Identification of a novel downstream binding protein implicated in late-phase-specific activation of the adenovirus major late promoter

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ABSTRACT

The adenovirus major late promoter (MLP) is induced to very high levels after the onset of the viral DNA replication. Previous studies have identified sequence elements located downstream of the MLP startsite (DE1, between +85 and +98; DE2, between +100 and +120) implicated, together with the upstream promoter element, in this late-phase-specific transcriptional activation. One protein (DEF, now renamed DEF-A), induced during the late phase of viral infection, has been identified and shown to bind to the DE1 element (Jansen-Durr et al., 1989, J. Virol. 63, 5124–5132). Here we report about a distinct late-phase-specific protein (DEF-B) and its interactions with DEF-A. DNA-binding studies reveal that DEF-B interacts with the 5' part of DE2 (DE2b), whereas DEF-A, besides its interaction with DE1, also binds to the 3' portion of DE2 (DE2a), but with a lower affinity than for DE1. Furthermore, when added together, DEF-A and DEF-B cooperatively assemble onto the DE2 element as a heteromeric complex which is substantially more stable than the complexes formed by each protein alone. Using an *in vivo* transcriptional assay of the MLP, we show that DEF-A and DEF-B both have intrinsic transactivating properties.

INTRODUCTION

DNA replication and cell proliferation are often linked to processes which involve alteration of specific transcription patterns and which may ultimately lead to cell differentiation (see 1 and 2 for reviews). Elucidation of the molecular mechanisms controlling the replication-dependent transition of gene expression may therefore help understanding the events governing the fate of eukaryotic cells during normal and pathological development. Viral gene control during the progression of DNA virus infection through the lytic cycle provides a convenient model system to study the molecular aspects of the early-to-late switch of the transcriptional program.

The major late promoter (MLP) of adenovirus types 2 and 5 is one of the viral promoters that has been most extensively studied as a model for eukaryotic transcription (see 3 and 4 for reviews). Efficient constitutive transcription from this promoter depends on a series of well defined promoter elements (5–14), among which: a properly positioned TATA box, an upstream element (UE) located between positions –67 and –49, relative to the MLP transcriptional startsite, a CAAT box between –80 and –76 which can functionally substitute for UE *in vivo*, and an initiator element spanning the transcription startsite. Biochemical studies have shown that the assembly of active initiation complexes involves, in addition to RNA polymerase, the sequential binding of the general transcription factors to the polymerase or their cognate promoter elements (see 15 and 16 for recent reviews).

Although the MLP is active during the early phase of lytic infection, its activity is strongly increased after the onset of viral DNA replication (17). Superinfection experiments have shown that viral DNA introduced into late infected cells exhibits the early gene expression pattern until it undergoes its own replication (18). It has been deduced that replication of the viral genome results in cis-acting modifications which selectively stimulate MLP transcription from the replicated template molecules.

In addition to these putative template alterations, sequence elements located downstream of the MLP startsite have been identified and shown to be essential *in vivo* for promoter activation (19–22). Indeed, DNase I protection and DMS-interference mapping experiments have delineated two main sequence elements (DE1 and DE2), located between +85 and +98 and between +100 and +120, respectively and which specifically bind proteins only present in adenovirus-infected cells, late in infection. The binding of these proteins correlates with the transcriptional activation of the MLP, as measured by cell-free transcription (23, 24) or during infection with recombinant viruses (20, 22). Footprinting experiments combined with *in vitro* transcriptional analyses of selected promoter mutations suggested that the late-phase-specific stimulation of the MLP results, at least in part, from a cooperative action of the UE and DE elements (25).

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We have previously characterized a virus-induced factor (DEF, now called DEF-A) which binds to DE1 (24). Here we identify a second DNA-binding activity (DEF-B) which specifically binds to the 5' portion of the DE2 element and triggers the binding of DEF-A to the 3' portion of DE2. The role of these proteins in the transcriptional activation from the MLP has been investigated.

MATERIAL AND METHODS

Cell extracts and partially purified fractions

HeLa cells, grown in Eagle medium supplemented with 5% calf serum, were infected with 10 PFU per cell of adenovirus type 5 (wt) or the E1a-defective derivative dl312 (dl). In some experiments DNA replication was blocked by addition of cytosine arabinoside (AraC) to the growth medium, at a final concentration of 20 μ g/ml, just after adsorption of the virus and 12 h later (23). Whole cell extracts were prepared 20 h post-infection (wt20hpi, wt20hpi+AraC or dl20hpi), exactly as described (25).

Crude extracts (wt20hpi) were fractionated by chromatography over heparin-Ultrogel (IBF), as described (26). Proteins were recovered by successively washing the column with buffers containing 0.1, 0.3 (H0.3 fraction), 0.6 (H0.6 fraction) and 1M KCl. DEF-A and DEF-B activities were eluted in fractions H0.6 and H0.3, respectively (see Results). The eluted fractions were dialyzed against a buffer (DB) containing 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 100 mM KCl and 20% glycerol, and stored at $-80^\circ C$ before use. Fractions H0.3 and H0.6 were further chromatographed over calf thymus DNA-cellulose prepared essentially as described (27), but including a 1 hr UV (312 nm) cross-linking step. The heparine fractions (15 ml) were loaded on DNA-cellulose columns (12 ml, 2 cm diameter) equilibrated in a buffer containing 10 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 100 mM KCl and 20% glycerol, washed with 30 ml of this buffer and successively eluted with 30 ml of the same buffer containing 0.2 (DC0.2) and 0.3 M KCl (DC0.3). DEF-A (after rechromatography of fraction H0.6) and DEF-B (after rechromatography of fraction H0.3) were eluted in fractions DC0.3 and DC0.2, respectively (C.T., unpublished results). The eluted fractions were dialyzed against buffer DB.

