JOURNAL OF VIROLOGY, Dec. 1989, p. 5124–5132
0022-538X/89/125124-09$02.00/0
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Vol. 63, No. 12

Replication-Dependent Activation of the Adenovirus Major Late Promoter Is Mediated by the Increased Binding of a Transcription Factor to Sequences in the First Intron

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Received 15 May 1989/Accepted 2 August 1989

During lytic infection, the adenovirus major late promoter (MLP) is primarily activated after the onset of viral DNA replication. Using a combination of DNA binding and in vitro transcription assays, we delineated a discrete MLP element spanning positions +80 to +106 which is essential for the replication-dependent activation of this promoter. We also identified a 40-kilodalton protein (the downstream element factor [DEF]) which binds to the +86-TTGTCAGTTT-+95 motif within this element. Whereas the DEF-binding activity is barely detectable in uninfected cells, it is readily visualized in adenovirus-infected cells, but only after the onset of viral DNA replication. Preventing the interaction of DEF with the MLP template impairs the in vitro transcriptional stimulation. We conclude that this replication-dependent activation of the MLP is, at least in part, mediated by induction of the specific binding of DEF to the MLP downstream element.

Accurate, efficient, and regulated transcription of class II promoters depends on the combinatorial interaction of particular nuclear proteins with specific DNA sequence elements (for a review, see reference 29). With the exception of enhancer elements, which may be found at largely variable distances upstream or downstream of the transcription start site, most promoter elements are located within about 100 base pairs upstream of the cap site. In several cases, transcriptional control elements positioned within the transcribed sequence have been described (5, 17, 23, 44, 45, 51; and references in reference 21), but their cognate factors have been only poorly characterized.

The major late promoter (MLP) of human adenovirus type 2 or type 5 is typical of many cellular promoters in possessing two essential elements located 5′ to the start site, the so-called upstream element (UE) between positions −61 and −51 and the TATA box between positions −31 and −25 (24) with respect to the MLP start site (+1). In addition to these upstream elements (6, 10, 15, 16, 27), several observations suggest the contribution of downstream promoter elements to maximal MLP activity (2, 9, 36, 42).

Low levels of transcription from the MLP dependent on the products of the viral immediate-early gene E1a are detectable during the early phase of lytic infection (8). The sequences required for this E1a-mediated activation have been localized between −66 and +33 by transient expression studies (26). In addition, MLP expression is strongly stimulated after the onset of viral DNA replication (12, 41). The molecular basis of this replication dependence of the MLP activity is still unclear. Thomas and Mathews (46) using superinfection experiments have demonstrated that the early-to-late transition is a function of the particular state of the viral DNA template, as induced by its own replication. Besides these essential *cis*-acting modifications, Thomas and Mathews (46) did not rule out the additional contribution of *trans*-acting components. Mansour et al. (31) using in vivo expression systems have defined a sequence element, the 3′ border of which is located between +33 and +195 of the MLP, that is essential for efficient transcription initiation from this promoter in replicating virus or plasmid constructs. Their results clearly show that a *trans*-acting factor(s) encoded or induced by adenovirus is required in addition to DNA replication. Using a cell-free transcription system initially developed by Leong and Berk (25) and based on extracts from cells infected with the wild-type adenovirus type 5 (wt) or its E1a-defective *dl*312 derivative (dl), we (21) recently confirmed that sequences upstream of +33 were involved in the E1a responsiveness of the MLP in vitro and demonstrated that sequences between +33 and +131 were implicated in the replication-induced activation of the MLP.

In the present report, we further extend this study and show that the efficient binding of a 40-kilodalton (kDa) protein (downstream element factor [DEF]) to an element located between +80 and +106 is responsible for the transcriptional stimulation of MLP observed in wt-infected cell extracts compared with dl-infected cell extracts. Our results indicated that induction of this DEF-binding activity depends on viral DNA replication before extract preparation and suggested that the E1a gene products are dispensable for at least part of this induction.

MATERIALS AND METHODS

Preparation of whole-cell extracts. HeLa cells grown in Eagle medium supplemented with 5% calf serum were infected with 10 PFU of adenovirus type 5 (wt) or its E1a-defective *dl*312 derivative (dl) per cell. For replication-blocked extracts, cytosine arabinoside (araC) was added at a final concentration of 20 μg/ml to the growth medium right after adsorption of the virus and 12 h later (25). Extracts were prepared 20 or 48 h postinfection (p.i.) by the method of Manley et al. (30), but the final dialysis was against buffer A (21) containing 50 mM Tris hydrochloride (pH 7.9), 12.5 mM $MgCl_2$, 40 mM ammonium sulfate, 0.1 mM EDTA, 2 mM dithiothreitol, and 17% glycerol. Protein concentration was adjusted to 6 μg/μl after dialysis.

Partial purification of DEF. A whole-cell extract (70 ml, 420 mg of protein) prepared from about 10^{10} wt-infected cells harvested at 20 h p.i. was chromatographed on a 100-ml

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heparin-agarose column. The column was eluted stepwise with a buffer (TGK) containing 10 mM Tris hydrochloride (pH 8), 10% glycerol, and increasing KCl concentrations as indicated (52). A 50-ml portion of the 0.6 M KCl eluate (H0.6; 70 ml, 105 mg of protein) was passed over a DEAE 5PW high-pressure liquid chromatography column and eluted with TGK buffers as indicated. A 3-ml sample of the 0.25 M KCl eluate (D0.25; 10 ml, 10 mg of protein) was chromatographed on a 3-ml DEF affinity column containing 6 μg of multimerized DE1 oligonucleotide per ml (20). The column was eluted with TGK buffers as indicated, and 2 ml of the 0.6 M KCl eluate [A0.6(1), 3 ml] was passed a second time over the same column and eluted as before. DEF activity was recovered again in the 0.6 M KCl eluate [A0.6(2), 3 ml].

In vitro runoff transcription. Transcription reactions were done in 16 μl of final volume with 8 μl of extract as previously described (21). In competition experiments, the extracts were preincubated for 5 min at 25°C with various amounts of the double-stranded competitor oligonucleotides, together with a constant amount (50 ng per assay) of sonicated salmon sperm DNA. After addition of the template, the preincubation was prolonged for 10 min at 25°C. The reaction was started by adding the nucleotides and carried out for 45 min at 30°C. After synthesis, RNA was extracted and analyzed by 5% polyacrylamide-urea gel electrophoresis. Specific transcripts were quantitated by densitometry of autoradiograms (standard deviations calculated from independent experiments were in the range of 10 to 20%).

