ability to confer specific protection against DNase I digestion to the EIIaE promoter region. As shown in Fig. 6*B*, a clear footprint, between positions −82 and −68, was generated by this

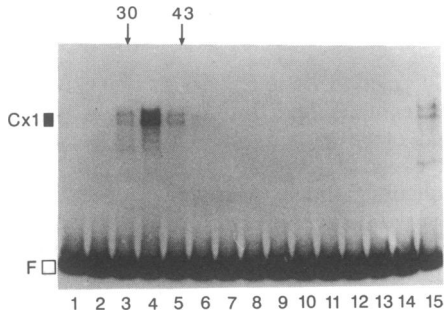

FIG. 4. Glycerol gradient analysis of RT1.00 fraction. One hundred micrograms of RT1.00 was centrifuged for 16 hr at 60,000 rpm (SW 60 rotor) through a 5–20% glycerol gradient in 50 mM Tris·HCl, pH 7.9/300 mM KCl/0.5 mM dithiothreitol/0.1 mM EDTA. Dialyzed gradient fractions (lanes 1–14) and an aliquot of RT1.00 (lane 15) were assayed for specific band-shifting activity as described in the legend to Fig. 3. The positions of carbonic anhydrase (30 kDa) and ovalbumin (43 kDa) run in a parallel gradient are indicated.

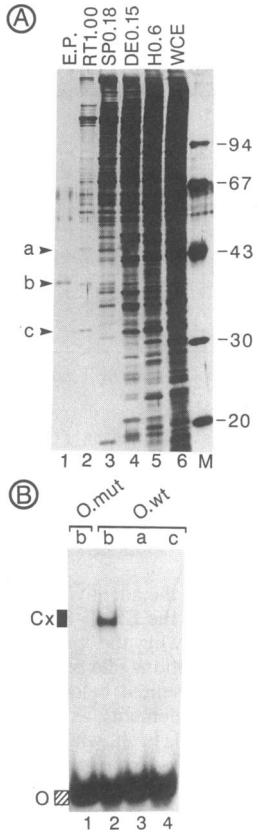

FIG. 5. Protein analysis of the EIIaE-B-containing fractions. (*A*) Aliquots of the various protein fractions (4 μg of WCE, 3 μg of H0.6, 2 μg of DE0.15, 1.5 μg of SP0.18, 0.5 μg of RT1.00) were mixed with a sample buffer (22) containing 5% 2-mercaptoethanol and 1% $NaDodSO_4$ and heated at 100°C for 5 min before loading on a 10% $NaDodSO_4$/polyacrylamide gel (22). Protein staining was as described (23). Three individual bands (a, b, and c) in the RT1.00 sample are indicated by the arrowheads. The bands corresponding to bands a–c in lane 2 were excised from a separate gel loaded with 10 μg of RT1.00 and electroeluted as described (24) for DNA samples, and 10% of the eluted band b (≈35 ng) was run on lane 1 (E.P.) under the same conditions as above. Protein size markers are shown (in kDa) in lane M. (*B*) The eluted bands a–c were renatured (25) and aliquots (≈1% of the eluates) were assayed for band-shifting activity in the presence of the ^{32}P-end-labeled oligonucleotides (O.mut or O.wt, see Fig. 1*B*) and 5 μg of carrier thyroglobulin. Cx (■) and O (▨) refer to the specific complex and free oligonucleotide, respectively.

fraction, when tested under the standard conditions, either in the presence (lanes 3–5) or absence (lanes 8–10) of nonspecific competitor DNA. The fact that this protection corresponds to that obtained with crude WCE (8) nuclear extracts or DE0.15 fraction (7) further stresses the selectivity of the DNA binding activity present in fraction A0.6(1) and demonstrates the correlation with the 40-kDa protein identified here.

