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Nuclear Factor EF-1A Binds to the Adenovirus E1A Core Enhancer Element and to Other Transcriptional Control Regions

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We have identified a cellular enhancer-binding protein, present in nuclear extracts prepared from human and rodent cells, that binds to the adenovirus E1A enhancer element I sequence. The factor has been termed EF-1A, for enhancer-binding factor to the E1A core motif. EF-1A was found to bind to two adjacent, related sequence motifs in the E1A enhancer region (termed sites A and B). The binding of EF-1A to these adjacent sites, or to synthetic dimerized sites of either motif, was cooperative. The cooperative binding of EF-1A to these sites was not subject to strict spacing constraints. EF-1A also bound to related sequences upstream of the E1A enhancer region and in the polyomavirus and adenovirus E4 enhancer regions. The EF-1A-binding region in the E1A enhancer stimulated expression of a linked gene in human 293 cells when multimerized. Based on the contact sites for EF-1A binding determined by chemical interference assays, this protein appears to be distinct from any previously characterized nuclear binding protein.

Transcriptional enhancer regions have been identified that regulate a variety of viral and cellular genes in either a constitutive or an inducible manner (reviewed in references 21 and 35). By definition, enhancers augment the expression of a linked gene in an orientation- and location-independent fashion. In a number of instances, enhancer regions are composed of multiple, functional domains; each domain may contain several elements (motifs). Recent studies with the simian virus 40 (SV40) enhancer region and the response elements of the hormone receptor family have defined several classes of enhancer elements (references 2, 10, 13, and 38 and references therein). Certain motifs function singly, others function when dimerized or multimerized, and others function only in conjunction with distinct elements. The term enhanson has been used to describe a motif that requires dimerization, multimerization, or an additional element for activity (38). A number of studies have shown that enhansons are the target sites for specific DNA-binding proteins (reviewed in references 28 and 37). A synergistic action of multimerized enhancer elements has been observed (13, 26, 38, 42).

The adenovirus type 5 (Ad5) E1A transcriptional control region contains a complex array of regulatory elements. We previously identified an enhancer region (Fig. 1A) that is located between −304 and −146 relative to the E1A cap site at +1 (Ad5 nucleotides [nt] 195 to 353 relative to the left end of the virus [22]). This enhancer region is composed of at least three distinct enhancer elements. Enhancer element I is repeated at −300 and −200 (Fig. 1) and shares sequence similarity with elements in several eucaryotic enhancer regions (22). Element I specifically enhances E1A transcription in virus-infected HeLa cells (24). A second enhancer element, element II (Fig. 1B; −250 to −220) is located between the two copies of element I and enhances transcription of the entire adenovirus chromosome during infection (24). A third enhancer element, the E2F-binding site, is repeated at −285 and −220 (Fig. 1B). The E2F site at −285 has been shown to activate the transcription of a heterologous, linked gene in an E1A-dependent fashion in a transient expression assay (31); E2F DNA-binding activity is induced by adenovirus infection (30). E1A transcription in infected HeLa cells, however, is not detectably influenced by deletion of the two E2F sites in the context of the intact E1A enhancer region (P. Hearing, unpublished data).

In this report, we describe the identification of a cellular enhancer-binding protein, termed EF-1A, that binds to the E1A enhancer region in vitro. EF-1A-binding activity was detected in a number of different human and rodent cell types. The results from binding and competition experiments and chemical interference assays show that EF-1A binds to two adjacent and related sequence motifs, one of which is the upstream copy (−300) of enhancer element I. The binding of EF-1A to these adjacent sites, or to synthetic dimerized sites of either motif, is cooperative. This binding domain displays enhancer activity in human 293 cells when multimerized. Our analyses also demonstrate that EF-1A binds to element I-like sequences present upstream of the E1A enhancer, in the adenovirus E4 transcriptional control region, and in the polyomavirus A enhancer region.

MATERIALS AND METHODS

Cells, transfections, and nuclear extracts. Suspension cultures of HeLa cells were grown in suspension-modified minimum essential medium containing 7% calf serum. Monolayer cultures of 293 cells (20) were grown in Dulbecco modified Eagle medium containing 10% calf serum. DNA transfections were performed by the calcium phosphate precipitation method (47). The cells were split the day before transfection. The following day, the cells were transfected with 1 μg of plasmid DNA and 19 μg of salmon sperm carrier DNA per 100-mm-diameter dish. After incubation overnight with the calcium phosphate precipitate, the cells were washed with Tris-buffered saline solution (TBS), TBS containing 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and TBS. Fresh medium was added, and cell extracts were prepared 48 h later. Chloramphenicol acetyltransferase (CAT) enzyme levels were assayed as described previously (19). Transfections were performed six independent times. The results were quantitated by excision of the acetylated and nonacetylated

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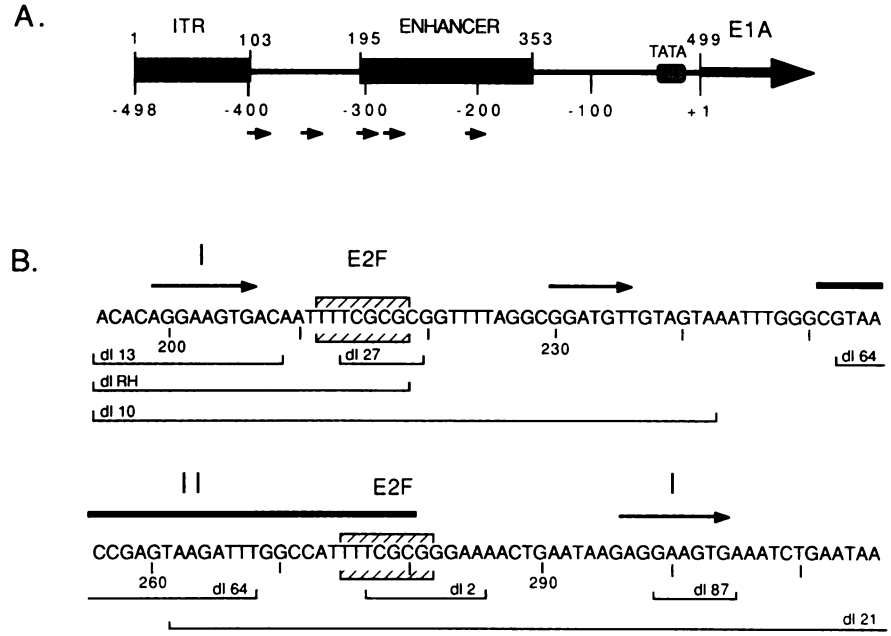


FIG. 1. (A) A schematic view of the left end of the adenovirus genome containing the E1A transcriptional control region. The E1A transcription initiation site is located at Ad5 nt 499 (1) and is designated +1. ITR, Inverted terminal repeat. Numbers above the line represent Ad5 nucleotides from the left end of the viral genome; numbers below the line represent Ad5 nucleotides relative to the E1A cap site at +1. ➡, Enhancer element I repeats and related sequence motifs described in the text. (B) Nucleotide sequence of the Ad5 E1A enhancer region from nt 195 to 316. Numbers below the sequence represent Ad5 nucleotides from the left end of the viral genome; arrows indicate the enhancer element I repeats and a related sequence motif; boundaries of enhancer element II are designated by a bar above the sequence; the two E2F-binding sites in the E1A enhancer region (31) are boxed; brackets represent deletion mutations described in the text (each bracket includes the deleted nucleotides); mutant designations are given within the brackets. The deletion in mutant dl 21 extends rightward to Ad5 nt 355.

[^{14}C]chloramphenicol forms from the chromatogram and direct scintillation counting.

