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The Product of the Adenovirus Intermediate Gene IVa2 Is a Transcriptional Activator of the Major Late Promoter

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During the course of lytic infection, the adenovirus major late promoter (MLP) is induced to high levels after replication of viral DNA has started. We had previously shown that sequence elements located downstream of the MLP start site were implicated in this late-specific transcriptional activation (DE1, between +85 and +98; DE2, between +100 and +120). Two positive transcription factors involved in this activation have been detected. DEF-A, which specifically binds to DE1 and also to the 3′ portion of DE2 (DE2a), and DEF-B, which interacts with the 5′ part of DE2 (DE2b). When present together, these two proteins cooperatively assemble onto the DE2 element. We now report the purification of DEF-B and show that it is identical to the product of the adenovirus IVa2 gene product. This conclusion is based on microsequence analysis of DEF-B as well as on the inhibitory effect of antibodies against IVa2 on the DNA-binding activity of DEF-B and also on DE-dependent in vitro transcription. In addition, we show that bacterially synthesized IVa2 protein binds to the DE sequences with the same specificity as DEF-B. Finally, in transfected cells, a recombinant IVa2 protein stimulates MLP activity in a DE-dependent fashion. The physiological implications of these findings are discussed.

The adenovirus infection cycle constitutes an attractive model system for the study of temporal control of initiation at different transcription units, including the early-to-late switch of the viral expression program. Lytic infection has been divided into four stages of gene expression: pre-early, early, intermediate, and late (5, 20, 42). Pre-early and early genes are transcribed before the onset of viral DNA synthesis, which starts at 6 to 8 h postinfection. Although the promoter for the major late transcription unit (MLP) is active at early times, only transcripts corresponding to the promoter-proximal set (L1) of late messengers are produced (43, 36). Full expression of the late genes requires DNA replication. The expression of intermediate genes like IVa2 and IX begins at about the same time as DNA synthesis, with peak levels of these transcripts being found after DNA replication (6, 11, 25, 45). The MLP from adenovirus types 2 and 5 (Ad2 and Ad5) has been most extensively studied as a model for eucaryotic transcription (for reviews see references 4 and 27). Basal transcription from this promoter depends on a set of well-defined promoter elements: a properly positioned TATA box, an upstream element (UE) located between positions −67 and −49 relative to the MLP transcriptional start site, a CAAT box between −80 and −76, an initiator element spanning the start site, and additional elements located beyond position +140 (10, 17, 21, 29, 31, 39, 40, 44, 47).

The activity of the MLP is strongly increased after the onset of viral DNA replication (43). Sequence elements located downstream of the MLP start site have been identified and shown to be essential in vivo for promoter activation (3, 24, 28, 29). DNase I protection and dimethyl sulfate interference mapping experiments have delineated two main downstream sequence elements (DE1 and DE2), located between +85 and +98 (DE1) and +100 and +120 (DE2). These elements specifically bind proteins that are detected only in adenovirus-infected cells in the late phase of infection. The binding of these proteins correlates with the transcriptional activation of the MLP, as measured by cell-free transcription (18, 19) or during infection with recombinant viruses (24, 29). 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 (32).

The presence of two virally induced factors, DEF-A and DEF-B, correlates with late-phase MLP stimulation. DEF-A binds specifically to DE1 and also, with lower affinity, to the 3′ portion of DE2 (DE2a). DEF-B interacts with the 5′ part of the DE2 element (DE2b). When added together, DEF-A and DEF-B were found to cooperatively assemble onto the DE2 element as a complex that was more stable than that formed by each protein alone (19, 33).

Here we report the isolation and purification of DEF-B by fractionation of late-infected cell extracts through a series of ionic exchange and hydrophobic chromatographic steps. Peptide sequence analysis of proteolytic cleavage products of the purified protein which retained DEF-B-specific band shift activity revealed sequence identities with the product of the adenovirus intermediate gene IVa2. Several experimental approaches, including immunological reactions, DNA-binding assays, as well as in vitro and in vivo transcription tests established the regulatory function of the viral IVa2 protein in the late-phase transcription and confirmed its identity to the DEF-B factor.

MATERIALS AND METHODS

Cell extracts and protein fractionation. HeLa cells, grown at a density of 10^6 cells per ml in Eagle medium supplemented with 5% calf serum, were infected with Ad5 (wild type) or its E1a-defective derivative *dl*312 at 10 PFU per cell. Whole-cell extracts (WCE) were prepared 20 h after infection with the

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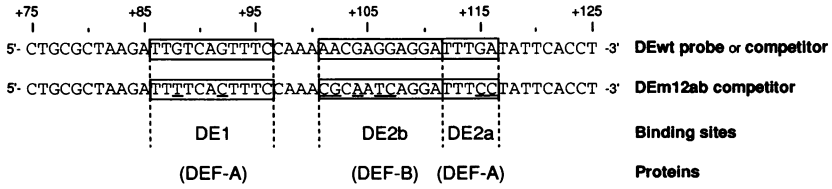

FIG. 1. Nucleotide sequence of the MLP DE. The sequences and coordinates (with respect to the MLP start site at +1) of the nontranscribed strands of the oligonucleotides used as the probe (DEwt) or competitors (DEwt and DEm12ab) in electrophoretic band shift assays are shown. Competitor oligonucleotides were either the same as the probe oligonucleotide but unlabeled (DEwt competitor) or with nucleotide alterations (underlined) within elements DE1, DE2a, and DE2b (33) that impair specific complex formation (DEm12ab). The boxed DE elements correspond to the minimal binding sites (DE1, DE2a, and DE2b) originally deduced from dimethyl sulfate interference mapping of specific complexes with the DEF-A and DEF-B proteins (33).

wild type (wt20hpi WCE) or *dl*312 (*dl*20hpi WCE) as described previously (32).

