Different binding site requirements for binding and activation for the bipartite enhancer factor EF-1A

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ABSTRACT

The human transcription factor EF-1A binds to the purine-rich E1A core enhancer sequence in the adenovirus E1A and E4 and polyomavirus enhancer regions. The consensus binding site for EF-1A resembles that of members of the *ets* domain protein family. EF-1A activation of transcription requires a dimeric binding site. Analysis of binding sites containing point mutations revealed that EF-1A binding is determined by the core nucleotides of the binding site, while transcriptional activation is determined both by the core and some peripheral nucleotides that do not affect binding. We have purified EF-1A and analyzed its two constituent subunits, EF-1A α and EF-1A β . EF-1A α (MW ~60 kD) makes the primary DNA contacts. EF-1A β (MW ~50 kD) forms a heteromultimeric complex with EF-1A α both in solution and on a dimeric binding site. Binding of both EF-1A subunits is necessary, but not sufficient, for transcriptional activation. We present immunochemical and functional evidence that EF-1A α is related to the murine *ets*-related protein GABP α and that EF-1A β is related to the murine protein GABP β .

INTRODUCTION

The initiation of transcription at eukaryotic class II promoters is stimulated by proximal and distal *cis*-acting regulatory sequences. The distal elements, called enhancers, are defined as sequences that augment transcription independent of their location and orientation relative to the promoter. Recent research has focused on the role of protein-protein interactions in the activation of transcription. Interactions between proteins bound to proximal and distal activator sequences and the basal transcription factors are important mechanisms of activation. Likewise, cooperative interaction between enhancer binding proteins have been found to be important for the binding and activating properties of a number of proteins, e.g. members of the leucine zipper family and basic helix-loop-helix family of proteins (9).

We previously described the binding of the cellular nuclear activity, enhancer factor 1A (EF-1A), to five sites in and upstream of the adenovirus (Ad) E1A enhancer, to two sites in the Ad E4 enhancer, and to three sites in the polyomavirus (Py) enhancer (10, 11). We have detected EF-1A-like activities in human, mouse and rat cells. Genetic studies have shown that EF-1A binding sequences are principal enhancer elements for early Ad and Py transcription and are important for Py DNA replication (10–13). In the Ad E1A enhancer, EF-1A binds cooperatively to two adjacent sites (Fig. 1, sites A and B), independent of the spacing of the sites. In the Py enhancer, two of the Py EF-1A sites, 1 and 3, overlap previously determined binding sites for the murine factor PEA-3 and members of the *ets* family of proteins (11, 14, 15). The *ets* family of nuclear proteins share a highly conserved 85 amino acid (aa) domain, in which the DNA binding region resides, with the *v-ets* oncoprotein from the avian retrovirus E26. *Ets* proteins have been characterized in a number of species that diverge significantly in aa sequence outside of the *ets* domain (16–21). The consensus DNA binding sequences for *ets*-proteins and EF-1A are strikingly similar, both being centered around a purine-rich core (Fig. 1; 22, 23). In this article, we demonstrate that efficient EF-1A binding to a dimeric binding site is essential, but not sufficient, for transcriptional activation. EF-1A binding efficiency is determined by the nucleotides in the purine core of the binding site. The ability of the site to act as an enhancer is determined by these nucleotides, but also by peripheral nucleotides that do not affect binding. We show that EF-1A is composed of two subunits: EF-1A α (~60 kD), which is responsible for the primary DNA contacts of the complex, and EF-1A β (~50 kD), which interacts with EF-1A α and is required for the activating properties of the complex. Analysis by sizing chromatography indicates that the α and β subunits of EF-1A are able to form a ~200 kD complex in the absence of DNA. However, stable formation of a multimeric $\alpha\beta$ complex on DNA *in vitro* requires a dimeric binding site and is associated with enhancer activity *in vivo*.

Recently, two murine transcription factors, GABP α and GABP β , were cloned and characterized (24, 25). These proteins bind

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as a complex to a repeated purine-rich binding site in the herpes simplex virus ICP4 promoter (26). The GABP α subunit is *ets*-related and is responsible for the primary DNA contacts of the ab complex. The β subunit contacts the α subunit and flanking nucleotides in the DNA binding site. GABP β contains ankyrin-repeats that are involved in the formation of the $\alpha\beta$ complex (25). Using anti-GABP subunit antibodies in gel mobility shift assays and Western blot analyses, we have found that EF-1A and GABP are related proteins.

