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Cooperative Binding of EF-1A to the E1A Enhancer Region Mediates Synergistic Effects on E1A Transcription during Adenovirus Infection

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A cellular nuclear factor, EF-1A, binds to a sequence motif which is repeated in the adenovirus type 5 E1A transcriptional control region. Previous genetic analyses demonstrated that two of these binding sites are predominant functional elements of the E1A enhancer region in vivo. In this report, we demonstrate that the cooperative binding of EF-1A to neighboring sites in the E1A enhancer region results in a synergistic activation of E1A transcription in infected cells.

The adenovirus E1A transcriptional control region is composed of multiple regulatory elements (5, 7–11, 13, 16, 17). Genetic analyses have revealed that a predominant functional enhancer element, element I, is active in viral infections and is repeated at positions −200 and −300 relative to the E1A cap site (Fig. 1, sites 1 and 3) (7, 10). A cellular nuclear factor, EF-1A, binds to both copies of element I and to three degenerate sites (Fig. 1, sites 2, 4, and 5) in the E1A 5′-flanking region (3). EF-1A binds cooperatively to sites 2 and 3 in the E1A enhancer region and to sites 4 and 5 upstream of the enhancer region (3). In this report, we demonstrate that EF-1A bound to site 2 can cooperate with EF-1A bound to site 1 or 3 to activate E1A transcription in virus-infected cells. This synergism is likely mediated by cooperative binding interactions.

EF-1A binding sites function to activate E1A transcription in virus-infected cells. To study the role of individual EF-1A binding sites in the activation of E1A transcription in virus-infected cells, we analyzed a series of viral mutants which contain specific deletions in and upstream of the E1A enhancer region. Initially, we examined the E1A steady-state mRNA levels in different cell lines infected with two viral mutants carrying large deletions in the E1A 5′-flanking region, *dl*309-2 and *dl*309-8 (Fig. 1, dl 2 and dl 8, respectively). Various cell lines (HeLa, 293, HepG2, and MG63) were coinfected with the wild-type or mutant viruses and *dl*310, a phenotypically wild-type virus which carries a small in-frame deletion in the E1A 3′ exon and which was used as an internal control for viral mRNA levels and to provide functional E1A protein in *trans* (9, 12). We previously found that these mutations reduced E1A expression approximately 10-fold in single infections of HeLa cells (7) and about 5-fold in coinfections of HeLa cells with *dl*310 (data not shown). The greatest effect of these deletions was observed in MG63 cells (a human osteosarcoma cell line) (Fig. 2A). Mutant *dl*309-2, which carries a deletion of the three cap site-proximal EF-1A binding sites, produced five times less E1A mRNA than did the wild-type virus. *dl*309-8 carries a deletion spanning all five EF-1A binding sites and produced about 20-fold less E1A mRNA than did the wild-type virus.

To more specifically define the sequences that are required for maximal E1A transcription in MG63 cells and to assess the role of EF-1A in E1A transcription in infected cells, we analyzed viruses with small internal deletions which remove one or more of the EF-1A binding sites in the E1A enhancer region (Fig. 1 and 2B). Deletion of cap site-proximal EF-1A binding site 1 (*dl*309-87) resulted in a twofold reduction in E1A mRNA levels. Deletion of EF-1A binding site 3 (*dl*309-13) resulted in a fivefold reduction in E1A mRNA levels. Viruses carrying deletions impinging on EF-1A binding site 2 (*dl*309-17 and *dl*309-128) produced 10 to 20 times less E1A mRNA than did the wild-type virus. These single EF-1A binding site mutations revealed an apparent hierarchy of EF-1A binding sites. EF-1A binding site 2 exerted the most dramatic effect on E1A mRNA levels, binding site 3 exerted an intermediate effect, and binding site 1 was only marginally required for activity.