Recombinant plasmids. Plasmid pML131 was constructed as previously described (21) by inserting into pBR322 an adenovirus type 2 segment between positions −245 and +131 (with respect to the MLP cap site). Plasmid pML80 was obtained by cloning the adenovirus type 2 DNA *Dde*I fragment comprising the MLP cap site (between positions −174 and +80) into the *Eco*RV site of pBR322. The pML128, pML106, and pML33 recombinants were constructed by inserting between the *Hin*dIII and *Bam*HI sites of pBR322 the *Hin*dIII-*Bam*HI fragments derived from pML131 after exonuclease *Bal*31 digestion from position +131 to positions +128, +106, and +33, respectively, and insertion of a *Hin*dIII linker at the deletion endpoints. The pG recombinant (19) contains the entire rabbit β-globin gene between positions −425 and +1700.

Electrophoretic band-shift assays. Gel retardation experiments were performed in a 10-μl final volume as described previously (18). Briefly, about 0.3 ng (5,000 cpm) of the ^{32}P-5′-end-labeled double-stranded oligonucleotide probe (DE1) was incubated with the fractions to be tested in the presence of poly(dI-dC) as a nonspecific competitor. For competition experiments, appropriate amounts of unlabeled oligonucleotides were preincubated with the protein fractions before addition of the labeled probe. After 10 min at 25°C, the complexes were separated by electrophoresis on a nondenaturing 4.5% polyacrylamide gel for 70 min at 180 V. Gels were transferred to Whatman DE81 paper and vacuum dried before autoradiography.

DMS interference analysis. Retardation assays scaled up 10- to 20-fold were performed with a DE1 oligonucleotide probe partially methylated by dimethyl sulfate (DMS) treatment (18). The band corresponding to complex I was excised, and the DNA was electroeluted and purified. Subsequently, the DNA was cleaved at G and A residues by a 30-min incubation at 90°C in 30 μl of 10 mM Na_2HPO_4 and 1 mM EDTA, followed by an additional 15-min incubation at 90°C after the addition of 3 μl of 0.1 N NaOH. DNA was purified, separated on a 16% sequencing gel, and exposed for autoradiography.

DNase I footprinting assays. Footprinting reactions were performed essentially as described previously (21). About 0.5 ng of the *Ava*II-*Eco*RI fragment of pML131 5′ end labeled at the *Ava*II site (nontranscribed strand) was incubated in buffer A for 10 min at 30°C in the presence of poly(dI-dC) and whole-cell extracts or chromatography fractions. When indicated, the protein samples were preincubated with specific double-stranded oligonucleotides before the addition of the probe DNA. DNase I (about 1,500 Kunitz units per mg of protein; Sigma Chemical Co., St. Louis, Mo.) was added. After further incubation for 5 min at 30°C, the DNA was purified and loaded on an 8% polyacrylamide sequencing gel next to DNase I-treated naked probe DNA and G and G+A reactions of the probe fragment. After electrophoresis, the gel was dried and exposed for autoradiography.

Southwestern blot (DNA-protein) analysis. Samples corresponding to 50 μg of protein of the D0.25 fraction were separated on a 9% polyacrylamide-sodium dodecyl sulfate (SDS) gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were saturated by incubation for 1 h at room temperature in a solution containing 5% nonfat dry milk and 10 mM HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8). After being washed in buffer B (10 mM HEPES [pH 8], 50 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.25% nonfat dry milk), the membranes were incubated for 60 min at room temperature with the multimerized oligonucleotide probes (about 2×10^7 cpm) in 20 ml of buffer B containing 150 μg of sonicated salmon sperm DNA. The membranes were then washed three times for 10 min in buffer B containing 200 mM NaCl, dried, and exposed for autoradiography. The multimerized probes were prepared as follows. Double-stranded oligonucleotides (2 μg) with 5′ protruding complementary ends were phosphorylated and, after tandem ligation of an average of 25 copies, nick translated to specific activities of about 5×10^7 cpm/μg.

UV cross-linking experiments. Gel retardation assays scaled up to 10- to 20-fold were performed with fraction D0.25, using as probe a 5′-end-labeled DE1 double-stranded oligonucleotide in which all T residues had been replaced by bromodeoxyuridine during chemical synthesis. Complex I, localized after 2 h of autoradiography at 4°C, was excised from the gel and irradiated on a UV transilluminator (312 nm). Appropriate irradiation times were determined empirically. The resulting protein-DNA adducts were electroeluted and analyzed on a 9% polyacrylamide-SDS gel.

RESULTS

Precise mapping of binding site of replication-dependent transcription factor DEF. In an earlier study, we mapped, by differential DNase I footprinting on the adenovirus MLP template, protein-binding sites selectively protected by extracts prepared from infected cells in which viral DNA had replicated. A major protection (corresponding to region DE1) was delineated between +86 and +92 with respect to the MLP transcription start site, and a weaker site (corresponding to region DE2) was found between +113 and +120 (Fig. 1). The appearance of these protections has been correlated with the replication-dependent transcriptional activation of the MLP (21).

To further characterize this replication-induced DNA-binding activity, we performed gel-shift experiments using a

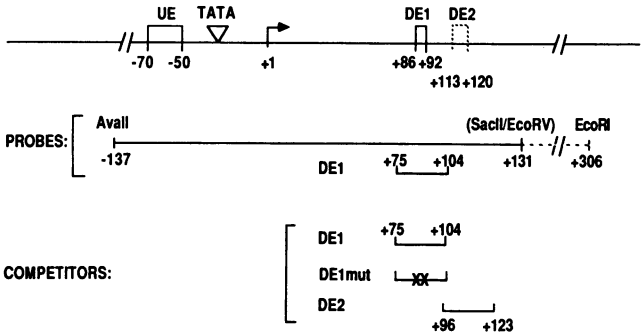

FIG. 1. Schematic representation of the adenovirus MLP and DNA fragments used for protein binding assays. The MLP region is depicted on the top, with the promoter elements discussed in the text positioned relative to the transcription start site (+1). The arrow points to the direction of transcription. The *Ava*II-*Eco*RI fragment excised from pML131 represents the probe fragment used for DNase I footprinting experiments. The solid line corresponds to MLP sequences between −137 and +131, and the dashed line corresponds to the adjacent *Eco*RV-*Eco*RI pBR322 sequences in pML131 (restriction sites in parentheses have been lost during cloning). The segment extending between positions +75 and +104, spanning the DE1 element, represents the chemically synthesized double-stranded oligonucleotide used as the probe in the gel-shift assays. The same fragment with the natural sequence (DE1) or containing G-to-T and G-to-C transversions (××) at positions +88 and +92 (DE1mut) and a fragment spanning the DE2 element between position +96 and +123 were used as unlabeled competitors.