MATERIALS AND METHODS

Cultured cells, nuclear extracts, and purification of EF-1A

Monolayer cultures of S3 HeLa cells were maintained in Dulbecco's modified Eagle medium containing 10% calf serum. Suspension cultures of S3 HeLa cells were maintained in suspension-modified minimal essential medium containing 7% calf serum. Nuclear extracts were prepared by the method of Dignam et al. (27), modified as described previously (11). Briefly, suspension cultures of HeLa cells were lysed by dounce homogenization, and isolated nuclei were extracted in a buffer containing 20 mM HEPES (pH 7.5), 420 mM NaCl, 20% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin. The nuclei were removed by centrifugation and the supernatant was dialyzed overnight against two changes of a dialysis buffer (DB.1) containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 20% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 1 mM PMSF. The supernatant of the dialysate was designated the crude nuclear extract. The crude HeLa cell nuclear extract (10 mg/ml) was fractionated on a DEAE-cellulose column with a bed volume of 1 ml per 10 mg loaded protein, equilibrated in DB.1. The column was washed with three bed volumes of DB 0.1, and the EF-1A containing fraction was eluted with DB containing 300 mM NaCl (DB.3). This fraction was designated HeLa DE.3 and was enriched five-fold for EF-1A activity. The HeLa DE.3 fraction was dialyzed to equilibrium against DB containing 50 mM NaCl (DB.05) and applied to a double-stranded calf thymus DNA column (ds CT-DNA), containing 0.21 g ds CT-DNA per g protein applied. The ds CT-DNA column was washed with three volumes DB.05; the flow-through (FT) was enriched 20-fold for EF-1A activity and was depleted considerably for other DNA binding activities. Typically, ds CT-DNA FT from a loading of 100 mg of DE.3 was concentrated to a final volume of 4 ml on Amicon Centriflo CF25 membrane cones and incubated O/N with 1 ml packed volume of DNA-coupled sepharose beads, containing ~300 mg ligated ds A-core oligonucleotide. The A-core oligonucleotide affinity column was washed with three volumes DB.05 and subsequently eluted with three volumes DB containing 1 M NaCl (DB1.0). The high salt eluate, termed B1, was highly enriched for EF-1A (> 1000-fold) and was used for further analysis or passed one or more additional times over the affinity column. For analytical purposes, the purified EF-1A was applied in DB1.0 to a phenyl-sepharose hydrophobic interaction column (Pharmacia, Sweden), the column was washed with DB containing 200 mM NaCl, and EF-1A was eluted with DB containing 0 mM NaCl. This fraction of EF-1A was enriched approximately 10,000-fold. For the sizing chromatography analysis, 2 mg of HeLa DE.3 fraction was loaded on a Superose 12 sizing column (Pharmacia, Sweden) that was equilibrated and eluted with DB.05 at a rate of 0.25 ml/min on a Pharmacia FPLC apparatus.

The procedure of Hager and Burgess was used for the elution of EF-1A activity for SDS-polyacrylamide gels (SDS-PAG) (28). HeLa DE.3 fraction was boiled for three minutes in the presence of 1 $\times$ loading buffer (1% SDS, 50 mM Tris (pH 6.8), 5% Glycerol, 5% β -mercaptoethanol) and electrophoresed on a conventional 12.5% SDS-PAG. The gel was sliced horizontally according to MW_r and proteins in the different slices were eluted by crush-and-soak O/N in elution buffer (20 mM Hepes (pH 7.5), 100 mM KCl, 5 mM DTT, 0.1% SDS, 0.1 mM EDTA and 0.1 mg/ml BSA). The proteins in the eluate fractions were acetone-precipitated, the pellets were rinsed twice with 80% acetone, and resuspended in denaturation buffer (6 M Guanidinium hydrochloride (Gu-HCl), 20 mM HEPES (pH 7.5), 100 mM NaCl, 20% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 1 mM PMSF). The fractions were then renatured by microdialysis against several hundred volumes of denaturation buffer without Gu-HCl.

In vitro DNA-protein binding and footprinting assays

Electrophoretic mobility shift assays were performed as previously described (10) in reactions of a total volume of 15 μ l containing 1 μ g of single-stranded calf thymus DNA, 25 mM HEPES (pH 7.5), 40 mM KCl, 1.5 mM MgCl₂, 2% glycerol, 10 μ M EDTA, 10 μ M EGTA and 300 μ M DTT. Protein was preincubated with non-specific CT-DNA for 10 min at room temperature (rt). 10,000 cpm (0.8 to 1.0 fmol) of ³²P-labeled probe and unlabeled competitor DNA subsequently were added simultaneously and the mixture was incubated for 20 min at rt. DNA-protein complexes were resolved on a 4% 30:1 (acrylamide:bisacrylamide) polyacrylamide gel in 0.5 $\times$ TBE (25 mM Tris (pH 8.3), 25 mM boric acid and 0.5 mM EDTA) at 4°C and detected by autoradiography. For antibody supershift assays, the DNA-protein binding reactions were combined as usual, but following the 20 min incubation at rt, diluted antibody was added and incubated with the reaction for 60 min at 4°C prior to loading on the gel.

1,10-Phenanthroline-Cu (OP-Cu) footprinting was performed as described by Kuwabara and Sigman (29). Binding reactions with affinity purified EF-1A and end-labeled probes were scaled up 15-fold and electrophoresed in preparative mobility shift gels, as described above. The gels were subjected to OP-Cu cleavage, and exposed to autoradiographic film O/N. The detected bands were eluted and electrophoresed on a 12.5% sequencing gel alongside purine and pyrimidine cleavage reactions of the probes. The gel was fixed in 10% acetic acid, dried and exposed to autoradiographic film.

Probe and competitor DNAs and oligonucleotides

The A-core dimer probe fragment (ACD) was generated by cloning a dimeric head-to-tail A-WT oligonucleotide into the polylinker region of pUC9, and excising the fragment with EcoRI and HindIII. The sequence of the A and B core wild type and mutant oligonucleotides used are listed in Fig. 4. The sequence of the A-X oligonucleotide is: 5'-TCGACACACCTA-GTGAC-3':5'-TCGAGTCACTGGATGTG-3', where the three underlined nucleotides were changed from the 5'-GGA-3' present in the upper strand of the A-WT sequence. The A and B probe fragments used in Fig. 2A were isolated from the E1A-CAT vector by excision with EcoRI and BsaAI. The sequence of the GABP oligonucleotide was derived from Thompson et al. (25) and is: 5'-TCGACGCGGAACGGAAGCGGAACCGC-3':5'-TCGAGCGGTTTCCGCTTCCGTTCCGCG-3'. The GABP

probe fragment was made by gel purifying and annealing the two GABP oligonucleotide strands. All the probe fragments were ^{32}P -labeled by incorporating (α - ^{32}P)-dATP and (α - ^{32}P)-dCTP using Klenow DNA polymerase, yielding specific activities of approx. 20,000 cpm/fmol. Competitor fragments were generated by multimerizing annealed oligonucleotides by ligation. The oligonucleotide competitors were quantitated spectrophotometrically.