To further investigate the apparent inequivalence of the three EF-1A binding sites in the E1A enhancer region, we examined the effect of double mutations in this region. When the site 1 and site 3 mutations were coupled, the resulting virus (*dl*309-13/87) showed a significant reduction (20- to 40-fold) in E1A transcription. This result indicates that EF-1A binding sites 1 and 3 are functionally redundant. When EF-1A binding site 2 mutations were coupled to binding site 1 mutations (*dl*309-128/87 and *dl*309-17/87) or to binding site 3 mutations (*dl*309-10), no further decrease in E1A mRNA levels was observed relative to that with the single site 2 mutations. These results indicate that in the absence of site 2, sites 1 and 3 function poorly to activate E1A transcription, whereas in the presence of site 2, sites 1 and 3 function well. These results suggest a functional synergism between EF-1A binding sites 1 and 2 and sites 2 and 3. The fact that EF-1A binds cooperatively to sites 2 and 3 (3) indicates that this synergistic effect is mediated by cooperative binding interactions.

EF-1A binding activity is present in MG63 cell nuclear extracts. We previously described EF-1A binding activity in HeLa and 293 cell nuclear extracts (3). To correlate transcriptional activity in infected MG63 cells with EF-1A binding, we tested MG63 cell nuclear extracts for EF-1A binding

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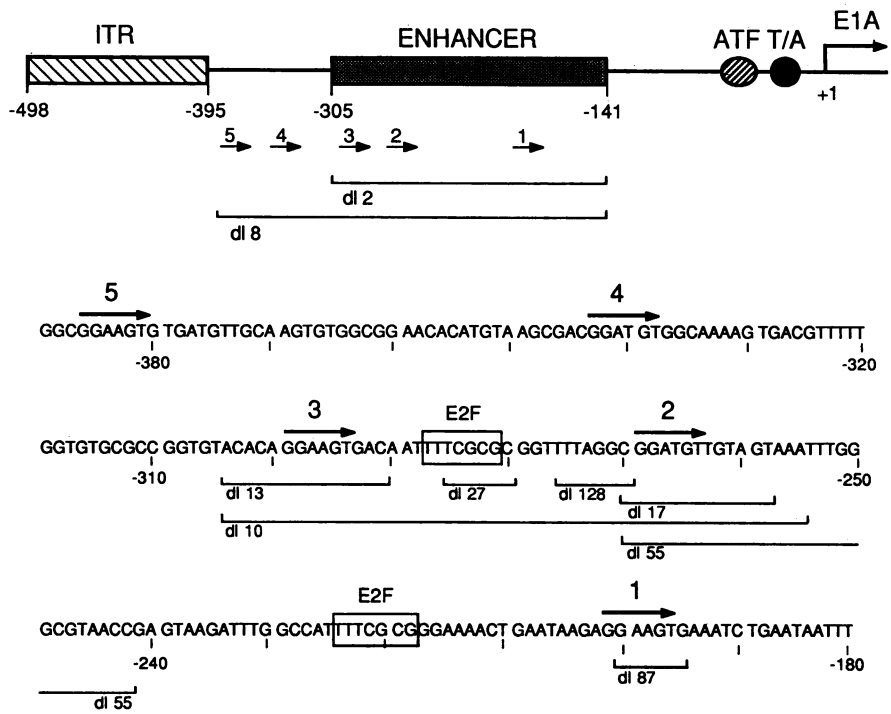

FIG. 1. Schematic view of the adenovirus E1A transcriptional control region. The top part of the figure displays a view of the left end of the adenovirus genome. The E1A transcription initiation site (+1) is located at nucleotide 499 (1). The site of TATA homology (T/A), activating transcription factor binding site (5), enhancer region (7, 10), and inverted terminal repeat (ITR) are indicated. The numbers below the line represent adenovirus nucleotides relative to the E1A initiation site at +1. The arrows represent the EF-1A binding sites (sites 1 to 5) described in the text. Deletion mutants *dl*2 and *dl*8 (7) are designated by brackets. The nucleotide sequence of the adenovirus E1A enhancer region is shown in the bottom part of the figure; nucleotide numbers below the sequence are relative to the E1A initiation site. The arrows (1 to 5) above the sequence correspond to the 5′-GGA(A/T)GT-3′ core sequence of the EF-1A binding sites (3). Two binding sites for the cellular transcription factor E2F (13) are indicated by boxes. The brackets below the sequence designate the extents of the individual deletion mutations described in the text. The name of each mutant is indicated below each bracket. The deletions were generated with D-loop mutagenesis (8), and the mutations were rebuilt into intact viruses as described previously (18).