An extract from a 56-liter culture of wt20hpi cells (300 ml at 7 mg of protein per ml) was applied to a 250-ml heparin-Ultrogel (IBF) column equilibrated in buffer A (10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing 0.1 M KCl. After the extract was washed with 3 column volumes of buffer A containing 0.1 M KCl, proteins were recovered by successive elutions with buffer A containing 0.25, 0.6, and 1 M KCl. The eluted fractions were dialyzed against buffer A, tested for the presence of DEF-B activity by an electrophoretic band shift assay (see below), and stored at −80°C before use. The 0.6 M eluate (240 ml), which contained the DEF-B activity, was precipitated with 50% ammonium sulfate (AS). After centrifugation, the pellet was resuspended in buffer A (H0.6 fraction; 160-ml final volume, 1 mg/ml) and applied onto a 200-ml DEAE-Spherodex (IBF) column equilibrated in buffer A containing 0.1 M KCl. The flowthrough fractions which contained the DEF-B activity (DEFT; 170 ml at 250 μg/ml) were pooled and directly loaded onto a 20-ml Sulfopropyl-Spherodex (IBF) column equilibrated in buffer A containing 0.1 M KCl. The DEF-B activity was recovered in the 0.2 M KCl eluate (SP0.2 fraction; 80 ml at 50 μg/ml). After addition of AS to a final concentration of 0.9 M, the SP0.2 fraction was applied onto a 10-ml phenyl-Sepharose (Pharmacia) column equilibrated in buffer B (25 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol) containing 0.9 M AS. The proteins were eluted stepwise in buffer B with decreasing AS concentrations, and the DEF-B activity was found in the low-pressure 0 M AS eluate [P(LP)0 fraction; 70 ml at 0.4 μg/ml]. This fraction was adjusted to 0.9 M AS and loaded onto a 3-ml TSK-phenyl-5PW high-pressure liquid chromatography (HPLC) column (Toso-Haas) equilibrated in buffer B containing 0.9 M AS. The proteins were eluted with a 35-ml linear AS gradient (0.9 to 0 M). The DEF-B activity was recovered in five fractions of 900 μl each at an AS concentration of about 0.25 M [P(HP {high pressure})0.25 fraction; about 6 μg/ml].

Electrophoretic band shift assays. Gel retardation assays were performed as previously described (33). Approximately 0.5 ng (30,000 cpm) of a ^{32}P-5′-end-labeled, synthetic double-stranded oligonucleotide, corresponding to the DE12 element (DEwt probe; Fig. 1), was incubated with WCE or the chromatographic fractions, in the presence of appropriate amounts of poly(dI-dC) as a nonspecific competitor. After 15 min at 25°C, the complexes were separated by nondenaturing 4.5% polyacrylamide gel electrophoresis (acrylamide/bisacrylamide, 80:1) at 4°C. Retardation competition experiments were carried out by preincubating the extracts with poly(dI-dC) and unlabeled competitor oligonucleotides (DEwt or DEm12ab; Fig. 1) for 15 min at 25°C before addition of the probe and further incubation (see above).

Protein sequencing. Between 5 and 10 μg of protein corresponding to the P(HP)0.25 fraction was electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and stained with Coomassie blue. The major band (55 kDa) was excised, cut into small pieces, and vacuum dried (Speedvac). In-gel proteolytic cleavage was performed by addition of trypsin (2 μg) in 0.3 ml of 0.2 M ammonium carbonate (pH 8.9). After 4 h at 30°C, the peptides were eluted from the gel fragments by two 20-min incubation periods in 0.4 ml of a mixture of 60% acetonitrile–0.1% trifluoroacetic acid (41). The eluates were pooled, lyophilized, and resuspended in 20 μl of 0.1% trifluoroacetic acid, and the peptides were fractionated by HPLC. Selected peptide peaks were collected and subjected to automatic Edman degradation sequencing (Applied Biosystems model 477/A sequencer).

Antibodies and immunoblot procedure. Polyclonal antibodies were raised against synthetic peptides derived from N-terminal (Pn; residues 25 to 42) and C-terminal (Pc; residues 428 to 443) portions of the IVa2 protein (Pn, RAARSAPL-HRDPDYADEDC; Pc, CIHRTLNDRDRWSRAYR [see Fig. 5]), with an additional Cys residue at the C or N terminus, respectively. The peptides were coupled to ovalbumin (16) before subcutaneous injection into rabbits. Antibodies were purified from positive sera by successive caprylic acid and AS precipitations (16) and resuspended in phosphate-buffered saline at a protein concentration of 3 mg/ml. Antibodies against bacterial glutathione *S*-transferase (GST) and against the F domain of the human estrogen receptor were gifts from H. Boeuf and D. Metzger, respectively. Immunoblot analysis of SDS-gel-separated proteins was performed as previously described (7).

