Interaction of Nuclear Factor EF-1A with the Polyomavirus Enhancer Region

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Enhancer factor 1A (EF-1A) is a mammalian nuclear protein that previously was shown to bind cooperatively to the repeated core enhancer element I sequence in the adenovirus E1A enhancer region. We now have characterized three binding sites for EF-1A in the polyomavirus A2 (Py) enhancer region. Site 1 resides in the Py A enhancer domain, and sites 2 and 3 reside in the Py B enhancer domain. EF-1A binding to Py site 1 is independent of cooperation with other EF-1A sites or the adjacent binding sites for PEA-1 and PEA-2, two murine nuclear factors that bind in the Py A enhancer domain. EF-1A binding to Py sites 2 and 3, in contrast, is cooperative, similar to the situation previously observed with binding sites in the adenovirus E1A enhancer region. In a transient replication assay, EF-1A site 1 functions synergistically with the PEA-1 and PEA-2 sites in the A enhancer domain to enhance Py replication. The functional cooperativity observed with the EF-1A, PEA-1, and PEA-2 sites *in vivo* does not reflect cooperative DNA binding interactions, as detected *in vitro*. Py EF-1A site 1 alone is capable of weakly stimulating Py replication. EF-1A site 1 overlaps with the binding sites for the murine nuclear protein PEA-3 and the *ets* family of oncoproteins.

Enhancers were originally described in simian virus 40 (1) and polyomavirus (4) as DNA domains that were essential for viral transcription and capable of augmenting transcription of a linked gene in a distance- and orientation-independent manner. Polyomavirus requires the presence of an active enhancer in *cis* for DNA replication (5). The polyomavirus type A2 (Py) enhancer is positioned from nucleotide (nt) 5021 to nt 5265 (numbering according to Tyndall et al. [29]) between the early and late start sites of transcription and immediately next to the origin of replication. The Py enhancer has been divided in two domains, the A enhancer domain (*BclI-PvuII*, nt 5021 to 5130) and the B enhancer domain (*PvuII-PvuII*, nt 5131 to 5265) (9). Three nuclear proteins bind to the A enhancer domain: PEA-1 (20, 23) (believed to be the murine analog to AP-1) binds at nt 5114 to 5120, PEA-2 (12, 23) binds at nt 5123 to 5129, and PEA-3 (15) binds at nt 5108 to 5113 (the PEA-3 A site) (Fig. 1A). Several proteins bind to the B enhancer domain including EF-C (19), PEB-1 (21, 22), and PEA-3 (the PEA-3 B site) (15) (Fig. 1A). While several functionally redundant subregions of the Py enhancer have been defined, no single subregion has been found essential for Py replication in all cell types (3, 16–18, 25, 29, 30).

The binding sites for PEA-1, PEA-2, and PEA-3 in the A enhancer domain all reside in a subdomain termed the A element (nt 5108 to 5130) (30). This element corresponds to sequences that have been found tandemly repeated in different polyomavirus strains (26). Several studies have shown that mutations which alter the binding sites for PEA-1 or PEA-3 in that region reduce Py early transcription and DNA replication *in vivo* and that these two factors cooperate functionally (15, 17, 28, 30). Additionally, PEA-1, PEA-2, and PEA-3 all are subject to activation by the nonnuclear oncogene *Ha-ras* and phorbol acetate, and PEA-1 and PEA-3 share the ability to be activated by a series of other

nonnuclear oncogenes and serum components (11, 33, 34, 36).

We previously described the binding of a cellular nuclear protein, enhancer factor 1A (EF-1A), to the adenovirus type 5 (Ad5) enhancer element I sequence in the Ad5 E1A and E4 enhancer regions (2). Element I specifically activates E1A transcription in Ad5-infected cells (8). EF-1A was found to bind to two adjacent, nonidentical sites in the E1A enhancer, termed A and B (Fig. 1B), in a cooperative manner without strict spacing constraints. Multimers, but not monomers, of oligonucleotides containing EF-1A binding sites strongly enhance the transcription of a heterologous, linked gene in a transient assay (2). EF-1A-like activities have been detected in human, mouse, and rat cells (2).

In this report, we describe the binding of nuclear factor EF-1A to three sites in the Py enhancer. One site is located in the Py A enhancer domain (site 1), and the other two sites are located in the Py B enhancer domain (sites 2 and 3; Fig. 1A). EF-1A site 1 overlaps and shares nucleotides with the previously described PEA-3 A site (15). As these proteins share binding motifs and functional properties, it is possible that EF-1A and PEA-3 are human and murine equivalents of the same activity. This binding motif is also shared by the products of the *ets* proto-oncogene family, which has been shown to bind to the PEA-3 site in the Py A enhancer (32). EF-1A binds to Py site 1 in the absence of other EF-1A sites, while EF-1A binding to sites 2 and 3 depends on cooperative interaction with the adjacent site (similar to the situation in the Ad5 E1A enhancer [2]). The independent binding of EF-1A to site 1 does not reflect an interaction with PEA-1, PEA-2, or sequences immediately upstream of the EF-1A/PEA-3 site. Our studies showed that the binding sites for PEA-1, PEA-2, and EF-1A/PEA-3 all are required for full enhancer function of the Py enhancer region. A single copy of EF-1A site 1 could enhance Py replication in a transient replication assay, although at very reduced levels compared with enhancer fragments which also contain the PEA-1 and PEA-2 sites.