Electrophoretic band-shift assays

Gel retardation assays were performed as previously described (25), with minor modifications. About 0.5 ng (30,000 cpm) of ^{32}P -5'-end-labelled, synthetic double-stranded oligonucleotides were incubated with wt or dl extract (about 10 μ g protein) or partially purified fractions (H0.3 or H0.6), in the presence of appropriate amounts of poly (dI-dC) as non-specific competitor. After 15 min at $25^\circ C$, the complexes were separated by non-denaturing 4.5% polyacrylamide gel electrophoresis (acrylamide: bis-acrylamide, 80:1) at $4^\circ C$.

Retardation competition experiments were carried out by preincubating the extracts with the poly (dI-dC) and unlabeled competitor oligonucleotides, for 15 min at $25^\circ C$, before adding the probe and further incubation (see above). Off-rate measurements were performed by adding a 400 molar excess of unlabeled competitor probe, after the formation of the complex. Aliquots of the reaction were removed after increasing times of incubation at $15^\circ C$ and analyzed by gel electrophoresis, as described above. Dimethyl-sulfate (DMS)-interference mapping experiments were performed as described earlier (24, 28).

In vivo expression assay

The BamHI fragment of pML553 (23) comprising the MLP sequences between positions -259 and $+553$ was inserted into the BamHI site of M13mp9. Oligonucleotide-directed mutagenesis was carried out on the resulting single-stranded M13 recombinant. The SacII fragment ($-246/+131$, relative to the MLP startsite) of the parental or mutated MLP derivatives and a HindIII-NcoI fragment (coordinates 5171/37) spanning the SV40 replication origin were respectively inserted into the BglII and HindIII sites of the polylinker of the pXP2-luciferase reporter plasmid (29). The MLP activity of the resulting recombinants was assayed essentially by following an infection/transfection protocol originally established by Mansour et al. (21). COS7 cells, grown in Dulbecco medium supplemented with 5% calf serum, were infected with adenovirus type 5 or its E1a-defective derivative (dl312), at 20 PFU per cell, in 9 cm Petri dishes. Two hours later, the MLP-luciferase recombinants (5 μ g), together with 10 μ g of carrier DNA (pSP65 plasmid) were transfected into these cells by calcium-phosphate co-precipitation (6). The cells were harvested about 24 hours later, extracts were prepared, normalized by protein concentrations and luciferase activity was measured, as described (29). All transfection assays were repeated at least four times in duplicate, with independent template preparations.

RESULTS

We have previously delineated, by DNase I protection and DMS interference mapping, sequence elements (DE1 and DE2) located downstream of the MLP startsite, between positions $+86$ and $+125$. We have shown that these elements bind factors specifically involved in the late-phase-dependent induction of the MLP and have partially characterized the factor which interacts with DE1, the most 5' of these elements (23, 24). Complex formation was originally analyzed by electrophoretic band-shift experiments with DNA probes restricted to the DE1 element. On the other hand, DNase I footprint competition studies indicated that the two downstream elements may bind the same factor (20, 24, 25). To further examine the protein-DNA interactions at the level of these downstream elements, we have analyzed complex formation on both DE1 and DE2 elements, using probes spanning the region between $+75$ and $+124$.

Downstream-complex formation is late-phase-specific

Whole-cell extracts were prepared 20 h post-infection (pi) from HeLa cells with the wild type adenovirus-5 (Ad5) or its dl312 derivative, referred to as the wt20hpi or dl20hpi extracts, respectively. At these times after infection, viral DNA undergoes replication only in the wt- but not in the dl-infected cells. Thus, comparative analyses of the corresponding cell extracts allow the *in vitro* study of replication-related or late-phase-dependent viral events.

A typical gel-shift experiment with a probe spanning the entire downstream element (DE12wt) is shown in Fig. 1A. Two major DNA-protein complexes with different mobilities ('a' and 'c') were obtained with the wt20hpi extract, but neither complex was observed with the dl20hpi extract or with an extract prepared from cells infected with wt virus, but where DNA replication was blocked with cytosine arabinoside (wt20hpi+AraC). The formation of both complexes was prevented by preincubation of the extract with the DE12wt competitor, but not with a mutated

Figure 1. Comparative analysis of MLP downstream and upstream DNA binding activities. Standard band-shift experiments were performed with whole cell extracts prepared from Ad5 or dl-infected cells which were blocked or not with AraC, using oligonucleotide probes which span the MLP downstream elements (DE12wt, +75 to +124, panel A) or the MLP upstream element (UE, -75 to -49, panel B). The non-specific competitor poly(dI-dC) was added at 10 μ g per assay. The specificity of the retarded complexes was determined by competition with a 400 molar excess of unlabeled oligonucleotides corresponding to the wild type downstream elements (DE12wt) or a mutated derivative thereof (DE12m12ab, see Fig. 4). Arrows point to DE-specific ('a' and 'c') and UE-specific (UEF) complexes. F refers to the unbound probe.

oligonucleotide altered within each DE element (DE12m12ab, see Fig. 4). These results indicate that complexes 'a' and 'c' are specific for the DE elements and that their formation is restricted to the late phase of viral infection.

As a control, we verified that, under the same conditions, a constitutive factor could be detected with similar efficiencies in each of these extracts. The extracts used in the experiment shown in Fig. 1A were probed with an oligonucleotide (UE) comprising the recognition site for the MLP upstream transcription factor (MLTF, USF or UEF) (30, 26, 12). One major specific complex was observed which was resistant to competition by any of the DE12 oligonucleotides (Fig. 1B, lanes 2 and 3), but sensitive to an excess of unlabeled UE oligonucleotide and resistant to a mutated UE oligonucleotide (not shown). In agreement with earlier findings (31, 25), roughly equal amounts of this complex were obtained with each extract (Fig. 1B, compare lanes 1, 4 and 5). This observation, further emphasizes our conclusion that the DE-specific complexes 'a' and 'c' are formed by proteins selectively activated during the late phase of viral infection.