Recombinant IVa2 protein production. The IVa2 cDNA was derived from the IVa2 genomic sequence of Ad2 (coordinates 5708 to 4084) by deletion of the intronic sequence (coordinates 5695 to 5418 [9]), using PCR-mediated mutagenesis of an Ad2-derived template. The primers used were oligonucleotides ending with appropriate restriction sites and spanning (i) positions 5708 to 5394, but lacking the intronic sequence, and (ii) positions 4084 to 4119, on the opposite strand. The amplified DNA fragment was cloned into a derivative of pGEX-3X (Pharmacia), in frame with the GST sequence located upstream and six His codons located downstream of the IVa2 sequence (gift of J. Acker). The structure of the resulting recombinant construct (pGST-IVa2-H6) was verified by nucleotide sequencing. An overnight starter culture (15 ml) of *Escherichia coli* NB42 transformed with pGST-IVa2-H6 was inoculated into 500 ml of culture medium and grown at 37°C to an optical density at 600 nm of about 1. After isopropylthiogalactopyranoside (IPTG) induction (0.5 mM, 2 h at 37°C), bacteria were collected and sonicated, and the recombinant IVa2 product, which was essentially produced as inclusion bodies, was solubilized in 6 M guanidinium-HCl–50 mM

Tris-HCl (pH 7.5) and purified by chromatography on a 1.5-ml column of metal chelate affinity agarose (Qiagen, Pharmacia). The recombinant IVa2 protein was eluted with 40 mM imidazole, with a recovery of about 1 mg of protein from a 1-liter culture.

In vitro run-off transcription. Transcription reactions were carried out under conditions previously described (32), adapted from a system originally developed by Leong and Berk (22). The DNA templates were obtained by PCR amplification of the MLP sequences between positions −137 and +314, either wild type (MLP DEwt) or deleted for the DE elements (MLP Δ12, lacking the DE1 and DE2 elements between +85 and +124) (32). Transcripts were analyzed by electrophoresis on 6% acrylamide–urea sequencing gels. All transcription assays were repeated at least three times with independent extract preparations.

In vivo expression assay. A reporter plasmid comprising the simian virus 40 replication origin and MLP sequences (MLP DEwt or MLP Δ12) between positions −246 and +131, inserted 5′ to the luciferase-coding sequence, was constructed as previously described (33). A IVa2 expression vector was constructed by inserting the Ad2 IVa2 cDNA sequence (see above) into the polylinker of the pAT3 vector (gift of M. Vigneron) located 3′ to the cytomegalovirus enhancer/thymidine kinase gene promoter and sequences encoding the F domain of the human estrogen receptor (2), so as to produce a IVa2 fusion protein tagged with the F epitope at its N terminus.

Transcriptional activity of the MLP-based reporters was assayed essentially by an infection-transfection protocol originally established by Mansour et al. (28). COS7 cells, grown in Dulbecco medium supplemented with 5% calf serum, were cotransfected with the IVa2 expression vector (100 ng) and the MLP-luciferase recombinants (5 μg), together with 10 μg of carrier DNA (pSP65 plasmid), by calcium phosphate coprecipitation in 9-cm petri dishes (17). Eighteen hours after transfection, the cells were infected with Ad5 or its E1a-defective derivative (*dl*312) at 20 PFU per cell. The cells were harvested either 6 or 20 h postinfection. Extracts were prepared and normalized by protein concentrations, and luciferase activity was measured as described previously (37). All transfection assays were repeated at least three times with independent template preparations. The values always agreed to within 20% from one experiment to the other.

RESULTS

Previous studies have demonstrated the induction of two proteins (DEF-A and DEF-B) during intermediate to late phases of adenovirus infection. These proteins contribute to the late-phase-dependent activation of the MLP by cooperatively binding to the MLP DE. Both proteins were recovered in wt20hpi WCE and could be separated in preliminary experiments by differential elution upon chromatography over Heparin-Ultrogel (33). The nature of either of these proteins was unknown. To identify the DEF-B protein, we set up a large-scale purification procedure (Fig. 2) starting from wt20hpi WCE to yield sufficient amounts of DEF-B to attempt partial sequencing of the purified protein.

Purification of DEF-B as a 55-kDa polypeptide. The purification of DEF-B was monitored through each chromatographic step by an electrophoretic band shift assay, using a probe that spans the entire DE region (see Materials and Methods). As shown in Fig. 3B (lane 1), three major retarded bands (a, b, and c) were observed with the wt20hpi WCE under the conditions used. The specificity of these complexes had