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FIG. 1. EF-1A binding sites in the Py and Ad5 enhancer regions. (A) Schematic representation of the central part of the Py enhancer region and the Py enhancer fragments used. The numbers below the line represent the nucleotide numbers according to reference 29. The A enhancer domain (nt 5021 to 5130) and the B enhancer domain (nt 5131 to 5265) are separated by the *Pvu*II site at nt 5130. The arrows represent the three binding sites for EF-1A, two of which overlap PEA-3 binding sites (15). The binding sites for PEA-1 (23), PEA-2 (23), EF-C (19), and PEB-1 (21, 22) are represented by boxes and ovals. The enhancer fragments used in the competition experiments are Py 1 (nt 5091 to 5128), Py 2 (nt 5129 to 5159), Py 3 (nt 5153 to 5265), Py 1+2 (nt 5091 to 5159), Py 2+3 (nt 5129 to 5265), and Py 1+2+3 (nt 5109 to 5265) and are shown as lines below the schematic representation. (B) Schematic representation of nt 189 to 270 in the Ad5 enhancer region and EF-1A sites A and B (2). The fragment Ad5 E1A wt (nt 189 to 270) comprises both sites, and the fragment Ad5 E1A RH (nt 189 to 195 and 219 to 270) contains an internal deletion mutation (—), deleting EF-1A site A but not site B (2).

MATERIALS AND METHODS

Cultured cells and nuclear extracts. For the replication assay, murine MOP-8 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, as described by Muller et al. (18). The cells were transfected with 1 μ g of DNA per 10^6 cells by the DEAE-dextran method (18). Cell lysates were harvested 24 h after transfection by the Hirt procedure (10), and low-molecular-weight DNA was digested with the restriction endonucleases *Eco*RI and *Dpn*I. The products were separated on a 1% agarose gel, transferred onto a nitrocellulose membrane, and visualized by hybridization to a 32 P-labeled probe, generated by randomly primed translation of linearized pPyNEO plasmid (14). The hybridized bands were subsequently detected by autoradiography. The replication assay was repeated six times.

Nuclear extracts were prepared by the method of Dignam et al. (6). Suspension cultures of human HeLa cells and murine MOP-8 cells were grown to a density of 4×10^5 to 5×10^5 cells per ml in suspension-modified minimum essential medium containing 7% calf serum. The cells were harvested by centrifugation, washed, and lysed in a hypotonic buffer by Dounce homogenization. All buffers used in the extraction contained 2 μ g of aprotinin per ml, 2 μ g of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride. Isolated nuclei were resuspended in an extraction buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 420 mM NaCl, 20% glycerol, 1.5 mM MgCl_2 , 0.2 mM EDTA, and 1 mM dithiothreitol and gently stirred on ice for 30 min. The nuclei were removed by centrifugation,

and the supernatant was dialyzed overnight against two changes of a dialysis buffer (DB) containing 20 mM HEPES (pH 7.5), 100 mM KCl, 20% glycerol, 5 mM MgCl_2 , 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The dialysate was clarified by centrifugation at $25,000 \times g$ for 20 min, and the supernatant was designated the crude nuclear extract. The crude extract (10 mg/ml) was fractionated on a DEAE-cellulose column with a bed volume of 1 ml/10 mg of loaded protein, equilibrated in DB. Following the loading, the column was washed with three bed volumes of DB, and the EF-1A-containing fraction was eluted with DB containing 300 mM KCl. This fraction was designated HeLa DE 0.3 and was enriched fivefold for EF-1A activity.

Plasmids, oligonucleotides, probes, and competitor fragments. The Ad5 E1A wild-type (wt) enhancer fragment, the deletion RH fragment (E1A RH) (Fig. 1B), and the fragment Ad5 238 (nt 195 to 238) were all residing in the polylinker region of the pUC9 plasmid (2). All the Py enhancer fragments originated from the wt Py A2 enhancer fragment (nt 5021 to 5262). The deletion mutants were generated by standard procedures. All the Py fragments were inserted in a vector, pPyNEO, which contains the Py origin of replication and early promoter fused to the *neo* gene in a pUC9 vector background. The enhancer fragments were inserted immediately adjacent to the Py replication origin at nt 5262, in their natural orientation. The fragments were excised from the vector for competition experiments either by digestion with *Eco*RI and *Hind*III or by using the restriction enzymes that

FIG. 2. Py enhancer fragments compete for the binding of EF-1A. The Py enhancer fragment Py 1+2 (Fig. 1) was ^{32}P labeled and used as a probe in binding reactions with the HeLa DE 0.3 fraction. The reaction mixtures contained the probe, single-stranded calf thymus DNA as a nonspecific competitor, and increasing concentrations of unlabeled competitor fragments. The competitors were added in different molar ratios (from 10- to 1,000-fold molar excess relative to the probe) as indicated above the lane. The bound complexes were resolved by electrophoresis on a 4% native acrylamide gel and were detected by autoradiography. The major specific band is marked by a bracket ([); two faster-migrating complexes were also specifically blocked. The bottom band is unbound probe fragment. The reaction mixture marked 0 did not contain any specific competitor DNA, and the pUC lane was insensitive to competition with a pUC9 DNA fragment. The competitor fragments were Ad5 E1A wt, Ad5 E1A RH, Py 1, Py 2, Py 3, Py 1+2, Py 2+3, and Py 1+2+3 (Fig. 1 and text).

cut in the Py enhancer region (*Pvu*II at nt 5130 and *Mse*I at nt 5133).