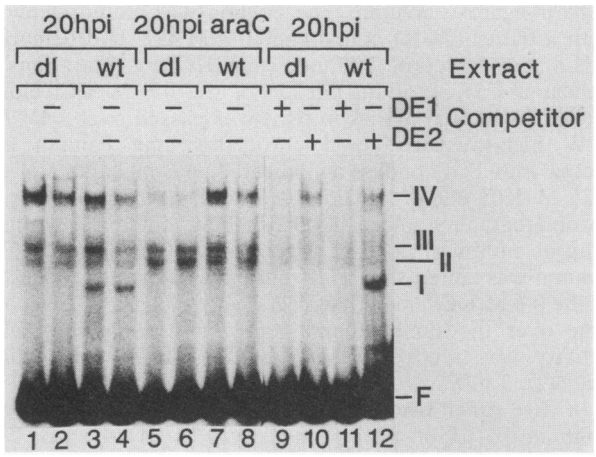

FIG. 2. Gel-shift analysis of 20 h p.i. cell extracts. Gel retardation assays were performed as described in Materials and Methods, using 1 μl (3 μg of protein) of 20 h p.i. dl or wt or of 20 h p.i. araC dl or wt extracts per assay in the presence of 1 μg (lanes 1, 3, 5, 7, and 9 to 12) or 2 μg (lanes 2, 4, 6, and 8) of poly(dI-dC). Where indicated (+), the extracts were preincubated with 100 ng of the DE1 or DE2 competitor oligonucleotides. F, Position of the free probe; I to IV, retarded nucleoprotein complexes.

synthetic double-stranded oligonucleotide spanning the MLP downstream element between +75 and +104 (probe DE1, Fig. 1). Whole-cell extracts were prepared 20 h p.i. from HeLa cells infected with adenovirus type 5 or its *dl*312 derivative, referred to hereafter as the 20 h p.i. wt or dl extracts, respectively. In control experiments, the wt- or dl-infected cells were grown in the presence of araC to prevent DNA replication. The corresponding extracts prepared at 20 h p.i. are referred to as the replication-blocked wt and dl extracts (20 h p.i. araC).

A comparative analysis of the gel-shift pattern generated by these extracts is shown in Fig. 2. Three major nucleoprotein complexes with different mobilities (II, III, and IV) were obtained with each type of extract (lanes 1 to 8). One additional complex (I) was uniquely detected with 20 h p.i. wt extracts. The fact that the formation of this latter complex was efficiently titrated only by oligonucleotide DE1 (lane 11) and not by oligonucleotide DE2 (lane 12) revealed that complex I corresponds to a protein which specifically binds to sequences located between +75 and +104. Altogether, these observations indicated that the protein in complex I most likely represents the major replication-dependent DNA-binding activity which we described previously (21). We will subsequently refer to this protein as DEF (for downstream element factor). While complexes II and III were titrated equally well by both DE1 and DE2 oligonucleotides, complex IV was titrated most efficiently by DE1, suggesting that, in contrast to complexes II and III (whose abundance often varies from one extract preparation to the other [see Fig. 7 and 9]), complex IV involves a specific DNA-binding protein. The fact, however, that complex IV was not restricted to 20 h p.i. wt extracts but also appeared in 20 h p.i. dl and replication-blocked wt extracts rules out the possibility that the corresponding protein is involved in the replication-dependent activation process. The reason for the reduction in complex IV formation with 20 h p.i. araC dl extracts has not been further investigated.

DMS interference mapping was used to delineate the DEF-binding site in complex I. From the position of the nucleotides whose methylation interfered with complex formation on both the nontranscribed (Fig. 3A) and transcribed (Fig. 3B) strands, it was deduced (Fig. 3C) that the minimal DEF interaction site corresponds to the decanucleotide +86-TTGTCAGTTT-+95, in good agreement with the results of our DNase I footprinting experiments (see reference 21 and Fig. 6).

Binding of DEF protein to its recognition sequence is required for MLP transcriptional stimulation. To demonstrate the implication of the DE1 element in the replication-dependent activation of the MLP, we performed a transcriptional analysis of a selected series of truncated MLP templates. As we had previously shown that the replication-responsive element was located between positions +33 and +131 (21), we constructed a series of plasmids (Fig. 4) with MLP fragments comprising the whole upstream promoter region (up to position −245 or −174) but extending to various positions downstream of the transcription start site (+33, +80, +106, +131). The relative template efficiencies of the resulting recombinants were tested by runoff transcription in the presence of 20 h p.i. wt and dl extracts. Under the conditions used in the experiment shown in Fig. 4, the recombinant retaining MLP sequences to position +131 (pML131) was transcribed about 5- to 10-fold more efficiently in 20 h p.i. wt extracts than in 20 h p.i. dl extracts, while the recombinant lacking the sequences between positions +131 and +33 (pML33) was transcribed at nearly the same level in both extracts, as was a control rabbit β-globin template (pG). These results confirmed the presence within the MLP +33 to +131 fragment of an element selectively activated by 20 h p.i. wt extracts. Deletion of sequences composing the DE2 element, as in pML106, did not significantly reduce this activation, indicating that the DE2 region is dispensable for this effect. By contrast, deletion of the DE1 element, as in pML80, reduced the stimulation to levels similar to that observed with pML33, which lacks all se-