Nuclear extracts were prepared from HeLa and 293 cells by one of two methods. For the experiments performed with unfractionated nuclear extracts, extracts were prepared by the procedure of Borgmeyer et al. (4) and modified as described previously (39). Briefly, the cells were harvested, washed with phosphate-buffered saline solution, and lysed in LB (0.3 M sucrose, 10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) containing 0.4% Nonidet P-40. Isolated nuclei were washed in LB minus Nonidet P-40, and nuclear proteins were extracted with 0.3 M NaCl as described elsewhere (4, 39) except that the 0.1 M NaCl wash was omitted. Eluted proteins were precipitated by using ammonium sulfate at 45% saturation. Extracts were dialyzed for 4 h against 20 mM Tris (pH 7.5)–50 mM NaCl–25% glycerol–0.1 mM EDTA–0.1 mM EGTA–0.5 mM DTT–0.5 mM PMSF. For the preparation of the HeLa cell DE 0.3 fraction, nuclear extracts were prepared by the procedure of Parker and Topol (40). Briefly, the cells were harvested, washed with phosphate-buffered saline, and lysed by Dounce homogenization in hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT). Isolated nuclei were suspended in buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) and lysed by the addition of ammonium sulfate to 0.4 M. Chromatin and debris were precipitated by centifugation at 100,000 × *g* for 2 h, and proteins were precipitated by using ammonium sulfate at 45% saturation. The precipitated proteins were resuspended and dialyzed against DB (20 mM HEPES [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF). All buffers during extraction contained 2 μg each of aprotinin and leupeptin per ml and 0.5 mM PMSF. The dialyzed material was clarified by centrifugation at 15,000 × *g* for 20 min and then loaded on a DEAE-cellulose column (10 mg of protein per ml of bed volume) equilibrated in DB. The column was washed with 3 column volumes of DB, and EF-1A activity was eluted by using DB plus 0.3 M KCl. The pool containing EF-1A-binding activity was dialyzed against DB. EF-1A-binding activity was enriched in the HeLa DE 0.3 fraction approximately fivefold relative to the activity of the starting material.

Plasmids, probes, competitor DNAs, and oligonucleotides. The following plasmid DNAs were used for generating probe and competitor DNAs. The wild-type E1A enhancer region, *Rsa*I to *Sst*II (Ad5 nt 195 to 353), was cloned into the polylinker region of pUC9; this plasmid is termed pUC-WT-ENH. Mutant E1A enhancer regions containing deletions (dl) RH, 87, RH/87, 27/2, and 64 (24) were cloned in a similar fashion. The deletions in the E2F-binding sites in the E1A enhancer region (Fig. 1B; dl 27 and dl 2) were generated by partial digestion of pUC-WT-ENH with *Fnu*dII, followed by limited digestion with S1 nuclease, a repair reaction using Klenow DNA polymerase, and subsequent ligation. Deletions progressing into the E1A enhancer region from the *Rsa*I site at Ad5 nt 195 (Fig. 1B; mutants dl 13 and dl 10) or the *Sst*II site at Ad5 nt 353 (Fig. 1B, mutants dl 21 and 195-244, −238, −233, and −223) were generated by standard procedures and will be described elsewhere (M. Graeble and P. Hearing, manuscript in preparation). DNA fragments containing EF-1A-binding sites upstream of the E1A en-

hancer region, in the E4 promoter region, and in the polyomavirus enhancer region were cloned into the polylinker region of pUC9. These fragments include −393 to −305 from the left end of Ad5 (*Hph*I to *Rsa*I; Ad5 nt 106 to 195), −195 to −92 from the right end of Ad5 (*Bam*HI to *Fnu*dII from plasmid pE4-330/−195 [34]), and DNA fragments from the polyomavirus A2 genome (43) containing the A enhancer region (nt 5104 to 5177) and the B enhancer region (nt 5133 to 5264). Deletions progressing from the *Rsa*I site (Ad5 nt 195) toward the left terminus of the genome (see Fig. 8A; LE 194, LE 155, and LE 110) were generated by standard procedures and will be described elsewhere (L. Hatfield and P. Hearing, manuscript in preparation). Deletions mutations from the right terminus and progressing toward the E4 cap site (see Fig. 8B, E4 −195, E4 −161, and E4 −131) were previously described (34). Other plasmid DNAs include pUC-E2 (Ad5 E2 sequences from −98 to −14 [*Nar*I to *Bss*HII] in pUC9) and pIFN-β-pro (the human β interferon [IFN-β] sequences from −210 to −14, derived from pBVIFN210 [48] by digestion with *Eco*RI and *Nco*I, in pUC18 [a kind gift of Miklos Toth, Institute of Biochemistry, Hungarian Academy of Sciences, Szeged, Hungary]). The authenticity of each plasmid construction and the endpoints of each deletion were verified by nucleotide sequence analysis.

The sequence of the 195-239/dl27 oligonucleotide is 5′-TCGAGACACAGGAAGTGACAATTTGGTTTTAGGCG GATGTTGTAG-3′: 5′-T CGACTACAACATCCGCCTAA AACCAAATTGTCACTTCCTGTGTC-3′. The sequence of the A oligonucleotide is 5′-TCGACGGTGTACACAGGAA GTGACAATTTC-3′: 5′-TCGAGAAATTGTCACTTCCTG TGTACACCG-3′. The sequence of the B oligonucleotide is 5′-TCGACGGTTTTAGGCGGATGTTGTAGTAAC-3′: 5′-TCGAGTTACTACAACATCCGCCTAAAACCG-3′. Mutant oligonucleotide A-X contains three point mutations in the site A enhancer core sequence (5′-AGGAAGT-3′ changed to 5′-ACCTAGT-3′). Mutant oligonucleotide B-X contains three point mutations in the site B enhancer core sequence (5′-CGGATGT-3′ changed to 5′-CCCTTGT-3′).

The 195-239/dl27 oligonucleotide was inserted in the vector pSV-dlOP-CAT (7) between the unique *Kpn*I and *Bam*HI sites. Plasmid pSV-dlOP-CAT contains one complete SV40 72-base-pair (bp) repeat flanked by unique *Kpn*I and *Bam*HI sites at the upstream and downstream ends, respectively, fused to the 21-bp repeats and early-promoter sequences of SV40 (SV40 nt 182 to 8) joined to the CAT-coding sequences (19). pSV-dlOP-CAT contains a deletion of T-antigen-binding site I (*Bgl*I-*Hin*dIII). The size and orientation of each oligonucleotide insertion were determined by nucleotide sequence analysis.

^{32}P-labeled probes were generated by repair reactions using pUC plasmid clones that had been linearized with *Eco*RI or *Hin*dIII, Klenow DNA polymerase, and ^{32}P-labeled dATP and dCTP. After the labeling reaction, the reactions were incubated at 70°C for 10 min, and the probes were released from the vector by restriction enzyme digestion (*Hin*dIII or *Eco*RI). The specific activity of the probes ranged from 0.5×10^4 to 2×10^4 cpm/fmol. The probe and competitor DNAs were isolated from polyacrylamide gels by electroelution and precipitated from ethanol three times.