activity by using a gel mobility shift assay (Fig. 3). The complexes observed with the MG63 cell nuclear extract and the EF-1A dimerized site 3 probe [MG63, lane (−)] were identical to the complexes detected with the partially fractionated HeLa cell extract (HeLa DE 0.3). These complexes were specifically competed for by oligomerized site 3 (A-WT) and site 2 (B-WT) but not by oligomerized site 3 containing three point mutations which disrupt EF-1A binding (A-X), as previously described for EF-1A binding with a HeLa cell extract (3). We previously demonstrated that complex 1 is formed by cooperative binding of EF-1A to adjacent binding sites (3). It is the formation of this dimeric EF-1A complex which correlates with enhancer function in vivo (see below and reference 3). The precise nature of the lower complexes in the mobility shift assay is under investigation; they appear to represent monomeric interactions of EF-1A or a related factor with the probe DNA (2). This analysis indicates that EF-1A binding activities in HeLa and MG63 cell nuclear extracts are similar, if not identical, and strengthens the suggestion that EF-1A activates E1A transcription in infected MG63 cells.

EF-1A binding to mutant enhancer region fragments. Since all three EF-1A binding sites in the E1A enhancer region are important for maximum levels of E1A transcription in infected cells but appear to foster functionally distinct interactions between EF-1A and each of these sites (Fig. 2), EF-1A binding to mutated E1A enhancer fragments which exhibited transcriptional defects in viral infections was analyzed. DNA fragments from adenovirus type 5 E1A positions −228 to −309 were used as probes in mobility shift assays with an MG63 cell nuclear extract (Fig. 4A). The major complex detected (indicated by the bracket in Fig. 4A) was specifically competed for by wild-type dimerized site 3 (panel A, WT) but not by the mutant site (A-X; data not shown). We previously demonstrated that the binding of EF-1A to sites 2 and 3 in this region was cooperative (3). The same result was found with an MG63 cell nuclear extract. Fragments with deletions which impinged on EF-1A binding site 2 (*dl*55, *dl*17, and *dl*128) all failed to form the EF-1A complex observed with the wild-type fragment. Similarly, a fragment carrying a deletion in EF-1A binding site 3 (*dl*13) failed to bind EF-1A. Conversely, a fragment carrying a 6-bp deletion between EF-1A binding sites 2 and 3 (*dl*27) bound EF-1A at a level similar to that of the wild-type fragment. Thus, in the absence of EF-1A binding site 1, fragments with deletions which impinged on binding site 2 or binding site 3 failed to exhibit cooperative EF-1A–DNA interactions, as expected. This analysis verifies the specificity of EF-1A binding to this region with the MG63 cell extract and the effect of specific deletions on EF-1A binding.

We next examined EF-1A interactions with the entire E1A enhancer region encompassing binding sites 1, 2, and 3 (Fig. 4B). Difficulty in clearly reproducing specific EF-1A binding with the large E1A enhancer region probe and the crude