The MLP downstream elements bind two proteins

To more extensively characterize the complexes assembled on the DE12wt probe, we fractionated the wt20hpi extract by heparin-Ultrogel chromatography as outlined in Fig. 2A. While the DNA-binding activity responsible for the formation of complex 'a' was eluted at 0.6 M KCl (fraction H0.6; Fig. 2B, lane 3), none of the fractions generated complex 'c' given by the starting material (Fig. 2B, lane 1). A distinct binding activity, producing complex 'b' (lane 2), was detected in the 0.3 M eluate (fraction H0.3). Mixing experiments with the various heparin fractions revealed that only a combination of fractions H0.3 and H0.6 was able to reconstitute complex 'c' (lane 4).

We next examined whether complex 'c' results from the simultaneous binding of the proteins responsible for the formation of complexes 'a' and 'b', or if it arises independently from the

Figure 2. Fractionation of the wt20hpi extract reveals the heteromeric nature of complex 'c'. A wt20hpi extract was fractionated by heparin-Ultrogel chromatography (A), as described in Materials and Methods. Comparative band-shifts were performed with fractions H0.3 and H0.6, either separately or combined, and the starting extract (B). Serial band-shifts were carried out (C) on increasing amounts of H0.6 (0.1 to 3 μ l), in the absence (lanes 1–5) or presence (lanes 6–10) of a fixed amount of H0.3 (1 μ l), or on increasing amounts of H0.3 (0.03 to 1 μ l), in the absence (lanes 17–22) or presence (lanes 11–16) of a fixed amount of H0.6 (3 μ l) and compared to the pattern yielded by the original wt extract (lane 23). The DE12wt oligonucleotide was used as probe (see legend to Fig. 1) in these experiments B and C). The non-specific competitor poly(dI-dC) was added at 10 μ g per assay in the presence of the 20hpi extract, at 0.25 or 0.5 μ g per assay in the presence of the H0.3 or H0.6 fractions alone, respectively, and at 0.75 μ g per assay in the mixing experiments (panel C, lanes 1–22). Specific bands are indicated by the arrows. The probe alone has been run in parallel (lane P).

association of other, distinct proteins present in fractions H0.3 and H0.6. As shown in Fig. 2C, the addition of increasing amounts of H0.6 to a fixed amount of H0.3 (lanes 6–10) progressively led to the disappearance of complex 'b', with a corresponding accumulation of complex 'c'. A similar enhancement of complex 'c' was observed at the expense of complex 'a', in the presence of increasing amounts of H0.3 (lanes 11–16), while increased levels of either H0.6 or H0.3 separately yielded no detectable complex 'c' formation (lanes 1–5 and 17–22, respectively). This clear precursor-product relationship between the formation of complexes 'a' and 'b' on one hand and that of complex 'c' on the other, suggests that complex 'c' corresponds to the simultaneous binding of complexes 'a' and 'b' to the same DNA molecule.

To determine the nucleotides involved in protein binding in each of these complexes, we performed a DMS-interference mapping. The experimental results are shown in Fig. 3A for each complex and summarized in Fig. 3B. From the position of the nucleotides whose methylation interfered with complex formation on both transcribed (T) and non-transcribed (NT) strands of the target DNA, minimal interaction sites could be assigned to each complex. Complex 'a' was restricted to DE1, the portion of the downstream element the most proximal to the transcriptional startsite: the interference pattern exhibited by this complex

Figure 3. DMS-interference mapping of nucleoprotein complexes 'a', 'b' and 'c'. (A) Free (F) and bound (B) probes were excised from preparative retardation assays performed with DMS-treated DE12wt oligonucleotide, 5'-end-labelled at position +75 (non-transcribed strand, NT) or +124 (transcribed strand, T) and either fraction H0.6 (Cx a), fraction H0.3 (Cx b) or both fractions together (Cx c). The DNA was recovered and analyzed by sequencing gel electrophoresis, after chemical cleavage at methylated G and A residues (24, 28). Residues whose methylation interferes with complex formation are denoted with open symbols and those whose methylation facilitates complex formation with closed symbols. Nucleotide coordinates relative to the MLP startsite (+1) are given. The bars next to 1 and 2, 3 and 4, and 9 and 10 refer to the portions of the gel which have been exposed for shorter (open) or longer (striped) times. (B) Summary of the DMS-interference pattern of complexes 'a' (○), 'b' (□) and 'c' (△). The position of methylation sites interfering (open symbols) or facilitating (closed symbols) complex formation are shown on the non-transcribed (N.T., above) and transcribed (T., below) strand of the MLP down-stream region (between +80 and +126). The regions covered by complexes 'a' and 'b' are referred to as DE1 and DE2b, respectively. The additional downstream extension of complex 'c' interference is denoted DE2a.

actually corresponded to that of the DEF protein previously identified (24). Complex 'b' mapped to a region (DE2b), further downstream, adjacent to DE1. Finally, the interference pattern of complex 'c' was very similar to that of complex 'b', but extended further downstream, spanning region DE2a in addition to DE2b (see Fig. 3B). The actual 3' boundary of this element may in fact extend up to position +124, as suggested by DNase I footprinting experiments (23, 20).

