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High-Level Transcription from the Adenovirus Major Late Promoter Requires Downstream Binding Sites for Late-Phase-Specific Factors

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The adenovirus major late promoter (MLP) is active during both the early and late phases of infection. During the early phase the activity of the MLP is similar to those of the other early viral promoters, but during the late phase the rate of transcription from the MLP becomes much greater by comparison. We report here that sequence-specific binding proteins are induced during the late phase which interact with three regions in the first intron of the MLP transcription unit from positions +37 to +68, +80 to +105, and +105 to +125 relative to the transcription initiation site. To measure the significance of these binding sites for transcription during the late phase, we constructed MLP-β-globin fusions and substituted them for early region 3 in adenovirus recombinants. Deletion of the binding sites caused significant reductions in the rate of transcription, specifically during the late phase of infection. Deletion of all three sites reduced the rate of transcription 25- to 50-fold and the accumulation of cytoplasmic MLP-β-globin RNA 200-fold. These results indicate that the high rate of transcription from the MLP during the late phase of infection results from the interaction of virus-induced transcription factors with three binding sites in the first intron of the major late transcription unit.

The adenovirus type 2 (Ad2) major late promoter (MLP) is an extraordinarily strong promoter during the late phase of infection. However, the MLP is active during both the early and late phases of infection (32). During the early phase, the rate of transcription is similar to that of the other early promoters (28, 32), and the primary transcript is processed into a single, promoter-proximal mRNA (10, 21). Following the onset of viral DNA replication, the rate of transcription from the MLP increases greatly to a level at least 20 times higher than those for other early viral promoters (32). During the late phase, the polymerase elongates further than during the early phase, and the longer primary transcript is processed into 1 of more than 30 possible mRNAs by alternative cleavage-polyadenylation and alternative RNA splicing (1, 29, 32). These late mRNAs encode most of the viral proteins required for the assembly of virions.

Two upstream sequences have been identified as important elements of the MLP during the early phase of infection. They were discovered through directed mutagenesis and analysis of transcription following the transfection of plasmid DNAs into uninfected cells (14, 20) and infection with mutant adenoviruses (6, 22). These same promoter elements are required for maximal transcription in vitro with extracts of uninfected cells (9, 11, 15, 25, 37). One of these promoter elements, the TATA box (positions −31 to −25 relative to the transcription initiation site) is the binding site for a host cell transcription factor called TFIID (31) or BTF 1 (26). The other element, mapping from −63 to −52, is the binding site for a host cell transcription factor called MLTF (7), USF (31), or UEF (26).

Recent results indicate that the high rate of transcription from the MLP during the late phase of infection requires virus-induced *trans*-acting factors which interact with sequences downstream from the transcription start site, within the first intron of the major late mRNAs. Studying a set of defective adenovirus-simian virus 40 hybrid viruses constructed to express high levels of simian virus 40 T antigen, Mansour et al. (24) found that the insertion of simian virus 40 sequences at position +33 relative to the MLP initiation site resulted in 5 to 10 times less MLP T-antigen message than observed when the same sequences were inserted at +190 or further downstream. Pulse-labeling studies showed that the decreased RNA concentration resulted from a decrease in transcription rate. Further, transfection experiments indicated that the higher rate of transcription dependent on sequences mapping between +33 and +190 required *trans*-acting factors expressed only in adenovirus-infected cells (24). Jansen-Durr et al. (16) reported that a sequence-specific DNA-binding protein(s) was expressed during the late phase of Ad5 infection which protected the sequence from +76 to +120 downstream from the MLP initiation site in a DNase I footprinting analysis. Moreover, these investigators found that whole-cell extracts prepared from HeLa cells during the late phase of infection with Ad5 were more active for in vitro transcription from the MLP than were extracts prepared from cells infected with mutant *dl*312, which is blocked in viral DNA replication. At a low template concentration, most of the increased transcriptional activity of the whole-cell extracts prepared from infected cells during the late phase of infection depended on sequences in the template mapping between positions +33 and +131 (16).

In this work, we report that there are at least three binding sites for virus-induced factors expressed during the late phase of infection which protect DNA sequence in the region from positions +37 to +125 from DNase I digestion. To measure the significance of these binding sites for transcription during the late phase of infection, we constructed and analyzed nondefective adenovirus recombinants in which MLP region sequences were fused to the human β-globin coding region and substituted for nonessential early region 3 sequences. Our results indicate that each of the three binding sites is required for maximal rates of transcription from the

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MLP during the late phase of infection. The entire sequence from +33 to +192 stimulates transcription 25- to 50-fold during the late phase.

MATERIALS AND METHODS

DNase I footprinting. Whole-cell extracts (23) were prepared from mock-infected and adenovirus-infected HeLa cells, as described previously (18). Unless indicated otherwise in the figure legend, 100 μg of extract protein was incubated at 30°C for 60 min with 5 to 8 ng of end-labeled probe plus 1 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) in 20 μl of 50 mM KCl–2.5 mM $MgCl_2$–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9)–1 mM dithiothreitol–50 μM EDTA–8.5% glycerol. The reaction mixture was then diluted with an equal volume of 5 mM $CaCl_2$–10 mM $MgCl_2$–freshly diluted DNase I (20 μg/ml; Organon Teknika) and incubated for 1 min at room temperature, and then the reaction was terminated by the addition of 150 μl of 7 M urea–0.1 M LiCl–0.5% sodium dodecyl sulfate–10 mM EDTA–10 mM HEPES, pH 7.9, plus 5 μg of yeast tRNA. The solution was extracted with phenol-chloroform, ethanol precipitated, and analyzed by electrophoresis on an 8% polyacrylamide–8 M urea sequencing gel. The wild-type (wt) probe was labeled on the 5′ end of the top (noncoding) strand or the 3′ end of the bottom (coding) strand at the *Hin*dIII site at position +192 relative to the MLP transcription start site and extended to the *Xho*I site at −260. The probes in Fig. 4 were prepared by labeling the 5′ end of the top strand at the *Hin*dIII site at +192 and then cleaving at an *Eco*RI site engineered at position −66. The competitor DNAs (see Fig. 5) were ligated double-stranded DNA multimers of the sequences (top strand shown here) CTCGAGTTGGGTGAGTACTCCCTCTCAAAAG (R1), GTCGACTTCTGCGCTAAGATTGTCAGTTTCCA (R2), and CTCGAGAAAAACGAGGAGGATTTGATATT (R3).

Construction of the MLP-β-globin recombinants. Ad2 sequence from the engineered *Eco*RI site at −66 to the *Hin*dIII site at +192 of plasmid pXB210 (15) was cloned into the equivalent sites of M13mp18. Regions R1 (positions +37 to +62), R2 (positions +72 to +98), R3 (positions +98 to +120), and R2+3 (positions +72 to +120) were deleted from the M13 clone by using the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.) and confirmed by DNA sequencing. The wt and mutagenized Ad2 sequences from −66 to +192 were then cloned into pUC18 for further manipulation. The *Eco*RI site at position −66 of each of these plasmids was converted to an *Xho*I site by filling in the *Eco*RI ends and ligating on *Xho*I linkers (Collaborative Research, Inc.). The *Eco*RI site at −66 of plasmid p−66 to +33 (19) was similarly converted to an *Xho*I site.

The *Bam*HI fragment from positions −1460 to +477 relative to the transcription start site of the human β-globin gene (30) was cloned into the *Bam*HI site of M13mp11. A *Hin*dIII restriction site was introduced at −6 by using the oligonucleotide-directed in vitro mutagenesis system (Amersham) to change the wt sequence from (−6) TTGCTT (−1) to AAGCTT. The mutated β-globin *Bam*HI fragment was then substituted for the wt fragment in pHpstβ, a clone of the 4.4-kilobase (kb) *Pst*I fragment from the human genome including all of the β-globin gene. β-Globin sequence from the engineered *Hin*dIII site at −6 to the *Pst*I site at +2138 was then cloned between these sites in pUC19 to create pHβgH3.

