Efficient Transcription, Not Translation, Is Dependent on Adenovirus Tripartite Leader Sequences at Late Times of Infection

FIRELLI V. ALONSO-CAPLEN,¹ MICHAEL G. KATZE,² AND ROBERT M. KRUG^{1*}

*Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021,¹ and
Department of Microbiology, University of Washington, Seattle, Washington 98195²*

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To determine whether the tripartite leader is required for efficient translation in adenovirus-infected cells at late times of infection, we constructed recombinant adenoviruses containing the influenza virus nucleocapsid protein (NP) gene expressed under the control of the adenovirus major late promoter (MLP). We chose the NP gene because previous results showed that the influenza virus NP mRNA was an extremely effective initiator of translation in cells which were superinfected with influenza virus at late times of adenovirus infection (M. G. Katze, B. M. Detjen, B. Safer, and R. M. Krug, *Mol. Cell. Biol.* 6:1741-1750, 1986). The NP gene in the adenovirus recombinants was inserted downstream of an MLP that replaced part of the early (E1A) region. The resulting NP mRNAs either lacked any tripartite leader sequences or contained at their 5' ends various portions of the tripartite leader: 33, 172, or all 200 nucleotides of the leader. The relative amounts of the NP protein synthesized by the recombinants were directly proportional to the amounts of the NP mRNA made, indicating that the presence of 5' tripartite leader sequences did not enhance the translation of NP mRNA. In addition, the sizes of the polysomes containing NP mRNA were not increased by the presence of tripartite leader sequences, indicating that the initiation of translation was not enhanced by these sequences. On the other hand, the presence of tripartite leader sequences immediately downstream of the MLP did enhance the transcription of the inserted NP gene, as shown by Northern (RNA) analysis of *in vivo* NP mRNA levels and by *in vitro* runoff assays with isolated nuclei. Our results indicate that more than 33 nucleotides of the first leader segment of the tripartite leader are required for optimal transcription from the MLP.

According to the scanning model for the mechanism of eucaryotic protein synthesis (17), a 40S ribosomal subunit binds at the 5' capped end of an mRNA and migrates down the chain until it reaches the first AUG initiation codon. Features that interfere with this linear movement, e.g., secondary structure in the 5' untranslated region (19, 29), have been shown to inhibit translation. In contrast, the presence of the cap and the base sequence directly flanking the AUG codon can enhance translation (1, 18). In one system, in adenovirus-infected cells at late times of infection, a specific 5' untranslated region, the tripartite leader, has been reported to be required for efficient translation. Most late adenovirus mRNAs contain this 5' tripartite leader sequence, which is generated by splicing of sequences from three distinct regions of the adenovirus genome (2, 36). The role of the tripartite leader in translation has been assessed by constructing recombinant adenoviruses in which a marker mRNA sequence (i.e., an mRNA not made at late times of adenovirus infection) was placed under the control of the major late promoter (MLP). Various portions of the tripartite leader were appended to the 5' ends of the marker mRNAs. With several marker mRNAs, adenovirus E1A mRNAs (26), hepatitis B surface antigen mRNA (6), and mouse dihydrofolate reductase mRNA (4), it was reported that the entire tripartite leader sequence was required for efficient translation at late times of adenovirus infection. In contrast, it was also reported that the tripartite leader did not enhance the translation of another marker mRNA, simian virus 40 (SV40) T antigen mRNA (28). Consistent with the latter result, the adenovirus polypeptide IX mRNA, which

lacks the tripartite leader, has been shown to be translated at late times more efficiently than the late adenovirus mRNAs containing the tripartite leader (20).

To address the role of the tripartite leader in translation in late adenovirus-infected cells, we used the influenza virus mRNA coding for the nucleocapsid protein (NP). In cells which were superinfected with influenza virus at late times of adenovirus infection, the influenza virus NP mRNA and other influenza virus mRNAs were extremely effective initiators of protein synthesis (13, 15). In fact, the NP mRNA was on larger polysomes than were several late, tripartite leader-containing adenovirus mRNAs with comparably sized coding regions, indicating that the NP mRNA was more efficiently initiated than these adenovirus mRNAs. The high efficiency of translation of the NP mRNA was maintained even when adenovirus protein synthesis was essentially shutdown in cells coinfecting with the adenovirus mutant *d1331* that does not synthesize VAI RNA (15). One interpretation of these results is that the NP mRNA, which does not have the tripartite leader, is intrinsically a good mRNA and can outcompete the late adenovirus mRNAs in translational initiation. Alternatively, the efficient translation of NP mRNA in doubly infected cells might conceivably be mediated by another influenza virus gene product.