The sequence of the PyA oligonucleotide is: 5'-AATTCA AGCAGGAAGTGACTAACTGACCGCAG-3'; 5'-AGCTTC TGCGGTCAGTTAGTCACTTCCTGCTT-3'. The oligonucleotide PyA M1 has two point mutations in the PEA-1/AP-1 site (5'...ACTAAC...-3' to 5'...ACGCACT...-3') and has the sequence 5'-AATTCAAGCAGGAAGTGACGCACTGACCGCAG-3'; 5'-AGCTTCTGCGGTCAGTGCGTCACTTCCTGCTT-3'. The oligonucleotide PyA M2 has two point mutations in the PEA-2 site (5'-GACCGCA...-3' to 5'-GAGCACA...-3'), and has the sequence 5'-AATTCAAGCAGGA AGTGACTAACTGAGCACAG-3'; 5'-AGCTTCTGTGCTC AGTTAGTCACTTCCTGCTT-3'. The oligonucleotide PyA M1/2 contains both the point mutations of PyA M1 and PyA M2. The oligonucleotide PyA M3 has three point mutations in the EF-1A/PEA-3 site (5'...CAGGAAG...-3' to 5'...CACCTAG...-3'), and has the sequence 5'-AATTCAAGCAGCT AGTGACTAACTGACCGCAG-3'; 5'-AGCTTCTGCGGTC AGTTAGTCACTAGGTGCTT-3'. DNA fragments were ^{32}P labeled with Klenow DNA polymerase to incorporate [α - ^{32}P] dATP and [α - ^{32}P] dCTP. This procedure yielded probes with a specific activity ranging from 10,000 to 12,500 cpm/fmol.

Competitor fragments were quantitated by ^3H labeling with Klenow DNA polymerase and [α - ^3H]dATP. Accurate quantities were then calculated by using trichloroacetic acid precipitation to determine the specific activities, which usually ranged from 60 to 70 cpm/fmol. The probe and competitor fragments were isolated from polyacrylamide gels by electroelution and ethanol precipitated. Oligonucleotide competitors were used in their crude form and were quantitated spectrophotometrically.

In vitro DNA-protein binding assay and DEPC interference assay. Electrophoretic mobility shift assays were performed in a total volume of 15 μl as described by Bruder and Hearing (2), with some modifications. A total of 1.5 to 5 μg

of HeLa DE 0.3 was preincubated with 1 μg of single-stranded calf thymus DNA in a solution containing 25 mM HEPES (pH 7.5), 40 mM KCl, 1.5 mM MgCl_2 , 2% glycerol, 10 μM EDTA, 10 μM EGTA, and 300 μM dithiothreitol for 10 min at room temperature. A total of 5,000 cpm (0.4 to 0.5 fmol) of ^{32}P -labeled probe and competitor DNA was added simultaneously, and the mixture was incubated for 20 min at room temperature. The complexes were resolved electrophoretically at 10 V/cm on a 4% 30:1 (acrylamide:bisacrylamide) polyacrylamide gel in 0.5 $\times$ TBE (25 mM Tris [pH 8.3], 25 mM boric acid, 0.5 mM EDTA) at 4°C. The gel was subsequently dried, and the bands were detected by autoradiography. Diethyl pyrocarbonate (DEPC) interference assays were performed as described by Sturm et al. (27).

RESULTS

Binding of EF-1A to the Py enhancer region. There are three EF-1A binding site homologies in the Py enhancer region. To map the binding of EF-1A to the Py enhancer region, we prepared six DNA competitors which contained the individual EF-1A sites and combinations of these sites (Fig. 1A). The enhancer fragment Py 1+2 (nt 5091 to 5159), which contains EF-1A sites 1 and 2, was radiolabeled and used as a probe in a mobility shift assay. The binding of DEAE-cellulose-fractionated HeLa cell nuclear extract (HeLa DE 0.3) to the Py 1+2 probe yielded three specific bands which were sensitive to competition by the homologous fragment (Fig. 2, lanes 0 and Py 1+2); this binding pattern is very similar to that described for the binding of EF-1A to the Ad5 E1A enhancer region (2). The upper DNA-protein complex (bracket in Fig. 2) correlates with the occupation of both EF-1A binding sites by bound protein (see below), and it is the formation of this complex which correlates with Ad5 E1A enhancer activity (2, 2a). Two E1A

enhancer fragments, E1A wt, containing Ad5 EF-1A sites A and B, and E1A RH, containing site B alone (Fig. 1B), both competed for the binding of EF-1A to the Py enhancer region. E1A wt competed fivefold better than E1A RH, reflecting the cooperative nature of EF-1A binding to these sites (2). In contrast, the Py enhancer fragments Py 1, comprising EF-1A site 1, and Py 1+2, comprising EF-1A sites 1 and 2, competed equally well, suggesting that EF-1A binding to site 1 was independent of cooperation with EF-1A site 2. A fragment which contained Py EF-1A sites 1, 2, and 3 was reduced for binding compared with Py site 1. We do not understand this result, but it may reflect the interaction of other abundant or high-affinity nuclear proteins with the intact Py enhancer region which may interfere with EF-1A binding. The fragment Py 2, containing only EF-1A site 2, was a poor competitor (reduced 10-fold) compared with Py 1 and Py 1+2. The fragment Py 3, comprising site 3 alone, was comparable to Py 2 in competition for EF-1A binding, while the fragment Py 2+3, comprising sites 2 and 3, showed an improved competition (increased fivefold) compared with the individual Py 2 and Py 3 sites. This suggested that EF-1A binding to sites 2 and 3 is relatively weak and depends on cooperation with another EF-1A site, as for the Ad5 E1A enhancer (2). The binding of EF-1A to site 1 is independent of cooperation with other EF-1A sites.