Antibodies and Western blot analysis

Polyclonal rabbit antibodies against recombinant GABP α and GABP β proteins were a generous gift of Dr. Steven McKnight (Carnegie Institution of Washington, Baltimore, Md). For Western blot analysis, protein samples were electrophoresed on a 12.5% SDS-PAGE and electroblotted onto a nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The membranes were blocked with 5% Blotto (5% dry milk, 20 mM Tris (pH 8.0) and 30 mM NaCl), incubated with primary antibody in 5% Blotto containing 0.2% Tween 20 and 0.5% NP 40 (Blotto-D), washed with 5% Blotto-D, and incubated with secondary antibody conjugated with horseradish peroxidase in 5% Blotto-D and washed in 5% Blotto-D. The bands were detected using enhanced chemiluminescence (ECL) (Amersham, UK).

Plasmid clones and transfection

The E1A-CAT expression vector contains E1A promoter region sequences from -141 to +49 inserted upstream of the CAT gene (30). Wild type and mutant oligonucleotides were inserted at -141, using a unique XhoI site, as head-to-tail dimers, unless otherwise noted. Monolayer cultures of S3 HeLa cells were transfected using the calcium phosphate precipitation method (31). Briefly, cells were split the day before transfection. The following day, cells were transfected with a calcium phosphate mix containing 1 μg E1A-CAT expression vector plus 19 μg salmon sperm DNA per 100 mm dish (approx. 5×10^5 cells). After incubation O/N with the mix, the cells were washed and fresh medium was added. Total cellular extracts were prepared 48 hrs. following the addition of fresh medium and CAT activity was assayed as previously described (30). The results presented represent the average of a minimal of six independent transfection assays using two independent preparations of purified plasmid DNAs.

RESULTS

Cooperative binding of EF-1A to a dimeric binding site is correlated with enhancer function

We previously demonstrated that HeLa cell EF-1A bound *in vitro* in a cooperative manner to adjacent and related copies of the E1A core enhancer element (Fig. 1, 5'-AGGAAGTG-3' and 5'-CGGATGTT-3', A and B sites, respectively; 10). Figure 2A displays the DNA-protein complexes that are detected using a gel mobility shift assay with HeLa cell EF-1A and monomeric and dimeric binding site probes. The binding of EF-1A to a monomeric A site (A-) resulted in the formation of two DNA-protein complexes (complexes 2 and 3, lanes 1-4). When the highest concentration of protein was added, a more retarded species, complex 1, was weakly formed. In contrast, dimeric A sites, with the two sites in a head-to-tail (A-/A-), head-to-head (A-/A-), or tail-to-tail (A-/A-) orientation, readily formed complex 1 in addition to complexes 2 and 3 upon EF-1A

binding (lanes 5-16). Likewise, the binding of EF-1A to a dimeric B site (B-/B-) resulted in the formation of complexes 1, 2 and 3, although the formation of complex 1 was reduced approximately 4-fold (lanes 17-20). The specificity of EF-1A interaction is demonstrated by competition for the formation of all three complexes using the homologous dimeric site (A-WT, lane 22) but not a mutant site containing a triple point mutation in the conserved GGA of the core sequence (A-X, lane 23).

These same sites were introduced upstream of the E1A promoter region in an expression vector (E1A-CAT) and assayed for their ability to activate transcription in HeLa cells *in vivo* (Fig. 2B). An A-WT monomer displayed little enhancer activity, while the insertion of direct dimeric A repeats, in either orientation (A-/A-, A-/A-), enhanced expression significantly (40 to 50-fold). The two non-direct dimers (A-/A- and A-/A-) both enhanced CAT expression (~10-fold) but at a reduced level compared to the direct repeats. A dimeric mutant site (A-X/A-X) did not display enhancer activity (data not shown). Since dimeric, but not monomeric, EF-1A binding sites efficiently formed complex 1 *in vitro* and activated E1A expression *in vivo*, we correlate the formation of complex 1 with enhancer competency. Unlike the A-type dimers, the B-type dimer (B-/B-) had little detectable enhancer function, even though it formed complex 1 in a binding assay. This result will be addressed in further detail below.

Different binding site nucleotides are required for efficient binding and enhancer function

A series of oligonucleotide competitors containing point mutations in the EF-1A A and B binding sites were generated (Fig. 3). The nucleotides in and around the GGAA core of the Ad E1A site A were assigned numbers from 1 to 10, and purines that were previously identified as interference sites in diethyl pyrocarbonate (DEPC) and dimethyl sulfate (DMS) footprinting of EF-1A were targeted for mutagenesis (Fig. 3; 10). We assessed the ability of multimerized oligonucleotides to compete for binding of EF-1A (Fig. 4). The A-WT competitor is a multimer of the EF-1A A site oligonucleotide. The ability of the A-WT competitor to compete for EF-1A binding (Fig. 4, lanes 2-4) was arbitrarily set to 1 (Fig. 3). The B-WT competitor, a multimer of the EF-1A B site oligonucleotide, was reduced 3 to 4-fold for EF-1A binding

Figure 1. The sequence of the two EF-1A binding sites in the adenovirus E1A enhancer region, EF-1A A and EF-1A B, used in the *in vitro* binding and *in vivo* enhancer assays and the binding site for the murine transcription factor GABP are shown. The shaded boxes indicate the purine-cores involved in factor binding, as determined by Bruder and Hearing and Thompson et al. (10, 25). Shown below these sequences are the consensus EF-1A binding site (10, 11) and the consensus *ets* binding site derived from Karim et al. (23). The purine cores are shaded.