β-Globin sequence from −4 to +2138 was excised from pHβgH3 via the *Hin*dIII and *Xba*I sites in the polylinker, mixed with the *Xho*I to *Hin*dIII fragment (positions −66 to +192) of the wt or mutagenized MLP sequence, and ligated to the pA5-130 cloning vector (5) between the *Xho*I and *Xba*I sites in a directional three-fragment ligation reaction. The −66 to +33 MLP sequence was similarly fused to the β-globin coding sequence in the pA5-130 vector. The no-promoter construct was obtained by filling in the engineered *Hin*dIII site at −6 in the β-globin sequence, ligating on an *Xho*I linker, and cloning between the *Xho*I and *Xba*I sites of pA5-130.

The pA5-130 plasmid DNAs containing these chimeric MLP-β-globin transcription units were cleaved at the *Cla*I site, treated with phosphatase, phenol-chloroform extracted, and digested with *Xba*I. After further phenol-chloroform extraction and ethanol precipitation, this DNA was ligated to the *dl*321 viral DNA large *Xba*I fragment as described elsewhere (5), and the ligated DNA products were transfected into 293 cells (13). Plaques were picked, viral stocks were grown on 293 cells, and Hirt DNA was prepared and analyzed by restriction analysis. Viruses (see genome structures diagrammed in Fig. 6) were plaque purified three times, and virus stocks were prepared and assayed by plaque titration on 293 cells.

Infection and preparation of cytoplasmic RNA. Three confluent 100-mm-diameter plates of 293 cells (~10^7 cells per plate) were infected at a multiplicity of infection of 40 PFU per cell with *dl*321 (5) or MLP-β-globin recombinant adenoviruses. Two plates were harvested at 6 h postinfection (p.i.) to process cytoplasmic RNA and nuclei. The remaining plate was harvested at 20 h p.i. Cytoplasmic RNA was isolated as described previously (3). Nuclei were prepared as described below for in vitro nuclear run-on analysis.

Primer extension. A 20-mer primer complementary to nucleotides +11 to +30 of human β-globin RNA was used to analyze the 5′ ends of the MLP-β-globin RNAs: AGTGAA CACAGTTGTGTCAG. A 5-μg amount of 20-h-p.i. cytoplasmic RNA plus 15 μg of yeast tRNA carrier was hybridized to 1.5 pmol of the 5′-^{32}P-labeled primer in 10 μl of 0.25 M KCl–10 mM Tris (pH 7.0)–1 mM EDTA at 37°C for 30 min after an initial 10-min incubation at 80°C. A 25-μl volume of 0.33 mM dATP, dTTP, dGTP, and dCTP–10 mM $MgCl_2$–5 mM dithiothreitol–50 mM Tris (pH 7.5)–50 μg of dactinomycin per ml–200 U of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) was added, and the incubation was continued at 37°C for 1 h. The reaction products were ethanol precipitated after the addition of sodium acetate to 0.3 M and centrifuged, and the pellet was rinsed with chilled 90% ethanol, air dried, dissolved in 7 μl of 98% formamide, incubated at 90°C for 5 min, and analyzed by electrophoresis in an 8% polyacrylamide–8 M urea sequencing gel followed by autoradiography.

S1 nuclease analysis. MLP-β-globin RNA was analyzed with a 3′-labeled probe extending from the labeled site at position +1398 to +2138. This probe was prepared from pHβgH3 by filling in the *Eco*RI site at +1398 in the β-globin sequence by using ^{32}P-labeled nucleotide triphosphates followed by *Pst*I digestion at +2138. RNAs polyadenylated at the β-globin poly(A) site, +1607, protect a fragment of 212 nucleotides (8). For analysis of late RNA, 5 μg of the 20-h-p.i. cytoplasmic RNA plus 15 μg of tRNA carrier was hybridized to excess probe at 47°C for 12 to 14 h in 80% formamide buffer, as described previously (4). For analysis of early RNA, 50 μg of 6-h-p.i. cytoplasmic RNA was similarly hybridized to the same probe. S1 digestion was performed at room temperature for 1 h, using 100 U of S1

nuclease (Bethesda Research Laboratories), as described previously (4).

L3 family RNA was analyzed by using a 3'-end-labeled probe extending from the labeled end at the *Bam*HI site at 59.5 map units on *dl*321 viral DNA to the *Xba*I site at 78.5 map units. Hybridization was to 0.1 μg of the 20-h-p.i. cytoplasmic RNA plus 25 μg of carrier tRNA at 50°C for 14 h in 80% formamide hybridization buffer. L3 family RNAs generate an S1-protected fragment of 837 nucleotides. Early region 4 RNA was analyzed with a 3'-end-labeled probe extending from the labeled *Xma*I site at 91.9 map units to the *Xho*I site at 83 map units and was prepared by using plasmid pA5-130 (5). Hybridization reaction mixtures contained 12 μg of 6-h-p.i. cytoplasmic RNA plus 15 μg of tRNA carrier and excess probe and were hybridized at 50°C for 12 to 14 h. E4 RNAs protect a 284-nucleotide fragment from S1 nuclease digestion. S1 digestion conditions for L3 and E4 RNA analyses were as described above for β-globin RNA analysis.

In vitro nuclear run-on analysis. Nuclei isolated from one 100-mm-diameter plate of infected 293 cells following Nonidet P-40 lysis and low-speed centrifugation (3) were washed gently in 4 ml of ice-cold isotonic Nonidet P-40 lysis buffer (3). Nuclei were pelleted by centrifugation at 1,000 rpm for 3 min in a DPR 6000 centrifuge (International Equipment Co.) at 4°C, suspended in 40 μl of nuclei storage buffer (0.5 M sorbitol, 2.5% Ficoll [M_W 400,000], 10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.3 mM spermidine, 1 mM dithiothreitol, 50% glycerol), and stored at −70°C until use. Nuclei stored at −70°C were thawed on ice, and in vitro nuclear run-on reactions were performed and nuclear RNA was isolated as described elsewhere (36). Each nuclear run-on reaction incorporated ~10^7 cpm into trichloroacetic acid-precipitable material.

Nuclear RNA was hybridized to nitrocellulose filters (Schleicher & Schuell, Inc.) prepared as follows. M13 DNA clones were incubated at 100°C in 10 mM Tris (pH 7.5)–1 mM EDTA for 2 min, quick-chilled on ice, and added to ice-cold ammonium acetate to give a final concentration of 1 M. A 4.5-μg sample of DNA in 100 μl was applied to a nitrocellulose filter presoaked in 1 M ammonium acetate under a gentle vacuum in a slot blotter (Schleicher & Schuell). The nitrocellulose filter was air dried and baked under a vacuum at 80°C for 2 h. The nitrocellulose filter was then prehybridized in 5 ml of hybridization buffer containing 0.3 M NaCl, 10 mM Tris (pH 8.0), 20 mM EDTA, 100 μg of sheared and denatured salmon sperm DNA per ml, 100 μg of yeast tRNA per ml, 1× Denhardt solution (0.2 μg of Ficoll per ml, 0.2 μg of polyvinylpyrrolidone per ml, 0.2 μg of bovine serum albumin per ml), and 50% formamide in a 50-mm-diameter petri dish on a shaker inside a 42°C incubator for 2 h. Then 10^6 Cerenkov cpm of in vitro nuclear run-on RNA was added to the hybridization buffer, and hybridization was continued at 42°C for 48 h with shaking. After removal of the hybridized filter, a duplicate filter to which similar single-stranded probes had been applied was added and subjected to a second round of hybridization for 48 h to ensure that >90% of the labeled cRNA had hybridized to the first filter. If greater than ~10% of the nuclear RNA isolated from a nuclear run-on analysis using nuclei from a 100-mm-diameter plate was used, substantial hybridization was observed on the second filter. Hybridized filters were washed for 30 min with shaking at room temperature with 500 ml of 1× SET buffer (0.15 M NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA) plus 0.1% sodium dodecyl sulfate and then with 500 ml of 0.1× SET plus 0.1% sodium dodecyl sulfate at 60°C for 1 h. Next the filters were incubated in 2× SET containing 5 μg of RNase A for 20 min at room temperature. Finally, the filters were washed two times for 30 min in 500 ml of 0.1× SET plus 0.1% sodium dodecyl sulfate at 60°C, blotted dry, and exposed to a preflashed X-ray film with an intensifying screen (17).