In vitro DNA-protein binding reactions and chemical interference assays. DNA-protein complexes were assayed by using an electrophoretic mobility shift assay (5, 12, 16). For the binding reaction mixtures (30 μl), 10 to 20 μg of unfractionated nuclear extract or 2 to 5 μg of the HeLa DE 0.3 fraction was preincubated with 2 to 4 μg of homopolymer DNA (partly single-stranded, partly double-stranded DNA [9]) or poly(dI-dC) in 20 mM HEPES (pH 7.5)–40 mM KCl–1 mM $MgCl_2$–10% glycerol–0.5 mM DTT–0.1 mM EGTA at room temperature for 10 min. Then 10,000 cpm of ^{32}P-labeled probe DNA (0.5 to 2 fmol) and the competitor DNAs indicated in the figures were added simultaneously, and the reaction mixtures were incubated for 20 min at room temperature. DNA-protein complexes were resolved in a 4% polyacrylamide gel (30:1, acrylamide/bisacrylamide), electrophoresed in 25 mM Tris (pH 8.3)–25 mM boric acid–0.5 mM EDTA at 4°C (10 V/cm), and detected by autoradiography. Dimethyl sulfate (DMS) and diethyl pyrocarbonate (DEPC) interference assays were performed as described previously (44).

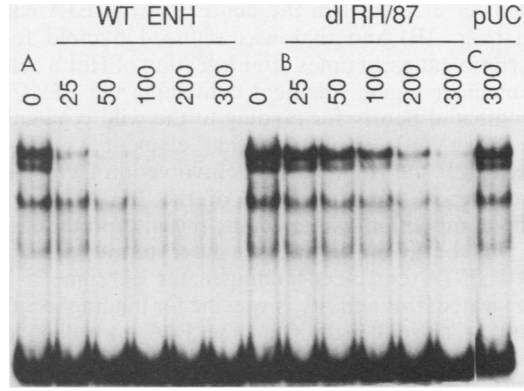


FIG. 2. Binding of EF-1A to the E1A enhancer region. A ^{32}P-labeled probe of the E1A enhancer region (Ad5 nt 195 to 353, corresponding to −304 to −146 relative to the E1A cap site) was used in binding reactions with a nuclear extract prepared from HeLa cells. Binding reaction mixtures contained the ^{32}P-labeled E1A enhancer region probe, nuclear extract, homopolymer DNA as a nonspecific competitor, and increasing molar concentrations of competitor DNA fragments (25-, 50-, 100-, 200-, and 300-fold molar excess). The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel. The competitor DNAs were the wild-type E1A enhancer region (nt 195 to 353) (A), dl RH/87 (the E1A enhancer region containing deletions RH and 87; see Fig. 1B) (B), and pUC (a nonspecific competitor DNA fragment from pUC9, *Eco*RI to *Pvu*II (C). The bracket corresponds to the complex referred to in the text.

RESULTS

The EF-1A-binding sites in the E1A enhancer region. We have previously shown that enhancer element I is required for maximal E1A transcription at early times after infection (22, 24). E1A transcription occurs immediately after infection with adenovirus and in the absence of any known viral gene products (3). Transcription of E1A would be expected to rely on cellular transcription factors present at the time of infection. We used an electrophoretic mobility shift assay (5, 12, 16) to detect nuclear proteins that interact with the E1A enhancer region. Nuclear extracts prepared from uninfected HeLa cells were incubated with a ^{32}P-labeled probe corresponding to the wild-type E1A enhancer region (nt 195 to 353 [Fig. 1B]; −305 to −144 [Fig. 1A]), and DNA-protein complexes were resolved in a polyacrylamide gel (Fig. 2). A DNA-protein complex (doublet; see below) was detected that was specifically competed against by increasing molar concentrations of the homologous enhancer region fragment (Fig. 2A) but was not competed against by a nonspecific competitor DNA (Fig. 2C). We previously described a viral mutant, dl309-RH/87, that contains deletions of both copies

of enhancer element I in the context of the E1A enhancer region (Fig. 1B) and that was reduced fivefold for E1A transcription at early times after infection of HeLa cells (24). The enhancer region fragment containing the RH/87 mutation competed poorly for binding to the wild-type enhancer region (Fig. 2B), suggesting that element I, and/or the adjacent E2F site (Fig. 1B), was involved in factor binding. At the highest concentrations of the RH/87 competitor, specific competition was evident; the interpretation of this result will be given below. We have termed this binding activity EF-1A (enhancer-binding factor 1A) since, as will be demonstrated, this activity is specific for binding to enhancer element I-like sequences (i.e., the E1A core enhancer sequence and related sequence motifs).

Identical binding activities and competition patterns were obtained by using HeLa cell nuclear extracts prepared at early times after adenovirus infection and from human 293 cells (data not shown). EF-1A binding activity also was detected in nuclear extracts prepared from a human liver cell line (HepG2), rat liver cells, and mouse L cells (G. Bolwig and P. Hearing, unpublished data). As will be described, the contact sites for DNA-protein interaction were the same for the lower and upper species of the doublet observed in Fig. 2. The intensity of the lower species varied, depending on the preparation of extract and the extraction procedure used to isolate nuclear proteins (compare Fig. 2 with Fig. 3B and Fig. 6 to 8); it appears likely that the lower species in the doublet reflects proteolysis of EF-1A during extract preparation. Several other complexes are evident in Fig. 2; the specificity of these complexes has not been determined, but on the basis of competition analyses, they appear to be nonspecific DNA-protein interactions.

Enhancer region fragments with specific mutations in either copy of element I (dl RH and dl 87; Fig. 1B), in element II (dl 64; Fig. 1B), and in both copies of the E2F-binding site (dl 27/2; Fig. 1B) were used as ^{32}P-labeled probes in binding reactions with the HeLa cell nuclear extract (Fig. 3A). A complex (doublet) was detected with the wild-type enhancer region probe that was not observed with the dl RH/87 probe; this result was expected on the basis of competition binding reactions (Fig. 2). When probes containing the individual element I mutations were used in the assay, it was evident that deletion of the upstream copy of element I (at −300; dl RH) reduced complex formation considerably, whereas deletion of the downstream copy of element I (at −200; dl 87) only marginally affected binding. Deletion of both E2F-binding sites (dl 27/2) or enhancer element II (dl 64) did not affect complex formation. In other studies, we have demonstrated that E2F does not bind to the dl 27/2 fragment (M. Huang and P. Hearing, Genes Dev., in press).

To define the binding site further, we performed binding reactions using ^{32}P-labeled probes containing deletions that progress into the enhancer region from either the upstream or the downstream side (Fig. 3). Mutant dl 13 (nt 209 to 353) contains a deletion of the upstream copy of element I that does not impinge on the −285 E2F-binding site (Fig. 1B). This mutant was reduced in complex formation to a level similar to that of mutant dl RH. A fragment containing the sequences between nt 195 and 261 (dl 21 [Fig. 1B]; −304 to −238) formed the complex nearly as well as did the wild-type enhancer region, whereas deletion of the sequences between nt 195 and 242 (dl 10; Fig. 1B) abolished binding. Binding reactions using probes with deletions approaching the binding site from the downstream side demonstrated that efficient binding was observed by using a fragment with an endpoint

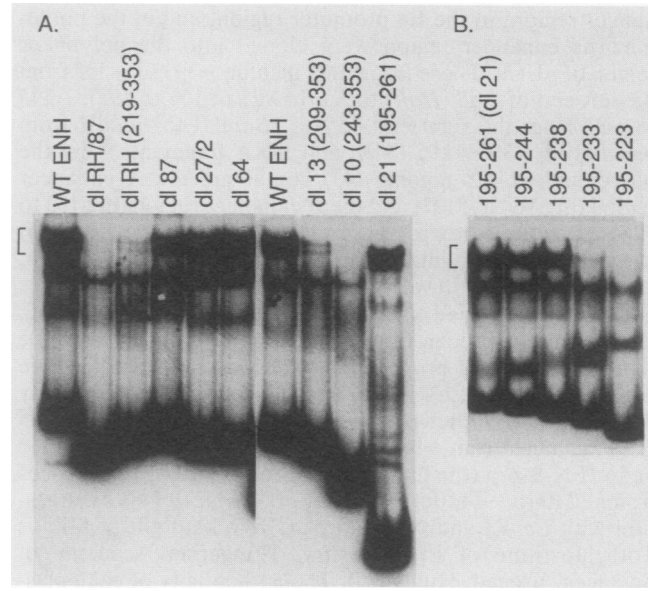


FIG. 3. Binding of EF-1A to mutant E1A enhancer regions. The wild-type (WT ENH) and mutant E1A enhancer regions were ^{32}P labeled and used as probes (as indicated above the lanes) in binding reactions with HeLa cell nuclear extract and homopolymer DNA. The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel. The bracket corresponds to the complex referred to in the text.

at nt 238 (−261 [Fig. 3B]; 195 to 238), whereas deletion of an additional 5 nt dramatically reduced complex formation (Fig. 3B; 195 to 233). These results defined the upstream boundary for efficient EF-1A binding between nt 195 and 209 (−304 to −290) and the downstream boundary between nt 233 to 238 (−266 to −261). Identical results were obtained in competition binding experiments using the E1A enhancer region as a ^{32}P-labeled probe and the different enhancer region mutants as competitor DNAs (data not shown).