To discriminate between these two alternatives, we have expressed the NP mRNA in late adenovirus-infected cells in the absence of other influenza virus gene products. We have inserted the NP gene into the adenovirus genome under the control of the MLP, with either none or various portions of the tripartite leader appended to the 5' end of the NP mRNA. We showed that the tripartite leader is not required for the efficient translation of NP mRNA in late adenovirus-infected

* Corresponding author.

FIG. 1. Construction of the recombinant adenoviruses AdNP-L172 and AdNP-L0. (a) Structure of the recombinant plasmids pMPCV2-NP and pMPCV3-NP. PKG, Ad5 packaging sequences extending from 0 to 1 map unit; MLP, Ad2 MLP; L1-3 represents 172 out of 200 nucleotides of the tripartite leader (only for pMPCV2-NP); $\square$, sequences encoding the influenza virus NP; $\square$, Ad5 sequences from 7.9 to 15.5 map units; —, pBR322 sequences; *Amp^r*, ampicillin resistance. The presumed structure of the NP mRNA is shown outside the plasmid circle. The solid box represents the coding region of the NP gene, and the arrowhead denotes the position of poly(A) addition within the E1B region. (b) Generation of recombinant adenoviruses by in vivo recombination between the linearized plasmid and *Xba*I-cleaved *dl309* DNA extending from 3.8 to 100 map units. The recombination occurs between the homologous 7.9 to 15.5 map unit sequences in each DNA to yield the recombinant viruses, AdNP-L172 and AdNP-L0.

cells. On the other hand, the presence of tripartite leader sequences immediately downstream of the MLP enhanced the transcription of the inserted NP gene.

MATERIALS AND METHODS

Cells and viruses. Cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (293 cells) or with 10% calf serum (MDBK and MDCK cells). The adenovirus type 5 (Ad5) mutant H5 *dl309* (12) was propagated in 293 cells (9) and purified as previously described (30). The titer of adenovirus stocks was determined by plaque assay on 293 cells (21). The A/WSN/33 strain of influenza virus was grown in MDBK cells and titrated on MDCK cells (7).

Construction of recombinant adenoviruses containing the NP gene. The pAPR502 plasmid containing the entire NP gene of influenza A/PR8/34 virus (35) was provided by P. Palese. Plasmid pMPCV2, which was provided by J. Logan and T. Shenk (26), contains the Ad2 MLP beginning at the *Sst*II site (Ad2, 16.5 map units), the intact first and second Ad2 late leader, and about two-thirds of the third leader (60 of 88 base pairs). These leader sequences do not contain introns since they were derived from a cDNA clone (pJAW-43) of the Ad2 fiber mRNA 5' domain (36). The Ad2 MLP and tripartite leader sequences are flanked on the left by the 353-base-pair packaging sequences of Ad5 and on the right by Ad5 sequences from 7.9 to 15.5 map units. All of these sequences were inserted into pBR322. Plasmid pMPCV3, also provided by J. Logan and T. Shenk, is similar to plasmid

pMPCV2 except that it does not contain any tripartite leader sequence. The recombinant plasmids pMPCV2-NP and pMPCV3-NP (Fig. 1a) were formed by excision of NP from the *Eco*RI site of pAPR502 and by blunt-end ligation to the large fragment of plasmids pMPCV2 or pMPCV3, extending from *Bgl*II to *Xho*I. Plasmid pMPCV2-NP was linearized with *Clal*I, and plasmid pMPCV3-NP was linearized with *Eco*RI. Each linearized DNA was combined with a sucrose gradient-purified large fragment of *Xba*I-cleaved *dl309* DNA (12), extending from 3.8 to 100 map units (Fig. 1b). The DNA mixture was transfected onto 293 cells by using calcium phosphate precipitation (10), and the cells were overlaid with agar for plaque detection. Recombinant adenoviruses were produced by in vivo recombination between homologous adenovirus sequences.