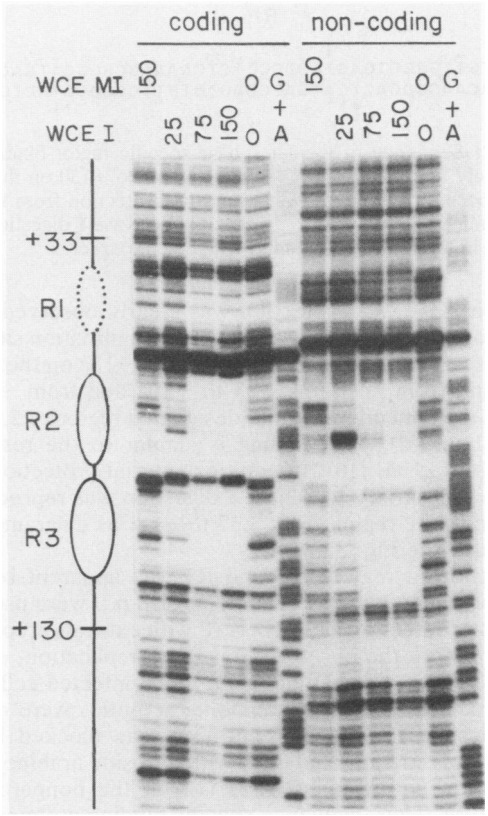

FIG. 1. Binding sites for late-phase-specific factors in the first intron of the MLP transcription unit. DNase I footprinting was performed with the indicated amounts (in micrograms) of whole-cell extract protein prepared from HeLa cells infected with Ad5 pm975 (WCE I) or mock-infected cells (MI) at 20 h p.i. The coding strand was labeled at the 5' end; the noncoding strand was labeled at the 3' end. Maxam-Gilbert G+A sequencing reaction products for each strand are shown as markers.

The M13 clone used to detect β-globin RNA was a clone of the coding strand from the *Bam*HI fragment from nucleotides −1460 to +477 in M13mp11. The noncoding β-globin strand was obtained by placing the *Hin*dIII-*Bam*HI fragment from pHβgH3 (nucleotides −6 to +477) between the same sites in M13mp11. The L1 *r* and *l* single-stranded probes were constructed by cloning the *Sst*I-*Hin*dIII fragment (Ad2 nucleotides 12039 to 13636) from *dl*321 viral DNA into M13mp11 and M13mp10, respectively.

RESULTS

Three binding sites for late-phase-specific factors in the first intron of the major late transcription unit. Figure 1 shows the results of DNase I footprinting analysis of the downstream region of the MLP, using whole-cell extracts from adenovirus-infected and mock-infected cells. Extracts were prepared at 20 h p.i. with the Ad5 derivative pm975, which is defective in the expression of the 12S mRNA from E1A but otherwise exhibits an infectious cycle very similar to that of

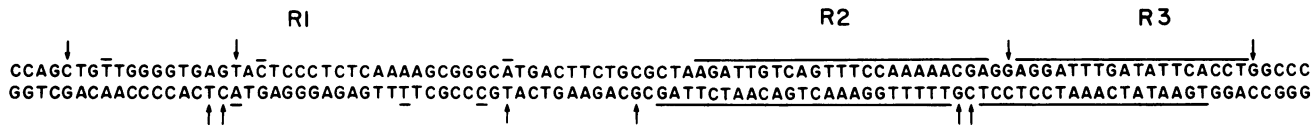

FIG. 2. Sequence of the late-phase-specific factor-binding sites. The top and bottom strands are the noncoding and coding strands, respectively, extending from +30 on the left to +130 on the right, relative to the MLP transcription initiation site. Horizontal lines drawn above a base indicate complete or partial protection from DNase I digestion at the 5′ phosphodiester bond of the indicated base. Vertical arrows indicate increased (hypersensitive) DNase I digestion at the 5′ phosphodiester bond of the indicated base, relative to the extent of digestion in the absence of added whole-cell extract.

wild-type Ad5 (27). Protection was clearly observed in the region downstream from the transcription initiation site from positions +80 to +102 and from +105 to +122 on the coding strand and from positions +83 to +105 and from +108 to +125 on the noncoding strand (designated regions R2 and R3 in Fig. 1 and 2). This finding is similar to the results of Jansen-Durr et al. (16). In addition, partial protection from and hypersensitivity to DNase I digestion was reproducibly observed in the region from +37 to +68, as diagrammed in Fig. 2 (also see Fig. 3 and 4).

Protection in regions R2 and R3 was apparent but less complete when extracts prepared at 12 h p.i. were used. No significant protection was observed with extracts prepared at 6 h p.i., before the onset of viral DNA replication, or with extracts prepared in parallel from mock-infected cells (Fig. 3). These sequence-specific binding activities were not observed when viral DNA replication was blocked by the treatment of Ad2-infected cells with cytosine arabinoside or by infection with mutant *ts*125 (12) at the nonpermissive temperature. Thus, sequence-specific binding proteins interacting with regions R1, R2, and R3 were observed only in extracts of cells prepared during the late phase of adenovirus infection.

To test whether factors bound to these regions independently, deletion mutants were constructed in which region R2 or R3 was deleted, and the footprinting analysis was repeated on the mutant DNAs (Fig. 4). The deletion of region R2 did not interfere with the binding of late-phase-specific factors to regions R1 and R3. Footprints very similar to those observed for the wt probe were seen over these regions. In addition, new hypersensitive sites just downstream from region R1 were observed when regions R1 and R3 were juxtaposed in the R2 deletion mutant. Similarly, the deletion of region R3 did not interfere with the interaction of sequence-specific binding proteins with regions R1 and R2. We conclude that there are at least three binding sites for factors induced during the late phase of infection which bind to sequences from positions +37 to +68, +80 to +105, and +105 to +125. It is also possible that more than one late-phase-specific protein binds in each of these three regions.