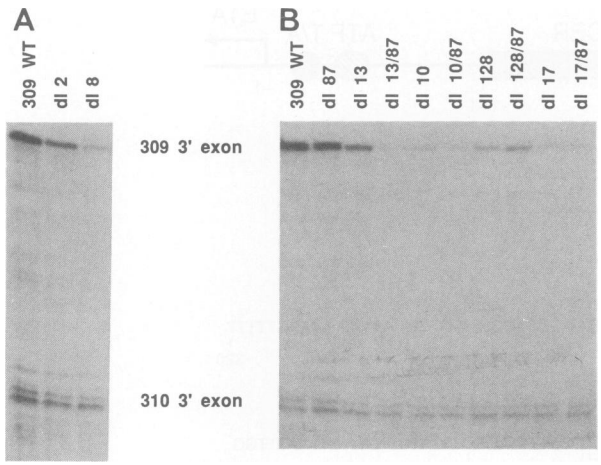

FIG. 2. RNase protection analysis of E1A mRNAs synthesized in virus-infected MG63 cells. MG63 cells were coinfected with *dl*310 and the viruses indicated above each lane at a multiplicity of 10 PFU per cell. Total-cell RNA (4) was isolated 24 h after infection, and E1A transcripts were mapped with a T7-E1A riboprobe which distinguishes the 3′ exon of E1A transcripts of *dl*310 from that of the coinfecting virus (9). The products of RNase protection were analyzed in a denaturing polyacrylamide gel. Protected bands corresponding to the *dl*309 (wild-type parent of the deletion mutants) and *dl*310 3′ exons are indicated. (A) Analysis of E1A RNAs from cells infected with *dl*309 (wild type) and deletion mutants *dl*309-2 and *dl*309-8. (B) Analysis of E1A RNAs from cells infected with viruses carrying small deletions in the EF-1A binding sites in the E1A enhancer region.

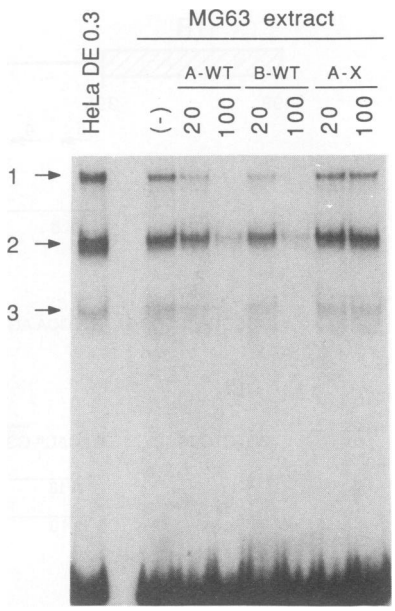

FIG. 3. Presence of EF-1A in MG63 cell nuclear extracts. A ^{32}P-labeled dimerized EF-1A binding site 3 probe was used in a gel mobility shift assay to detect EF-1A binding as previously described (3). The first lane (HeLa DE 0.3) displays the binding pattern obtained with a partially fractionated HeLa cell nuclear extract (3). The second lane [MG63 (−)] displays the binding pattern obtained with an MG63 cell nuclear extract. The adjacent lanes display the products of binding reactions done with a 20- or 100-fold molar excess of specific competitor DNAs: A-WT, multimerized EF-1A site 3; B-WT, multimerized EF-1A site 2; A-X, multimerized EF-1A site 3 containing three point mutations which disrupt EF-1A binding. The probe and competitor DNAs and the procedure for the preparation of nuclear extracts have been described previously (3). The EF-1A complex is indicated by an arrow and labeled 1; complexes 2 and 3 are discussed in the text.

MG63 cell nuclear extract prompted us to use a more purified EF-1A preparation from HeLa cells (HeLa DE 0.3). EF-1A in the HeLa DE 0.3 fraction bound to the E1A enhancer region probe (adenovirus type 5 E1A positions −144 to −309) to form the same complexes as those that were observed with the dimerized site 3 probe and HeLa and MG63 cell extracts (Fig. 3). Deletion of EF-1A binding site 1 (*dl*87) or binding site 3 (*dl*13) resulted in a two- to fivefold decrease in the formation of the EF-1A complex (complex 1), while deletion of both sites 1 and 3 (*dl*13/87) severely reduced the formation of this complex. Deletion of binding site 2 (*dl*17 and *dl*128) resulted in a more dramatic decrease in the ability of these fragments to form the EF-1A complex relative to the single site 1 or site 3 deletions. Double mutants, with deletions of binding site 1 in addition to binding site 2, showed no additional decrease in EF-1A binding over that in enhancer fragments carrying the single site 2 deletions (Fig. 4B; compare *dl*17 with *dl*17/87 and *dl*128 with *dl*128/87). As expected, deletion of binding sites 2 and 3 (*dl*10) severely reduced EF-1A binding.