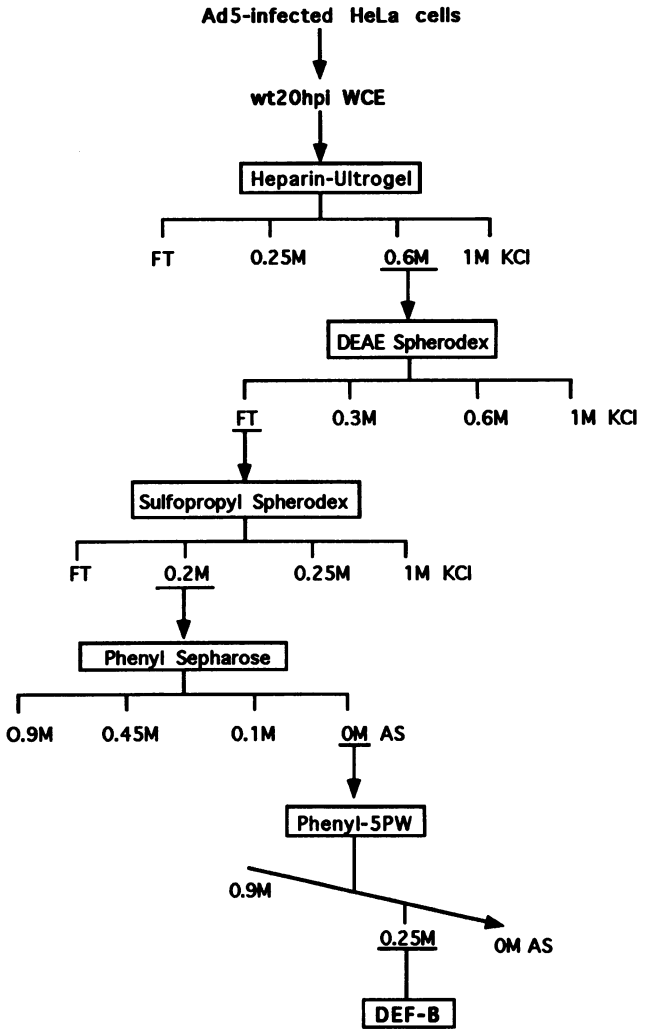

FIG. 2. Fractionation of wt20hpi WCE. WCE were prepared from Ad5-infected HeLa cells, collected 20 h postinfection, and fractionated over a series of columns (boxed) as described in Materials and Methods. Elutions were performed with increasing KCl concentrations in buffer A (first three columns) or decreasing AS concentrations in buffer B (last two columns), as indicated. DEF-B-containing fractions, as determined by electrophoretic band shift assays, are underlined. FT (flowthrough) corresponds to unadsorbed material.

been previously ascertained by competition and DMS dimethyl sulfate interference mapping experiments (33): complex a reflects the binding of DEF-A to the DE1 segment (+86 to +97) of the probe; complex b corresponds to the binding of DEF-B to the DE2b portion (+101 to +111) of the probe; complex c represents the simultaneous binding of both proteins to the DE2a and DE2b domains (+101 to +116) of the probe.

Analytical-scale purification tests revealed that DEF-A and DEF-B could be separated on DEAE-Spherodex columns. We therefore decided to coelute both activities from Heparin-Ultrogel (H0.6 fraction; Fig. 3B, lane 2) before separating them in the second step by chromatography over DEAE-Spherodex. DEF-B (complex b) was recovered in the DEFT fraction (Fig. 3B, lane 3), whereas DEF-A was retained on the column (not shown). The DEFT fraction was directly applied onto Sulfopropyl-Spherodex, where DEF-B was recovered in the 0.2 M eluate (SP0.2 fraction). Further purification of

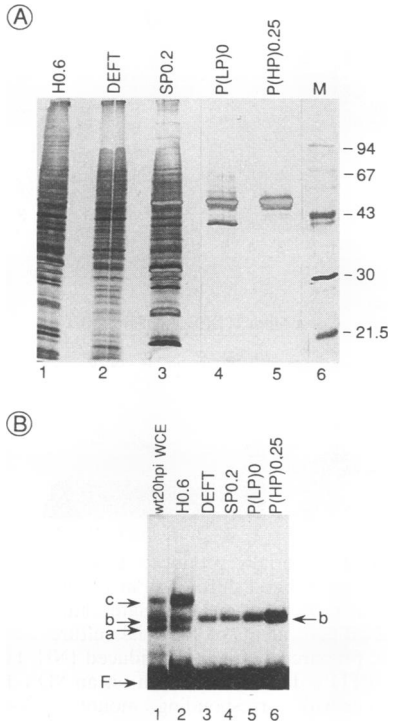

FIG. 3. Purification of DEF-B. (A) Protein analysis. Aliquots of the various DEF-B-containing chromatographic fractions [about 2 μg of H0.6, 2 μg of DEFT, 2 μg of SP0.2, 0.2 μg of P(LP)0, and 0.2 μg of P(HP)0.25] were diluted in sample buffer, heat denatured (3 min at 100°C), and loaded on an SDS–10% polyacrylamide gel, as indicated. About 150 ng of each molecular weight marker protein (Pharmacia) was run in parallel (lane M; positions indicated in kilodaltons). Proteins were revealed by silver staining. (B) Electrophoretic band shift analysis. Starting material (wt20hpi WCE, about 1 μg protein) and aliquots of the chromatographic fractions (about 500, 200, 100, 2, and 1 ng, respectively) were tested for DEF-B-binding activity in the presence of labeled probe and poly(dI-dC) (1 μg, 3 μg, 1 μg, 0.5 μg, 0.2 μg, and none for the samples in lanes 1 to 6, respectively). Arrows point to DE-specific complexes a, b, and c (see text). F, unbound probe.

DEF-B was achieved by successive hydrophobic chromatography over phenyl-Sepharose (at low pressure) and phenyl-5PW (at high pressure). As shown in Fig. 3A, only one major protein band with an apparent molecular mass of about 55 kDa could be detected in the P(HP)0.25 fraction.

Peptide sequencing of DEF-B identifies it as the Ad5 IVa2 gene product. Examination of the protein content and corresponding DNA-binding activity of the relevant P(HP) fractions revealed that DEF-B activity (Fig. 4B) paralleled the elution profile of the major 55-kDa protein (Fig. 4A). This observation strongly suggested that the DNA-binding activity could be attributed to this protein and prompted us to attempt to determine its identity by peptide sequencing. The P(HP) fractions of interest were pooled and submitted to SDS-polyacrylamide gel electrophoresis. The band corresponding to the 55-kDa protein (estimated to about 100 pmol) was excised and treated with trypsin, and the resulting digestion products were eluted and resolved by HPLC. Sequences of two DEF-B peptides which perfectly matched the sequences deduced from the open reading frame of the Ad2 and Ad5 IVa2 genes were obtained (Fig. 5). These two adenovirus serotypes encode IVa2 proteins with calculated molecular masses of 55 kDa that differ by only 4 out of a total of 449 residues.

DEF-B and IVa2 have identical DNA-binding properties.