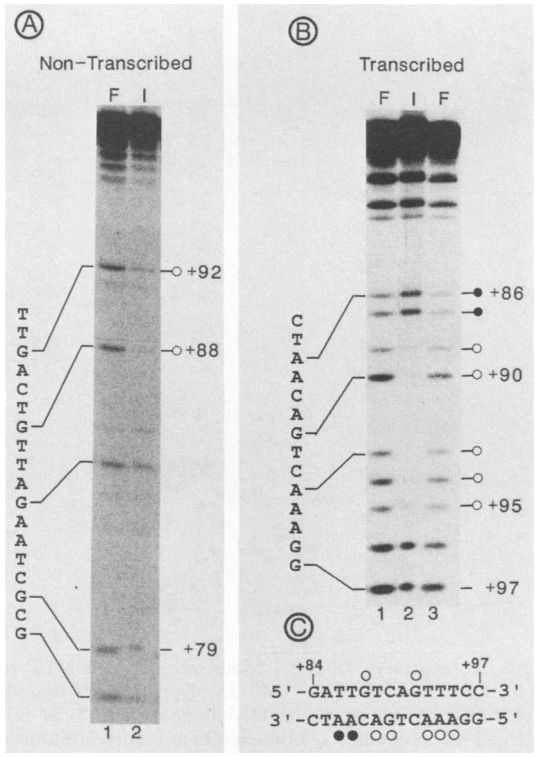

FIG. 3. DMS interference mapping of nucleoprotein complex I. Free probe (F) and complex I (I) were excised from preparative retardation assays (performed as described in Materials and Methods) with the DMS-treated oligonucleotide DE1 5′ end labeled at position +75 (nontranscribed strand) (A) or +104 (transcribed strand) (B). The DNA was recovered and, after chemical cleavage at methylated G and A residues, analyzed by gel electrophoresis. Residues whose methylation interferes with complex formation are denoted by open circles, and those whose methylation facilitates complex formation are shown by closed circles. Nucleotide coordinates relative to the MLP start site are given on the right, with the corresponding sequence on the left. The DMS interference pattern is summarized on the relevant sequence fragment in panel C.

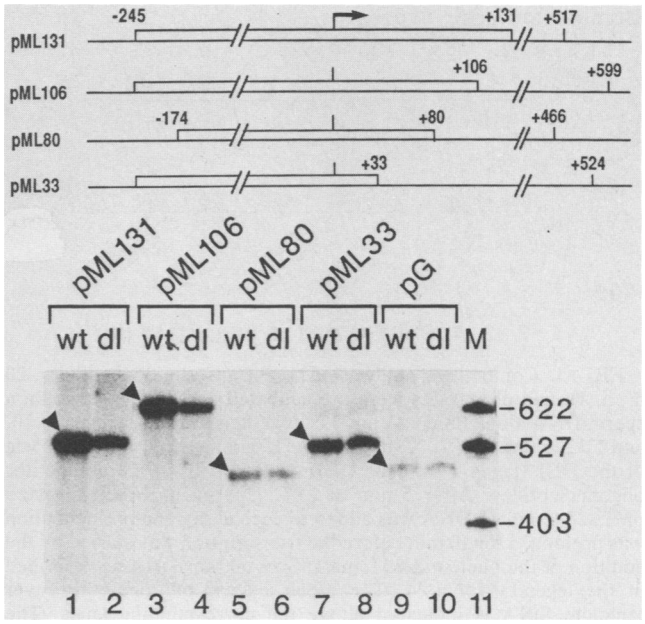

FIG. 4. Mutational analysis of MLP activity in 20 h p.i. cell extracts. A schematic representation of the various templates tested, with open boxes representing the MLP sequences retained in each recombinant (see Materials and Methods), is shown on the top. Coordinates relative to the MLP start site (arrow at +1) indicate the limits of the MLP sequences or refer to the positions of the restriction sites used to generate the runoff templates. In vitro transcription (shown below) was done as described in Materials and Methods with 20 h p.i. wt extracts (wt) or dl extracts (dl) in the presence of 200 ng of *Hinc*II-digested pML106 (lanes 1 and 2) or pML33 (lanes 3 and 4), or *Ssp*I-digested pML131 (lanes 5 and 6) or pML80 (lanes 7 and 8), or *Bam*HI-digested pG (lanes 9 and 10). The arrowheads point to the specific runoff transcripts in each lane pair (599, 524, 517, 466, and 475 nucleotides, respectively). Lane 11, *Msp*I-digested pBR322 DNA used as a size marker, with nucleotide lengths given on the right.

quences downstream of +33. This drop was not related to the absence of the MLP sequences between positions −245 and −174 (see top of Fig. 4), since we have previously shown (21) that sequences located upstream of position −137 were dispensable for the MLP activation in wt extracts. Furthermore, the lower overall template efficiency of pML80 in both wt and dl extracts was not systematically observed with other DNA preparations of the same plasmid. Altogether, these results indicated that the element which mediates this transcriptional stimulation must be located between +106 and +80, in agreement with the conclusion that region DE1, defined by the binding experiments, corresponds to this element.

To establish more directly that binding of DEF to its recognition site is required for maximal promoter activity in 20 h p.i. wt extracts, we performed competition experiments in which this protein was specifically titrated from the cell extracts. To this end, 20 h p.i. wt and dl extracts were preincubated with increasing amounts of oligonucleotides corresponding either to the natural DE1 sequence or to a mutated sequence (DE1mut, containing two nucleotide alterations selected on the basis of the DMS interference pattern [Fig. 1 and 3]) before in vitro transcription of the pML128 template was allowed to proceed (Fig. 5). Clearly, whereas the DE1mut oligonucleotide had no significant effect on the transcriptional stimulation exerted by the wt extracts (lanes 10 to 15), the wild-type DE1 oligonucleotide progressively diminished this stimulation (lanes 2 to 9) without affecting the transcriptional activity detected with 20 h p.i. dl extracts.

In a similar experiment, aimed at monitoring changes in the footprint pattern during DEF titration, we verified that the DE1 oligonucleotide specifically competes for the DEF protein and does not disrupt other nucleoprotein complexes essential for MLP activity. Figure 6A shows a DNase I footprint analysis of extracts preincubated with the DE1 oligonucleotides. While the DE1mut oligonucleotide had no effect on the protection patterns generated by both the 20 h p.i. dl and wt extracts (lanes 2 and 4), the wild-type DE1 oligonucleotide competed for the DE1-specific protection observed with the wt extract (compare lanes 3 and 4). It did not, however, affect the binding observed over the upstream promoter element (UE region; see references 7, 33, 38). Altogether, these observations confirmed that the binding of DEF to the MLP downstream region is required for the transcriptional stimulation observed in wt extracts. The DE1 oligonucleotide also reduced the usually weaker interaction occurring between +113 and +120, suggesting that the DEF protein interacts with both DE1 and DE2 sites (see Discussion).