From these results, it appears that complex 'c' does not correspond to the simple juxtaposition of complexes 'a' and 'b' on the DE region, but to a specific rearrangement between these complexes. This conclusion was further substantiated by the results of a gel-shift analysis of a series of DE mutants. Sets of

Figure 4. Mutational analysis of complex formation on the downstream elements. (A) Schematic representation of the DE12wt oligonucleotide (non-transcribed strand), with the boxed DE elements as deduced from the DMS-interference study (Fig. 3). Nucleotide alterations with respect to the wt sequence are indicated above each element. The bracketed sets of alterations (m1, m2b and m2a) correspond to the mutations (X) introduced individually or in various combinations into the probes used for band-shift analysis, as schematically symbolized on top of each series of retardation (B and C). Standard band-shift assays were carried out with the H0.3 and H0.6 fractions (isolated or combined), and the original wt20hpi extract. The concentration of poly (dI-dC) was as indicated in the legend to Fig. 2. Where indicated (lanes P), the probe alone has been run in parallel. Arrows point to specific bands. The bracket next to lanes 15 and 16 (B) refers to unidentified complexes, not reproducibly observed.

critical nucleotides, chosen on the basis of the DMS-interference patterns, were altered in each DE region (see Fig. 4A). The effects of the resulting mutations on specific protein binding were tested by the gel-shift assay. The mutations were assayed individually (Fig. 4B) or in any possible combination (Fig. 4C), in the presence of either wt20hpi extract, fractions H0.3 or H0.6, or both fractions together. Mutations in DE1 abolished formation of complex 'a', while mutations in DE2a prevented formation of complex 'c'. Mutations in DE2b precluded formation of both complexes 'b' and 'c'. On the other hand, double mutants with alterations in any pair of DE elements showed phenotypes corresponding to the additive effect of each mutation separately, as visualized in this assay. Finally, as expected, a mutant with all three DE elements modified has lost any specific protein binding capacity.

Figure 5. Specific complex formation on the downstream elements. A schematic representation of the DE12wt oligonucleotide, with the DE1, DE2a and DE2b elements, is shown. Interactions with specific DNA-binding proteins (DEF-A and DEF-B) present in the heparin fractions H0.6 and H0.3, respectively, are symbolized next to the corresponding retarded bands ('a', 'b' and 'c') of a typical gel-shift pattern. F refers to the unbound probe.

Figure 6. Off-rate measurements of complexes 'a', 'b' and 'c'. Specific complexes were formed by incubating, under standard band-shift conditions, the DE12wt oligonucleotide probe with either fraction H0.3 (A), H0.6 (B) or both fractions together (C). After 15 min at 25°C, a 400 fold molar excess of unlabeled DE12wt oligonucleotide was added and incubation temperature was decreased to 15°C. At the indicated time (min) following competitor addition, equal aliquots were removed from the incubation mixture and loaded on a gel that was already running. The concentration of poly (dI-dC) was as indicated in the legend to Fig. 2.

The additional retarded bands (bracketed in Fig. 4B, lanes 15 and 16) sometimes observed with a mutant lacking a functional DE2b element may correspond to H0.6 proteins binding simultaneously to both DE1 and DE2a elements. Similarly, the residual weak complexes found with the mutant altered in both DE1 and DE2b elements (Fig. 4C, lanes 5–8) may be related to a weak binding capacity of the DE2a element alone. Such possibilities have not been further investigated, but are, at least in part, supported by the results presented in Fig. 8 (see below).

Taken together these results indicate that the wt20hpi extract contains two DNA binding activities (see Fig. 5): the activity separated in heparin fraction H0.3 (DEF-B) binds to the DE2b element (complex 'b'); the activity present in fraction H0.6 (DEF-A) binds to DE1 on its own (complex 'a'), but mainly interacts with the DE2a element when combined with fraction H0.3 (complex 'c'). The detection of only complexes 'a' and 'c' with wt20hpi extracts (see Fig. 1, lane 1 for an example) most likely reflects the greater abundance of DEF-A over DEF-B in these extracts. No specific retarded bands with lower mobilities than

complex 'c' could be detected on the DE12wt probe at higher DEF-A and DEF-B concentrations, suggesting that binding to the DE2 elements precludes any further binding to the DE1 element. It is possible however that such multiprotein complexes, if present, could not be detected for technical reasons linked to their instability under the gel-shift assay conditions. Finally, as stated above, complexes 'a', 'b' and 'c' were selectively detected in wt20hpi extracts. Treatment of dl20hpi extracts with detergents like deoxycholate and Nonidet P40 under conditions which dissociate potential protein-protein interactions (32) did not reveal proteins with DE-specific binding activity in these extracts. Similarly, supplementing such extracts with either partially purified DEF-A or DEF-B fractions did not allow detection of complex 'c'. These observations (G.M., data not shown) rule out the possibility that the DEF-A and DEF-B proteins are present in dl20hpi extracts, but undetectable because complexed to inhibitory proteins.

The DEF-A and DEF-B proteins bind cooperatively to the DE elements

The observation that, in the presence of DEF-B, the DEF-A protein preferentially associates to the DE2a site suggests a cooperative binding of the two proteins to form complex 'c'. This possibility was in fact raised by the results of the experiment presented in Fig. 2C, showing a non-linear accumulation of complex 'c' relative to increasing concentrations of fractions H0.3 and H0.6.

Also in agreement with this conclusion is the higher stability of complex 'c', compared to complexes 'a' and 'b' which dissociated more readily than complex 'c', after addition of an excess of specific competitor, as shown in Fig. 6 by the measurement of the off-rates of each complex. Finally, we have performed competition experiments in which wt20hpi extract was preincubated with increasing amounts of the wt or mutated competitor DE oligonucleotides, before addition of the wt probe and analysis of residual complex formation by the band-shift assay (Fig. 7A). Under these conditions, oligonucleotides retaining just the DE2a (lanes 15–18) or DE2b (lanes 10–13) elements were only slightly more efficient competitors for complex 'c' than a control oligonucleotide mutated in all three elements (lanes 20–23). By contrast, a competitor containing both DE2a and DE2b elements (lanes 6–9) was far more effective (see Fig. 7B), further supporting the conclusion that the DEF-A and DEF-B proteins cooperate in their binding to these elements. The fact that the unmodified oligonucleotide (DE12wt) is yet a better competitor (Fig. 7A, lanes 1–5 and Fig. 7B), is most likely due to the presence of an intact DE1 element which may contribute to the overall protein binding activity of the DE region by providing an additional target for DEF-A and thereby facilitating the transfer of this protein to the DE2a element. The observation that a competitor only retaining an intact DE2a element (DE12m12b) readily competed for complex 'a' formation (Fig. 7A) reinforces the conclusion that the same factor (DEF-A) binds to the DE1 and DE2a elements.