Figure 2. A. *In vitro* binding of EF-1A to different E1A core enhancer element binding site probes. Binding reactions contained four different concentrations of HeLa DE.3 fraction (80, 160, 320 and 640 μ g/ml), as indicated by the open triangles, and the probe DNAs identified above the lanes: A monomer, lanes 1–4; A dimer, head-to-tail orientation, lanes 5–8; A dimer, head-to-head orientation, lanes 9–12; A dimer, tail-to-tail orientation, lanes 13–16, and B dimer, head-to-tail orientation, lanes 17–20. Lanes 21–23 contain the A core dimer probe (ACD, head-to-tail orientation) in a binding reaction that lacked specific competitor DNA (lane 21), or that contained a 250-fold molar excess of the A-WT (lane 22) or A-X mutant (lane 23) ligated oligonucleotides. The three specific DNA-protein complexes described in the text, 1, 2 and 3, are indicated at the left. The bottom band in the gel represents the unbound probe. **B.** Enhancer activity of EF-1A binding sites. Monomeric and dimeric A and B sites were introduced upstream of the E1A promoter region and analyzed for enhancer activity in transfected HeLa cells. The fold-enhancements relative to the enhancerless vector are given along with standard deviations.

(lanes 5–7), as previously observed (Fig. 2A; 10). The difference in A versus B binding efficiency reflects the nucleotide difference between these sites only at position 7 (Fig. 3, A vs. T) since the A 7A-T mutant site was reduced for EF-1A binding to a level comparable to the B-WT site (Fig. 4, lanes 20–22) while the B 7T-A mutant site was increased for EF-1A binding to the level of the A-WT site (Fig. 4, lanes 29–31). Differences between these sites at positions 3 and 10 (Fig. 3, A vs. C and G vs. T, respectively) did not influence EF-1A binding efficiency (Fig. 4, lanes 11–13 and 26–28). Two mutations in the purine core of the EF-1A binding site (Fig. 3, A 4G-C and A 7A-C) dramatically decreased EF-1A binding (Fig. 4, lanes 14–19), while mutations in flanking nucleotides (Fig. 3, A 1A-T and A 10G-C) had a modest or no effect on binding (Fig. 4, lanes 8–10 and 23–25).

The EF-1A site mutant oligonucleotides were dimerized in a head-to-tail orientation, inserted in the E1A-CAT vector, and assayed for their ability to activate E1A expression *in vivo*. The enhancer strengths of these sites are listed in Fig. 3. The A-WT dimer activated expression 45-fold in HeLa cells, compared to the enhancerless construct. As previously discussed, the B-WT dimeric site was only 3 to 4-fold reduced for binding relative to the A-WT site, but it was virtually incapable of transcriptional activation *in vivo* (Fig. 2B and 3). With most of the mutant binding sites, there was a very good correlation (within 2-fold) between the level of EF-1A binding *in vitro* and enhancer activity *in vivo* (Fig. 3, A 1A-T, A 3A-C, A 4G-C, A 7A-C and A 7A-T). The functional distinction between the dimeric A and B binding sites *in vivo* does not reflect the 3 to 4-fold difference in relative EF-1A binding *in vitro* between these sites, since the B 7T-A site bound EF-1A as well as the A-WT site *in vitro* yet

was still inactive as an enhancer *in vivo*. Rather, this functional distinction appears to reflect the primary sequence difference between these sites at the 3' region of the E1A core enhancer motif. Specifically, while the A 10G-C and A 10G-T mutations did not reduce EF-1A binding efficiency *in vitro* (Fig. 4), both of these mutations abolished the enhancer activity of these sites *in vivo* (Fig. 3).

EF-1A is composed of two proteins that form a heteromultimeric complex in solution

We purified an activity from HeLa cells that forms the three complexes 1, 2 and 3 with the dimeric binding site probe using conventional and DNA binding site affinity chromatography (see Materials and Methods). A series of protein species migrating between 40 kD and 70 kD MW_r on SDS-PAGE correlated with binding activity in the most purified fraction (phenyl-Sepharose fraction), but no single protein species correlated specifically with EF-1A activity (data not shown). To examine the composition of EF-1A binding activity, proteins were isolated by gel electrophoresis. Enriched HeLa cell nuclear extract (the DE.3 fraction) was electrophoresed on an SDS-PAGE and proteins corresponding to different molecular weight classes were eluted and renatured. The different fractions were tested for the ability to bind to a dimeric A-core probe using a mobility shift assay (Fig. 5A). Fraction 3, corresponding to ~ 60 kD MW_r , contained an activity that could form complexes 2 and 3 (lane 6); these complexes were competed specifically by the A-WT, but not the mutated A-X, competitor DNAs (lanes 12–14). No individual fraction gave rise to the formation of complex 1. Fraction 7, corresponding to a ~ 50 kD MW_r , contained no appreciable probe binding activity (lane 10), but upon mixing

		Competition for Binding <i>in vitro</i>	Relative Enhancer Function <i>in vivo</i>
A-WT	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GTGTCCTTCACTGAGCT	1	1
B-WT	1 2 3 4 5 6 7 8 9 10 TCGACAGCGGAAGTTG GTCCGCTTCAACAGCT	0.3	0.02 ± 0.01
A 1A-T	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GAGTCCTTCACTGAGCT	0.25	0.29 ± 0.09
A 3A-C	1 2 3 4 5 6 7 8 9 10 TCGACACCGGAAGTGAC GTGGCCTTCACTGAGCT	1	0.48 ± 0.06
A 4G-C	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GTGTCTTCACTGAGCT	0	0.02 ± 0.01
A 7A-C	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GTGTCTTCACTGAGCT	0.05	0.04 ± 0.02
A 7A-T	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GTGTCTTCACTGAGCT	0.25	0.57 ± 0.07
A 10G-C	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTGAC GTGTCTTCACTGAGCT	1	0.06 ± 0.02
A 10G-T	1 2 3 4 5 6 7 8 9 10 TCGACACAGGAAGTTAC GTGTCTTCACTGAGCT	1	0.06 ± 0.01
B 7T-A	1 2 3 4 5 6 7 8 9 10 TCGACAGCGGAAGTTG GTCCGCTTCAACAGCT	1	0.05 ± 0.01