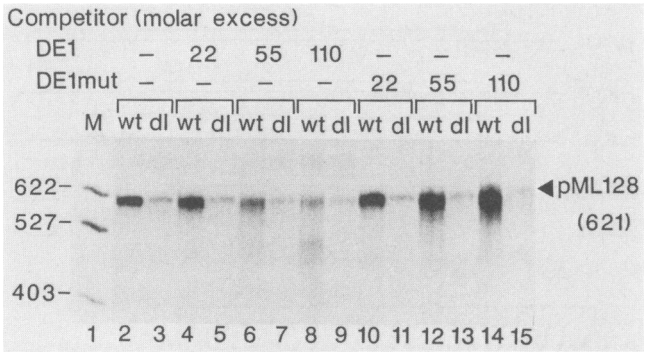

FIG. 5. Competition analysis of MLP transcriptional activity. 20 h p.i. wt and dl extracts were preincubated with sonicated salmon sperm DNA alone (lanes 2 and 3) or together with 30 (lanes 4, 5, 10, and 11), 75 (lanes 6, 7, 12, and 13), or 150 (lanes 8, 9, 14, and 15) ng of the DE1 (lanes 4 to 9) or DE1mut (lanes 10 to 15) competitor oligonucleotides. After 5 min at 25°C, 200 ng of *Hin*cII-digested pML128 template DNA was added to each assay and preincubation was prolonged for 10 min before the transcription was started by the addition of the nucleotides. Lane 1, Size marker DNA as described in the legend to Fig. 4. The molar excess of competitor over template DNA is indicated above the corresponding lanes. The specific runoff transcript (621 nucleotides) is marked.

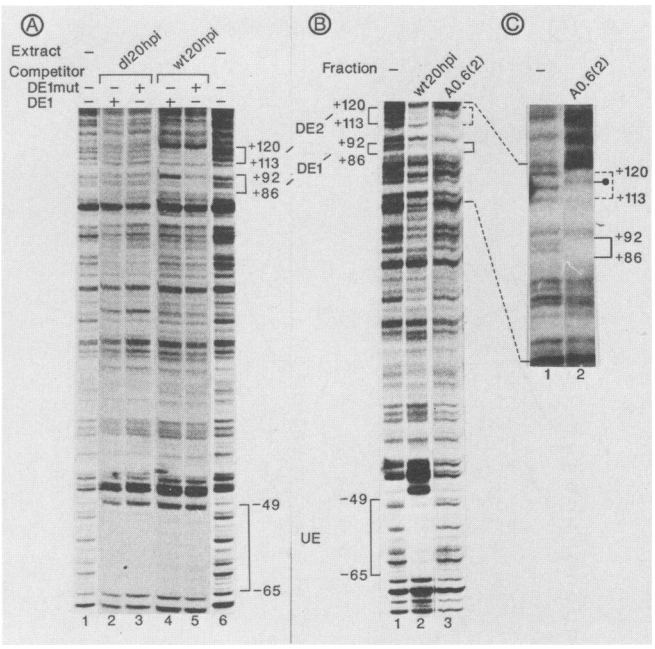

FIG. 6. Comparative DNase I footprinting of the MLP region. (A) 20 h p.i. dl and wt extracts (40 μg of protein per assay) were preincubated with 100 ng of the DE1 (lanes 1 and 3) or DE1mut (lanes 2 and 4) competitor oligonucleotides before addition of the probe fragment and DNase I digestion (see Materials and Methods). The positions relative to the MLP start site of the upstream element (UE) and DE1 and DE2 regions are indicated next to lane 5 (naked probe digestion pattern). (B) The footprints of 20 h p.i. wt extract (40 μg of protein, lane 2) or fraction A0.6(2) (3 μl, lane 3) are compared. The protected regions apparent in lanes 2 and 3 are bracketed on the left and right, respectively, compared with the naked probe digestion pattern (lane 1). The dashed bracket refers to a partial protection over DE2. (C) The protection pattern of fraction A0.6(2), from an experiment similar to that shown in panel B, is presented, slightly enlarged. The closed circle refers to DNase I-hypersensitive sites. Other symbols are as in panel B.

Molecular characterization of DEF protein. To further characterize the DEF activity in the absence of major contaminating DNA-binding proteins, we undertook a partial purification of this protein. A 20 h p.i. wt extract was fractionated as outlined in Materials and Methods by chromatography over heparin-Ultrogel and DEAE 5PW and two successive chromatographies over a specific DNA-Sepharose column. The DEF-binding activity observed by the electrophoretic band-shift assay was eluted at 0.6 M KCl from the heparin column, at 0.25 M KCl from the DEAE 5PW column (fraction D0.25), and at 0.6 M KCl from the affinity columns [fractions A0.6(1) and A0.6(2)].

A gel-shift analysis of the various chromatography fractions is presented in Fig. 7A, showing that fractions D0.25 and A0.6 produced one major complex indistinguishable from complex I. That this complex indeed corresponds to the binding of DEF was demonstrated by its selective titration with the wild-type DE1 oligonucleotide (Fig. 7B, lanes 1 to 4) and the lack of competition by the DE1mut oligonucleotide (lanes 5 to 7). In this experiment, the relatedness of DEF to the PEA2 and AP1 binding activities was also examined. The weak but significant competition observed with oligonucleotides comprising the corresponding binding sites (lanes 8 to 11) may reflect the strong sequence homology between these sites and the DEF-binding site (see legend to Fig. 7) but rules out the direct correspondence of DEF to any of these factors (see Discussion).

A comparative DNase I protection analysis of the crude 20 h p.i. wt extract and the A0.6(2) fraction is shown in Fig. 6B. The affinity-purified DEF, which was separated from the component generating the UE protection, produced the expected DEF-specific footprint over the DE1 element. Strikingly, the purified fraction also interacted with the DE2 element but generated at this position a protection pattern different from that obtained with the original cell extract (Fig. 6C).