Plasmid pD1, which was provided by K. Berkner (3), contains the Ad5 packaging sequence, the Ad2 MLP, the complete tripartite leader with its normal 5' splice site, a 3' splice site from an immunoglobulin gene, and the SV40 polyadenylation signal. Plasmid pDL1 was derived from plasmid pD1 by removal of 167 nucleotides of the tripartite leader between a *Pvu*II site within the first leader segment and the *Bam*HI subcloning site. The latter was restored by the addition of a *Bam*HI linker to the *Pvu*II site. The recombinant plasmids pD1-NP and pDL1-NP (Fig. 2a) were constructed by excision of NP from the pAPR502 *Eco*RI site and blunt-end ligation to the *Bam*HI site of pD1 or pDL1. Both plasmids were digested with *Eco*RI and *Xba*I to remove the modular transcription unit which was then ligated to a sucrose gradient-purified fragment of *Xba*I-cleaved

FIG. 2. Construction of the recombinant adenoviruses AdNP-L200 and AdNP-L33. (a) Structure of the recombinant plasmids pD1-NP and pDL1-NP. PKG, Ad5 packaging sequences extending from 0 to 1 map unit; MLP, Ad2 MLP; L1-3 + 5', complete tripartite leader with its normal 5' splice site (only in pD1-NP); 3', 3' splice site from an immunoglobulin gene (only in pD1-NP); $\square$, sequences encoding the influenza virus NP; pA, SV40 early polyadenylation signal. Plasmid pDL1-NP has only part (33 of 41 nucleotides) of the first leader segment, L1. —, pBR322 sequences; Amp^r, ampicillin resistance. The presumed structure of the NP mRNA is shown outside the plasmid circle. The solid box represents the coding region of the NP gene, and the arrowhead denotes the position of poly(A) addition using the SV40 early polyadenylation signal. (b) Generation of the recombinant adenoviruses AdNP-L200 and AdNP-L33 by in vitro ligation of the *Eco*RI-*Xba*I fragment of the plasmid (containing the modular transcription unit) with *Xba*I-cleaved *dl309* DNA.

dl309 DNA (Fig. 2b). The ligation mixture was transfected onto 293 cells as described above.

Approximately 10 to 15 days after transfection, plaques were picked and amplified by infection of 293 cells in 35-mm-diameter dishes. Culture lysates were prepared when cytopathic effect was almost complete (3 to 5 days). Cytoplasmic RNA obtained from these lysates was analyzed by dot blot hybridization (34) by using the ³²P-labeled NP DNA probe described below. Plaque-purified recombinant adenoviruses were propagated in 293 cells.

RNA extraction and analysis. Cells were collected at 20 h postinfection from 293 cells infected with adenovirus (recombinant or *dl309*) at a multiplicity of infection of 20 PFU per cell and from 293 cells infected for 16 h with adenovirus *dl309* and superinfected with influenza virus for an additional 4 h at a multiplicity of infection of 50 PFU per cell. Cytoplasmic poly(A)⁺ RNA was purified from infected cells as described previously (8, 16). Poly(A)⁺ RNA was analyzed by Northern (RNA) blot analysis. After electrophoresis on a formaldehyde-agarose (1%) gel, the gel was blotted onto a nitrocellulose filter. The nitrocellulose filters were hybridized to ³²P-labeled probes (33). For detecting NP mRNA, the probe used was nick-translated pAPR502 (31). The adenovirus probe was a nick-translated pBR322 plasmid containing the L3 region, the *Hind*III-*Bam*HI fragment from 50.1 to 59.5 map units. Where indicated, various dilutions of cytoplasmic poly(A)⁺ RNA was directly blotted onto nitrocellulose membranes, which were then hybridized to a ³²P-labeled probe. The resulting labeled dots were cut out and counted in a liquid scintillation counter.

Determination of the transcription rate. For in vitro run-off

transcription (16), nuclei were prepared from cells collected at 20 h postinfection with an adenovirus recombinant. The nuclei were washed twice in transcription reaction buffer (10 mM Tris hydrochloride [pH 7.9], 140 mM KCl, 14 mM 2-mercaptoethanol, 5 mM MgCl₂, 1 mM MnCl₂, 1 mM each ATP, CTP, and GTP, 1 mM *S*-adenosylmethionine, 10% glycerol), suspended in 0.2 ml of this buffer containing 0.5 mCi of [α -³²P]UTP (3,000 Ci/mmol), and incubated at 33°C for 15 min. The reaction was terminated by washing the nuclei with ice-cold Hanks balanced salt solution containing 3 mM UTP and suspending the nuclear pellet in 10 mM EDTA-10 mM Tris hydrochloride (pH 7.4)-0.2% sodium dodecyl sulfate (SDS). After treatment with 20 μ g of RNase-free DNase per ml (11) followed by digestion with 500 μ g of pronase per ml, nuclear RNA was extracted with phenol-chloroform and ethanol precipitated. Cell-equivalent amounts of ³²P-labeled nuclear RNA were hybridized to filters containing excess amounts of the specified probes of cloned DNA for 24 h at 65°C in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.15 M sodium citrate)-3 $\times$ Denhardt solution-0.5% SDS. Filters were then washed extensively with 2 $\times$ SSC-0.5% SDS at 65°C and subjected to treatment with 20 μ g of RNase A per ml for 30 min at 37°C. The amount of labeled RNA on the filter was determined by liquid scintillation counting.