To define contacting nucleotides for EF-1A on the Py enhancer, we performed DEPC binding interference assays. Both strands of the Py 1+2 enhancer fragment were ^{32}P labeled and partially modified with DEPC on adenine (A) and guanine (G) residues. The modified probes were incubated with the HeLa DE 0.3 fraction, and the DNA-protein complexes were resolved by electrophoresis. The DNA from the bound and unbound bands was eluted, subjected to piperidine cleavage at the modified nucleotides, and resolved on sequencing gels. The resulting autoradiograms are shown in Fig. 3, and the results are summarized in Fig. 4A. DEPC carboxylation of A and G residues at positions 5108 to 5113, 5142, and 5144 on the upper strand and at positions 5114 and 5144 to 5149 on the lower strand interfered with EF-1A binding (Fig. 4A). The eight contacting nucleotides in Py site 1 are all identical to corresponding nucleotides in EF-1A site A in the Ad5 E1A enhancer (Fig. 4B) (2). Likewise, six of the eight contacts in Py site 2 are in the same position as the corresponding contacts in the EF-1A site B in the Ad5 E1A enhancer (Fig. 4B).

EF-1A binding to site 1 does not depend on upstream sequences or proteins binding to the PEA-1 or PEA-2 site. The independent binding of EF-1A to Py site 1 may reflect a relatively strong interaction with a single site or the interaction of EF-1A with another protein that binds to an adjacent site. To test these possibilities, we constructed two left-end deletion mutations, L1 and L2, in the region immediately upstream of EF-1A site 1 (Fig. 4A). Mutant L1 carries a deletion of upstream sequences ending at nt 5109 (retained), leaving EF-1A site 1 intact but for the first A contact. Mutant L2 carries a deletion of upstream sequences ending at nt 5114 (retained), deleting all but one of the contacting nucleotides in EF-1A site 1. The fragments L1-*Pvu*II (nt 5109 to 5130) and L2-*Pvu*II (nt 5114 to 5130) were used as competitors for EF-1A binding against the Py 1+2 probe in a mobility shift assay (Fig. 5A). The L1-*Pvu*II fragment displayed strong competition, comparable to that of Py 1 relative to the E1A wt competitor fragment (Fig. 2), whereas the L2-*Pvu*II fragment displayed no competition. Therefore, upstream sequences played no important role in the binding of EF-1A to Py site 1.

FIG. 3. DEPC interference analysis of EF-1A binding sites in the Py enhancer region. The fragment Py 1+2 (Fig. 1) was ^{32}P labeled on the lower (A) and upper (B) strands and partially modified on A and G residues by DEPC. The modified probes were used in EF-1A binding reactions and electrophoresed on a 4% acrylamide gel. The DNA from the complexed and uncomplexed bands was eluted, subjected to piperidine cleavage, and separated on 12 and 16% denaturing acrylamide gels. The U (unbound) lanes show the fragments arising from the uncomplexed bands; the B (bound) lanes show those arising from the complexed bands. Contacting nucleotides can be seen as present in the U lane but absent from the B lane and have been marked with dots (●). The AG and CT lanes contain the A+G and C+T specific Maxam-Gilbert reaction products and the D lane in panel B contains the piperidine cleavage products of the modified upper-strand probe.

To investigate the possibility that the independent binding of EF-1A to Py site 1 could reflect an interaction with the neighboring PEA-1 and PEA-2 sites, we generated a series of mutant oligonucleotides corresponding to nt 5103 to 5130 in the Py enhancer (Fig. 4C). The PyA wt oligonucleotide encompasses the EF-1A, PEA-1, and PEA-2 sites. PyA M1 has point mutations in the PEA-1 site, and PyA M2 has point mutations in the PEA-2 site (as described by Piette and

FIG. 4. EF-1A binding sites. (A) Nucleotide sequence of the Py enhancer from nt 5100 to 5153 showing DEPC interference contact sites and the two left-end deletions, L1 and L2. EF-1A contact sites determined by DEPC interference are shown with closed triangles (▲). The left-end deletion L1 includes nt 5108, and L2 includes nt 5113. The two competitor fragments L1-PvuII (nt 5108 to 5130) and L2-PvuII (nt 5113 to 5130) have the PvuII site as the downstream border. (B) Comparison among the EF-1A binding site consensus sequence (2), Ad5 EF-1A sites A and B (2), Py EF-1A sites 1 and 2, and the proposed site 3. (C) Sequence of the oligonucleotides PyA wt, PyA M1, PyA M2, and PyA M3 corresponding to nt 5103 to 5130 in the Py enhancer. The oligonucleotides contain point mutations (indicated with boldface letters in shaded boxes) in the binding sites of PEA-1 (PyA M1), PEA-2 (PyA M2), and PEA-3/EF-1A (PyA M3). The oligonucleotide PyA M1/2 has both the PEA-1 and PEA-2 sites mutated, while the PyA wt oligonucleotide has all three sites intact.