As revealed by SDS polyacrylamide gel electrophoresis of the D0.25 and A0.6 fractions, a number of prominent polypeptides with a wide size distribution were recovered in the purest fraction (data not shown). In an attempt to identify the polypeptide(s) responsible for DE1-binding activity, the proteins of fraction D0.25 were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Blots were incubated with either the specific DE1 probe or an AP1 oligonucleotide used as nonspecific probe (Fig. 8A). While each probe bound with roughly equal affinities to two bands of high molecular mass (>100 kDa), the DE1 probe specifically lighted up a protein of about 40 kDa (lane 3), suggesting that the DEF-specific activity is associated with this single polypeptide. The nature of the high-molecular-weight material which was revealed by both probes (Fig. 8A) and not detected in uninfected cell extracts (P. Jansen-Durr, unpublished observation) has not been further investigated.

In an alternative approach to visualize the DEF protein, a preparative gel retardation experiment was performed with the D0.25 fraction, using a bromodeoxyuridine-substituted DE1 probe. After UV irradiation of the gel, the specific complex was electroeluted and analyzed by SDS-polyacrylamide gel electrophoresis. A single DNA-protein adduct with an apparent molecular mass of about 40 kDa was revealed (Fig. 8B). This finding, together with the results of the Southwestern analysis, clearly indicated that DEF corresponds to a protein of approximately 40 kDa.

DEF activity induced by DNA replication in the absence of

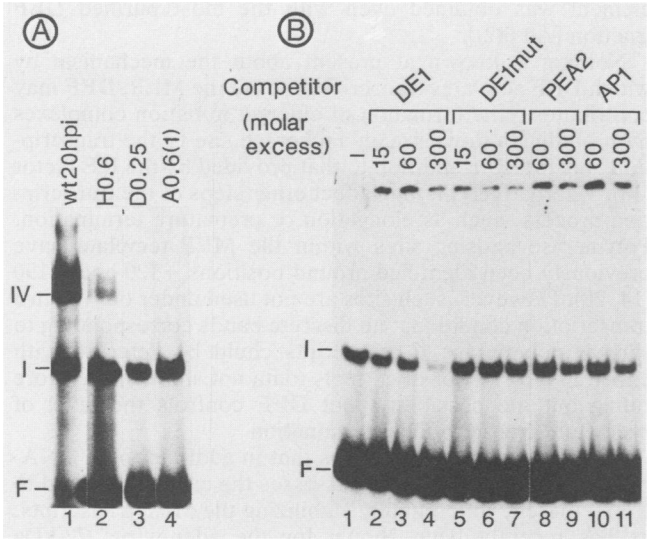

FIG. 7. Gel-shift analysis of the partially purified DEF fractions. (A) Gel retardation assays were performed with 0.5 μl (3 μg of protein) of 20 h p.i. wt extract and 1 μl (1.5 μg of protein) of H0.6, 1 μl (1 μg of protein) of D0.25, and 1 μl (equivalent to 3 μl of D0.25) of A0.6(1) fractions, as indicated, in the presence of 1, 1, 0.5, and 0.3 μg of poly(dI-dC) in lanes 1 to 4, respectively. Bands corresponding to free probe (F) and to nucleoprotein complexes I and IV are indicated. (B) The D0.25 fraction (1 μl) was used in a standard retardation assay (lane 1) or preincubated with unlabeled competitor oligonucleotides at the indicated molar excesses (lanes 2 to 11). The DE1 and DE1mut oligonucleotides were as indicated in Fig. 1. The PEA2 oligonucleotide corresponded to the double-stranded sequence (5′-TCGACTGCGGTCAGTTACTTACTTCC-3′) spanning the polyomavirus PEA2-binding site (34, 49). The AP1 oligonucleotide corresponded to the double-stranded sequence (5′-AGCTT GATTAGTCAGCCG-3′) spanning the AP1-binding site of simian virus 40 (3). Nucleotides homologous to the DEF-binding site are underlined.

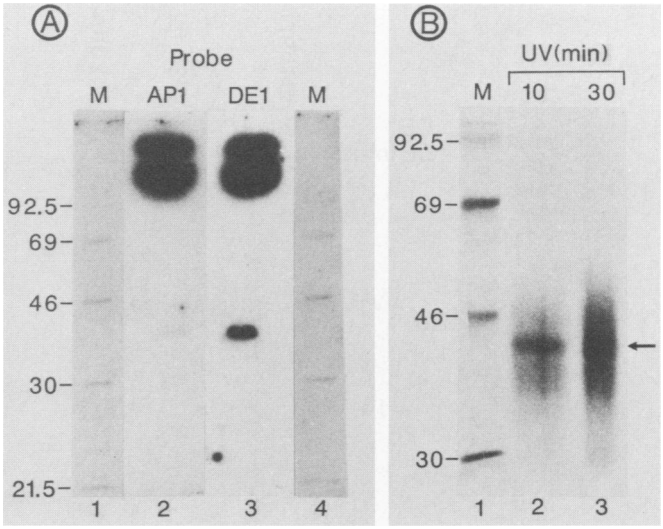

FIG. 8. Molecular characterization of DEF. (A) Southwestern analysis of the D0.25 fraction probed with the multimerized AP1 (lane 2) and DE1 (lane 3) oligonucleotides as described in Materials and Methods. Lanes 1 and 4, ^{14}C-labeled protein size markers (kilodaltons). (B) UV cross-linking analysis of complex I excised from a preparative retardation assay (equivalent to the assay shown in panel 8A, lane 3, but scaled up 20-fold) was performed as described in Materials and Methods. UV irradiation was for 10 min (lane 2) or 30 min (lane 3). Lane 1, Protein size markers as in panel A. The arrow points to the 40-kDa protein-DNA adduct.