To determine whether the same or different proteins interact with these binding sites, we performed DNase I protection analyses in the presence of specific DNA competitors (Fig. 5). When DNase I digestion was performed in the presence of increasing concentrations of synthetic DNA composed of multimerized R1 sequence, no alteration of the R2 or R3 footprints was observed. However, multimers of either site R2 or site R3 competed for binding to both sites R2 and R3 similarly (Fig. 5). These results suggest that the same factor interacts with both sites R2 and R3. The inverted repeat sequence TGANNNTCA occurs in footprint R3, while a similar sequence, AGANNNTCA, is found in R2. This may be the sequence recognized by the late-phase-specific factor which binds to regions R2 and R3. A second

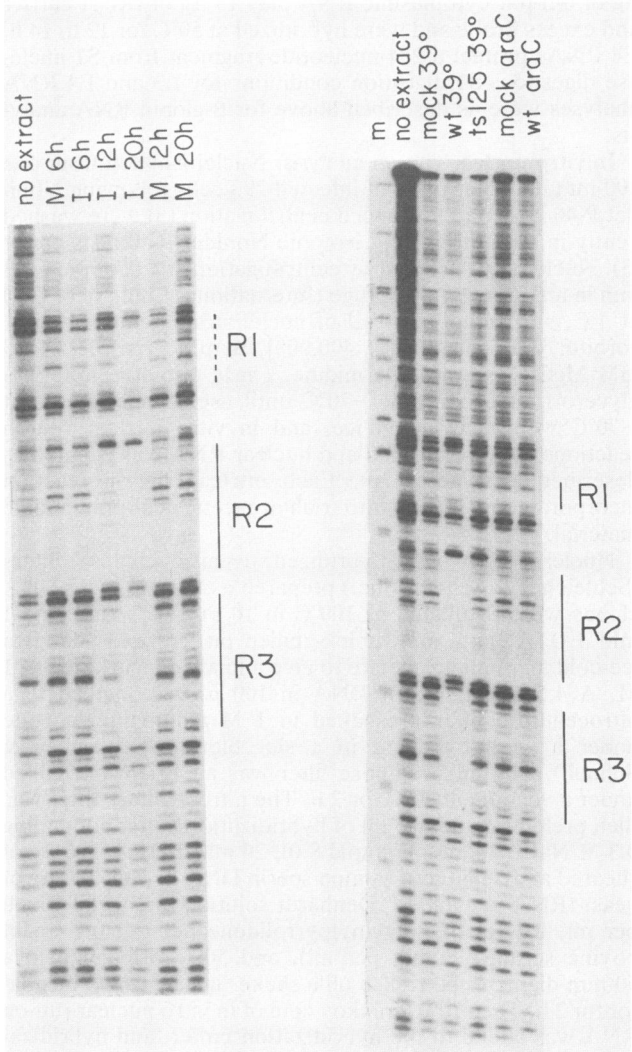

FIG. 3. Factors binding downstream from the start site of transcription of the MLP are induced during the late phase of infection. (Left) DNase I footprinting performed with whole-cell extract prepared at the indicated times p.i. with pm975 (lanes I) or post-mock infection (lanes M). (Right) DNase I footprinting was performed with whole-cell extracts prepared at 20 h post-mock infection at 39°C (lane mock 39°), after infection with Ad5 at 39°C (lane wt 39°) or with ts125 at 39°C (lane ts125 39°), after mock infection in the presence of 20 μg of cytosine arabinoside per ml (lane mock araC), or after infection with Ad5 in the presence of 20 μg of cytosine arabinoside per ml (lane wt araC), as indicated. Size markers are shown (lane m). DNase I digestions in the absence of added whole-cell extract are shown in both panels (lanes no extract). The coding strand was labeled at the 5′ end.

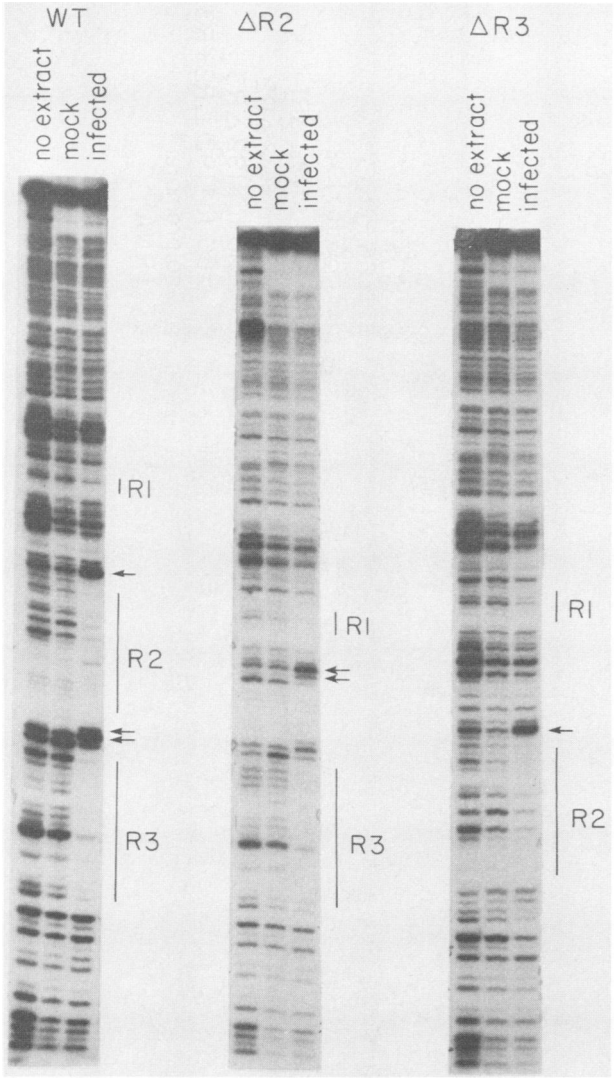

FIG. 4. Late-phase-specific binding proteins bind independently to regions R1, R2, and R3. DNase I footprinting was performed without added whole-cell extract (lanes no extract) or with whole-cell extract from mock-infected cells (lanes mock) or pm975-infected cells at 20 h p.i. (lanes infected). The left, middle, and right panels show footprinting on the wt Ad2, ΔR2 mutant (nucleotides +72 to +98 deleted), and ΔR3 mutant (nucleotides +98 to +120 deleted) sequences, respectively. The probes extend to −66 at the top of the gel. The protection of the regions at the top of the gel for both mock-infected and infected cell extracts is due to binding of MLTF (7). Arrows indicate hypersensitive sites.

late-phase-specific factor may interact with site R1. Alternatively, the same factor which interacts with sites R2 and R3 may also bind, with low affinity, to site R1.

Deletion of regions R1, R2, and R3, individually or in combination, reduces expression from the MLP during the late phase specifically. To test the significance of these binding sites for transcription from the MLP, we constructed adenovirus recombinants which contained sequences from the MLP region fused to the human β-globin coding region. These MLP-β-globin fusions were inserted into the adenovirus genome (Fig. 6A) so that we could analyze their transcription from replicated adenovirus templates during the late phase of an adenovirus infection.

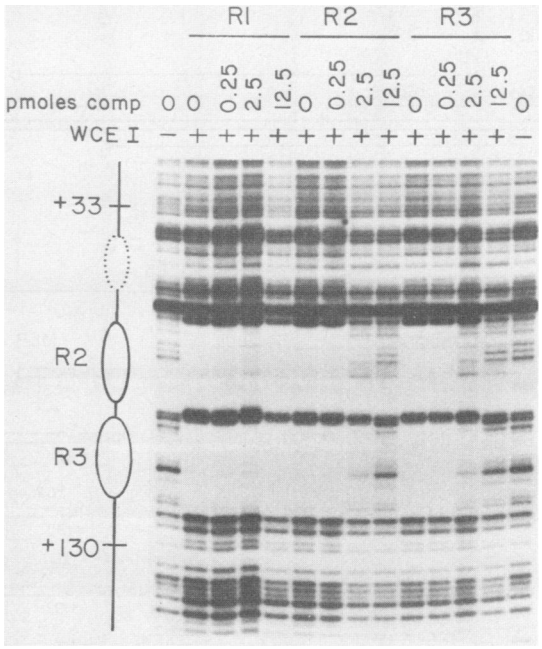

FIG. 5. Binding competition. DNase I footprinting was performed by using whole-cell extract prepared 20 h p.i. with pm975, except for the outside lanes, which contained no added extract protein. The indicated numbers of picomoles of synthetic DNA equal to binding sites R1 (positions +37 to +62), R2 (positions +72 to +98), or R3 (positions +98 to +120) were added as indicated before the addition of DNase I. Factor binding at site R1 was not evident in this footprint.