Figure 3. EF-1A binding and enhancer activity of A and B sites containing point mutations. A list of the EF-1A binding site mutant oligonucleotides used for *in vitro* mobility shift binding assays and transient expression assays are shown at the left. The nucleotides around the GGAA core were assigned numbers from 1 to 10 that are listed above the mutants; the mutated nucleotides are shaded and are indicated by the respective oligonucleotide names given at the very left. The triangles (▽) indicate previously determined DEPC and DMS interference sites (10). Column 1 gives the relative binding affinity of the mutants as determined by using multimerized oligonucleotides as competitors for EF-1A binding in mobility shift assays (summary of data displayed in Fig. 4). Column 2 gives the enhancer activity of each mutant, with standard deviation, relative to the A-WT dimeric oligonucleotide in the transient expression assay; the A-WT dimer augmented E1A transcription 45-fold.

with fraction 3, complex 1 was restored in a specific fashion (lanes 11–13). We therefore deduce that the EF-1A activity is composed of at least two proteins, a ~60 kD protein, that we term EF-1A α , and a ~50 kD protein, that we term EF-1A β . The two proteins form a heteromultimeric complex on a dimeric DNA binding site. EF-1A α is responsible for the primary DNA contacts of the activity and can form complexes 2 and 3 on a monomeric or dimeric site probe. This interpretation is supported by DEPC interference footprinting of the upper DNA strand in EF-1A complex 3, which only involves the α subunit, where all of the same interference sites as those found in EF-1A complex 1 ($\alpha + \beta$ subunit) were observed (5'-ACACAGGAAGTG-3', underlined letters indicate DEPC interference sites, data not shown; see also Fig. 9). EF-1A β interacts with EF-1A α and enables the formation of the more retarded complex 1 efficiently only with the dimeric site probe.

To investigate whether the two EF-1A subunits were complexed in the absence of a DNA binding site, enriched HeLa nuclear extract was fractionated on a Superose 12 sizing column (Fig. 5B). An EF-1A activity that could form complexes 1, 2 and 3 eluted in fractions corresponding to a MW_r ~200 kD

Figure 4. Effect of binding site mutations on *in vitro* binding of EF-1A. Multimerized oligonucleotides were used as competitors in binding reactions containing HeLa cell EF-1A (DE.3 fraction). Binding reactions contained the ACD probe and increasing concentrations (10-, 50- and 250-fold molar excess, as indicated by the open triangles) of the competitors listed across the top of the figure. DNA-protein complexes displayed in lane 1 are from binding reactions containing no specific competitor DNA. Complexes 1, 2 and 3 are indicated at the left. The specific competitor DNAs used in the assays are listed in Figure 3.

(lanes 5 and 6). An activity that could form complex 3 eluted corresponding to a MW_r ~65 kD (lane 10). Since the formation of complex 1 requires the presence of both EF-1A α and β subunits, the ~200 kD activity possibly represents an (EF-1A α)₂ (EF-1A β)₂ heterotetramer. These data demonstrate that the two proteins associate in the absence of DNA.

EF-1A and GABP are related activities

Recently, two murine transcription factors that bind to the HSV ICP4 promoter, GABP α and GABP β , were cloned and characterized (24, 25). The GABP binding site resembles a dimeric EF-1A consensus binding site, containing repeated purine-rich core sequences (Fig. 1). The *ets*-related GABP α can form two retarded complexes with a GABP probe in a gel mobility shift assay. Upon addition of GABP β , a more slowly migrating complex is formed (24, 25), a situation identical to that of EF-1A. We compared the binding of the HeLa DE.3 fraction and affinity purified EF-1A to the A core dimer (ACD) probe and a GABP probe (GA) in a gel mobility shift assay. Both the EF-1A enriched HeLa DE.3 fraction (data not shown) and affinity purified EF-1A formed similar complexes (1, 2 and 3) with the ACD and GA probes (Fig. 6A, lanes 1 and 4). These complexes were specifically competed by the A-WT, but not the A-X, competitor DNAs (lanes 2, 3, 5 and 6).

Western blot analyses were performed using the HeLa DE.3 fraction and affinity purified EF-1A and probed with polyclonal antisera (pAb) directed against recombinant GABP α and GABP β (Fig. 6B). The GABP α pAb detected a prominent ~60 kD MW_r species and a few smaller species in the two EF-1A containing fractions (left, lanes 2 and 4), but failed to do so in two EF-1A depleted fractions (left, lanes 1 and 3). The GABP β pAb detected two groups of proteins of MW_r ~50 kD and ~40 kD, respectively, in the EF-1A containing fractions (right, lanes 2 and 4), but not in the EF-1A depleted fractions (right, lanes 1 and 3). The molecular weights of the bands detected by the GABP α and β antisera correspond to those of the gel isolated EF-1A α and β subunits, respectively, suggesting that the respective α and β subunits of EF-1A and GABP are related. We are uncertain of the nature of the ~40 kD MW_r anti-GABP β detected band, but it could represent an alternatively spliced or degradation product of EF-1A β , and suggests that EF-1A β , like GABP β , may exist in two different forms (24).