The results from the binding studies displayed in Fig. 4 correlate extremely well with the transcriptional activities of the various mutants in infected cells (Fig. 2). EF-1A binds cooperatively to sites 2 and 3 (Fig. 4A and reference 2) and apparently to sites 1 and 2 (Fig. 4B), and stimulation of transcription in vivo is dependent on the integrity of these sites (Fig. 2). EF-1A binds weakly to individual binding sites, and individual sites have marginal enhancer activity. These studies indicate that binding site 2 mediates the cooperative binding and transcriptional synergy of this enhancer region. In the absence of site 2, sites 1 and 3 function poorly. This poor function may reflect a suboptimal spacing between the two remaining elements with the specific deletion mutants used in these experiments or a functional distinction between EF-1A binding sites. Current analyses are directed toward addressing these possibilities. Support for the latter possibility is based on the considerable flexibility that is tolerated between adjacent sites for the cooperative binding of EF-1A to sites 2 and 3: the deletion of 6, 7, or 13 bp between these sites or the inversion of either site with respect to the other did not significantly reduce EF-1A binding (3; data not shown). The finding that the EF-1A binding activities displayed by the various mutants reflected the abilities of these mutants to activate E1A transcription strongly suggests that the EF-1A binding motif is a major constituent of the E1A enhancer region and that EF-1A binding to the enhancer region activates E1A transcription in infected MG63 cells.

Our results show that small deletions in the E1A enhancer region had a greater effect on transcription than did larger deletions in this enhancer region (Fig. 2A and B). This apparent contradiction is likely explained by the transcriptional activity of the inverted terminal repeat (6, 14, 15) and sequences between the inverted terminal repeat and enhancer region which contain two additional EF-1A binding sites. The contribution of these elements to E1A transcription appears to be minimal in their natural context, but-

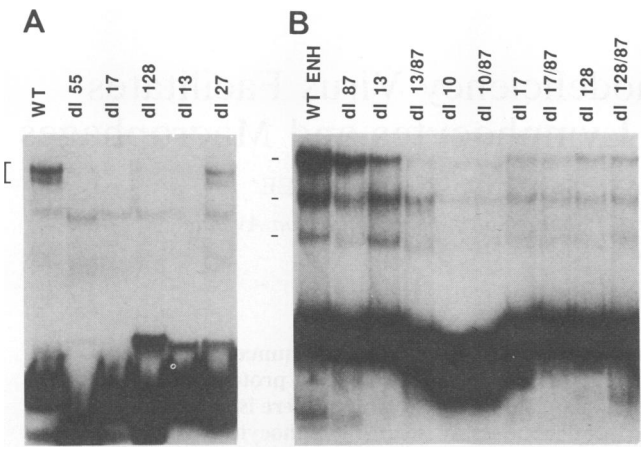

FIG. 4. EF-1A binding to mutant enhancer region fragments. Wild-type and mutant enhancer region fragments were used as probes in binding reactions with an MG63 cell nuclear extract (A) or the HeLa cell DE 0.3 fraction (B). The products of the reactions were analyzed with a mobility shift assay (3). (A) The enhancer region probes were as follows: WT, wild-type enhancer region probe from nucleotides −228 to −309; adjacent lanes, same DNA fragment but containing deletions *dl*55, *dl*17, *dl*128, *dl*13, and *dl*27 diagrammed in Fig. 1. The bracket on the left designates the EF-1A complex. The doublet appears to arise by proteolysis during extract preparation. (B) The enhancer region probes were as follows: WT ENH, wild-type enhancer region probe from nucleotides −144 to −309; adjacent lanes, same DNA fragment but containing single and double deletions *dl*87, *dl*13, *dl*13/87, *dl*10, *dl*10/87, *dl*17, *dl*17/87, *dl*128, and *dl*128/87 diagrammed in Fig. 1. Specific complexes are indicated by dashes.

transcriptional activity becomes apparent when these se quences are juxtaposed with the E1A TATA box region in the larger deletion mutants (6, 7).

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