Primer extension analysis. An oligonucleotide primer complementary to nucleotides 52 to 84 from the 5' end of NP mRNA was labeled at its 5' end by using [γ -³²P]ATP and polynucleotide kinase (27). This 5'-labeled primer was hybridized to poly(A)⁺ cytoplasmic RNA for 3 h at 55°C in 10 mM Tris hydrochloride (pH 7.4)-1 mM EDTA-750 mM

FIG. 3. Determination of the 5' ends of recombinant NP mRNAs by primer extension. (a) An oligonucleotide complementary to nucleotides 52 to 84 from the 5' end of influenza virus NP mRNA was labeled at its 5' end with [γ - 32 P]ATP and T4 polynucleotide kinase. This labeled primer was hybridized to cytoplasmic poly(A)⁺ RNA from 293 cells infected with either AdNP-L172 (lane 1), AdNP-L0 (lane 2), AdNP-L200 (lane 3), or AdNP-L33 (lane 4). After reverse transcription, primer-extended products were electrophoresed on a 5% denaturing polyacrylamide gel containing 8 M urea. The arrows point to the positions of the largest primer-extended products. Lane M shows 32 P-labeled *Hinf*I-digested pBR322 fragments with their nucleotide lengths. (b) Schematic diagram of each of the four recombinant NP mRNAs showing the position of each 5'-labeled oligonucleotide primer relative to the 5' end of the NP mRNA. The solid boxes represent adenovirus tripartite leader sequences.

NaCl in 100 μ l. After ethanol precipitation, reverse transcription was carried out for 1 h at 37°C in 50 μ l containing 50 mM Tris hydrochloride (pH 8.3)–75 mM KCl–10 mM dithiothreitol–3 mM MgCl₂–50 μ g of actinomycin D per ml–0.5 mM of each of the four dNTPs–100 μ g of nuclease-free bovine serum albumin per ml–4,000 U of Moloney murine leukemia virus reverse transcriptase per ml. The RNA was hydrolyzed by the addition of NaOH to 0.1 N and incubation for 30 min at 68°C. After neutralization with 2 M

sodium acetate (pH 5.0), DNA was isolated by phenol-chloroform extraction and ethanol precipitation. After being heated and fast cooled, the extended primer was separated from the unextended primer by electrophoresis on a 5% polyacrylamide gel containing 8 M urea.

Protein analyses. Infected cells were labeled for 1 h with [35 S]methionine in methionine-free Dulbecco modified Eagle medium at the times indicated in the text. After being washed two times with cold Hanks balanced salt solution, cells were disrupted on ice with lysis buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and aliquots were analyzed on 14% polyacrylamide gels.

For immunoprecipitation reactions, infected cell extracts in lysis buffer were incubated with protein A-Sepharose beads precoated with pooled monoclonal antibodies directed against the NP protein of influenza virus. After extensive washing of the beads, first with lysis buffer, then twice with cell dissociation buffer (10 mM Tris hydrochloride [pH 8.0], 250 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate), the proteins on the beads were eluted by being boiled in gel-loading reducing buffer (6.25 mM Tris hydrochloride [pH 6.8], 2.3% SDS, 10% glycerol, 0.05% 2-mercaptoethanol, 0.005% bromophenol blue). The proteins were resolved on a 14% polyacrylamide gel.

Preparation of polyribosomes. Cytoplasmic extracts were prepared and analyzed on 10 to 50% linear sucrose gradients in 10 mM Tris hydrochloride (pH 7.5)–5 mM magnesium acetate–500 mM KCl as described previously (5, 14, 22). Gradient fractions were pooled as described in the text, and the poly(A)⁺ RNA from these pools was analyzed by Northern blot analysis as described above.