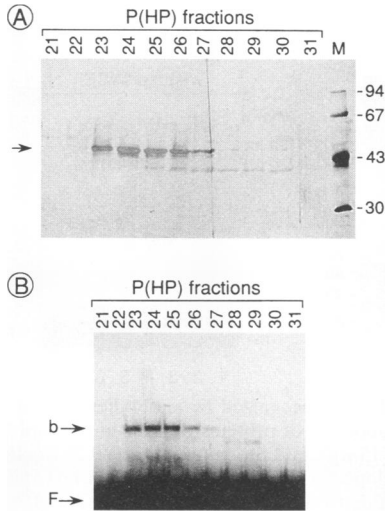

FIG. 4. HPLC of the P(LP)0 fraction. (A) The P(LP)0 fraction collected after low-pressure phenyl-Sepharose chromatography was loaded onto a phenyl-5PW HPLC column [P(HP)] and chromatographed as described in Materials and Methods and depicted in Fig. 2. Fractions 21 to 31, eluted from the P(HP) column, were analyzed (1/36 of each fraction) on an SDS–10% polyacrylamide gel as described in the legend to Fig. 3. The arrow points to the major band with DEF-B-binding activity. (B) The same fractions (1/6,000 of each) were assayed by electrophoretic band shifting as described in the legend to Fig. 3. Band b corresponds to DEF-B-specific complexes, as verified by competition experiments (not shown).

Antibodies against synthetic peptides derived from N-terminal (Pn) and C-terminal (Pc) potential antigenic domains of the Ad5 IVa2 protein were raised. We first used the anti-Pn antibodies (which turned out to be the more potent ones) to visualize the IVa2 protein. A single band corresponding to a polypeptide of 55 kDa could be detected in crude extracts from cells infected with wild-type Ad5 (wt20hpi WCE; Fig. 6A, lane 1). Similarly, a single band of the same size was detected in the purified DEF-B fraction [P(HP)0.25; Fig. 6A, lane 3]. No band was detected in extracts from cells infected with *dl*312 (*dl*20hpi WCE; Fig. 5A, lane 2), consistent with the delayed expression of viral genes in *dl*312-infected cells (12, 20) and with the lack of DEF-B activity in these cells (33). Altogether, these observations demonstrate the selectivity of the anti-Pn antibodies and suggest the relatedness of DEF-B to IVa2.

Further evidence for relatedness was provided by the effect of the anti-IVa2 antibodies on specific band shifts generated by wt20hpi WCE or the P(HP)0.25 fraction (Fig. 6B and C). Addition of increasing amounts of the anti-Pn antibodies to

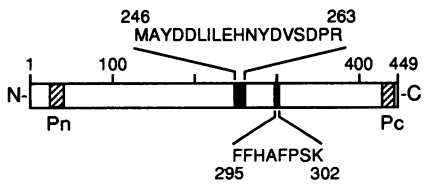

FIG. 5. Positions of sequenced DEF-B peptides in the IVa2 protein. The amino acid sequences determined from two DEF-B peptides (dark boxes) are given and positioned relative to the amino (N-) and carboxy (-C) termini of the sequence of the IVa2 protein (open box, 449 residues) from Ad2 or Ad5. The positions of the peptide sequences (Pn and Pc; see Materials and Methods) used for antibody production are also indicated (hatched boxes).

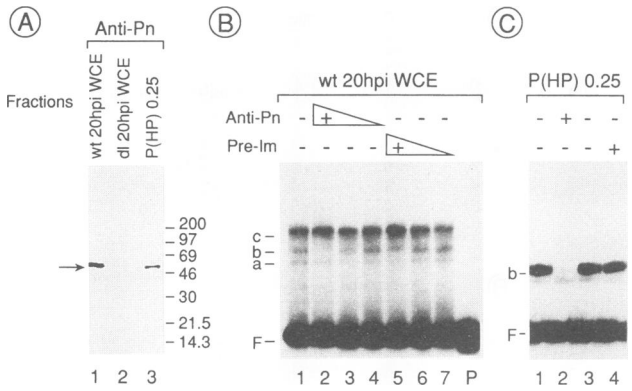

FIG. 6. DEF-B is recognized by antibodies against the IVa2 protein. (A) Immunoblots of proteins present in wt20hpi WCE (15 μg), *dl*20hpi WCE (15 μg), and the pooled P(HP)0.25 fractions (about 0.1 μg) were developed with the antibodies (anti-Pn) raised against an 18-residue peptide from the N terminus of the Ad5 IVa2 protein (see Materials and Methods). The arrow points to the single band revealed in wt20hpi WCE and purified DEF-B fraction. Molecular sizes (Rainbow markers; Amersham) are given in kilodaltons on the right. (B) Electrophoretic band shift assays of wt20hpi WCE were performed as for Fig. 3B in the presence of no (lane 1) or increasing amounts (from 0.03 to 3 μg) of anti-Pn (lanes 4 to 2) or preimmune (Pre-Im) antibodies (lanes 7 to 5). P, probe alone. (C) Electrophoretic band shift assays of the purified DEF-B protein [P(HP)0.25 fraction] were performed as for Fig. 3B in the presence of no (lanes 1 and 3) or 3 μg of anti-Pn (lane 2) or Pre-Im antibodies (lane 4).