To determine the DNA-protein contact sites, we used DMS and DEPC interference assays. The minimal binding region (nt 195 to 238; −304 to −261) was end labeled on each strand and treated with DMS to modify guanines or DEPC to modify adenines and guanines. These modified fragments were incubated with HeLa cell nuclear extract, and the DNA-protein complexes were resolved by electrophoresis. The bound (upper form of the doublet) and unbound fragments were eluted from the gel, cleaved at the modified bases, and analyzed on a sequencing gel. The results from the DMS interference assay are shown in Fig. 4A and B; those from the DEPC interference assay are shown in Fig. 4C and D. The absence of a specific band in the bound lane indicates that the modification of that nucleotide interfered with binding. The results of the interference assays are summarized schematically in Fig. 5A.

Two binding sites are evident from these analyses. DMS methylation of guanines at positions 200, 201, 230, and 231 on the upper strand (Fig. 4A) and at positions 198 and 229 on the lower strand (Fig. 4B) interfered with binding. DEPC carbethoxylation of adenines and guanines at positions 197, 199 through 204, 226, 227, 230, 231, 232, and 234 on the upper strand (Fig. 4C) and at positions 205, 233, and 235 on the lower strand (Fig. 4D) interfered with binding. We have designated the two sites revealed by these analyses as sites A and B (Fig. 5A). Seven of nine contacts in site A correspond to nucleotides in the E1A core enhancer motif

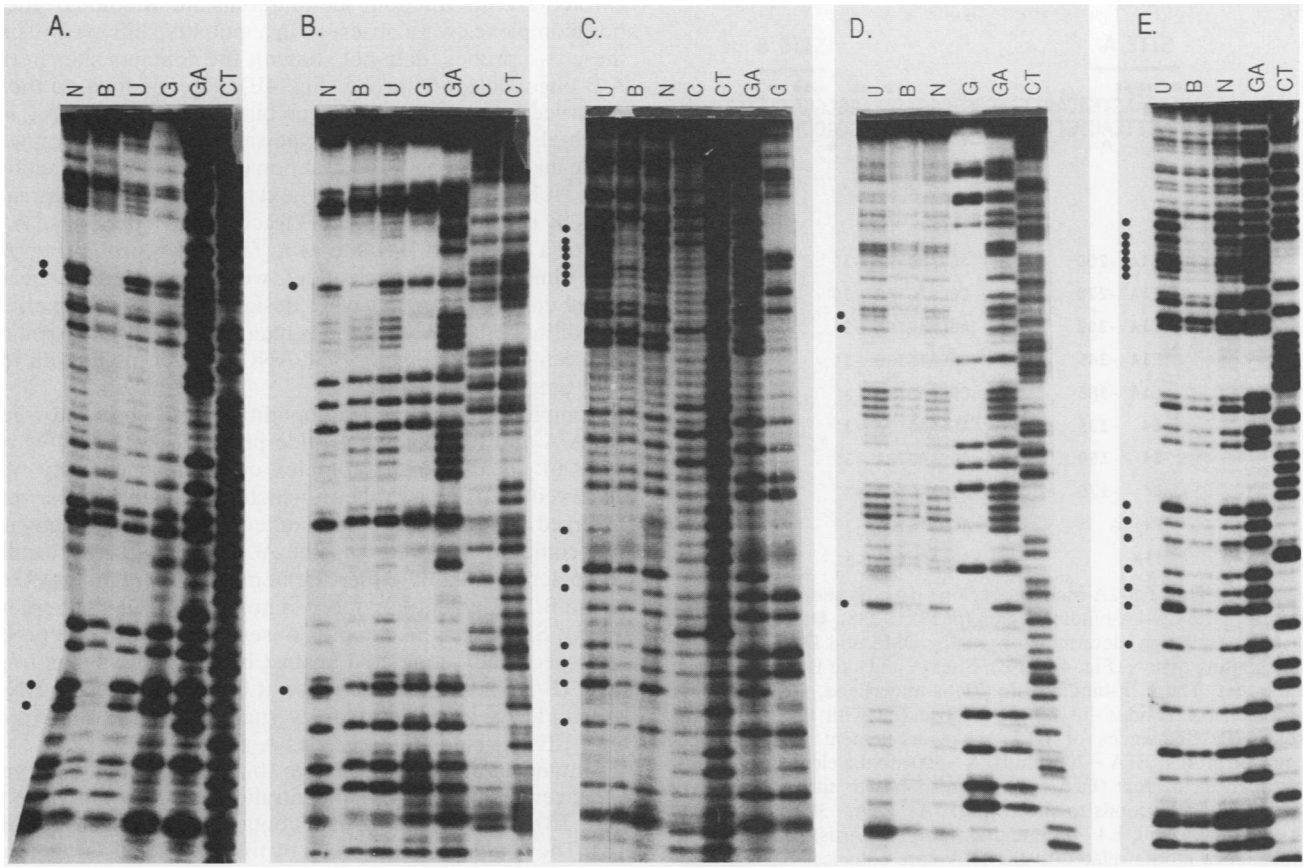


FIG. 4. Analysis of the EF-1A-binding region by using DMS and DEPC interference assays. The EF-1A-binding region (nt 195 to 238) was ^{32}P labeled on the upper strand (A, C, and E) and lower strand (B and D) and partially modified at G residues by using DMS (A and B) or partially modified at A and G residues by using DEPC (C, D, and E). The modified probes were used in binding reactions with the HeLa cell nuclear extract (A to D) or the HeLa DE 0.3 fraction (E). The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel and visualized by autoradiography. The unbound (U; free) and bound (B; complexed) DNAs were eluted, cleaved at the modified bases with piperidine, and analyzed in an 8% denaturing polyacrylamide gel. The probe DNAs were nt 195 to 238 (Fig. 5A) (A to D) and A/B oligonucleotide (see text) (E). Lanes: N, modified probes cleaved with piperidine; G, GA, C, and CT, homologous sequencing ladders. ●, Modified nucleotides that interfere with EF-1A binding.

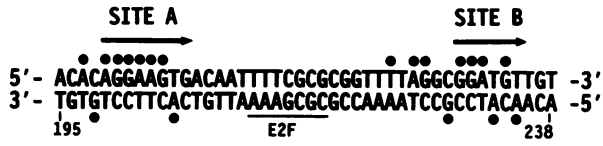
(5′-AGGAAGTGA-3′ [22, 24]). Site B shares sequence similarity with site A (5′-GGAtGT-3′; Fig. 5B). While several of the contact sites in sites A and B were distinct, six contact sites were in comparable positions (5′-$^{A}_{G}$NNGGANGT-3′; Fig. 5A). Identical results were obtained when the contact sites in the lower species of the doublet were determined (data not shown).