The EIIaE-B Factor Present in the Crude WCE and in the Purified RT1.00 Fraction Is Transcriptionally Active. We next examined whether EIIaE-B, which has so far been functionally characterized only by its specific DNA binding activity, is indeed an essential component of the EIIaE transcription complex. Its effect on specific EIIaE transcription was first tested in an *in vitro* transcription system by using the HeLa WCE as a source of RNA polymerase and transcription factors. As previously shown (7), a deletion of sequences between −91 and −62 (as in the Δ-9162 recombinant, see ref. 5), which span the EIIaE-B binding site, decreased specific EIIaE *in vitro* transcription efficiency up to 10-fold [Fig. 7*A*, compare the transcription products of the

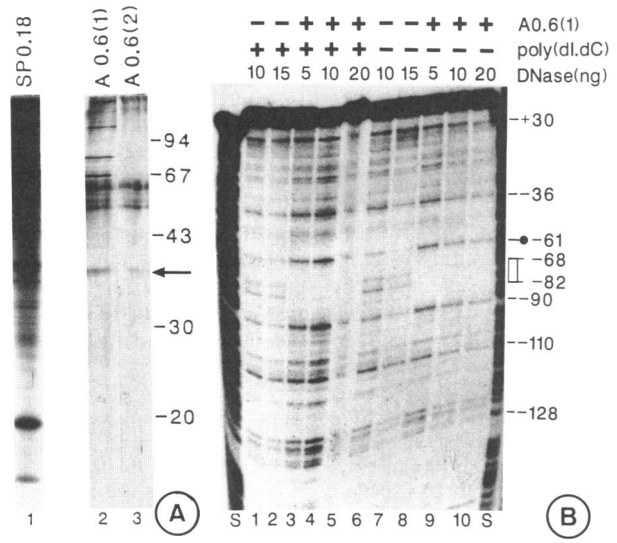

FIG. 6. Affinity chromatography purification of EIIaE-B. Fraction SP0.18 (2 mg) was chromatographed on the specific DNA-Sepharose column (5.5 ml) as described in the legend to Fig. 2. The 0.6 M KCl eluate [A0.6(1)] was collected, diluted to 0.2 M KCl with a buffer containing 5 μg of carrier insulin per ml, and reapplied on the same column. After extensive washing at 0.2 M KCl, the EIIaE-B activity was reeluted [A0.6(2)]. (*A*) $NaDodSO_4$ gel electrophoresis of equivalent aliquots of the indicated fractions. The arrow points to the major band at 40 kDa in A0.6(1) and A0.6(2), also visible in SP0.18. Positions of protein size markers (kDa) are given. (*B*) DNase I footprinting of the A0.6(1) fraction on the noncoding strand of the *Sma* I (−250)–*Pvu* II (+62) EIIaE promoter fragment (7) in the presence (lanes 1–5) or absence (lanes 6–10) of carrier poly(dI·dC). Digestion with DNase I (ng, as indicated) was for 5 min (7). The open box highlights the protected region (lanes 3–5 and 8–10). The dot indicates a hypersensitive site. Lanes S are G + A sequence ladders of the probe with nucleotide coordinates given on the right.