In the construct designated −66 to +192, Ad2 MLP sequence from positions −66 to +192 (containing the MLTF, TATA box, and R1, R2, and R3 binding sites) was fused to human β-globin sequences from −5 base pairs to +2.1 kb (relative to the β-globin transcription initiation site). Other constructs contained the −66 to +192 region from which the R1, R2, R3, or R2+3 regions were deleted (Fig. 6B). Construct −66 to +33 deleted all of the downstream binding sites. These MLP-β-globin fusions were substituted between map units 78.5 and 83 in the nonessential E3 region (Fig. 6A) of the Ad5 derivative *dl*321 (5). In a no-promoter control construct, the β-globin coding region alone from −5 to +2.1 kb was substituted for the viral E3 region (Fig. 6). The resulting recombinants were propagated in 293 cells (13), which constitutively express E1A and E1B functions, since *dl*321 has a deletion of the E1A region. Each of these recombinants replicated efficiently, since they grew to a high titer equivalent to that of wt Ad5 and formed similar-size plaques on 293 cells.

Insertion into the E3 region keeps the MLP promoter elements relatively well insulated from other adenovirus transcription control regions (Fig. 6A). The E3 promoter region lies 2.7 kb to the left of the inserted MLP, and the E4 promoter region lies 5.8 kb to the right. The MLP-β-globin transcription unit was oriented so that the direction of transcription was opposite to that of the endogenous MLP (Fig. 6A). This allowed analysis of in vitro nuclear run-on transcription, using strand-specific probes cloned in M13, since transcription through this region from the endogenous MLP would transcribe the noncoding β-globin strand as opposed to the coding β-globin strand transcribed from the inserted MLP region.

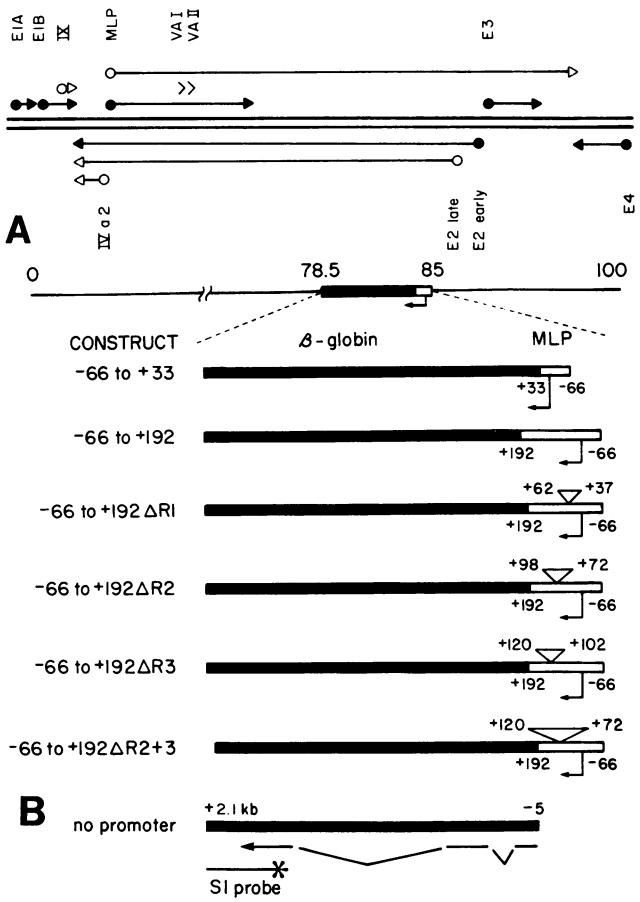

FIG. 6. Diagrams of the MLP-β-globin recombinants. (A) Transcription map of wt Ad5. The double line at the center represents the 36-kb genome. Promoters active during both the early and late phases of infection (●) and promoters active during the late phase only (○) are shown. Arrowheads indicate the direction of transcription. Promoters transcribed to the right and left are indicated above and below the genome, respectively. The VAI and VAII RNAs are transcribed by RNA polymerase III. See reference 2 for a review. (B) Structures of recombinants containing MLP-β-globin fusions substituted into E3. Numbers at the top refer to map units of *dl*321 (5). The structures of the inserted sequences are diagrammed below for each of the constructs named at the left. Ad2 sequences from the indicated regions of the MLP are shown (▭), with arrows indicating the transcription start site and direction. The β-globin coding sequence from −5 to the *Pst*I site at +2.1 kb is indicated (■). The no-promoter construct is an insertion of the β-globin coding region only. The β-globin exons are diagrammed at the bottom. Also shown is the map position of the 3′-end-labeled probe used in S1 nuclease analyses of the MLP-β-globin RNAs.

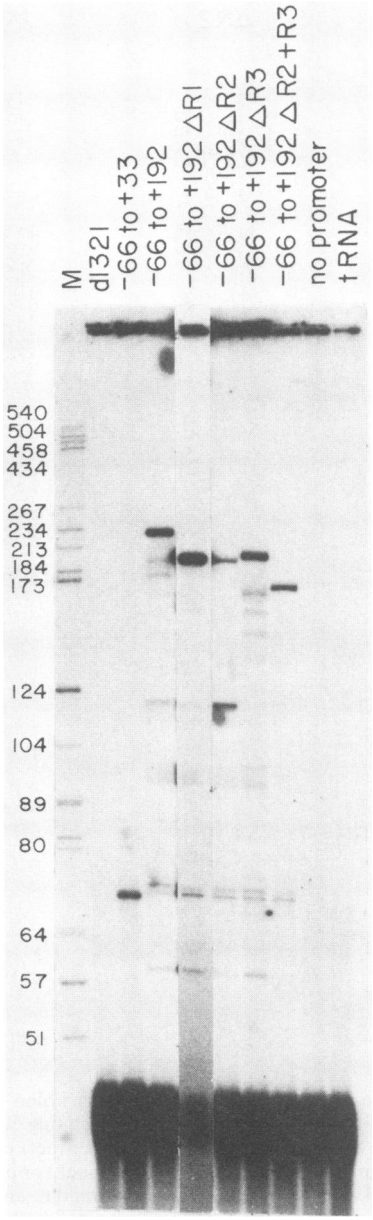

FIG. 7. Primer extension mapping of the transcription start site. Primer extension was performed as described in Materials and Methods by using cytoplasmic RNA isolated 20 h p.i. with the indicated virus constructs. Lane M, Markers, with sizes (in kilobases) indicated at the left; lane tRNA, control primer extension containing yeast tRNA only. The exposure was for 12 h, except for lane −66 to +192ΔR1, which was 1.5 h.

These recombinant viruses and the parental virus *dl*321 were infected into 293 cells, and cytoplasmic RNA was isolated during the late phase of infection (20 h p.i.). To confirm that transcription initiated from the expected start site of the inserted MLP, primer extension analysis was performed by using a primer complementary to β-globin coding sequences from positions +10 to +30. MLP-β-globin fusion RNAs which are not spliced before this position should generate primer extension products of 69, 228, 202, 201, 209, and 179 nucleotides for recombinants −66 to +33, −66 to +192, −66 to +192ΔR1, −66 to +192ΔR2, −66 to +192ΔR3, and −66 to +192ΔR2+3, respectively. The expected primer extension products were observed, indicating that transcription did initiate at the +1 position from the inserted MLP in these recombinants (Fig. 7).