Enhancer activity of the EF-1A-binding region. To test the functional enhancer activity of the EF-1A-binding region defined by the assays described above, we inserted one or multiple copies of an oligonucleotide containing this region upstream of the SV40 early promoter in the vector pSV-dlOP-CAT (7). The oligonucleotide (termed 195-239/dl27) corresponds to the sequences between nt 195 to 239 (−304 and −260) and also contains the dl 27 mutation (Fig. 1B) that disrupts the E2F-binding site at −285. Deletion 27 did not reduce the binding of EF-1A to the E1A enhancer region (Fig. 3A). This mutation was used to eliminate the possible contribution of E2F in the enhancer assays. Mobility shift and competition experiments demonstrated that EF-1A bound to this oligonucleotide with comparable efficiency to the wild-type binding domain (nt 195 to 238; data not shown).

Plasmids containing one, two, or four copies of the oligonucleotide upstream of the SV40 21-bp repeats were used in transient expression assays in HeLa and 293 cells, two cell lines that contained EF-1A binding activity. We found that insertion of the oligonucleotide did not increase CAT expression above the basal level (enhancerless vector) in HeLa cells either in the presence or in the absence of the adenovirus E1A gene products (data not shown). In contrast, the oligonucleotide stimulated CAT expression in 293 cells when multimerized. In 293 cells, one copy of the binding region (oligonucleotide) did not stimulate transcription, but insertion of two or four copies stimulated CAT expression 5- to 10-fold in either orientation. (Specific fold values ranged from 5.6 ± 1.1 to 10.1 ± 1.2.) We conclude that the EF-1A binding region, defined by in vitro binding experiments, contains intrinsic enhancer activity.

Cooperative binding of EF-1A to sites A and B. To test the possibility that the same protein was binding to sites A and B, oligonucleotides to these two sites were generated. Ligation of the A and B oligonucleotides (A/B) regenerated the wild-type EF-1A binding site (nt 190 to 239; −309 to −260) except that the E2F site at −285 was disrupted (5′-TTTC GCG-3′ to 5′-TTCTCGA-3′; see Materials and Methods). A ^{32}P-labeled A/B probe and the wild-type enhancer region probe (nt 195 to 238; −304 to −261) were used in binding reactions with a partially fractionated HeLa cell nuclear

A.



B.

E1A -200	5'- AGGAAGTGA -3'
E1A -270	5'- CGGATGTTG -3'
E1A -300	5'- AGGAAGTGA -3'
E1A -345	5'- CGGATGTGG -3'
E1A -385	5'- CGGAAGTGT -3'
E4 -135	5'- AGGAAGTTG -3'
E4 -150	5'- CGGAAGTGA -3'
E4 -175	5'- GGGAAGTGA -3'
Py A	5'- AGGAAGTGA -3'
IFN	5'- AAGTGA -3'

FIG. 5. The EF-1A-binding region. (A) Sequences of both strands of the EF-1A-binding region (nt 195 to 238). ●, Contact sites for EF-1A binding, determined by using DMS and DEPC interference binding assays (Fig. 4A to E). Sites A and site B are described in the text. The E2F-binding site (31) is underlined. DEPC modification of a T residue (nt 224) was observed with the nt 195-238 probe. (B) Sequences of E1A enhancer element I and related sequence motifs. E1A −200 and E1A −300 are the element I repeats described in the text (E1A −300 corresponds to site A; Fig. 5A); E1A −270 corresponds to site B (Fig. 5A); E1A −345, E1A −385, E4 −135, E4 −150, E4 −175, and Py (polyomavirus) A correspond to sequence motifs related to the E1A core enhancer element that are described in the text; IFN corresponds to the hexamer motif present in the human IFN-β regulatory region (11, 15).

extract (DE 0.3 fraction; see Materials and Methods). Identical complexes were observed in a mobility shift assay using these two probes (data not shown); the contacts sites in the A/B oligonucleotide probe (Fig. 4E) were identical to those observed by using the wild-type binding site (Fig. 4C). This result also demonstrates that the same binding activity was being analyzed with the unfractionated or partially fractionated HeLa cell extracts. We next performed binding reactions by using the HeLa DE 0.3 fraction and a dimerized A/A site or a dimerized B/B site as a ^{32}P-labeled probe (Fig. 6). The dimerized oligonucleotides were oriented in a head-to-tail configuration and were designed such that dimerization of individual A and B sites maintained the same spacing between contact points (5'-GGANGT-3') as found with the wild-type binding region.

Complexes with identical mobilities were observed when the A/A and B/B oligonucleotide probes were used (Fig. 6, lanes 0). In addition, a complex of identical mobility was observed by using a probe in which the A and B sites were reversed with respect to each other (B/A; data not shown). The complex observed with the A/A probe was specifically competed against by either the homologous fragment (A/A) or the B/B fragment but not by a nonspecific DNA fragment (pBR). Similarly, the complex observed with the B/B probe was specifically competed against by the homologous fragment (B/B) or the A/A site but not by the nonspecific DNA (pBR). These analyses strongly suggest that the same protein, EF-1A, binds to both sites A and B.

Mutant RH/87 competed for binding to the wild-type enhancer region at high concentrations of competitor DNA (Fig. 2B). The RH/87 fragment contains an intact site B (Fig. 1B). The competition observed by using the RH/87 fragment, therefore, likely reflects the binding of EF-1A to site B, yet clearly the binding to this fragment was reduced compared with binding to the wild-type enhancer region (approximately 10-fold from this and other experiments). To test the possibility that two adjacent sites were required for efficient binding in vitro, monomer and dimer A and B sites were

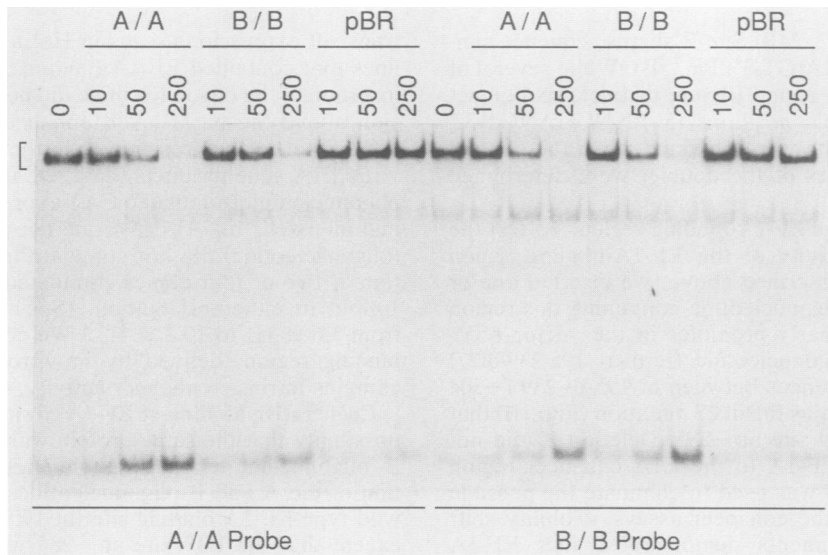


FIG. 6. Binding of EF-1A to dimerized A and B sites. ^{32}P-labeled probes of the dimerized A oligonucleotide (A/A; see text) or the dimerized B oligonucleotide (B/B; see text) were used in binding reactions with the HeLa DE 0.3 fraction, poly(dI-dC), and increasing molar concentrations of competitor DNA fragments (10-, 50-, and 250-fold molar excess). Competitor DNAs were A/A, B/B, and pBR (a nonspecific competitor DNA fragment from pBR322, *Eco*RI to *Eco*RV). The bracket corresponds to the complex referred to in the text.