To confirm this relationship, the anti-GABP α and GABP β antibodies were used in EF-1A binding reactions with the ACD

Figure 5. A. EF-1A is composed of two subunits. HeLa DE.3 fraction was electrophoresed on a 12.5% SDS-polyacrylamide gel and proteins corresponding to different MW_r were eluted and renatured. The renatured proteins in gel fractions were used in binding reactions containing the ACD probe. Lane 1 shows a binding reaction with the starting material, HeLa DE.3; lanes 2 and 3 show competition binding reactions containing HeLa DE.3 and a 1250-fold molar excess of the A-X or A-WT competitor DNAs. Lanes 4–11 show the DNA-protein complexes formed by proteins in gel fractions 1–8. Lanes 12 shows the binding activity in fraction 3 (EF-1A α) and lanes 13 and 14 are reactions using fraction 3 containing the A-X and A-WT competitor DNAs. Lane 15 is a binding reaction containing fraction 7. Lane 16 shows the binding activity when proteins in fractions 3 and 7 were mixed in a binding reaction. Lanes 17 and 18 are reactions containing fractions 3 and 7 and the A-X and A-WT competitor DNAs. Complexes 1, 2 and 3 are indicated at the left. **B.** EF-1A subunits exist in a heteromultimeric complex in the absence of DNA binding sites. HeLa DE.3 fraction was chromatographed on a Superose 12 sizing column, fractions (indicated across the top) corresponding to different relative molecular weights (MWs, marker proteins indicated across the top) were collected and used in a mobility shift assay with the ACD probe. Lane 1 shows a binding reaction with the starting material, HeLa DE.3, and the formation of the three EF-1A specific complexes 1, 2 and 3 (indicated at the left). Lanes 2–14 show the binding activities obtained using Superose fractions 3–16. Fraction 1 corresponds to the first fraction following the void.

probe (Fig. 6C). The GABP α pAb retarded the mobility of all three complexes 1, 2 and 3 formed by affinity purified EF-1A, while the GABP β pAb supershifted complexes 1 and 2, but not complex 3 (data not shown and Fig. 7, lanes 1, 5, and 9). A binding reaction containing gel isolated EF-1A α subunit and the ACD probe was supershifted by the anti-GABP α , but not the GABP β , antibodies (Fig. 6C, lanes 4–6). However, a reconstituted binding reaction containing both EF-1A α and EF-1A β was susceptible to both anti-GABP α and GABP β antibodies (Fig. 6C, lanes 8–10). Neither of these antibodies altered the mobility of a DNA-protein complex containing a distinct DNA binding activity (EF-C; data not shown).

Since the molecular weights of the ~ 60 kD MW_r anti-GABP α detected band and the ~ 50 kD MW_r anti-GABP β detected band correspond with the deduced sizes of the gel purified EF-1A α and β subunits (Fig. 5A) and based on their susceptibility to the pAbs in the supershifting assay (Fig. 6C), we conclude that the subunits of EF-1A and GABP are very likely human and murine equivalents of the same activity. Like the situation with GABP, the EF-1A α subunit is present in all 3 complexes 1, 2 and 3, while the β subunit is present in only in complexes 1 and 2 (24, 25).

Both EF-1A subunits are necessary, but not sufficient, for enhancer activity

Thompson et al. (25) demonstrated that the GABP α subunit contacted nucleotides in the purine core of the binding site while

the GABP β subunit contacted nucleotides 3' of this sequence. This suggested that the loss of enhancer activity by the inactive A 10G-C and A 10G-T sites could reflect altered or lack of binding of the EF-1A β subunit to these sites and hence a loss of enhancer activity since both subunits are required for function. To address this possibility, the transcriptionally active dimeric A-WT site and inactive B-WT, A 10G-C and A 10G-T dimeric sites were used as probes in a mobility shift assay with partially fractionated EF-1A (Fig. 7). All four of these binding site probes were capable of binding EF-1A to generate complexes 1, 2 and 3 (lanes 1–4). Additionally, both EF-1A α and EF-1A β subunits were present in these complexes as indicated by the appropriate supershifts using the anti-GABP α and β antibodies (lanes 5–12). Lastly, the stability of the DNA-protein complexes 1, 2 and 3 formed on the A-WT, A 10G-C and A 10G-T probes were equivalent as measured by susceptibility to increasing NaCl and urea concentrations and off-rate analyses *in vitro* (data not shown). We therefore conclude that the formation of EF-1A complex 1 is necessary, but not sufficient, for enhancer activity.

We performed copper-phenanthroline (OP-Cu) footprinting of affinity purified EF-1A bound to dimeric A-WT, A 10G-C and A 10G-T probes. The result obtained with the WT probe is shown in Figure 8, and the results are summarized in Figure 9. The EF-1A footprint did not extend beyond nt position 9 on both strands. Thus, the EF-1A complex does not appear to interact with the nucleotides downstream of position 9 that are critical for function. Identical results were obtained using the A 10 G-C

Figure 6. EF-1A and GABP are related activities. **A.** Affinity purified EF-1A from HeLa cells (ACL B1) was used in mobility shift binding and competition assays with the EF-1A A core dimer (ACD) and GABP (GA) probes (lanes 1–3 and 4–6, respectively). Complexes 1, 2 and 3 are indicated at the left. Binding reactions contained no specific competitor DNA (lanes 1 and 4), or a 250-fold molar excess of the A-WT (lanes 2 and 5) or A-X (lanes 3 and 6) competitor DNAs. **B.** Different fractions obtained during purification of EF-1A from HeLa cell nuclear extracts (DE.1, DE.3, ACL FT-A core ligated oligonucleotide affinity column flow-through fraction, and ACL-B1-affinity column bound fraction 1, lanes 1–4, respectively) were electrophoresed on a 12.5% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and were probed using anti-GABP α antiserum (left panel) and anti-GABP β antiserum (right panel). The position of protein marker proteins are indicated on both sides of the autoradiogram. **C.** HeLa DE.3 and gel purified EF-1A α and β subunits were used in binding reactions with the ACD probe. Lanes 1–3 are reactions containing HeLa DE.3 incubated with no antiserum (lane 1), anti-GABP α antiserum (lane 2) and anti-GABP β antiserum (lane 3). Lanes 4–6 are reactions containing gel purified EF-1A α subunit (fraction 3, see Fig. 5A and text) incubated with no antiserum (lane 4), anti-GABP α antiserum (lane 5) and anti-GABP β antiserum (lane 6). Lane 7 is a reaction containing the gel purified EF-1A β subunit (fraction 7, see Fig. 5A and text). Lanes 8–10 are reactions containing reconstituted EF-1A ($\alpha + \beta$, fractions 3 + 7) incubated with no antiserum (lane 8), anti-GABP α antiserum (lane 9) and anti-GABP β antiserum (lane 10).

and A 10G-T probes on both strands (results not shown; Fig. 9). It is therefore possible that an activity that is not part of the EF-1A complex characterized so far may interact with the downstream part of the EF-1A binding site to confer enhancer activity.