Yaniv [23]). PyA M1/2 has point mutations in both the PEA-1 and PEA-2 sites, and PyA M3 has point mutations in EF-1A site 1 (as described in reference 2). These oligonucleotides were used to compete against the Py 1+2 probe in a mobility shift assay (Fig. 5B). The competitors PyA M1/2 (Fig. 5B) and PyA M1 and PyA M2 (data not shown) all displayed a competition level identical to that of the competitor PyA wt, indicating that the binding of EF-1A to site 1 is independent of the binding of the factors PEA-1 and PEA-2 to neighboring sites. In contrast, the competitor PyA M3, which has three point mutations in the EF-1A site, did not display any competition for EF-1A binding.

Murine EF-1A-like activity binds to EF-1A binding site 1 and is important and sufficient for Py replication. To test the role of the Py EF-1A binding sites in enhancer region activity, we analyzed a series of different Py enhancer region fragments for their ability to enhance Py DNA replication in a transient assay. The fragments were inserted into a plasmid that contains the Py origin of replication (pPyNEO). The plasmid constructs were transfected into MOP-8 cells, which express the Py large T antigen (18), and replication was measured by Southern hybridization analysis (Fig. 6). The results of the transient replication assay showed that while

several elements of the Py enhancer are important for Py DNA replication, none are essential. A fragment spanning nt 5104 to 5177 (plasmid 8/1, lane 3) enhanced replication to nearly the level of the entire Py enhancer region (nt 5021 to 5262, plasmid wt Py enhancer, lane 2). The deletion of EF-1A site 1 in the plasmid 8/1 background reduced replication efficiency three- to fivefold (plasmid L2/1, lane 5), and the additional deletion of the PEA-1 site nearly abolished enhancer activity (plasmid 4/1, lane 6). In accordance with previous studies (30), we found that deletion of the EF-C binding site reduced replication considerably (plasmid 8/G8, lane 7, 10-fold reduction). The deletion of EF-1A site 2 in an EF-C mutant background did not reduce replication further (plasmid 8/pvu, lane 8), and in fact, replication was slightly enhanced, which may reflect a spacing phenomenon. Thus, when EF-1A site 1 is intact, EF-1A site 2 is not required.

We analyzed the individual contributions of the EF-1A, PEA-1, and PEA-2 sites in the context of the A enhancer element (nt 5104 to 5130). Each of these binding sites was important for optimal enhancer activity of the A element (Fig. 6, lanes 9 to 16). Individual point mutations in each of these binding sites reduced replication efficiency significantly (plasmids PyA M3, M1, and M2, Fig. 6, lanes 13 to

FIG. 5. Independent binding of EF-1A to Py site 1. (A) Fragments Ad5 E1A wt, Py L1-*Pvu*II, and Py L2-*Pvu*II (Fig. 4A) were used as competitors for EF-1A binding in a mobility shift assay. A ³²P-labeled Py 1+2 probe (Fig. 1A) was used in reaction mixtures containing HeLa DE 0.3 nuclear extract, single-stranded calf thymus DNA, and increasing concentrations of the competitor fragments (10- to 250-fold molar excess) as indicated above the lanes. The major specific band is marked by a bracket (I). The 0 lane shows a reaction mixture without specific competitor DNA, and the 1AX lane shows a reaction mixture containing a 1,250-fold molar excess of a multimerized Ad5 EF-1A site A oligonucleotide 32-mer containing the same point mutations as the PyA M3 oligonucleotide (2). (B) Oligonucleotides PyA wt, PyA M1/2, and PyA M3 (Fig. 4C) were used as competitors for EF-1A binding in a mobility shift assay as described for panel A.

15). A DNA fragment that carried an intact EF-1A site 1 but mutated PEA-1 and PEA-2 sites (PyA M1/2, lane 16) displayed weak, but detectable, enhancer activity.

We analyzed MOP-8 cell nuclear extracts for binding activities to the EF-1A sites. The binding of MOP-8 cell nuclear extract to the Py 1+2 probe resulted in two major specific bands in a mobility shift assay (Fig. 7) which correlated with the upper two species observed with a HeLa cell extract (Fig. 2). These bands were specifically blocked by the PyA wt but not by the mutated PyA M3 oligonucleotide (Fig. 4C), showing that murine MOP-8 cells contain an activity which binds to EF-1A sites that is similar to the human HeLa cell form.