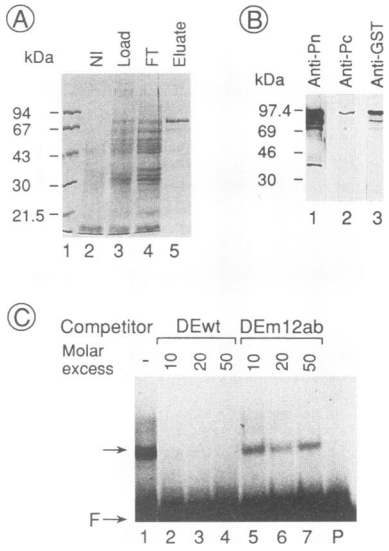

FIG. 7. Purified bacterially produced IVa2 recombinant protein generates a DE-specific band shift. (A) Purification of the His-tagged IVa2 recombinant protein by metal chelate affinity chromatography. Extracts from 2-ml equivalents of bacterial cultures transformed with pGST-IVa2-H6, prepared before (noninduced [NI]; lane 2) or after (load; lane 3) IPTG induction, were run on an SDS–10% polyacrylamide gel together with corresponding amounts of flowthrough (FT; lane 4) and eluate (lane 5). The gel was stained with Coomassie blue. Molecular weight markers (Pharmacia) were run in parallel (lane 1). (B) Immunoblot analysis of the metal chelate affinity chromatography eluate. The same amounts of protein as in lane 5 (of panel A) were separated by SDS-gel electrophoresis and revealed by antibodies against an N-terminal (anti-Pn) or a C-terminal (anti-Pc) IVa2 peptide or an anti-GST antibody, as indicated. Positions of Rainbow markers (Amersham) are given on the left. (C) Specific DNA-binding activity of the recombinant IVa2 protein. About 40 μg of eluate was electrophoresed on an SDS–10% polyacrylamide gel. The major upper band (85 kDa; see lane 5 in panel A) was excised, eluted, and renatured as previously described (15a). The renatured protein (now migrating as a single 85-kDa polypeptide, as verified by immunoblot analysis [not shown]) was assayed in the absence of poly(dI-dC) in band shift reactions with the DEwt probe alone (lane 1) or in the presence of increasing amounts of a competitor oligonucleotide (DEwt or DEm12ab), as indicated. The arrow points to a specific retardation complex. F, unbound probe; P, probe alone.

the retardation reaction with wt20hpi extract selectively impeded complex b formation (Fig. 6B, lanes 1 to 4), while control immunoglobulins had no effect (lanes 5 to 7). Surprisingly, complex c was not disrupted by the anti-Pn antibodies under the conditions used, suggesting that the Pn epitopes were hardly accessible when DEF-A was bound to DEF-B. As expected, the anti-Pn antibodies efficiently precluded specific complex assembly with the highly purified DEF-B protein (Fig. 6C), in agreement with their selective effect on complex b of the original extract (Fig. 6B).

A recombinant IVa2 protein, fused to the GST moiety (N terminus) and a polyhistidine tract (C terminus), was produced in bacteria. The fusion protein was extracted from inclusion bodies and purified by metal chelate affinity chromatography under denaturing conditions (Fig. 7A). As revealed by Coomassie blue staining (Fig. 7A, lane 5) and immunoblot analysis (Fig. 7B), one major polypeptide of about 85 kDa was recovered in the imidazole eluate, but additional bands of lower molecular mass, most likely resulting from internal translation initiations or partial degradations, were also detected. The full-length IVa2 fusion protein (85 kDa) was excised from a preparative SDS-polyacrylamide gel, renatured, and assayed for specific DNA-binding activity. As shown in Fig. 7C, a major retarded band was obtained with the recombinant protein (lane 1) that was efficiently competed for with an oligonucleotide comprising the DEF-B recognition site (DEwt; lanes 2 to 4) and much less with an oligonucleotide altered within each DE element (DEm12ab; lanes 5 to 7) or only on the DE2b element (not shown). The slight weakening of the signal in the presence of a mutated competitor is due to a nonspecific effect of the increased amount of DNA. The nature of the minor, more slowly migrating, specific complexes also detected by this assay is presently unknown, but the possibility exists that they correspond to alternate configurations of IVa2 that may have been generated during the renaturation process. These results indicate that the bacterially synthesized IVa2 product has the same DNA-binding specificity as the purified DEF-B protein, strongly supporting the conclusion that DEF-B and IVa2 are the same protein.

Specific inhibition of in vitro MLP activity by anti-IVa2 antibodies. We next examined the effects of antibodies against IVa2 on the DE-dependent transcriptional activation of the MLP in vitro. To this end, we compared the effects of increasing amounts of anti-Pn antibodies on the template efficiencies of MLP DEwt and MLP Δ12. In agreement with earlier observations (32), deletion of the DE elements resulted in a 5- to 10-fold-lower promoter activity (Fig. 8; compare lanes 1 and 5). While the antibodies did not significantly affect transcription from the MLP Δ12 template (lanes 6 to 8), they reduced MLP DEwt promoter efficiency (lanes 2 to 4) to levels close to that of the deleted promoter. Together with the observation that the anti-IVa2 antibodies impaired specific DEF-B DNA-binding activity, the results of this transcriptional analysis further confirm the molecular identity of DEF-B.

Expression of recombinant IVa2 in transfected cells stimu-

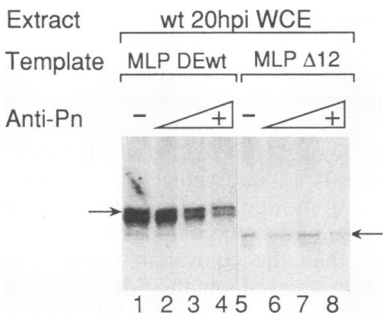

FIG. 8. Antibodies against the IVa2 protein abolish DE-mediated MLP activation in vitro. Cell-free transcription experiments were performed with wt20hpi WCE, using as the template either MLP DEwt or MLP Δ12, as indicated. Reactions were carried out in the absence (lanes 1 and 5) or presence of increasing amounts of anti-Pn antibodies (0.6 μg [lanes 2 and 6], 1.5 μg [lanes 3 and 7], and 3 μg [lanes 4 and 8]). Arrows point to the specific transcripts expected from the corresponding templates.