That this is indeed the case is further supported by results of band-shift experiments with brominated probes corresponding to the DE1 or DE2a elements (Fig. 8). We have previously observed (Jansen-Dürr, Boeuf, Mondésert and Kédinger, unpublished results) that bromodeoxyuridine-substituted oligonucleotides were much more efficient probes than their unsubstituted counterparts for the detection of specific, but weak or unstable nucleoprotein complexes. As illustrated in Fig. 8A,

Figure 7. Preferential disruption of complex 'c' by competitor oligonucleotides retaining both DE2a and DE2b elements. (A) Retardation competition experiments were performed as described in Materials and Methods, with the wt20hpi extract and DE12wt probe, in the presence of the indicated molar excess of competitor oligonucleotides (depicted on the top of each series). Uncompeted binding reactions are shown in lanes 1, 14 and 19. Poly (dl-dC) concentrations, symbols and annotations are as in Fig. 4. (B) The relative amounts of probe in complex 'c' have been determined by densitometry of various exposures of the autoradiogram and plotted against the molar excess of competitor oligonucleotide used in each assay (symbolized on the right, next to the corresponding curves).

a roughly twenty-fold stronger retardation signal corresponding to complex 'a' was obtained with the brominated DE1 probe (compare lanes 1 and 4). Whereas a complex of the same mobility could barely be detected with the unsubstituted DE2a probe (lane 7), a clear retarded band was observed when the corresponding brominated probe was used (lane 10). This complex was clearly late-phase-dependent, since it was detected in the presence of the wt20hpi extract but not with the dl20hpi extract (lane 11). The weak retarded bands in lanes 5 and 11 have not been reproducibly observed from one extract to the other and have therefore not been further investigated. The specificity of the late-phase-dependent complex has been verified by its sensitivity to DE12m12b and DE12m2ab competitors and resistance to DE12m12ab competitor (data not shown). These results strongly suggest that the DEF-A protein binds to both DE1 and DE2a sites, although its affinity appears highest for the former site. This conclusion was further supported by experiments with a partially purified DEF-A fractions, DC0.3, devoid of DEF-B activity (see Fig. 2 and 8B). As shown in Fig. 8C, a major retarded complex ('a') was obtained with brominated probes keeping either an intact DE1 element (DE12m2abBr) or an intact DE2a element (DE12m12bBr), in the presence of the 0.3M eluate of the H0.6 material retained on DNA-cellulose (DC0.3). This complex was in each case competed by brominated oligonucleotides retaining either an intact DE1 or DE2a element,

Figure 8. DEF-A binds to the DE1 and DE2a elements. (A) Standard band-shift experiments were performed with whole cell extracts prepared from Ad5 (wt20hpi) or dl-infected cells (dl20hpi), using synthetic oligonucleotide probes which span the DE1 (DE1wt or DE1Br, +75 to +104) or the DE2a (DE2awt or DE2aBr, +104 to +131) elements. Where indicated (Br), all T residues had been replaced by bromodeoxyuridine during chemical synthesis of each strand of the probes. Other conditions were as described in legend to Fig. 1. (B) A wt20hpi extract was fractionated by heparin-Ultrogel chromatography, as outlined in Fig. 2A. Fraction H0.6 was further chromatographed on DNA-cellulose, as described in Materials and Methods. (C) Band-shifts were performed with 2 μ l of fraction DC0.3, in the presence of 0.5 μ g of poly (dl-dC) per assay and 100 ng (i.e. about 200-fold molar excess) of the brominated competitors, as indicated. Probes were synthetic oligonucleotides (+75 to +124, see Fig. 4a) corresponding to the DE12m2ab (lanes 1-4) and DE12m12b (lanes 5-8) mutants and brominated within the DE1 and DE2a elements, respectively (see above). Probes and competitors are schematized as in Fig. 4BC.

whereas an oligonucleotide mutated in all three DE elements had no effect. As expected from the relative affinity of DEF-A for the DE1 and DE2a elements, the DE12m2ab oligonucleotide was a slightly better competitor than the DE12m12b oligonucleotide (Fig. 8C, compare lanes 2 and 3). These experiments, showing that DEF-A can interact, in the absence of DEF-B, with either the DE1 or DE2a elements, strongly support the conclusion that this double binding activity is an intrinsic property of DEF-A itself. These results also indicate that the DE2a element is not just an extension of the DEF-B binding area caused by the DEF-A/DEF-B interaction, but that it corresponds to a genuine binding site which contributes to the stability of this interaction.