DISCUSSION

We described the binding of nuclear protein EF-1A (2) to three sites in the Py enhancer region. EF-1A binding site 1 (nt 5108 to 5114) resides in the Py A enhancer domain, and EF-1A site 2 (nt 5142 to 5150) and site 3 (likely at nt 5201 to 5209, discussed below) reside in the Py B enhancer domain (Fig. 1). We previously reported that EF-1A bound in a cooperative fashion to adjacent sites in the Ad5 E1A enhancer region (2). A similar observation was made with respect to Py EF-1A sites 2 and 3, where the binding to an individual site was weak, while EF-1A bound fivefold better to a DNA fragment which contained both binding sites (Fig. 2, Py 2 and Py 3 versus Py 2+3). In contrast, EF-1A bound independently to Py site 1 in the absence of adjacent EF-1A binding sites (Fig. 2, Py 1 versus Py 1+2 and Py 1+2+3). Mutational analysis of the sequences surrounding EF-1A site 1 revealed that neither deletion of sequences upstream of that site nor point mutations in the two downstream binding sites for PEA-1 and PEA-2 reduced the binding of EF-1A to

site 1 (Fig. 5). The reason for the independent binding of EF-1A to Py site 1 remains unclear since both Py sites 1 and 2 are structurally very similar to the A and B sites in the Ad5 E1A enhancer where cooperative binding was observed. The lack of cooperative interactions between EF-1A bound at Py sites 1 and 2 may reflect an unfavorable alignment of these sites with respect to each other. This, however, was not found to be the case for EF-1A binding to the Ad5 enhancer region, where the precise spacing and orientation of individual EF-1A sites did not appear to be critical (2). EF-1A appeared to bind to Py site 1 with intrinsically higher affinity than most other single binding sites that we tested.

The mutation of EF-1A site 1 (the PEA-3 A site) led to a marked reduction in the enhancement of replication in all the constructs tested (Fig. 6). While EF-1A binding to site 1 as measured with human extracts is independent of the neighboring PEA-1 and PEA-2 sites, these three sites cooperate to enhance Py replication in murine cells. Murine PEA-1 and human AP-1 are likely related activities, and the Py PEA-1 site is functional in both murine and human cells (11, 20, 23, 33, 34, 36). In contrast, PEA-2 activity has been identified in murine cells but not in human cells (36). Our replication data are in agreement with the recent reports which demonstrated that the PEA-1, PEA-2, and PEA-3 sites are all important for the enhancement of Py transcription and DNA replication (3, 15, 17, 25, 30, 31, 36). Martin et al. (15) previously proposed that PEA-1 and PEA-2 cooperate for binding. Our results support this conclusion since plasmid mutants having either or both of the PEA-1 and PEA-2 sites mutated replicated equally poorly (Fig. 6). Deletion of EF-1A site 2 did not lead to a reduction in replication, and a construct containing the PEA-2, EF-1A site 2, and EF-C sites displayed a very low level of replication, suggesting that EF-1A site 2 makes a

FIG. 6. Elements involved in enhancement of Py DNA replication. A schematic representation of Py enhancer fragments and oligonucleotides that were inserted into the pPyNEO plasmid replication vector is shown on the right. The plasmid constructs were transfected into MOP-8 cells, cell lysates were harvested by the Hirt procedure (10), and recovered plasmid DNA was digested with *EcoRI* and *DpnI*. The products were separated on a 1% agarose gel, transferred to a nitrocellulose membrane, and visualized by hybridization to a ³²P-labeled pPyNEO probe and subsequent autoradiography. The resulting autoradiogram is shown on the left, and the replicated band is indicated with a bracket (I). Lane 1 shows the replication of an enhancerless plasmid, and lane 2 shows the replication of a plasmid containing the entire Py enhancer region (nt 5021 to 5265). Lanes 3 to 16 show the replication of plasmids containing the enhancer region fragments and mutants described in the text. The endpoints of each enhancer region fragment are indicated in parentheses.

very small contribution to enhancer activity in these assays (Fig. 6). EF-1A site 3 very likely corresponds to the PEA-3 B site (nt 5203 to 5208) discussed by Martin et al. (15). This site shares a 7 of 8 nt homology with the EF-1A consensus sequence (Fig. 4B) with a single C-to-T transition. EF-1A site 3 does not seem to play a major role in the tested system either, since deletion of the downstream half of the enhancer region resulted in only a minor decrease in replication. Our results showed that the interaction between the proteins binding to the A enhancer region (PEA-1, PEA-2, and EF-1A/PEA-3) and EF-C in the B enhancer region are important for attaining a wt level of replication in MOP-8 cells.

The independent binding of EF-1A to Py site 1 correlates with the importance of this site for enhancer function. In contrast, EF-1A sites 2 and 3 bound EF-1A more weakly, and these sites did not play an important role in enhancer activity in the replication assays. The functional distinction of EF-1A binding sites is analogous to the situation with the Ad5 E1A enhancer region (2). EF-1A was found to bind cooperatively to adjacent A and B sites in the E1A enhancer region. EF-1A also was found to bind cooperatively to dimerized A or B sites. While EF-1A was found to bind both A and B sites at similar levels, only the A sites, but not the B sites, augment the transcription of a heterologous, linked gene (2, 2a). Thus, it seems that both the Ad5 E1A and Py enhancers have two types of EF-1A binding sites. In the Ad5 system, EF-1A site B seems to serve as an activator indi-

rectly by supporting the binding of EF-1A to the Ad5 A site. Py sites 2 and 3, however, do not appear to play an auxiliary role for the binding of EF-1A to the activating site 1, so the function of these sites is less clear. The functional redundancy of enhancer elements within the intact Py enhancer region has been well documented (16, 18, 25, 30), and this may reflect the evolution of the enhancer region to utilize a subset of different enhancer binding proteins that are available in different cell types. For example, EF-1A bound at Py sites 2 and 3 may functionally interact in vivo with other enhancer binding proteins that were not detected in our studies due to the nature of the assay (enhancement of DNA replication) or the cell line used in the analysis (MOP-8 cells). The Py enhancer functions in a variety of cells and tissues (3, 5, 9, 25, 28, 31), and EF-1A sites 2 and 3 may be important in the context of different cell types. Alternatively, clustered binding sites for a particular binding protein may serve as a sink to initially engage the factor with a region of DNA followed by translocation of the protein to a particular target site. Such a mechanism has been proposed for the function of the repeated spacer and gene promoters within the *Xenopus* rRNA transcription unit (24).