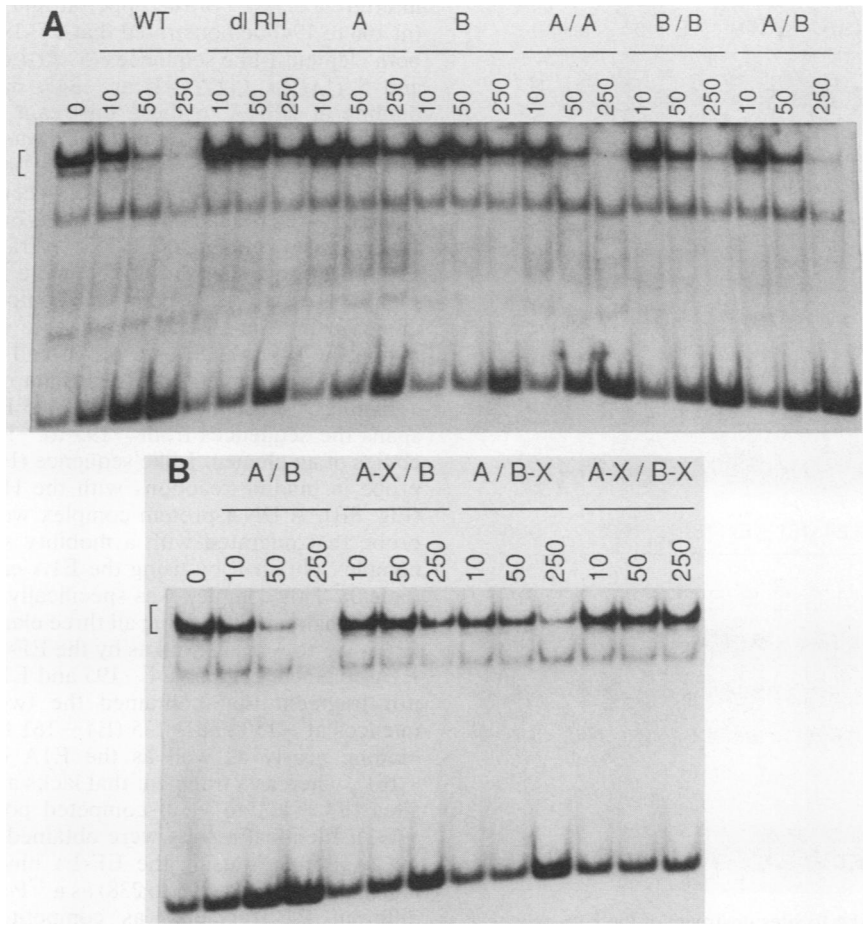


FIG. 7. Cooperative binding of EF-1A to a dimerized binding site. (A) A ^{32}P-labeled probe containing the EF-1A-binding region (nt 195 to 238) was incubated with the HeLa DE 0.3 fraction, poly(dI-dC), and increasing concentrations of competitor DNA fragments (10-, 50-, and 250-fold molar excess). The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel. The competitor DNAs are indicated above the lanes and explained in the text. WT, the EF-1A-binding region (nt 195 to 269). The bracket corresponds to the complex referred to in the text. (B) The binding reactions were performed as described in for panel A. The competitor DNAs (indicated above the lanes) are described in the text.

tested in competition binding reactions. A ^{32}P-labeled probe of the wild-type binding site (nt 195 to 238; −304 to −261) was incubated with the DE 0.3 fraction in the absence or presence of increasing molar concentrations of different competitor DNAs (Fig. 7A). The homologous binding site (WT in Fig. 7A) competed efficiently for EF-1A binding, whereas a fragment containing the RH deletion (dl RH) competed poorly for binding. Similarly, monomeric A and B sites also were weak competitors for EF-1A binding, whereas dimeric A and B sites (A/A, B/B, and A/B) competed with similar efficiencies to the wild-type site (increased approximately 10-fold relative to values for the monomeric sites).

Because the competition efficiencies may have been affected by the length of the competitor DNAs, we tested the binding of EF-1A to dimeric A/B sites that contain point mutations in site A, site B, or both. Mutant site A-X/B is the same length as the wild-type A/B binding site and contains three point mutations in the site A enhancer core sequence (5′-AGGAAGT-3′ changed to 5′-ACCTAGT-3′). Similarly, mutant site A/B-X contains three point mutations in the site B enhancer core sequence (5′-CGGATGT-3′ changed to 5′CCCTTGT-3′). Mutant site A-X/B-X contains both sets of point mutations. A ^{32}P-labeled probe of the wild-type binding site (nt 195 to 238; −304 to −261) was incubated with the DE 0.3 fraction in the absence or presence of increasing molar concentrations of the different dimeric wild-type and mutant binding sites (Fig. 7B). As was observed in competition experiments using the monomeric A and B sites (Fig. 7A), the dimeric competitors that contain mutations in either site A or site B were reduced approximately 10-fold in EF-1A-binding efficiency (Fig. 7B). We conclude from these analyses that EF-1A binds cooperatively to two adjacent and related sequence motifs. In these and other binding reactions, the monomeric and dimeric B sites had binding efficiencies about twofold lower than that of site A.

EF-1A binds to other enhancer regions. We tested the binding of EF-1A to other viral and cellular enhancer regions that contain enhancer element I-like sequences. The IFN regulatory element of the human IFN-β gene contains a hexamer repeat (5′-AAGTGA-3′; Fig. 5B) that is important for the induction of IFN-β expression by poly(I-C) and by viruses (11, 15). A multimerized hexamer repeat acts as an inducible enhancer element (11, 15) and resembles the E1A element I core sequence (e.g., 5′-AAGTGAAAGTGAA AGTGA-3′). Sequences that are similar to EF-1A sites A and B also are found upstream of the E1A enhancer region (at −385 and −345), in the adenovirus E4 transcriptional