Transcriptional analysis of the DE mutations

To examine the effects of the individual and combined DE alterations on the late-phase-specific MLP transcriptional activation, the promoter efficiencies of the corresponding mutants were measured as follows. To analyze the functional role of the

	ELEMENTS RETAINED				RELATIVE BASAL MLP ACTIVITY	RELATIVE LATE-PHASE-SPECIFIC MLP ACTIVATION			
	UE	DE1	DE2b	DE2a		(a)	(b)	(c)	(d)
wt	+	+	+	+	5	100	100	100	
m1	+	-	+	+	4.8	52	34	48	
m2a	+	+	+	-	3.1	70	40	73	
m2b	+	+	-	+	3	38	35	35	
m12a	+	-	+	-	6	52	45	45	
m12b	+	-	-	+	4.5	45	35	40	
m2ab	+	+	-	-	4	30	52	52	
m12ab	+	-	-	-	5.8	25	25	30	
Δ 12	+	-	-	-	4.3	23	22	22	
mU Δ 12	-	-	-	-	1.1	7	6	7	

Figure 9. Transcriptional analysis of MLP recombinants. The MLP is diagrammed with promoter elements essential for late-phase-specific stimulation. The MLP elements which were retained (+) or altered (-) in each of the MLP-luciferase recombinants used in the transfection assay (see Materials and Methods) are indicated. The values of basal reporter activity (column a), as measured in dl312-infected cells, are given for each MLP recombinant relative to the wt construct (5): numbers correspond to the average of five independent experiments. The values given in columns b-d represent the ratios of luciferase activity of each MLP recombinant in Ad5 versus dl312-infected cells, relative to those of the wt MLP constructs (100), for three independent experiments. The actual wt levels of late-phase-specific activation in experiments b, c and d were 42, 12 and 15 fold, respectively.

DE elements in a cellular context we have inserted the various DE mutations into a MLP-luciferase chimeric reporter plasmid containing a minimal SV40 origin of replication. The recombinant constructs were transfected into COS cells which were infected with either wild type Ad5 or dl312. As a prerequisite for these experiments, we verified that extracts, prepared 20 hours post-infection from Ad5-infected COS cells, contained the DEF-A and DEF-B DNA-binding activities (not shown). Furthermore, these cells which constitutively express SV40 T antigen, support replication of SV40 origin-containing plasmids (21). In both dl312 and Ad5-infected cells, a significantly higher expression level was obtained from the MLP-luciferase recombinants in the presence of this replication origin (C.T., unpublished observation), most likely reflecting increased template copy numbers. The results of three independent transient expression experiments are tabulated in Fig. 9 (columns b-d). Although the levels of late-phase-specific stimulation, as defined by the ratio of luciferase expression in Ad5 versus dl312-infected cells, varied between 10 and 40 fold, for the wt MLP recombinant, very similar levels of relative stimulations were found, from one set of experiments to the other, with the various MLP recombinants. Importantly, when the MLP sequences of the MLP-luciferase reporter plasmid were replaced by the rabbit β -globin promoter (-109/+11), the level of luciferase expression was the same in Ad5 and dl312-infected cells (results not shown), indicating that the late-phase-dependent stimulation observed in this assay is MLP-specific.

As shown in Fig. 9, the simultaneous alteration or deletion of the DE elements (as in m12ab or Δ 12) decreased the late-phase-specific stimulation by about 70 to 80% (columns b-d), without significantly affecting the MLP-directed expression in the dl312-infected cells (column a). The additional alteration of the UE element (as in mU Δ 12) further reduced this stimulation (columns b-d) and, as expected from the UE alteration, decreased basal promoter activity (column a). These *in vivo* results confirm the critical involvement of the entire DE region in the replication-dependent activation of the MLP (24, 31, 25).

They also reinforce our previous conclusion, initially based on *in vitro* experiments (25), that the UE element actively contributes to this effect. When the DE1, DE2a and DE2b elements were altered separately (as in m1, m2a and m2b, respectively), the activation of the MLP was significantly affected (columns b-d), indicating that each DE element is important for maximal late phase responsiveness of the promoter. This is further illustrated by the fact that each element alone (as in m12a, m12b and m2ab) retains a low level of activity (columns b-d). Altogether, these results indicate that both DEF-A and DEF-B proteins are transcriptionally active and that both contribute to full promoter activation.

DISCUSSION

Comparison of available nucleotide sequences from different adenovirus serotypes reveals striking conservation of the DE1 and DE2 elements (10/12 and 15/16 nucleotides, respectively), within members of three distinct groups of adenovirus: the non-oncogenic serotypes 2, 4 and 5, the weakly oncogenic type 7 and the highly oncogenic type 12 (24 and our unpublished observation). This homology, which closely correlates with the DMS interference mapping of the DE protein binding sites, further stresses the functional importance of these elements and suggests that similar mechanisms are involved in the control of late transcription of these viral genomes. Mason et al. (22) have shown in fact that MLP sequences between +82 and +148 in adenovirus-4, and within the first intron in adenovirus-7 (both comprising the conserved DE elements described here) play essential roles in expression from this promoter in adenovirus-based vectors. On the other hand, following the same argument, the poor sequence conservation over a weaker protein binding site (R1, between +37 and +68) previously identified by Leong et al. (20) tends to rule out a major contribution of this element in MLP regulation. By contrast, as expected from their essential requirement, a good conservation is observed for the sequences spanning the inverted CAAT box at -76, the major upstream promoter element at -62 and the TATA box at -31. The initiator around +1 is less conserved.

Leong et al. (20) have constructed recombinant adenoviruses harboring the MLP promoter fused to the human β -globin coding sequences and inserted into the non-essential E3 region of the virus. Functional analysis of deletion mutations of the downstream region of this chimeric gene, during infection, indicated that the elements corresponding to DE1 (R2) and DE2 (R3) are essential for maximal rates of transcription from the MLP during the late phase of infection. In agreement with our earlier (25) and present results which suggest that the DE1 and DE2 elements are partially redundant, these authors observed that only a deletion removing both DE1 and DE2 elements abolished MLP activation. Similarly, Reach et al. (11), who have introduced a series of mutations into defined elements of the MLP within the natural viral context, found that point mutations altering nucleotides essential for specific protein contacts with the DE1 element had no detectable effect on MLP activity. It remains to be established whether mutations simultaneously altering either of the upstream elements (UE or CAAT box) and both DE1 and DE2 elements will abolish MLP activation in infected cells, as observed in our transfection assays.