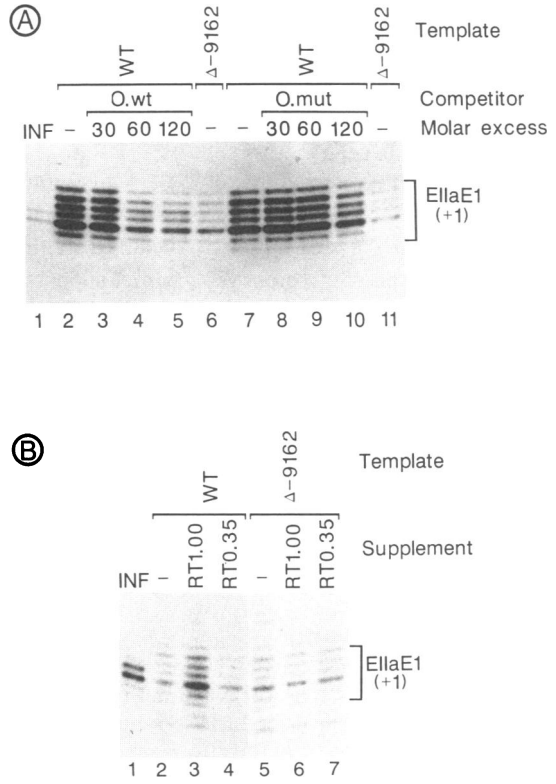

FIG. 7. Transcriptional activity of EIIaE-B. (*A*) WCE (6 μl) was preincubated (16) for 10 min at 30°C in the presence of 50 ng of poly(dI·dC) alone (lanes 2, 6, 7, and 11) or together with increasing amounts of O.wt (lanes 3–5) or O.mut (lanes 8–10) as indicated. The nucleotides (0.5 mM each) and 200 ng of the LS wild type (WT) or Δ-9162 EIIaE template (5) were added and, after 45 min at 30°C, RNA was extracted and analyzed for specific transcripts initiated at the EIIaE major cap site (EIIaE1). Cytoplasmic RNA from adenovirus type 2-infected HeLa cells (INF) was analyzed in parallel with the same probe (lane 1) to confirm the specificity of the EIIaE1 signals. (*B*) The basic reconstitution assays (22 μl) contained the enriched STF (4 μg), BTF1 (2.5 μg), BTF2 (2.2 μg), and BTF3 (1 μg) fractions, 0.002 unit of purified HeLa cell RNA polymerase (see Fig. 2 and ref. 16), and 300 ng of the LS wild type (WT) or Δ-9162 template, without or with RT1.00 (0.2 μg) or RT0.35 (0.2 μg), as indicated. After 15 min of preincubation at 24°C, the nucleotides (2.5 mM each) were added and incubation was prolonged for 60 min at 24°C before RNA extraction and analysis.

wild type (WT), lane 2 or 7, with those of the mutant (Δ-9162) template, lane 6 or 11]. Similarly, addition of increasing amounts of the oligonucleotide corresponding to the EIIaE-B binding site (O.wt) to the incubation reaction progressively reduced EIIaE transcription from the wild-type template to a level comparable to that of the Δ-9162 template (compare lanes 2–5 with lane 6). As expected, the mutated oligonucleotide (O.mut) did not impair the wild-type template efficiency (compare lanes 7–10). These results indicate that the binding of EIIaE-B to its recognition site is required for maximal transcription from the EIIaE promoter.

To determine whether this transcriptional stimulatory activity was preserved throughout the purification, we tested the effect of the RT1.00 fraction on EIIaE template activity in a reconstructed transcription system (Fig. 7*B*). A base level of specific EIIaE transcription could be reconstituted by adding to the reaction mixture highly purified fractions lacking substantial EIIaE-B DNA binding activity, as preliminarily verified by the specific band-shift assay [see Fig. 3, lanes 4 and 5, and ref. 8 for fractions SP0.34 (BTF2), SP0.60 (BTF3), and DE0.25 (BTF1), respectively]. That indeed no endogenous EIIaE-B factor contributed to the level of transcription detected in this system was further supported by the equal efficiencies of the EIIaE wild-type and Δ-9162 templates (Fig. 7*B*, lanes 2 and 5). Supplementation of the incubation mixture with the RT1.00 fraction containing the EIIaE-B DNA binding activity increased specific transcription from the wild type ≈8-fold (lane 3) but not from the Δ-9162 recombinant (lane 6). By contrast, addition of an equal amount of protein from a fraction devoid of EIIaE-B activity (RT0.35) did not stimulate specific transcription from either template. These results strongly suggest that the EIIaE-B factor contained within fraction RT1.00 has retained specific DNA binding and transcription activities.

DISCUSSION

We have characterized a host-cell transcription factor, EIIaE-B, that binds to the major upstream element of the adenovirus EIIaE promoter (element B) located between positions −90 and −70. After extensive purification, the factor appears as a polypeptide of 40–43 kDa that exhibits the expected specific DNA binding activity and stimulates a reconstituted *in vitro* transcription system. Despite the existence of a symmetrical motif, within the EIIaE-B binding site (see Fig. 1*A*), it is unlikely, however, that the active form of the factor is a dimer: the factor binds to EIIaE with first-order kinetics (8) and the glycerol gradient analysis and $NaDodSO_4$ gel electrophoresis indicate that it is solubilized as a monomer, under our experimental conditions.