In unpublished experiments, we have identified two different HeLa cell nuclear proteins that bind to monomeric and dimeric EF-1A binding site oligonucleotide probes. The EF-1A activity described in this report corresponds to a protein with an approximate molecular size between 46 and 50 kDa. A second activity of 56 to 60 kDa binds to the same

FIG. 7. EF-1A-like binding activity is evident in MOP-8 cell extracts. Murine MOP-8 cell nuclear extract was incubated with 32 P-labeled Py 1+2 probe (Fig. 1), single-stranded calf thymus DNA as a nonspecific competitor, and oligonucleotide competitors (Fig. 4B). The 0 lane shows a binding reaction mixture without specific competitor DNA. The PyA M3 lane shows a reaction which contained a 1,250-fold molar excess of the oligonucleotide competitor PyA M3, containing a mutated EF-1A site (Fig. 4C). The PyA wt oligonucleotide competitor was added in increasing amounts (50- to 1,250-fold molar excess) as indicated above the lanes.

site and gives rise to faster-migrating species in the mobility shift assay. Preliminary observations correlate EF-1A (46 to 50 kDa), but not the second activity (56 to 60 kDa), with the ability to activate transcription in vivo (1a). It is presently unclear what relationship these two proteins may have with the murine activity PEA-3. Gutman and Wasylyk (7) recently demonstrated that the size of PEA-3 is approximately 65 kDa, and that this protein also binds to sequences homologous to the Py PEA-3 A binding site in the serum-responsive element of human collagenase and *c-fos* promoters. PEA-3 is likely related to members of the *ets* oncogene family. Wasylyk et al. (32) recently demonstrated that p68^{c-ets-1}, p54^{c-ets-1}, and p58-64^{c-ets-2} bind to the PEA-3 A site in the Py enhancer and are capable of activating transcription of a gene linked to the Py A element in LMTK⁻ fibroblasts. Last, Watabane et al. (35) have described a transcription factor, E4TF1, that binds to a previously defined EF-1A site (2) in the Ad5 E4 promoter. E4TF1 consists of two subunits. A 60-kDa HeLa cell protein binds to the specific DNA sequence on its own but does not stimulate transcription, and a 53-kDa protein, which does not bind DNA on its own, associates with the 60-kDa protein and confers transcriptional activation. It seems unlikely that E4TF1 and EF-1A are identical based on the difference in size and DNA binding properties.

EF-1A binding activity has been detected in a series of different human cell lines—HeLa (epithelial), 293 (fibroblast), HepG2 (liver), Namalwa (B lymphocyte)—and in different species (human, mouse, rat) and it therefore appears to be a ubiquitous activity. Current data (2, 13, 15, 32, 33, 35) suggest that a family of related proteins with the same binding specificity bind to the E1A enhancer core motif and

related sequences. Some members (e.g., EF-1A) seem to display constitutive enhancer activity, while the activity of other members (e.g., PEA-3) may be inducible by activating agents that appear to converge on the protein kinase C pathway.

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REFERENCES

1. Banerji, J., S. Rusconi, and W. Schaffner. 1981. Expression of a β -globin gene is enhanced by remote SV40 sequences. *Cell* 27:299–308.
- 1a. Bolwig, G., and P. Hearing. Unpublished data.
2. Bruder, J. T., and P. Hearing. 1989. Nuclear factor EF-1A binds to the adenovirus E1A core enhancer element and to other transcriptional control regions. *Mol. Cell. Biol.* 9:5143–5153.
- 2a. Bruder, J. T., and P. Hearing. Unpublished data.
3. Campbell, B. A., and L. P. Villareal. 1988. Functional analysis of the individual enhancer core sequences of polyomavirus: cell-specific uncoupling of DNA replication from transcription. *Mol. Cell. Biol.* 8:1993–2004.
4. de Villiers, J., and W. Schaffner. 1981. A small segment of polyoma virus DNA enhances the expression of a cloned β -globin gene over a distance of 1400 base pairs. *Nucleic Acids Res.* 9:6251–6264.
5. de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen. 1984. Polyomavirus DNA replication requires an enhancer. *Nature (London)* 312:242–246.
6. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475–1489.
7. Gutman, A., and B. Wasylyk. 1990. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA-3 and AP-1 binding sites. *EMBO J.* 9:2241–2246.
8. Hearing, P., and T. Shenk. 1986. The adenovirus type 5 E1A enhancer contains two functionally distinct domains: one is specific for E1A and the other modulates all early units in *cis*. *Cell* 45:229–236.
9. Herbolme, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* 39:653–662.
10. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365–369.
11. Imbler, J. L., C. Schatz, C. Wasylyk, B. Chatton, and B. Wasylyk. 1988. A Harvey-ras responsive transcription element is also responsive to a tumour-promoter and to serum. *Nature (London)* 332:275–278.
12. Kamachi, Y., E. Ogawa, M. Asano, S. Ishida, Y. Murakami, M. Satake, Y. Ito, and K. Shigesada. 1990. Purification of a mouse nuclear factor that binds to both the A and B cores of the polyomavirus enhancer. *J. Virol.* 64:4808–4819.
13. Karim, F. D., L. D. Urness, C. S. Thummel, M. J. Klemsz, S. R. McKercher, A. Celada, C. Van Beveren, R. A. Maki, C. V. Gunther, J. A. Nye, and B. J. Graves. 1990. The ETS-domain: a new motif that recognizes a purine-rich core DNA sequence. *Genes Dev.* 4:1451–1453.
14. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. Martin, M. E., J. Piette, M. Yaniv, W.-J. Tang, and W. R. Folk. 1988. Activation of the polyomavirus enhancer by a murine activator protein 1 (AP-1) homolog and two contiguous proteins. *Proc. Natl. Acad. Sci. USA* 85:5839–5843.
16. Mueller, C. R., W. J. Muller, and J. A. Hassel. 1988. The polyomavirus enhancer comprises multiple functional elements. *J. Virol.* 62:1667–1678.