Two protein fractions (DEF-A and DEF-B) bind to the DE elements. We have previously characterized a protein, formerly called DEF, which specifically binds to an oligonucleotide

comprising only the DE1 element. Southwestern and UV cross-linking experiments have shown that it corresponds to a 40 kD protein (24). On the basis of several independent observations, we conclude that this protein actually corresponds to the DEF-A activity described here: (i) the specific DNA-binding activity of both proteins is detected only in extracts from cells where viral DNA has replicated; (ii) using a purification scheme similar to that previously described (24), both activities copurify, as revealed by comparative band-shift analysis of the chromatography fractions with probes containing only the DE1 or both DE1 and DE2 elements; (iii) the DMS interference patterns generated by both proteins are identical (24 and present results); (iv) in the retardation pattern produced by the wt20hpi extract with a probe comprising both DE1 and DE2 elements, complexes 'a' and 'c' (but not 'b') are selectively competed by oligonucleotides retaining only the DE1 element (not shown), in agreement with specific footprint competition experiments (20, 25).

In the absence of DEF-B, DEF-A binds most efficiently to the DE1 element. It also binds to the distal portion of the DE2 element (DE2a), but with much lower affinity (see Fig. 7 and 8). Surprisingly, there is no obvious sequence homology between these elements. It will be of interest to determine if the same protein domain of DEF-A recognizes both of these distinct elements or if separate domains of its polypeptide chain are involved. Other factors (the mammalian TEF-1 and Oct2 factors, the yeast HAP1 factor, the drosophila Paired gene product) have been described which are able to bind to two apparently unrelated DNA sequence elements (33–38). In the case of the HAP1 factor and the Paired product the DNA-binding domains have been delineated: while a single domain in HAP1 recognizes both DNA elements (39), two distinct domains seem to be involved in the other protein (38).

The nature and physico-chemical properties of the protein responsible for the DEF-B activity remain to be established. Our results show that this protein, when mixed with the DEF-A protein, enhances the binding of DEF-A to the DE2a site, leading to the assembly of the heteromeric complex 'c'. In addition, we have examined whether stereo-specific alignment of the DE2a and DE2b elements was critical for complex 'c' formation. We have recently observed (data not shown) that a 5 bp insertion (i. e. about one half helical turn) between these elements severely impaired formation of complex 'c' and that partial restoration of this complex was observed after an additional 5 bp insertion. These results, revealing the existence of spatial constraints for complex 'c' formation, suggest that particular protein-protein interactions are involved and further stresses the cooperativity between DEF-A and DEF-B for binding to the DE2 elements. Despite the cooperativity between these two proteins for the formation of complex 'c', the overall dissociation rate of this complex is high (bound half-life below one minute, at 15°C) compared to that of complexes involving the E2F or E4F proteins, for example, which show much longer half-lives at 25°C, but under otherwise comparable experimental conditions (40–42). This weak apparent affinity is surprising given the pronounced effect of the DE elements on the MLP activity, but may reflect the necessity for this downstream complex to allow progression of transcribing polymerases through the DE region. In this respect, it may also be relevant that DEF-A has two target sites within the DE region: the one to which it binds on its own (DE1) and the other (DE2) to which it binds in concert with DEF-B.

Multiple binding sites for particular factors have previously been reported (43 and references therein) and shown to contribute to the higher occupancy of a critical cognate promoter element. Similarly, the double binding possibility of DEF-A may facilitate its access to the promoter despite its weak affinity.

The results of the mutational analyses of the MLP template activity confirm the contribution of each of these DE elements to maximal promoter stimulation. Surprisingly however, a mutation (m1) which abolishes binding of DEF-A to the DE1 element was generally more deleterious to MLP activation than a mutation (m2a) which prevents DE2a occupancy (see Fig. 9). This observation suggests that the cooperative binding of DEF-A and DEF-B to the DE2 element, as measured by the band-shift assay, does not correlate with maximal *in vivo* MLP activation. We have no explanation for this apparent discrepancy between the results of the DNA-binding and transcriptional assays. The possibility exists, for example, that some DE mutations (like m2a) may be partially complemented *in vivo* by additional MLP elements (located within the MLP –246/+131 domain used in this study) still to be identified. In this respect, it may be of interest to assay the effects of the DE mutations in the context of an altered UE element. Alternatively, an adenovirus-2 element, functionally related to the adenovirus-12 'mitigator' element recently described by Zock and Doerfler (44), may alter some of the properties of the DEF-A and DEF-B proteins in the monkey COS7 cells used in our *in vivo* assay. Elucidation of the precise mechanisms underlying this promoter activation clearly awaits the isolation of the factors involved, among which DEF-A and DEF-B.

We have shown (25, and present results) that the factors which bind to the DE elements cooperate with the factor (UEF or USF) bound to the upstream element (UE or UPE) to achieve maximal MLP activity. Reach et al. (10) have previously reported that the UE and the inverted CAAT box located just upstream are functionally redundant promoter elements, when assayed in the viral context. More recently, these authors have presented evidence suggesting that the factors which bind to these upstream elements (USF and CP1, respectively) may each interact with the TATA box recognition factor TFIID to achieve MLP activation (11). Although we have not examined whether the DE-binding proteins may cooperate with the CAAT factor, these results suggest that late-phase-dependent transcriptional stimulation of the MLP requires the joined action of USF or CP1, TFIID and the DEF-A and DEF-B proteins.

The present results demonstrate that both DEF-A and DEF-B proteins are involved in the late-phase-dependent activation of the MLP. The molecular mechanisms by which these factors actually mediate their effect is not yet understood, but most likely imply particular interactions between these proteins themselves and with the basal transcription apparatus. The availability of an *in vitro* system allowing the faithful reproduction of this transcriptional stimulation and its reconstruction from the purified components of the active initiation complex will help elucidating these mechanisms.