DISCUSSION

We have purified EF-1A using conventional, sizing and DNA binding site affinity chromatography. The EF-1A binding activity can be accounted for by two proteins, EF-1A α (MW ~ 60 kD) and EF-1A β (MW ~ 50 kD) that can coexist in a ~ 200 kD heteromultimeric complex in the absence of DNA (Fig. 5B). The gel purified EF-1A α subunit can only form the retarded complexes 2 and 3 with a dimeric binding site probe in a gel retardation assay, while the EF-1A β subunit possesses no apparent DNA binding properties. Upon the mixing of EF-1A α and β in a binding reaction, binding activity was restored for the formation of EF-1A complex 1. Complex 2 can be formed by gel purified EF-1A α (Fig. 5A, lane 6), but contains both the α and β subunit when formed by affinity purified EF-1A (Fig. 7, lanes 5–12), suggesting that the α subunit may homodimerize on a dimeric binding site in the absence of the β subunit. The properties of EF-1A α and β highly resemble those of the previously described human factor E4TF-1 (32), and the recently described and cloned murine transcription factors GABP α and β (24, 25). E4TF-1 and EF-1A are both composed of a ~ 60 kD DNA binding protein and a ~ 50 kD associated factor that is required for binding site mediated enhancer activity. Likewise, GABP is also composed of two subunits—the GABP

Figure 7. EF-1A α and β subunits bind to both transcriptionally active and inactive EF-1A binding sites. Direct (head-to-tail) dimeric fragments of the A-WT (A/A), B-WT (B/B), and A 10G-C and A 10G-T mutants were excised from the respective E1A-CAT plasmids and used as probes in binding reactions containing HeLa DE.3 fraction. Complexes 1, 2 and 3 are indicated at the left. Lanes 1–4 display complexes observed from binding reactions containing the A/A (lane 1), B/B (lane 2), A 10G-C (lane 3) and A 10G-T (lane 4) probes. Lanes 5–8 are identical to lanes 1–4, but the reactions were incubated with anti GABP α antiserum. Lanes 9–12 are identical to lanes 1–4, but the reactions were incubated with anti GABP β antiserum.

Figure 8. 1,10-Phenanthroline-Cu (OP-Cu) protection footprinting analysis of EF-1A binding to the A-WT dimeric binding site probe. Probe fragments were ^{32}P labeled on the upper or lower strands, and used in an *in vitro* binding reaction with affinity purified EF-1A. The binding reaction was electrophoresed in a 4% PAG and subjected to OP-Cu cleavage. The upper EF-1A complex 1 (see Fig. 2) and the unbound probe were excised and electrophoresed on a 12% denaturing PAG. The B lanes show the fragments arising from the complexed bands; the U lanes show the fragments arising from the free probe. The AG and CT lanes show the ladders arising from purine and pyrimidine cleavage of the probes. Nucleotides that were protected from OP-Cu cleavage by EF-1A are indicated by the vertical bars. The left hand panel shows the results for the upper strand; the right hand panel shows the results for the lower strand. The results are summarized in Figure 9.

α subunit is related to the *ets* domain protein family and is responsible for the primary DNA contacts of the GABP $\alpha\beta$ complex, while the GABP β subunit interacts with the β subunit and makes additional contacts 3' of the purine-rich sequence in the binding site (Fig. 9). Like the EF-1A subunits, the GABP subunits coexist in α complex in the absence of DNA (25). Western blot analyses of EF-1A containing fractions and affinity purified EF-1A demonstrated that the anti-GABP α pAb detected an ~ 60 kD protein, corresponding to the apparent MW of EF-1A α , indicating that EF-1A α is related to GABP α and the *ets* domain family of proteins (Fig. 6B). The GABP β antiserum detected two species of ~ 50 and ~ 40 kD (Fig. 6B), and it therefore seems that there may be two subspecies of EF-1A β , as is the case with the GABP β_1 and β_2 subunits (24, 25). While the ~ 50 kD EF-1A β species was capable of interaction with EF-1A β , the ~ 40 kD EF-1A β species apparently was not (Fig. 5A and data not shown). This result may be due to the inability of the ~ 40 kD EF-1A β species to correctly refold following denaturation during the gel elution procedure since this species is present in the affinity purified fraction that was selected for specific DNA binding (Fig. 6B).

The A monomer (A $\rightarrow$) had poor enhancer activity *in vivo* and weakly formed the retarded complex 1 *in vitro*, while the A dimers (A $\rightarrow$ /A $\rightarrow$ and A $\rightarrow$ /A $\leftarrow$) readily formed complex 1 and were potent enhancer elements (Fig. 2). We therefore correlate

Figure 9. A schematic representation of OP-Cu protection and DEPC interference footprinting of EF-1A complex 1, formed by dimeric A-WT and A 10 G-C and A 10 G-T mutant probes. The boldface residues in the A-WT sequence indicate DEPC interference points (10). The shaded bars indicate the extent of the OP-Cu protection footprint on each strand. Nucleotide position 10 is boxed on the A 10 G-C and A 10 G-T mutant probe sequences. The likely DNA interactions of EF-1A α and β are indicated at the top, and are based on our observations for the α subunit (DEPC interference sites) and those of Thompson et al. for the β subunit (25). The proposed interactions of the putative coactivator are indicated by arrows.