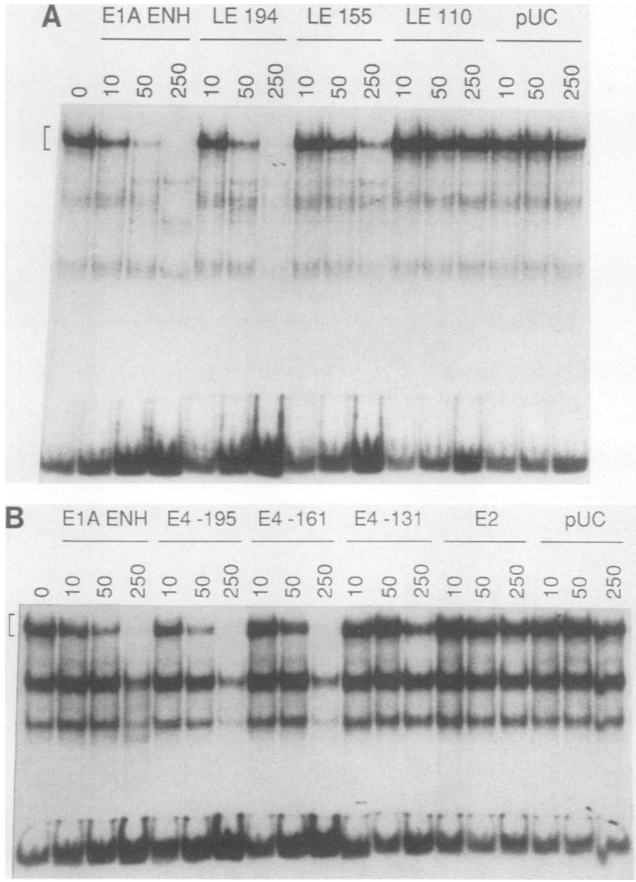


FIG. 8. Binding of EF-1A to sites upstream of the E1A enhancer region and in the E4 transcriptional control region (A) A ^{32}P-labeled probe containing sequences upstream of the E1A enhancer region (nt 106 to 194 [Fig. 1A]; −393 to −305) was incubated with the HeLa DE 0.3 fraction, homopolymer DNA, and increasing concentrations of competitor DNA fragments (10-, 50-, and 250-fold molar excess). The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel. Competitor DNAs: E1A ENH, the EF-1A-binding region in the E1A enhancer (nt 195 to 238); LE 194, nt 1 to 194; LE 155, nt 1 to 155; LE 110, nt 1 to 110; pUC, same as for Fig. 2. (B) A ^{32}P-labeled probe containing sequences from the Ad5 E4 transcriptional control region (−195 to −92) was incubated with the HeLa DE 0.3 fraction, homopolymer DNA, and increasing concentrations of competitor DNA fragments (10-, 50-, and 250-fold molar excess). The products of the binding reactions were electrophoresed in a 4% polyacrylamide gel. Competitor DNAs: E1A ENH, as for panel A; E4 −195, E4 sequences from −195 to −10; E4 −161, E4 sequences from −161 to −10; E4 −131, E4 sequences from −131 to −10); E2, the E2 promoter region from −98 to −14); pUC, same as for Fig. 2). Brackets correspond to the complexes referred to in the text.

control region (at −170, −150 and −135), and in the polyomavirus A enhancer region (45; Fig. 5B).

A DNA fragment containing the sequences upstream of the E1A enhancer region from nt 106 to 194 (−393 to −305), which contains two element I-like copies at −385 and −345 (Fig. 5B), was used as a probe in binding reactions with the HeLa DE 0.3 fraction (Fig. 8A). A complex was observed that had a mobility similar to that of the complex obtained with the E1A enhancer region (lane 0). This complex was efficiently competed against by the EF-1A-binding site in the E1A enhancer region (E1A ENH in Fig. 8A). A DEPC interference assay of the upper strand of the left-end probe (nt 106 to 194) demonstrated that EF-1A made contacts with both element I-like sequences (5′-GGCGGAAGTG-3′ at −385 and 5′-GACGGATGTG-3′ at −345; data not shown). The binding of EF-1A to these upstream sites appeared to be cooperative. Binding to this region was efficiently competed against by a fragment that contains the left end of the Ad5 genome (nt 1 to 194; LE 194), whereas the competition efficiency was reduced fivefold when one of the two element I-like cores was deleted (LE 155). A fragment that lacks both E1A core sequences did not compete for binding (LE 110). Identical results were obtained in competition binding experiments using the EF-1A binding site in the E1A enhancer region (nt 195 to 238) as a probe and the different left-end fragments as competitor DNAs (data not shown).

Similarly, a fragment from the Ad5 E4 control region that spans the sequences from −195 to −92 and contains three copies of an element I-like sequence (Fig. 5B) was used as a probe in binding reactions with the HeLa DE 0.3 fraction (Fig. 8B). A DNA-protein complex was observed with this probe that migrated with a mobility similar to that of the complex obtained by using the E1A enhancer region probe (lane 0). This complex was specifically competed against by an E4 fragment containing all three element I-like sequences (E4 −195 to −10) as well as by the EF-1A-binding site in the E1A enhancer region (E4 −195 and E1A ENH). A competitor fragment that contained the two element I-like sequences at −150 and −135 (E4 −161 to −10) competed for binding nearly as well as the E1A enhancer region (E4 −161), whereas a fragment that lacks all three element I-like sites (E4 −131 to −10) competed poorly for binding (E4 −131). Identical results were obtained in competition binding experiments using the EF-1A binding site in the E1A enhancer region (nt 195 to 238) as a ^{32}P-labeled probe and the different E4 fragments as competitor DNAs (data not shown). Two other complexes are evident in Fig. 8B that were weakly competed against by the EF-1A-binding site in the E1A enhancer region as well as by several of the E4 DNA fragments. The nature of these binding sites has not been determined.

We also examined the binding of EF-1A to other viral and cellular promoter and enhancer regions. A DNA fragment containing the human IFN-β transcriptional control region (−210 to −14) did not compete for binding of EF-1A to the E1A enhancer region (data not shown). A DNA fragment that contains the polyomavirus A enhancer region (45) competed for binding of EF-1A to the E1A enhancer region probe but at a level that was reduced about fivefold compared with competition using the homologous E1A site (data not shown). In contrast, the polyomavirus B enhancer region (45) and the SV40 enhancer and early-region promoter did not compete for EF-1A binding (data not shown). Competitor DNAs containing the Ad5 E2 early promoter (−98 to −14; Fig. 8B) or the adenovirus major late promoter (−244 to −9; data not shown) did not compete for EF-1A binding. Taken together, the binding experiments, competition experiments, and interference assays identify the following consensus motif for EF-1A binding: 5′-$^{A}_{G}$N$^{A}_{C}$GGA$^{A}_{T}$GT-3′.

DISCUSSION

We have identified a cellular nuclear protein, termed EF-1A, that binds to the upstream copy of the E1A enhancer element I core motif (at −300; site A [Fig. 5A]) and to an adjacent and related sequence element (at −270; site B [Fig. 5A]). EF-1A bound only weakly, however, to the down-

stream copy of the E1A core enhancer sequence (at −200; Fig. 3A, WT ENH and dl 87 versus dl RH and dl 10; J. Bruder and P. Hearing, unpublished data). EF-1A-binding activity was detected in nuclear extracts prepared from human HeLa and 293 cells, the HepG2 human liver cell line, rat liver cells, and mouse L cells. The results from binding and competition experiments and chemical interference assays demonstrated that EF-1A contacts two adjacent and related sites (termed sites A and B; Fig. 5A). The fact that EF-1A bound efficiently to probes that contain the natural arrangement of these sites (A/B) and to dimeric copies of either individual site (A/A and B/B; Fig. 6) suggests that the same protein binds to both sites. This possibility is strengthened by the observation that the binding of EF-1A to a dimeric A site (A/A) was competed against by a dimeric B site (B/B) and likewise that the binding of EF-1A to a dimeric B site (B/B) was competed against by a dimeric A site (A/A; Fig. 6). EF-1A binding to single site A or site B motifs was reduced approximately 10-fold compared with the binding observed to dimeric A and B sites (Fig. 7A). Similarly, EF-1A binding to dimeric A/B sites that contain point mutations in either site A or B was reduced approximately 10-fold compared with the binding observed to the wild-type A/B site (Fig. 7B). These results, and the results of binding experiments using enhancer region fragments that contain mutations in either site A or site B (Fig. 2 and 3), strongly suggest that the binding of EF-1A to adjacent A and B sites is cooperative. The fact that EF-1A bound poorly to the downstream copy of enhancer element I (at −200) likely reflects the fact that only a single binding site is present in this region, whereas the upstream copy of element I (at −300) is situated adjacent to a second binding site (site B; Fig. 5A) that stabilizes EF-1A interaction with the DNA. Mutant probes dl RH and dl 13 (Fig. 1B) contain an intact site B (at −270; Fig. 5A) and the downstream copy of enhancer element I (at −200) yet bound EF-1A very weakly, suggesting that cooperative binding to these two sites (−270 and −200) does not occur. This could reflect an improper alignment of these sites with respect to each other. Alternatively, another protein present in the fractions containing EF-1A activity may bind to a site that overlaps the enhancer element I copy at −200 and block the interaction of EF-1A with this site in vitro.