E1a expression. Since replication-blocked wt extracts failed to produce the DEF-specific complex (complex I in Fig. 2) and did not induce the transcriptional stimulation of the MLP template (21), we concluded that replication of the viral DNA in the infected cells before extract preparation is necessary for DEF activation. To examine whether in addition to DNA replication viral E1a expression is also required, we prepared extracts from HeLa cells infected with *dl*312 but collected at 48 h p.i. (48 h p.i. dl extracts). At this time p.i., despite the absence of the E1a proteins, viral DNA undergoes replication but with a roughly seven-fold-reduced overall efficiency compared with wt-infected cells at 20 h p.i., as deduced from thymidine incorporation measurements (P. Jansen-Durr, unpublished data). Furthermore, it has been previously shown that MLP transcripts become readily detectable in dl-infected cells at 48 h p.i. (1; unpublished observation). A comparative analysis of the gel retardation pattern produced by the 20 h p.i. wt (replication- and E1a-positive), the 48 h p.i. dl (replication-positive and E1a-negative), and the 20 h p.i. dl (replication- and E1a-negative) extracts is shown in Fig. 9. The DEF-specific complex I, which was barely visible with 20 h p.i. dl extracts (lanes 4 to 6), was detected in the presence of 48 h p.i. dl extracts (lanes 1 to 3), although the amount of this complex remained about 10-fold lower than that with 20 h p.i. wt extracts (lane 7). Similarly, when comparing the transcriptional activities of these extracts, we detected a low but significant stimulation of MLP transcription in the presence of the 48 h p.i. dl extract (data not shown). These results are therefore consistent with the conclusion that the DEF binding activity is induced to significant levels by viral DNA replication alone. However, full activation may require high rates of replication which could be reached only in the presence of the E1a gene products.

DISCUSSION

We have previously demonstrated the implication of a MLP sequence element located between +33 and +131 in the replication-dependent stimulation of the transcription from this promoter (21). We now establish the direct correlation between this transcriptional stimulation and the binding of a cellular protein, DEF, to a sequence element located between positions +80 and +106 of the MLP downstream region. Deletion of this element drastically reduces the specific stimulation. Although the DEF-binding site resembles that of other transcription factors, our competition experiments clearly indicated that DEF is a distinct entity. As deduced from Southwestern and UV cross-linking experiments, the DEF binding activity corresponds to a protein of approximately 40 kDa. Finally, activation of this factor, whose activity is barely detectable in noninfected or dl-infected cells, requires components induced upon viral DNA replication but is not directly achieved by the E1a products themselves.

DEF is a DNA-binding transcriptional activator. That the DEF protein, as identified by gel retardation and DNase I footprinting, is a specific transcriptional activator is demonstrated by the following complementary observations. (i) Analysis of external deletion mutants mapped the 3′ border of a downstream sequence element essential for maximal in vitro MLP activity between positions +80 and +106. (ii)

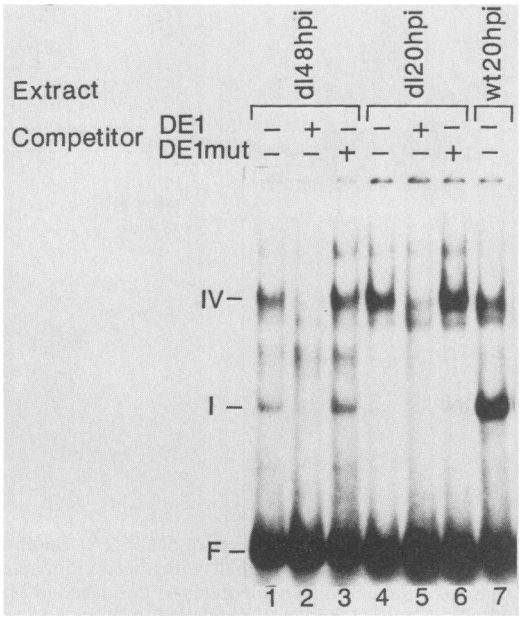

FIG. 9. Comparative DNA binding analysis of 20 h p.i. and 48 h p.i. extracts. Standard gel retardation assays were performed with 0.5 μl (3 μg of protein) of each extract: 48 h p.i. dl extract (lanes 1 to 3), 20 h p.i. dl extract (lanes 4 to 6), and 20 h p.i. wt extract (lane 7). Where indicated (+), the extract was preincubated with 100 ng of the DE1 or DE1mut competitor oligonucleotides. The positions of complexes I and IV are indicated. F, Free probe.

DNase I protection experiments delineated a protein-binding site between +86 and +95, occupied only in the presence of the 20 h p.i. wt cell extracts which exhibit the transcriptional activation. (iii) Specific depletion of 20 h p.i. wt extracts for DEF protein by oligonucleotide-mediated competition simultaneously abolished the appearance of the +86 to +92 (DE1) footprint and the transcriptional stimulation, while the mutated binding site had no effect on either of them. Conversely, the transcriptional activity of 20 h p.i. dl extracts, in which DEF binding activity is barely detectable, was not affected by titration with the same competitor oligonucleotides. (iv) Finally, the transcription activities of the DEF-depleted extracts on MLP templates retaining the DEF-binding site (pML128, Fig. 5) were similar to the activities of nondepleted extracts on templates lacking the DEF-binding site (pML33 or pML80, Fig. 4); this observation indicates that DEF binding may account for most if not all of the effect of the downstream element (DE1).

A weaker binding site (DE2, between +113 and +120) was detected next to the DE1 site by DNase I footprinting of 20 h p.i. wt extracts. Strikingly, both DE1 and DE2 protections were simultaneously relieved by competition with an oligonucleotide spanning only the DE1 site. If the DE2 protection is due to the interaction of the MLP sequences with an additional protein, then the binding of this protein would depend on the occupancy of the DE1 site. Alternatively, the binding of DEF to its own recognition site could induce protection of adjacent sequences from DNase I digestion. This latter hypothesis is actually supported by the following observations: deletion of the DE2 element (as in pML106) had no significant effect on MLP activity; competition with an oligonucleotide comprising only the DE2 sequences did not affect the footprint pattern obtained with 20 h p.i. wt extracts (data not shown); some protection over the DE2 element was obtained even with the most purified DEF fraction [A0.6(2)].

Nothing is known at present about the mechanism by which DEF activates transcription from the MLP. DEF may contribute to the formation of efficient initiation complexes by providing a downstream anchorage site to the transcription apparatus, in addition to that provided by the UE factor (38). Alternatively, it may affect other steps of the transcription process, such as elongation or premature termination. Polymerase pausing sites within the MLP template have previously been identified around positions +120 and +190 (14, 28). However, such sites are not used under our in vitro transcription conditions; no discrete bands corresponding to shorter polymerase II transcripts could be detected with either 20 h p.i. wt or dl extracts (data not shown), therefore ruling out the possibility that DEF controls the level of premature transcriptional termination.