17. Muller, W. J., D. Dufort, and J. A. Hassell. 1988. Multiple subelements within the polyoma enhancer function synergistically to activate DNA replication. *Mol. Cell. Biol.* **8**:5000–5015.
18. Muller, W. J., C. R. Mueller, A.-M. Mes, and J. A. Hassel. 1983. Polyomavirus origin for DNA replication comprises multiple genetic elements. *J. Virol.* **47**:586–599.
19. Ostapchuk, P., J. F. X. Diffley, J. T. Bruder, B. Stillman, A. J. Levine, and P. Hearing. 1986. Interaction of a nuclear factor with the polyoma virus enhancer. *Proc. Natl. Acad. Sci. USA* **83**:8550–8554.
20. Piette, J., S.-I. Hiari, and M. Yaniv. 1988. Constitutive synthesis of activator protein 1 transcription factor after viral transformation of mouse fibroblasts. *Proc. Natl. Acad. Sci. USA* **85**:3401–3405.
21. Piette, J., M.-H. Kryszke, and M. Yaniv. 1985. Specific interaction of cellular factors with the B enhancer of polyoma virus. *EMBO J.* **4**:2675–2685.
22. Piette, J., and M. Yaniv. 1986. Molecular analysis of the interaction between an enhancer binding factor and its DNA target. *Nucleic Acids Res.* **14**:9595–9611.
23. Piette, J., and M. Yaniv. 1987. Two different factors bind to the α -domain of the polyoma enhancer, one of which also interacts with the SV40 and c-fos enhancers. *EMBO J.* **6**:1331–1337.
24. Reeder, R. H., J. G. Roan, and M. Dunaway. 1983. Spacer regulation of *Xenopus* ribosomal gene transcription: competition in oocytes. *Cell* **35**:449–456.
25. Rochford, R., B. A. Campbell, and L. P. Villarreal. 1990. Genetic analysis of the enhancer requirements for polyomavirus DNA replication in mice. *J. Virol.* **64**:476–485.
26. Ruley, H. E., and M. Fried. 1983. Sequence repeats in a polyoma virus DNA region important for gene expression. *J. Virol.* **47**:233–237.
27. Sturm, R., T. Baumruker, B. R. J. Franza, and W. Herr. 1987. A 100-kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV 40 enhancer. *Genes Dev.* **1**:1147–1160.
28. Tang, W. J., S. L. Berger, S. J. Triezenberg, and W. R. Folk. 1987. Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. *Mol. Cell. Biol.* **7**:1681–1690.
29. Tyndall, C., G. Lamantia, C. M. Thacker, J. Favaloro, and R. Kamen. 1981. A region of the polyomavirus genome between the replication origin and the late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res.* **9**:6231–6249.
30. Veldman, G. M., S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. *Mol. Cell. Biol.* **5**:649–658.
31. Wasylyk, B., J. L. Imler, B. Chatton, C. Schatz, and C. Wasylyk. 1988. Negative and positive factors determine the activity of the polyoma virus enhancer α domain in undifferentiated and differentiated cell types. *Proc. Natl. Acad. Sci. USA* **85**:7952–7956.
32. Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. 1990. The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature (London)* **346**:191–193.
33. Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk. 1989. PEA-3 is a nuclear target for activation by non-nuclear oncogenes. *EMBO J.* **8**:3371–3378.
34. Wasylyk, C., J. L. Imler, and B. Wasylyk. 1988. Transforming but not immortalizing oncogenes activate the transcription factor PEA-1. *EMBO J.* **7**:2475–2483.
35. Watanabe, H., T. Wada, and H. Handa. 1990. Transcription factor E4TF-1 contains two subunits with different functions. *EMBO J.* **9**:841–847.
36. Yamaguchi, Y., M. Satake, and Y. Ito. 1989. Two overlapping sequence motifs within the polyomavirus enhancer are independently the targets of stimulation by both the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate and the Ha-ras oncogene. *J. Virol.* **63**:1040–1048.