the ability to form EF-1A complex 1 with enhancer activity. Since the binding study of the gel purified subunits revealed that both EF-1A α and β are involved in the formation of complex 1, both subunits must be required for enhancer activity. Dimeric EF-1A sites enhanced transcription *in vivo* best in a head-to-tail configuration—the orientation these sites possess in the Ad E1A and E4 enhancer regions. Is EF-1A the activity that is responsible for activation of transcription via the E1A enhancer core sequence *in vivo*? The data from our laboratory and others strongly support this idea. First, a panel of 9 different viruses carrying mutations in and around EF-1A sites A and B in the Ad E1A enhancer region were analyzed for E1A transcription *in vivo* in infected Hela cells. A direct correlation was observed between E1A enhancer region activity *in vivo* and EF-1A binding *in vitro* with each of the mutants; the panel of mutants ranged in E1A expression *in vivo* and EF-1A binding *in vitro* from wild type to 25-fold reduced levels (12). Second, point mutation of nucleotides 1, 3, 4 and 7 in the A site all led to a concomitant decline in EF-1A binding *in vitro* and enhancer function *in vivo* of the dimerized sites (Fig. 3). Additionally, two other distinct EF-1A dimeric binding sites, the HSV GABP site (data not shown) and the Ad E4 E4TF-1 site (32), also possess intrinsic enhancer activity. Third, the HeLa cell factor E4TF-1, which shares identical binding properties and subunit composition with EF-1A and is very likely the same activity, is capable of activation of E4 transcription *in vitro* (32). The binding of EF-1A to a dimerized site is necessary, but not sufficient, for enhancer activity. This conclusion is based on the following observations: The B $\rightarrow$ /B $\rightarrow$ dimer was only reduced 3-fold for binding, but had no enhancer activity (Fig. 2). Second, point mutation of nucleotide G-10 in the A site to a T residue, which is present at that position in the transcriptionally inactive B site, or to a C residue did not affect EF-1A binding but abolished enhancer activity altogether (Fig. 3). Third, mutation of nucleotide T-7 in the B-site to an A residue increased EF-1A binding to the level of A-WT, but failed to restore enhancer activity (Fig. 4). Thus, EF-1A binding efficiency is largely determined by the nucleotides in the purine core of the binding site. The ability of the site to

act as an enhancer is determined by these nucleotides, but also by peripheral nucleotides that do not affect binding, notably that in position 10 (Fig. 3) and position 11 (data not shown), which differs between the active A-site and the inactive B-site.

We have detected no difference in off-rate or susceptibility to NaCl or urea between EF-1A binding to transcriptionally active (A-WT) and inactive (B-WT, A 10G-C and A 10G-T) dimeric binding site probes (data not shown). EF-1A α and β are constituents of complex 1 and 2 on both transcriptionally active and inactive dimeric binding site probes, as testified by the retardation of these complexes by both GABP α and β antisera (Fig. 7). The OP-Cu footprint of EF-1A on both the active and inactive binding sites has the same extent on both strands and does not cover nucleotide position 10 (Fig. 9) which is critical for transcriptional activation. It is possible that minute interactions between the EF-1A complex and nucleotide position 10 may confer subtle, undetected, conformational changes to the complex that result in activating properties on the A-WT site. However, we speculate that an ancillary activity may interact with the downstream sequences of the binding site, and confer activation by interacting with EF-1A and those sequences on the A-WT site. Three EF-1A/E4TF-1 sites have been demonstrated to function as activators: EF-1A/PEA-3 site 1 in the Py enhancer, the EF-1A A site in the Ad E1A enhancer and the EF-1A/E4TF-1 site between nt -150 and -140 in the Ad E4 regulatory region (10, 11, 32). In addition to their activating properties, these sites all share the same five residues following the purine core: GGAA-GTGAC. We find it likely that a putative coactivator interacts with the 3' GAC stretch, since the EF-1A footprint never extends to cover these nucleotides. It seems less likely that such an activity might be inhibiting, as there is divergence among the inactive EF-1A and point mutated A sites. Such an activity might interact optimally with EF-1A bound to direct A repeats (A $\rightarrow$ /A $\rightarrow$), allowing one unit to interact with each A repeat. The A $\rightarrow$ /A $\rightarrow$ and A $\rightarrow$ /A $\rightarrow$ repeats are reduced for activation, as is an A $\rightarrow$ /B $\rightarrow$ repeat (data not shown), perhaps because the putative coactivator interacts less favorably with EF-1A bound to the non-direct repeats. The A $\rightarrow$ /B $\rightarrow$ repeat may be reduced for activation compared to the A $\rightarrow$ /A $\rightarrow$ repeat, because it can only interact with one unit of the coactivator.

Other *ets* proteins (*ets-1*, *ets-2*, *elk*, *erg* and PU.1) have been identified as transcription/enhancer factors (15, 20, 33–37). In the collagenase promoter and Py A enhancer domains, *ets*-dependent transcriptional activation is achieved via a monomeric *ets* protein binding site but only in conjunction with an adjacent AP1 site (38, 39). This result suggests that different *ets*-related binding proteins may activate transcription in conjunction with different auxiliary factors. One such interaction takes place on the serum response element (SRE) of the *c-fos* promoter, where an association between an ~62 kD *ets*-related protein (*elk-1*) and p67^{SRE} has been reported (40, 41). A second nuclear protein that is associated with enhancer activity has been shown to interact with PU.1 at the immunoglobulin enhancer (42). The *ets*-related proteins EF-1A α and GABP α augment transcription in association with different proteins, EF-1A β and GABP β , respectively. It seems likely that the modular composition of these and other enhancer activities may be part of a regulatory scheme, allowing the mating of different 'sub-factors' in different cell types to engender activity. Examples of this type previously have been demonstrated with the leucine zipper family and basic helix-loop-helix family (9 and references therein).

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