Finally, the possibility exists that in addition to its DNA-binding activity, DEF also possesses the capacity to bind to RNA, thereby differentially stabilizing the MLP transcripts, as has recently been shown for the adenovirus 72-kDa DNA-binding protein (39). This seems unlikely, however, since we could not detect any difference in the half-life of the specific RNA when measured in the presence or absence of DEF. In agreement with this conclusion, DEF did not bind to single-stranded oligonucleotides corresponding to DE1 (P. Jansen-Durr, unpublished observations).

DEF is activated during viral DNA replication. Activation of the DEF protein was readily visualized by the gel retardation assay, which revealed a striking increase of the DNA-binding activity of this protein in wt-infected cells compared with dl-infected cells at 20 h p.i. (Fig. 9). Our observation that in some extract preparations from noninfected cells a low level of DEF binding activity could be detected (data not shown) suggests that DEF is not a virus-encoded factor but a host-cell protein whose concentration or binding constant (or both) are increased in wt-infected cells.

A weak but significant activation of DEF also occurs in cells in which adenovirus DNA replication has been allowed to proceed in the absence of the E1a gene products, as in dl-infected cells collected at 48 h p.i. (Fig. 9). On the other hand, extracts from wt-infected cells expressing normal amounts of E1a but in which DNA replication was blocked by the presence of araC exhibited very poor DEF binding (Fig. 2) or transcriptional activity (21). It appears, therefore, that the strong DEF activation seen in 20 h p.i. wt extracts is not directly achieved by the E1a products themselves but rather by diffusible factors induced upon viral DNA replication. However, since the efficiency of viral DNA replication is linked to the extent of early gene expression, the E1a proteins do contribute indirectly to the DEF induction process by raising the level of this expression. We cannot exclude the formal possibility that, given a certain level of viral DNA replication, E1a exerts a direct effect on DEF activation as well. Mansour et al. (31), using a transient expression assay of MLP-containing plasmids, have previously shown that replication of the transfected recombinants was not sufficient to induce any stimulation of MLP activity but that adenovirus infection was required in addition. This observation, which stresses the essential role of viral DNA replication, further supports the involvement of some *trans*-acting factor(s) encoded or induced by the adenovirus.

Involvement of DEF in transcription of other adenovirus genes. In addition to the major late transcription unit, two other adenovirus genes, those encoding polypeptides IX and

IVa2, are turned on after viral DNA replication has started (11, 32, 47, 48), therefore representing potential targets for the DEF protein. Sequences with significant homologies (underlined below) with the MLP DEF-binding site are found downstream of the RNA initiation site of these genes (+52-ATTGTGAGCT-+61 for the IX gene and +63-GTCT GTAT-+70 and +85-TGTCCGT-+79 for the IVa2 gene). It will be of interest to examine whether the expression of these viral late genes involves a common control mechanism.

Relationship of DEF with other transcription factors. DMS interference analysis of the DEF-binding site identified an essential decanucleotide, 5'-TTGTCAGTTT-3', within the DE1 region. This motif shares striking homologies with a series of viral sequence elements comprised within the simian virus 40, polyomavirus, hepatitis B virus, and cytomegalovirus enhancers (50). When oligonucleotides corresponding to these sequences were tested for their ability to compete for the specific binding of DEF in the gel retardation assay, only the AP1- and PEA2-binding sites displayed some competition activity (Fig. 8B; data not shown). While excluding the identity of DEF with these factors, this observation raises the possibility that DEF belongs to a set of related *trans*-acting factors. In keeping with this conclusion, it may be worth recalling the recent finding of Sharma et al. (40) that a 43-kDa protein involved in transcription of the hamster histone H3.2 gene and recognizing a sequence distinct from but homologous to the AP1-binding site is immunologically related to the AP1 protein.

In addition to DEF, we detected by the gel retardation assay another protein (corresponding to complex IV) which specifically binds to the DE1 oligonucleotide. However, since this protein was found at roughly equal amounts in both dl- and wt-infected cell extracts, its direct involvement in the observed transcriptional activation of the MLP is unlikely. In fact, its titration by competitor oligonucleotides did not significantly affect the basal level of in vitro transcription (Fig. 5). Furthermore, a protein producing complex IV was also detected in uninfected HeLa cells as well as in other cell lines (data not shown). This protein is, however, most likely distinct from the factors previously identified in uninfected cells by Reinberg et al. (36) and Cohen et al. (9). These factors also bind to sequences located downstream of the MLP start site, but in contrast to the complex IV protein, they are required for optimal MLP activity.

The interesting possibility exists that the complex IV protein could correspond to an inactive form of DEF which has retained its specific DNA-binding capacity but not its transcription-activating competence. It is then tempting to speculate that this protein is activated by some unknown mechanism during viral DNA replication. Precedents for the existence of such inactive forms of transcription factors have been documented for hormone receptors (13, 37) and the immunoglobulin kappa enhancer binding protein (4). DNA binding and subcellular mobilization of these factors are activated by specific hormone binding or phorbol ester treatment, respectively, which results in the dissociation of inhibiting protein moieties from the inactive factors. It will be of interest to explore the possibility that the complex IV protein corresponds to a similar type of association between DEF and an inhibitory protein.

Alternatively, DEF may correspond to a bifunctional replication-transcription factor which would be actively recruited for viral DNA replication and simultaneously activated for late gene transcription. The host-coded nuclear factors I and III (NF-I and NF-III), which are involved in both adenovirus DNA replication and cellular and viral promoter function (through their CCAAT box and octamer motif binding activity), constitute representative examples of such bifunctional proteins (22, 35, 43). Although DEF is likely not identical to these nuclear factors, based on their DNA recognition sequences, the characterization of their molecular and genetic structures will be required to establish whether or not these factors are related.

ACKNOWLEDGMENTS

We thank C. Hauss for excellent daily technical assistance, J. L. Weickert for large-scale cell culture, A. Staub and F. Ruffenach for synthesis of oligonucleotides, and M. Chipoulet and J. M. Egly for help and advice in protein purification. We thank H. Boeuf and I. Davidson for critical reading of this report and the entire secretarial staff for manuscript preparation.

This work was supported by grants from CNRS, INSERM, and the Association pour la Recherche sur le Cancer. P.J.-D. was supported by the Deutsche Forschungsgemeinschaft.

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