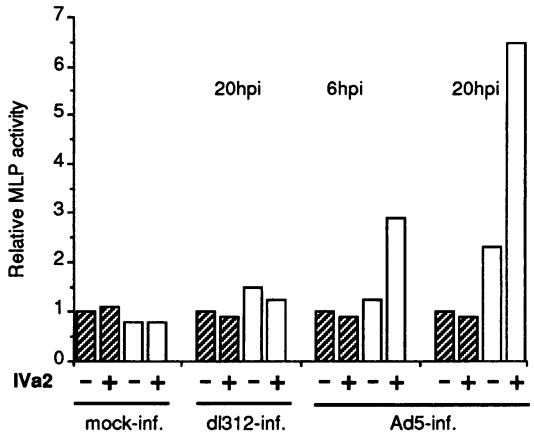

FIG. 9. Overexpression of a IVa2 recombinant protein in Ad5-infected cells increases the late-phase-dependent activation of the MLP. COS7 cells were transfected with an MLP-luciferase reporter bearing either MLP DEwt (open columns) or MLP Δ12 (hatched columns) in the absence (−) or presence (+) of an IVa2 expression vector (see Materials and Methods). Eighteen hours later, the cells were infected either with Ad5 (Ad5-inf.) or *dl*312 (dl312-inf.) or not infected (mock-inf.), as indicated. The cells were collected 6 h postinfection (6hpi), 20 h postinfection (20hpi), or 20 h after mock infection, and extracts were assayed for luciferase activity. The values of reporter activity (corresponding to the means of three independent experiments) are expressed relative to those of MLP Δ12, as measured in the absence of cotransfected IVa2 vector, which have been adjusted to 1. The actual relative activities of MLP Δ12, in the absence of IVa2, were 1, 1.1, 1.5, and 2 in mock-infected, *dl*312-infected, and 6- and 20-h postinfection Ad5-infected cells, respectively.

lates MLP activity. To investigate the effect of the IVa2 protein on MLP activity in a cellular context, we cotransfected COS cells with a IVa2 expression vector together with MLP-luciferase reporter plasmids with or without the DE sequences. The cells were subsequently infected with either *dl*312 or Ad5 before extract preparation and monitoring of luciferase activity. In previous experiments, we had verified that the DEF-A and DEF-B activities were both present in Ad5-infected COS cells at 20 h postinfection (33, 44a). Furthermore, these cells, which constitutively express simian virus 40 T antigen, support replication of simian virus 40 origin-containing plasmids (28). In both *dl*312- and Ad5-infected cells, a significantly higher expression level was obtained from the MLP-luciferase recombinants in the presence of this replication origin (Fig. 9) than in its absence (not shown; see Discussion). Finally, we verified by Western blotting (immunoblotting) with antibodies directed against the human estrogen receptor epitope fused to the recombinant IVa2 that under the transfection conditions used, roughly the same amounts of IVa2 protein accumulated in the cells, whether collected at 24 or 38 h posttransfection (not shown).

The results of typical transfection-infection experiments are presented in Fig. 9. Overexpression of IVa2 significantly stimulated MLP activity only in Ad5-infected cells, and the stimulation was dependent on the presence of intact DE elements in the reporter. No stimulation of the MLP upon IVa2 cotransfection occurred in noninfected or *dl*312-infected cells, consistent with the requirement of additional factors for the DE-mediated activation. As suggested by the threefold MLP activation in early Ad5-infected cells, such factors may have started to accumulate at 6 h postinfection in these cells. The two- to threefold DE-dependent activation detected in Ad5-infected cells at 20 h postinfection, in the absence of transfected IVa2, most likely reflected the contribution of the endogenous factors revealed by this assay.

Together with the data presented in previous sections, these results show that the IVa2 protein exhibits all testable properties of the DEF-B factor. It therefore meets essentially all of the criteria to identify it as a DE-binding transcriptional activator of the MLP.

DISCUSSION

The results presented here establish that the product of the adenovirus IVa2 gene corresponds to one of the factors (DEF-B) implicated in the late-phase-dependent activation of the MLP. Our observations, based on peptide sequence analysis and specific DNA-binding and transcriptional experiments, demonstrate that the IVa2 protein directly contributes to the efficiency of viral production by allowing progression into the late phase of the lytic cycle of infection.

Replication-dependent activation of the MLP. A component of the viral capsid had originally been tentatively identified as the product of the IVa2 gene (13, 14, 15, 38). More recent immunoblot experiments confirmed that the IVa2 protein represents only a minor component of the viral particle (46). Unlike the herpesvirus late gene product VP16, a transcription activator which is also included in the viral particle during packaging, the IVa2 protein does not seem to be essential for early viral gene expression. Instead, the accumulation of IVa2 transcripts and protein in infected cells parallels the kinetics of MLP activation, as would be expected of a transcriptional activator of the MLP. Levels of IVa2 mRNA (6, 39) and protein (46) were found to reach detectable levels at about 12 h postinfection, while transcripts from the major late unit, such as those belonging to the L3 and L5 families, were increased at 14 h postinfection (6). Similarly, DEF-B activity could be detected by specific band shifting only beyond 12 h postinfection, at times when MLP activation became apparent in cell-free transcription systems (24, 33).