Our previous genetic analyses of the E1A transcriptional control region demonstrated that the repeated copies of the E1A core enhancer motif were functionally redundant, since the deletion of either element (dl RH or dl 87; Fig. 1B) did not reduce E1A transcription, whereas the deletion of both elements (dl RH/87; Fig. 1B) reduced E1A expression fivefold (24). These studies also demonstrated that the mutation of site B (Fig. 5A) or the deletion of the sequences between the E1A enhancer region and the viral terminal repeat, nt 103 to 194 (−396 to −305; Fig. 1A), did not reduce E1A transcription in vivo (22). The in vitro binding studies demonstrated that EF-1A bound efficiently to the upstream copy of enhancer element I (site A; Fig. 5A) in conjunction with site B (Fig. 5A) but poorly to the downstream copy (at −200) of this sequence motif. Furthermore, there are two additional EF-1A-binding sites in the region between nt 103 and 194 (−396 to −305; Fig. 8A and data not shown). Thus, there is an apparent contradiction between results of the enhancer studies in vivo and the binding studies in vitro. We do not understand the basis of this discrepancy, but we note several points. First, the E1A core enhancer element I motifs were defined by using adenovirus mutants in HeLa cell infections. The region containing the EF-1A-binding motifs at −385 and −345 did not display enhancer activity in these assays (22). In contrast, a 24-bp segment centered at −330 (and overlapping the −345 element I motif) did enhance transcription in HeLa cells in a transient expression assay (25). Thus, the type of analysis used in expression studies may detect the activities of different regulatory elements. In addition, a number of other control elements have been identified in the E1A 5′-flanking region (22, 24, 25, 31) that may functionally substitute for one or more of the EF-1A-binding sites. Second, the upstream and downstream copies of enhancer element I share a perfect 9-bp repeated sequence (5′-ACAGGAAGTGA-3′ at −300 and 5′-AGAGGAAGTGA3′ at −200; Fig. 1B and 5B). The genetic studies demonstrated that at least one copy of this sequence is required for enhancer activity. The sites at −270, −345, and −385 are similar to the E1A core enhancer motif, but they are distinct (5′-GGCGGATGTTT-3′, 5′-GACGGATGTGG-3′, and 5′-GGCGGAAGTGT-3′, respectively; Fig. 5B). These sites may represent auxiliary elements that do not contain intrinsic enhancer activity but that serve as sites to stabilize the binding of EF-1A to the core enhancer motifs at −300 or at −200. Each of the E1A transcriptional control region mutants that maintained full enhancer region activity contained at least one copy of enhancer element I located at −300 or at −200 and one or more of the auxiliary binding sites (24). Recent studies have demonstrated that binding of the T3 receptor to the T3 response element activates transcription, whereas binding of the T3 receptor to a similar but distinct motif (the estrogen response element) does not augment expression (18). Other studies have demonstrated that the glucocorticoid receptor binds to two response elements upstream of the tyrosine aminotransferase gene; the distal binding site independently activates transcription, whereas the proximal site has no inherent enhancer activity but acts synergistically with the distal site to enhance transcription (27). It has been hypothesized that the binding of receptors to DNA may induce a conformation change in the receptor protein that alters transcriptional activity, depending on the binding site. A similar situation may exist with the EF-1A-binding sites in the E1A transcription control region. Further genetic and biochemical studies will be required to address this point.

The cooperative binding of EF-1A to sites A and B in the E1A enhancer region (at −300 and −270; Fig. 5A) does not appear to be subject to a strict spacing constraint. EF-1A bound efficiently to the dl 27 mutant DNA fragment (Fig. 3A), which contains a 6-bp deletion between these two adjacent sites (Fig. 1B). By using the first G residue of the EF-1A binding motif 5′-GGA$^{A}_{T}$GT-3′ within sites A and B as a reference point (the first G corresponding to Ad5 nt 200 and 230), the EF-1A binding sites are located 30 bp apart. A 6-bp deletion between sites A and B relocates these sites on essentially opposite faces of the DNA helix. In addition, we have observed that the deletion of 7 or 13 bp between sites A and B also does not significantly reduce the binding of EF-1A to this region (Bruder and Hearing, unpublished data). These results suggest that EF-1A may contain distinct domains involved in DNA binding and dimerization and that considerable flexibility exists between these domains of the protein. Similarly, binding of octamer transcription factors 1 and 2 to adjacent sites in the immunoglobulin heavy-chain promoter also appears to be cooperative and occurs when these sites are separated by either 2 or 14 bp (41). In contrast, binding of the HeLa cell protein TEF-1, which binds cooperatively to tandem repeats in the SV40 enhancer region (the Sph and GT- IIC motifs), displays a strict spacing

constraint: the insertion of 5 or 10 bp between the adjacent binding sites results in the loss of TEF-1 dimerization (8). Our data also indicate that EF-1A bound cooperatively to neighboring sites upstream of the E1A enhancer region (at −385 and −345; Fig. 8A), where the binding sites are separated by an additional turn of the helix compared with sites A and B in the E1A enhancer region.

Our data demonstrate that EF-1A binds to other regulatory regions. The EF-1A-binding region in the E4 promoter is located within a domain that has previously been shown to contain an E1A-responsive enhancer element (17, 32). This region also contains a binding site for the cellular transcription factor ATF (28, 32, 34); it is not clear at this point which element(s) is involved in the E1A response. Wanatabe et al. (46) have partially purified a protein from HeLa cells, termed E4TF1, which increases in vitro transcription from the E4 promoter and protects the sequences between −158 and −138 from digestion with DNase I (numbering system of Leza and Hearing [34]). This binding site overlaps the E1A element I core homology in the E4 promoter at −150 (Fig. 5B); E4TF1 may be the same factor as EF-1A. EF-1A also binds to the A enhancer domain of the polyomavirus enhancer region. A murine nuclear factor, termed PEA3, has been described that binds within the polyomavirus A enhancer domain (36). Point mutations that disrupt the E1A enhancer core motif in this region disrupt the binding of PEA3; a viral revertant with a point mutation that restores the E1A core motif restores PEA3 binding in vitro (36). Thus, PEA3 may represent the murine equivalent of EF-1A. We have detected EF-1A-binding activity to the E1A and polyomavirus enhancer regions by using a murine L-cell DE 0.3 fraction (Bolwig and Hearing, unpublished data).

Finally, our results demonstrate that the EF-1A binding site in the E1A enhancer region activates expression of a heterologous promoter in transfected 293 cells (see Results) but not in HeLa cells either in the absence or in the presence of the E1A gene products (data not shown). The conditions used for transfection of HeLa cells in the presence of the E1A gene products resulted in a 30-fold stimulation of the Ad5 E4 promoter (6; Hearing, unpublished data). We do not understand why expression from the SV40 early-region promoter was enhanced by the EF-1A binding site in 293 cells but not in HeLa cells. HeLa cells contain and express functional EF-1A activity, as determined from our previous experiments using virus infections (22, 24). The lack of enhancement in HeLa cells may reflect the levels of functional EF-1A in HeLa versus 293 cells; perhaps EF-1A activity is elevated in 293 cells and therefore more readily detected in a transient expression assay. Alternatively, a promoter-specific effect may account for the different effects on expression in the different cell line; a positive-acting factor may be present in 293 cells that is lacking or at low levels in HeLa cells, or an activity may be present in HeLa cells that blocks promoter or enhancer activity. Cell-specific transcriptional activation effects in 293 cells versus HeLa cells have previously been described (33). Finally, our previous analyses have shown that E1A expression is stimulated fivefold as the result of viral early region expression (23). It is possible that 293 cells express an adenovirus gene product (e.g., an early-region 1B polypeptide) that is required for optimal EF-1A activity that would be synthesized in HeLa cells during viral infection but that would not be expressed in a transient expression assay.

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