These primer extension products gave accurate mapping of the RNA cap site in the background of viral late mRNAs with the same 5′ sequence. However, they did not accurately measure the total cytoplasmic RNA expressed from the inserted MLP in these constructs. This result is because most of the MLP-β-globin RNA expressed from constructs −66 to +192, −66 to +192ΔR2, −66 to +192ΔR3, and −66 to +192ΔR2+3 were spliced from the adenovirus 5′ splice site at position +41 in the MLP sequence to the 3′ splice site

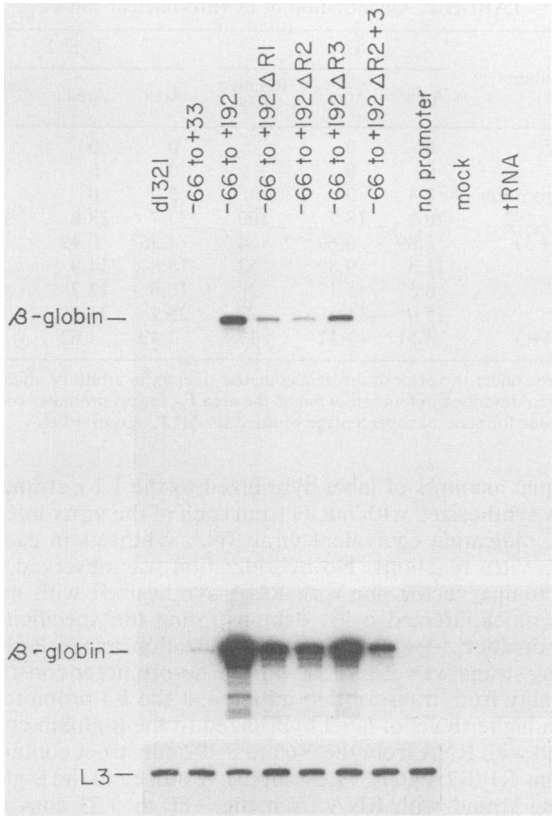

FIG. 8. MLP-β-globin RNA expression during the late phase of infection. (Top) S1 nuclease assays performed with the 3'-end-labeled β-globin coding strand diagrammed in Fig. 6, using 5 μg of cytoplasmic RNA prepared 20 h p.i.; the exposure was for 15 min. (Middle) A 3-h exposure of the same gel. (Bottom) S1 assay of L3 family RNA, using 0.1 μg of 20-h-p.i. cytoplasmic RNA; the exposure was for 3 h.

following the first intron in the β-globin gene (data not shown). Consequently, the RNA sequence complementary to the primer used in the primer extension analysis was spliced out of most of the cytoplasmic RNA expressed from these constructs. As a result, the primer extension products from these mutants greatly underestimate the total cytoplasmic RNA expressed. This splice did not occur in constructs −66 to +33 and −66 to +192ΔR1 because the adenovirus 5' splice site at +41 was deleted from these constructs. Figure 7 shows a shorter exposure of the primer extension product of −66 to +192ΔR1 RNA so that the intensity of this much more abundant primer extension product is similar to that of the neighboring lanes.

To accurately measure the relative expression of MLP-β-globin RNAs expressed by the different constructs, we performed S1 nuclease mapping, using a 3'-end-labeled probe which detects the third exon of the globin RNA (diagrammed in Fig. 6B). RNA from late region L3 was also analyzed as an internal control for the efficiency of infection and recovery of RNA (Fig. 8). All of the recombinants produced equivalent levels of L3 RNA from their endogenous MLPs. This finding demonstrates that they each expressed all the viral functions required for normal transcription from the wt MLP. Yet the recombinants expressed different levels of RNA from the MLP sequences inserted into E3 of the same DNA molecules. There was a profound difference in the level of β-globin RNA expressed from

TABLE 1. Expression of MLP-β-globin RNA

Construct	No. (%)[a] of Cerenkov cpm in S1-protected fragment	
	Expt 1	Expt 2
*dl*321	37	37
No-promoter	113 (0)	130 (0)
−66 to +33	260 (0.2)	285 (0.3)
−66 to +192	66,395 (100)	45,325 (100)
−66 to +192ΔR1	15,134 (22)	10,721 (23)
−66 to +192ΔR2	16,060 (24)	8,323 (18)
−66 to +192ΔR3	48,726 (73)	20,229 (44)
−66 to +192ΔR2+3	3,635 (5)	3,407 (7)

[a] Numbers in parentheses show the percentage of counts per minute relative to that of the −66 to +192 construct after subtraction of counts per minute in the no-promoter construct.

construct −66 to +192 compared with that of construct −66 to +33 (Fig. 8). The level of RNA expressed was sufficiently high to allow quantitation by cutting and counting the S1-resistant bands (Table 1). Although β-globin transcript from the no-promoter control construct was not detected in the primer extension assay (Fig. 7), a low level of β-globin RNA was detected in the S1 analysis (Fig. 8, middle panel). It is possible that this RNA was initiated at the E4 promoter. A small fraction of E2A RNAs have 5' exons from the E4 promoter. These are thought to result from primary transcripts extending leftward from the E4 promoter near the right end of the genome to 60 map units (10). Similarly, extended E4 primary transcripts from the no-promoter construct could be processed at the β-globin splicing and cleavage-polyadenylation signals to yield low-abundance RNAs containing the β-globin 3' exon. The −66 to +33 MLP expressed only about threefold more β-globin RNA than this background level (Table 1). The −66 to +192 MLP region expressed about 200 times more β-globin RNA. The ΔR1 and ΔR2 deletions each dropped the level of β-globin RNA to about 20% of the level of the −66 to +192 MLP. The ΔR3 deletion had a smaller effect, reducing β-globin RNA expression to 45 to 70% of the level of the −66 to +192 MLP, while deletion of ΔR2+3 reduced the level to only 5 to 7%.

Clearly, deletion of the binding sites for the late-phase-specific DNA-binding proteins greatly diminished expression from the MLP during the late phase of infection. We also analyzed β-globin RNA expressed from these constructs at 6 h p.i. of 293 cells, during the early phase of infection. Using the same S1 probe to detect the third β-globin exon, we observed that the MLP constructs expressed only slightly more β-globin RNA than the no-promoter control construct (Fig. 9). In striking contrast to the late phase of infection, the −66 to +192 construct did not express significantly more β-globin RNA than the −66 to +33 construct. E4 RNAs which were analyzed served as controls for infection and recovery of RNA. We conclude that these MLP constructs had very low promoter activity during the early phase of infection and that the sequences between positions +33 and +192 did not significantly increase this low activity during the early phase.

Regions R1, R2, and R3 stimulate transcription from the MLP during the late phase. To determine whether regions R1, R2, and R3 increase mRNA expression from the MLP during the late phase of infection by stimulating the rate of transcription, we performed in vitro nuclear run-on assays. Labeled RNA synthesized in vitro from infected cell nuclei isolated 20 h p.i. was hybridized to an M13 clone of the

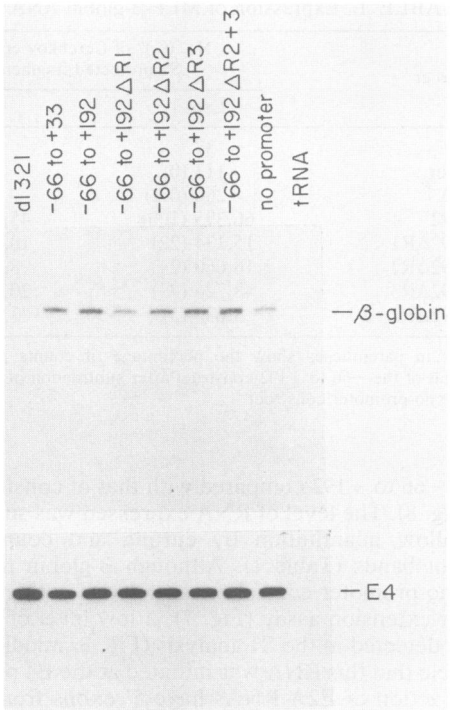

FIG. 9. MLP-β-globin RNA expression during the early phase of infection. (Top) S1 nuclease assays, using 50 μg of cytoplasmic RNA prepared 6 h p.i.; the exposure was for 40 h. (Bottom) S1 analyses of E4 RNA, using 12 μg of cytoplasmic RNA prepared 6 h p.i.; the exposure time was 2.5 h.