The timing of IVa2/DEF-B expression and MLP activation also correlates with viral DNA replication, which in wild-type Ad5-infected cells starts between 6 and 8 h postinfection and reaches maximal rates around 12 h after infection. Cells in which both cellular and viral DNA replication was blocked by chemicals such as cytosine arabinoside or hydroxyurea produced only the typical early transcripts; neither IVa2 nor

genuine late messengers (L2 to L5) could be detected (6, 46). In accordance with this observation, specific DNA binding to the DE sequences and MLP activation were also found to be impaired in these cells (18, 19, 24, 33). Similarly, when viral DNA replication was delayed by infection with *dl*312, an E1a-defective mutant of Ad5, no DE-binding activity and no MLP activation could be detected (18, 19). Our results showing that the IVa2 protein is critically involved in the late-phase-dependent activation of the MLP account for these observations. On the other hand, no MLP activation could be detected (6, 24) when viral DNA replication was selectively blocked by infection with H5 *ts*125, an Ad5 temperature-sensitive mutant of the viral DNA-binding protein, despite the presence of detectable amounts of IVa2 protein apparently related to increased translation efficiency of corresponding transcripts (46). These findings support our conclusion that the IVa2 protein is necessary but not sufficient for the late-specific activation of the MLP. Altogether, these observations suggest a model in which the IVa2 protein (among others) gains efficient access to the viral template during the process of replication and thereby contributes to the late-phase-dependent MLP activation.

Additional factors contribute to the late-phase-dependent activation of the MLP. Previous DNA-binding and transcriptional studies revealed that maximal MLP activation requires at least one additional infected-cell-specific protein, DEF-A (the nature of which is still unknown), that interacts with the IVa2/DEF-B protein, resulting in the cooperative binding of both late-phase-dependent factors to their cognate DE sequences. Our observation that expression of the IVa2/DEF-B protein in noninfected cells failed to activate the MLP (Fig. 9) further stresses the need for such a cofactor(s). Remarkably, significant DE-mediated activation of the MLP reporter plasmid occurred in Ad5-infected cells upon expression of the IVa2/DEF-B protein early after infection (i.e., at a time when viral DNA had not yet undergone replication). This activation may reflect the presence in these infected cells of low amounts of the necessary cofactor(s) which may be induced, at these early times postinfection, to levels sufficient for moderate MLP activation. Our results also indicate that the MLP-based reporter plasmid used in this study is transcriptionally competent at these early times after infection. Since this plasmid is able to autonomously replicate in COS cells (28, 44a), it may have acquired a nucleoproteic configuration comparable to that of replicated viral DNA and compatible with MLP activation (see above). Finally, it is worth recalling that constitutive factors, like Oct-1-related proteins, have also been shown (26) to bind to the DE sequences which share homologies (+85 to +92 and +111 to +119; Fig. 1) with the octamer consensus (5′-ATGCAAAT-3′). The interesting possibility that DEF-A corresponds to Oct-1 seems, however, unlikely on the basis of the following observations (27a): (i) complex a could not be competed for with oligonucleotides spanning the Ad5 Oct-1-binding site of the viral replication origin within the inverted terminal repeat; (ii) the DEwt oligonucleotide was a very poor competitor for the specific binding of Oct-1 to its target oligonucleotide; and (iii) anti-Oct-1 antibodies generated no detectable supershift or destabilization of complex a. On the other hand, these results do not exclude the possibility that the constitutive Oct-1 or Oct-1-related proteins contribute to the basal activation of the MLP, especially early in infection.

Control of the divergent IVa2 and MLP promoters. The start sites of the divergent MLP and IVa2 promoters are only about 200 bp apart on the viral genome. As already discussed, transcription from these promoters is temporally regulated during the late phase of infection, leading to the appearance of the IVa2 transcripts prior to MLP stimulation. Further, the IVa2 promoter is turned off at later times, while the MLP remains active till shortly before cell death. On the basis of in vitro transcription and footprinting experiments, it has been suggested (1, 34, 35) that the two promoters compete for the UE factor (UEF or MLTF/USF) which binds to two sites within the MLP-IVa2 intergenic region (30). In addition, Leong et al. (23) showed that a TFIID-containing fraction prepared from late-infected cells exhibited higher transcriptional activities than the equivalent fraction from mock-infected cells when assayed on the MLP. Together with our earlier finding that the UEF- and DE-binding factors act cooperatively on MLP transcription efficiency (32), these observations suggest that once activated by a proper combination of factors (including the IVa2/DEF-B protein), the MLP drives transcription toward its side, sequestering limiting transcription factors also involved in IVa2 promoter activity, with progressive down-regulation of the latter promoter. In apparent contradiction with this model, viruses with alterations of specific MLP elements did not show elevated IVa2 transcription in infected cells (40). The possibility therefore exists that the IVa2 promoter is directly inactivated by a specific inhibitor that accumulates later in infection. Evidence for such a negative regulatory protein has recently been provided, and its putative target site, between positions +11 and +27 within the IVa2 control region, has been identified (8). Whatever the precise mechanisms implicated, it clearly appears that the IVa2 protein is one of the factors that critically contribute to the control of these divergent promoters.

The identification of the IVa2 protein as one of the MLP-binding proteins raises new interest in the control of this promoter. The functional dissection of the IVa2 factor will be of particular importance, especially with respect to the detection of a highly conserved putative ATP/GTP-binding site which was revealed by computer analysis of the IVa2 peptide sequences from various adenovirus serotypes (not shown). The large-scale production of this factor should also allow the affinity purification of interacting proteins such as DEF-A or other cofactors of cellular or viral origin.

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