It has been shown by SivaRaman *et al.* (6) and in our laboratory (8) that the binding of EIIaE-B to its recognition site within the EIIaE promoter can be competitively inhibited by DNA fragments spanning the adenovirus EIa, EIII, and

EIV early promoter regions. On the other hand, Lee and Green (27) have shown that the binding of a factor, called E4F1, that interacts with various elements of the EIV promoter can be competitively inhibited by sequences present in the EIa, EIIaE, and EIII promoters. From these results, it seems very likely that the factors called EII-EF (6), EIIaE-B (8), and E4F1 (27) correspond in fact to the same protein. By sequence comparisons, putative recognition sites (ACGT-$^{A}_{C}$AC) for the E4F1 factor could be localized in the EIa, EIIaE, and EIII promoter regions (27), further supporting the involvement of a unique DNA binding factor in the transcriptional control of these viral early genes. The properties of the 40- to 43-kDa polypeptide that we have characterized strongly suggest that this unique DNA binding factor, indeed, corresponds to this protein.

We have previously shown that the EIIaE-B upstream promoter element, comprising the binding site for this factor, has the properties of a constitutive enhancer element (13). More recently we have demonstrated that this region is part of a domain that contributes to the maximal promoter activity of the nearby EIII transcription unit (35). From the DNA binding competition experiments (6, 8) it was concluded that EIIaE and EIII promoters bind the EIIaE-B factor but that the affinity for this factor was higher for the EIIaE promoter element than for a distinct element present within the divergently transcribed EIII promoter region. The possibility exists therefore that the strong binding site for the EIIaE-B factor on the EIIaE element increases the occupancy of the neighboring low-affinity site within the EIII promoter region required for maximal EIII promoter activity. It is striking, in this respect, that the distal binding sites that have been proposed for this factor in the EIa and EIV promoter regions (27) are located within elements that also exhibit enhancer properties (28, 29).

Recently Montminy and Bilezikjian (30) have reported the purification of a 43-kDa polypeptide (CREB) that binds to the cAMP-responsive element (CRE) of the somatostatin gene. From the striking similarity between the molecular masses and the recognition sites of the CREB (ACGTACA) and EIIaE-B (ACGT$^{A}_{C}$AC) factors, it may be deduced that they correspond in fact to the same or a related molecular entity. This conclusion raises the possibility that, like the CREB factor (30), EIIaE-B corresponds to a phosphoprotein.

The observation that the binding of EIIaE-B to the EIIaE promoter (ref. 6; unpublished observation) or the binding of the related E4F1 to the EIV promoter (27) is not competitively inhibited by the simian virus 40 enhancer sequences rules out the relationship between this (or these) protein(s) and the AP1 enhancer factor. Furthermore, Lee *et al.* (31), who report the purification of AP1, have established that it corresponds to a polypeptide of 47 kDa, therefore larger than the EIIaE-B factor.

Although EIIaE-B seems to be implicated in the expression of several adenovirus early genes, it does not appear to be directly involved in the EIa-mediated induction mechanism. Indeed, a deletion of the EIIaE-B binding site in the EIIaE promoter leads to an important decrease of the promoter constitutive activity, without impairing its inducibility by the EIa gene products (4, 5, 32). Accordingly, band-shift and DNase I footprinting experiments have shown that the binding of EIIaE-B to its recognition site in the EIIaE promoter was not affected by the EIa proteins (6, 8, 9, 11, 12). On the other hand, however, it has been shown that the enhancer activity of the EIIaE-B binding element is modulated by the sequences that flank this element (13). Both of these flanking regions (between −146 and −86 and between −71 and −29, respectively) contribute to the EIa inducibility of the central element and, together with it, can confer EIa responsiveness to an otherwise poorly responsive heterologous promoter (13, 32). Since the binding of a cellular protein to these sequences has been documented (7–12), it will be of interest to study the interactions between the EIIaE-B factor and this other host-cell component and, in particular, to examine how the EIa products affect these interactions. *In vitro* transcription systems reconstituted from purified cellular fractions, as described here, combined with cell-free systems mimicking the EIa-mediated effects (33, 34) will permit the dissection of the molecular mechanisms underlying these regulatory functions.

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