β-globin coding strand (Fig. 10; Table 2). As controls, the nitrocellulose filters also contained M13 clones of the L1 region *r* and *l* strands, the β-globin noncoding strand, and the M13mp11 cloning vector.

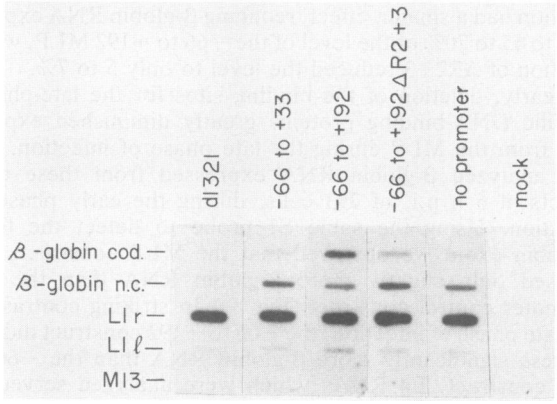

FIG. 10. In vitro nuclear run-on analyses of MLP-β-globin transcription rates. Nuclei were prepared from HeLa cells 20 h p.i. with the indicated adenovirus constructs, [α-^{32}P]GTP was incorporated in vitro, and RNA was isolated as described in Materials and Methods. A total of 10^6 acid-precipitable Cerenkov cpm from each in vitro nuclear run-on reaction was hybridized to nitrocellulose filters containing bound M13 clones of the β-globin coding (cod.) and noncoding (n.c.) strands and the *r* and *l* strands (coding and noncoding, respectively) of the L1 region of *dl*321, as well as M13 DNA. An autoradiogram of the washed and RNase A-treated filters is shown. The complementary region included in the β-globin M13 clones was 483 bases, while 1,598 bases of the L1 region was present in the L1 M13 clones.

TABLE 2. Quantitation of in vitro nuclear run-on

Mutant	Expt 1			Expt 2		
	Area[a]	Area'[b]	Relative (%)[c]	Area	Area'	Relative (%)
Mock	0	0		0	0	
*dl*321	0	0		0	0	
No-promoter	1.4	0		3.9	0	
MLP+192	20.1	18.7	100	32.7	28.8	100
MLP+33	2.09	0.69	4	4.36	0.49	2
*dl*R1	11.3	9.89	52	18.8	14.9	52
*dl*R2	6.57	5.17	28	16.6	12.7	44
*dl*R3	15.9	14.5	77	25.1	21.2	74
*dl*R2+R3	4.51	3.11	17	7.49	3.62	12

[a] Area under the peak of the densitometric tracing, in arbitrary units.
[b] Area described in footnote *a* minus the area for the no-promoter control.
[c] Value for area' as a percentage of area' for MLP −66 to +192.

Equal amounts of label hybridized to the L1 *r* strand for RNA synthesized with nuclei from each of the virus-infected cells, indicating equivalent viral RNA synthesis in each of the in vitro reactions. No hybridization was observed with the cloning vector nor with RNA synthesized with nuclei from mock-infected cells, demonstrating the specificity of hybridization. A low level of hybridization to the β-globin coding strand was observed with the no-promoter construct, probably from transcription initiated at the E4 promoter. A much higher level of label hybridized to the β-globin coding strand with RNA from the −66 to +192 construct containing regions R1, R2, and R3. Less label hybridized to the β-globin coding strand with RNA from the −66 to +33 construct, which lacks these regions, and with the −66 to +192ΔR2+3 construct RNA. A low level of hybridization to the L1 *l* strand was observed for *dl*321 RNA, as expected, since this region falls within the leftward E2 early and late transcription units which are both active during the late phase. In this experiment, somewhat more hybridization was observed for the constructs containing the leftward MLP inserts, perhaps because of additional leftward transcription from the inserted promoters, but this was not observed in all experiments.

The region in which the MLP β-globin constructs were inserted is included in the endogenous major late transcription unit (Fig. 6A). Consequently, the β-globin noncoding strand, fused to the viral *r* strand, was transcribed in each of the constructs containing β-globin sequences. Slightly less label hybridized to the β-globin noncoding strand for the −66 to +192 construct than for the other constructs. This may have been due to interference between polymerases transcribing this region in opposite directions in the case of the strong leftward promoter of the −66 to +192 construct. Alternatively, the decrease may have been due to RNA-RNA hybridization in solution above the filter of cRNA transcribed from each strand of the β-globin sequence. For the −66 to +192 construct, similar amounts of label hybridized to the β-globin coding strand as hybridized to the noncoding strand for each of the β-globin constructs. This result indicates that the leftward transcription rate from the inserted −66 to +192 MLP was similar to the rightward rate of transcription through this region from the endogenous MLP.

Quantitation of hybridization to the β-globin coding strand for each of the constructs in two experiments is shown in Table 2. After subtraction for the background transcription of the no-promoter construct, the −66 to +192 construct synthesized 25- to 50-fold more β-globin RNA than did the

−66 to +33 construct. The ΔR1, ΔR2, ΔR3, and ΔR2+3 constructs synthesized approximately 0.5, 0.4, 0.7, and 0.2 times as much β-globin RNA as did the −66 to +192 construct, respectively. These results for in vitro nuclear run-on transcription show somewhat smaller effects for these deletions than observed for quantitation of the cytoplasmic RNA concentrations (Table 1). This discrepancy may be due to differences in the efficiency of processing, transport, or stabilities of the different MLP-β-globin fusion RNAs. We conclude that the R1, R2, and R3 regions each stimulate the rate of transcription from the MLP during the late phase of infection. The entire region from positions +33 to +192 stimulates transcription 25- to 50-fold.

DISCUSSION

Mansour et al. (24) originally provided evidence that a late-phase-specific, virus-induced factor interacts with sequences in the region from positions +33 to +190 downstream from the Ad2 MLP to stimulate transcription during the late phase of infection. Jansen-Durr et al. (16) reported the binding of a late-phase-specific, virus-induced factor to the region from +76 to +120 by using DNase I footprinting. Further, they showed that in in vitro transcription reactions at a low template concentration, whole-cell extracts prepared from Ad5-infected cells during the late phase had higher activities on templates extending from −137 to +131 or further downstream than did extracts from E1A mutant-infected cells. Part of this higher activity appeared to be due to the effects of E1A expression as reported by us (18, 19), and part was due to late-phase-specific factors.

In this work, we further characterized the region between positions +33 and +192 downstream from the MLP which interacts with late-phase-specific, virus-induced factors and the influence of these binding sites on late-phase transcription from the MLP. We found that three independent regions mapping from approximately +37 to +68, +80 to +105, and +105 to +125, designated R1, R2, and R3, respectively, interact with factors which increase in activity between 12 and 20 h p.i. As shown by Jansen-Durr et al. (16), inhibition of viral DNA replication prevents the induction of these sequence-specific binding factors. The factors bind independently, since the deletion of R2 or R3 does not significantly affect the binding of factors to the remaining sites. Results of DNase I footprinting analyses performed in the presence of synthetic oligomers of sites R1, R2, and R3 (Fig. 5) suggest that the same factor binds to sites R2 and R3, despite their apparent lack of obvious homology. It is not yet clear whether the same factor binds to site R1 with low affinity or whether a second factor binds to site R1.

To characterize the influence of these binding sites on transcription during the late phase, we constructed adenovirus recombinants in which the wt MLP region including the downstream binding sites or mutants of these sites were fused to β-globin coding sequences and substituted into region E3. These constructs were capable of replicating in 293 cells, allowing us to measure the consequences of deleting the binding sites on transcription from replicated templates during the late phase of infection. We found that deletion of the entire region from positions +33 to +192 resulted in a 200-fold decrease in the accumulation of MLP-β-globin RNA in the cytoplasm at 20 h p.i. and a 25- to 50-fold decrease in the rate of transcription, as measured by in vitro nuclear run-on assays. Deletion of the individual sites had smaller effects.