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REFERENCES

1. Herbolme, P. (1990) *The New Biologist* 2, 937–945.
2. Villarreal, L. P. (1991) *Microbiol. Rev.* 55, 512–542.
3. Berk, A. J. (1986) *Ann. Rev. Genet.* 20, 45–79.
4. Flint, J. and Shenk, T. (1989) *Annu. Rev. Genet.* 23, 141–161.
5. Concino, M. F., Lee, R. F., Merryweather, J. P. and Weinmann, R. (1984) *Nucleic Acids Res.* 12, 7423–7433.
6. Hen, R., Sassone-Corsi, P., Corden, J., Gaub, M. P. and Chambon, P. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7132–7136.
7. Lee, R. F., Concino, M. F. and Weinmann, R. (1988) *Virology* 165, 51–56.
8. Logan, J. and Shenk, T. (1986) *Nucleic Acids Res.* 14, 6327–6335.
9. Miyamoto, N. G., Moncollin, V., Wintzerith, M., Hen, R., Egly, J. M. and Chambon, P. (1984) *Nucleic Acids Res.* 12, 8779–8799.
10. Reach, M., Babiss, L. E. and Young, C. S. H. (1990) *J. Virol.* 64, 5851–5860.
11. Reach, M., Xu, L.-X. and Young, C. H. S. (1991) *EMBO J.* 10, 3439–3446.
12. Sawadogo, M. and Roeder, R. G. (1985) *Cell* 43, 165–175.
13. Yu, Y. T. and Manley, J. L. (1984) *Nucleic Acids Res.* 12, 9309–9321.
14. Cohen, R. B., Yang, L., Thompson, J. A. and Safer, B. (1988) *J. Biol. Chem.* 263, 10377–10385.
15. Hariharan, N., Kelley, D. E. and Perry, R. P. (1991) *Proc. Natl. Acad. Sci. USA* 88, 9799–9803.
16. Greenblatt, J. (1991) *T. I. B. S.* 16, 408–411.
17. Roeder, R. G. (1991) *T. I. B. S.* 16, 402–408.
18. Shaw, A. R. and Ziff, E. B. (1980) *Cell* 22, 905–916.
19. Thomas, G. and Mathews, M. B. (1980) *Cell* 22, 523–533.
20. Alonso-Caplen, F. V., Katze, M. G. and Krug, R. M. (1988) *J. Virol.* 62, 1606–1616.
21. Leong, K., Lee, W. and Berk, A. J. (1990) *J. Virol.* 64, 51–60.
22. Mansour, S. L., Grodzicker, T. and Tjian, R. (1986) *Mol. Cell. Biol.* 6, 2684–2694.
23. Mason, B. B., Davis, A. R., Bhat, B. M., Chengelvala, M., Lubeck, M., Zandle, G., Kostek, B., Chodowski, S., Dheer, S., Molnar-Kimber, K., Mizutani, S. and Hung, P. P. (1990) *Virology* 177, 452–461.
24. Jansen-Durr, P., Boeuf, H. and Kédinger, C. (1988) *Nucleic Acids Res.* 16, 3771–3786.
25. Jansen-Durr, P., Mondésert, G. and Kédinger, C. (1989) *J. Virol.* 63, 5124–5132.
26. Mondésert, G. and Kédinger, C. (1991) *Nucleic Acids Res.* 19, 3221–3228.
27. Moncollin, V., Miyamoto, N. G., Zheng, X. M. and Egly, J. M. (1986) *EMBO J.* 5, 2577–2584.
28. Alberts, B. and Herrick, G. (1971) In Colowick, S. P. and Kaplan, N. O. (ed.), *Methods in Enzymology*. Academic Press, New York and London, Vol. XXI, pp. 198–217.
29. Jansen-Durr, P., Boeuf, H. and Kédinger, C. (1989) *EMBO J.* 8, 3365–3370.
30. Nordeen, S. K. (1988) *Biotechniques* 6, 454–457.
31. Carthew, R. W., Chodosh, L. A. and Sharp, P. A. (1985) *Cell* 43, 439–448.
32. Leong, K., Brunet, L. and Berk, A. J. (1988) *Mol. Cell. Biol.* 8, 1765–1774.
33. Baeuerle, P. A. and Baltimore, D. (1988) *Cell* 53, 211–217.
34. Davidson, I., Xiao, J. H., Rosales, R., Staub, A. and Chambon, P. (1988) *Cell* 54, 931–942.
35. Kemler, I., Schreiber, E., Muller, M. M., Matthias, P. and Schaffner, W. (1989) *EMBO J.* 8, 2001–2008.
36. LeBowitz, J. H., Clerc, R. G., Brenowitz, M. and Sharp, P. A. (1989) *Genes Dev.* 3, 1325–1338.
37. Pfeifer, K., Prezant, T. and Guarente, L. (1987) *Cell* 49, 19–27.
38. Poellinger, L., Yoza, B. K. and Roeder, R. G. (1989) *Nature* 337, 573–576.
39. Treisman, J., Harris, E. and Desplan, C. (1991) *Genes and Dev.* 5, 594–604.
40. Pfeifer, K., Kim, K. S., Kogan, S. and Guarente, L. (1989) *Cell* 56, 291–301.
41. Marton, M., Baim, S. B., Ornelles, D. A. and Shenk, T. (1990) *J. Virol.* 64, 2345–2359.
42. Raychaudhuri, P., Bagchi, S., Neill, S. D. and Nevins, J. R. (1990) *J. Virol.* 64, 2702–2710.
43. Rooney, R. J., Raychaudhuri, P. and Nevins, J. R. (1990) *Mol. Cell. Biol.* 10, 5138–5149.
44. Oehler, S., Eismann, E. R., Krämer, H. and Müller-Hill, B. (1990) *EMBO J.* 9, 973–979.
45. Zock, C. and Doerfler, W. (1990) *EMBO J.* 9, 1615–1623.