These binding factors induced specifically during the late phase of adenovirus infection are likely to be positively acting transcription factors which stimulate transcription from the MLP. Such late-phase-specific transcription factors could account for the much greater rate of transcription from the MLP during the late phase of infection compared with transcription rates from the other early viral promoters. It seems unlikely that the factors are involved in an antitermination mechanism, since deletion of the sites decreases downstream transcription. Also, no terminated transcripts from the wt template have been observed in in vitro transcription reactions using early- or late-phase extracts (16, 18, 19), even under conditions in which the downstream regions stimulate transcription (16; K. Leong and A. J. Berk, unpublished results).

Major questions raised by these studies concern the nature of these late-phase-specific binding factors. Are they in fact positively acting transcription factors? Are they virus-encoded late-phase-specific proteins, or are they host proteins modified by late viral functions? Another interesting question is why do they bind downstream from the transcription start site as opposed to upstream? Part of the reason may be that the leftward IVa2 promoter start site maps only 212 base pairs to the left of the MLP start site. Placing late-phase-specific factor-binding sites to the right of the MLP might focus the influence of these factors on the closer MLP. The next closest polymerase II promoter to the right of these late-phase factor-binding sites is the E2 late promoter, 20 kb away. Also, this entire region including the IVa2 promoter and the MLP and downstream sites all lie within the same DNA sequence which encodes the viral DNA polymerase, expressed from leftward transcripts initiated at the E2 early and E2 late promoters (31–35). This may impose constraints on the sequence which limit the possible locations of binding sites for late-phase-specific transcription factors.

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LITERATURE CITED

1. **Akusjarvi, G., and H. Persson.** 1981. Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature (London) **292**:420–426.
2. **Berk, A. J.** 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20**:45–79.
3. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
4. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
5. **Bhat, G., L. SivaRaman, S. Murthy, P. Domer, and B. Thimmappaya.** 1987. In vivo identification of multiple promoter domains of adenovirus EIIA-late promoter. EMBO J. **6**:2045–2052.
6. **Brunet, L. J., L. E. Babiss, C. S. H. Young, and D. R. Mills.** 1987. Mutations in the adenovirus major late promoter: effects on viability and transcription during infection. Mol. Cell. Biol. **7**:1091–1100.
7. **Carthew, R. W., L. A. Chodosh, and P. A. Sharp.** 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**:439–448.
8. **Charnay, P., R. Treisman, P. Melon, M. Chao, R. Axel, and T. Maniatis.** 1984. Differences in human α- and β-globin gene

expression in mouse erythroleukemia cells: the role of intragenic sequences. Cell **38**:251–263.

9. **Chodosh, L. A., R. W. Carthew, and P. A. Sharp.** 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol. **6**:4723–4733.
10. **Chow, L., J. B. Lewis, and T. Broker.** 1980. RNA transcription and splicing at early and intermediate times after adenovirus-2 infection. Cold Spring Harbor Symp. Quant. Biol. **44**:401–414.
11. **Concino, M., R. A. Goldman, M. H. Caruthers, and R. Weinmann.** 1983. Point mutations of the adenovirus major late promoter with different transcriptional efficiencies in vitro. J. Biol. Chem. **258**:8493–8496.
12. **Ensinger, M. J., and H. S. Ginsberg.** 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. **10**:328–329.
13. **Graham, F. O., J. Smiley, W. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
14. **Hen, R., P. Sassone-Corsi, J. Corden, M. P. Gaub, and P. Chambon.** 1982. Sequences upstream from the TATA box are required for in vivo and in vitro transcription from the adenovirus serotype 2 major late promoter. Proc. Natl. Acad. Sci. USA **79**:7132–7136.
15. **Hu, S.-L., and J. L. Manley.** 1981. DNA sequence required for initiation of transcription in vitro from the major late promoter of adenovirus 2. Proc. Natl. Acad. Sci. USA **78**:820–824.
16. **Jansen-Durr, P., H. Boeuf, and C. Kedinger.** 1988. Replication-induced stimulation of the major late promoter of adenovirus is correlated to the binding of a factor to sequences in the first intron. Nucleic Acids Res. **16**:3771–3786.
17. **Laskey, R. A.** 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. **65**:363–371.
18. **Leong, K., and A. J. Berk.** 1986. Adenovirus early region 1A protein increases the number of template molecules transcribed in cell-free extracts. Proc. Natl. Acad. Sci. USA **83**:5844–5848.
19. **Leong, K., L. Brunet, and A. J. Berk.** 1988. Factors responsible for the higher transcriptional activity of extracts of adenovirus-infected cells fractionate with the TATA box transcription factor. Mol. Cell. Biol. **8**:1765–1774.
20. **Lewis, E. D., and J. L. Manley.** 1985. Control of adenovirus late promoter transcription in two human cell lines. Mol. Cell. Biol. **5**:2433–2442.
21. **Lewis, J. B., and M. Mathews.** 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell **21**:303–313.
22. **Logan, J., and T. Shenk.** 1986. *In vivo* identification of sequence elements required for normal function of the adenovirus major late transcription control region. Nucleic Acids Res. **14**:6327–6335.
23. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a whole-cell extract. Proc. Natl. Acad. Sci. USA **77**:3855–3859.
24. **Mansour, S. L., T. Grodzicker, and R. Tjian.** 1986. Downstream sequences affect transcription initiation from the adenovirus major late promoter. Mol. Cell. Biol. **6**:2684–2694.
25. **Miyamoto, N. G., V. Moncollin, J. M. Egly, and P. Chambon.** 1985. Specific interaction between a transcription factor and the upstream element of the adenovirus-2 major late promoter. EMBO J. **4**:3563–3570.
26. **Moncollin, V., N. G. Miyamoto, X. M. Zheng, and J. M. Egly.** 1986. Purification of a factor specific for the upstream element of the adenovirus-2 major late promoter. EMBO J. **5**:2577–2584.
27. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping viral genes by site directed mutagenesis at a mRNA splice site. Nature (London) **295**:380–384.
28. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**: 213–220.
29. **Nevins, J. R., and M. C. Wilson.** 1981. Regulation of adenovirus-2 gene expression at the level of transcription termination and RNA processing. Nature (London) **290**:113–118.
30. **Proudfoot, N. J., M. H. M. Shander, J. L. Manley, M. L. Gefter, and T. Maniatis.** 1980. Structure and in vitro transcription of human globin genes. Science **209**:1329–1336.
31. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
32. **Shaw, A. R., and E. B. Ziff.** 1981. Transcripts from the adenovirus-2 major late promoter yield a single early family of 3′ co-terminal mRNAs and five late families. Cell **22**:905–916.
33. **Shu, L., M. S. Horwitz, and J. A. Engler.** 1987. Expression of enzymatically active adenovirus DNA polymerase from cloned DNA requires sequences upstream of the main open reading frame. Virology **161**:520–526.
34. **Shu, L., S. C. Pettit, and J. A. Engler.** 1988. The precise structure and coding capacity of mRNAs from early region 2B of human adenovirus serotype 2. Virology **165**:348–356.
35. **Stillman, B. W., J. B. Lewis, L. T. Chow, M. B. Matthews, and J. E. Smart.** 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell **23**:497–508.
36. **Weinheimer, S. P., and S. L. McKnight.** 1987. Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195**:819–834.
37. **Yu, Y.-T., and J. L. Manley.** 1986. Generation and functional analysis for base-substitution mutants of the adenovirus 2 major late promoter. Nucleic Acids Res. **12**:9309–9321.