RESULTS

Construction of adenovirus recombinants that express influenza virus NP mRNA with and without the tripartite leader. Our goal was to construct adenovirus recombinants that expressed influenza virus NP mRNAs containing at their 5' ends the entire (or almost entire) tripartite leader and to compare the translational efficiency of these mRNAs at late times of adenovirus infection to expressed NP mRNAs containing either no leader sequences or only a portion of the first leader. These recombinants contained an additional MLP replacing part of the E1A region of the adenovirus genome. The NP mRNA was inserted downstream of this MLP, so that the NP mRNA should be expressed at late times of adenovirus infection. Initially, we used vectors provided by Logan and Shenk (26) and in vivo recombination to generate adenovirus recombinants that expressed recombinant NP mRNAs containing at their 5' ends either no tripartite leader sequences (AdNP-L0) or almost the entire tripartite leader (172 of 200 nucleotides) (AdNP-L172) (Fig. 1). In these two constructs, the E1B polyadenylation site was used, resulting in about 700 additional nucleotides at the 3' ends of the NP mRNAs. Subsequently, for the reasons discussed below, we employed the vector system of Berkner et al. (3) and in vitro ligation to obtain adenovirus recombinants that expressed NP mRNAs containing either the entire tripartite leader (AdNP-L200) or only a portion (33 nucleotides) of the first leader (AdNP-L33) (Fig. 2). In addition, an SV40 polyadenylation site was present immediately downstream of the NP sequence, so that the NP mRNAs contained only 200 additional nucleotides at their 3' ends. The four recombinants were grown in 293 cells, which should complement the E1A defects of the recombinants.

FIG. 4. Sizes and relative amounts of the NP mRNAs synthesized by the four recombinant adenoviruses. One microgram of cytoplasmic poly(A)⁺ RNA prepared from 293 cells at 20 h postinfection with *d*/309 (lane 1), AdNP-L172 (lane 2), AdNP-L0 (lane 3), AdNP-L200 (lane 4), or AdNP-L33 (lane 5) and from 293 cells infected with *d*/309 for 16 h and an additional 4 h with influenza virus (lane 6) was denatured with formaldehyde and fractionated on a 1% agarose gel. The RNA was transferred to nitrocellulose and hybridized with either ³²P-labeled pAPR502 to probe NP mRNAs (panel a) or ³²P-labeled *Hind*III-*Bam*HI fragment from 50.1 to 59.5 map units encompassing the L3 region (panel b). Lanes 7 and 8 are 12-fold longer exposures of lanes 2 and 3, respectively. The 28S and 18S rRNA markers are shown.

To verify that the recombinant NP mRNAs had the predicted 5' ends, we carried out primer extensions, using an oligonucleotide complementary to nucleotides 52 to 84 from the 5' end of NP mRNA (Fig. 3). All the recombinant NP mRNAs initiated at the predicted sites. The three NP mRNAs that contained all or some tripartite leader sequence (AdNP-L200, AdNP-L172, and AdNP-L33) initiated at the 5' end of this sequence (lanes 1, 3, and 4), and the AdNP-L0 NP mRNA initiated at approximately the beginning of the *Eco*RI linker that joined the NP sequence to the MLP (lane 2). In addition to the expected extension product, one or more smaller products were generated. These represented premature terminations during reverse transcription, as S1 nuclease analysis indicated a single initiation site at the predicted site (data not shown).

The recombinants produced different levels of NP mRNAs.

The sizes and relative amounts of the NP mRNAs produced by the four recombinants were determined by Northern blot analysis of equivalent amounts of poly(A)⁺ cytoplasmic RNA from infected cells. The NP mRNAs were produced at late but not early times of infection. Increasing amounts were synthesized from 12 to at least 20 h postinfection. The steady-state levels of the NP mRNAs at 20 h postinfection are shown (Fig. 4a). The four recombinant NP mRNAs were larger than the NP mRNA synthesized in cells infected with both influenza virus and adenovirus (lane 6). The sizes of the recombinant NP mRNAs were those expected for mRNAs that initiated at the sites determined above and that utilized either the E1B polyadenylation site (AdNP-L172 and AdNP-L0) or the inserted SV40 polyadenylation site (AdNP-L200 and AdNP-L33). Different levels of NP mRNA were produced by the four recombinants, in contrast to the similar levels of representative late adenovirus mRNAs, L3 mRNAs, that were made by the recombinants (compare Fig. 4a and b). Because a larger amount of NP mRNA was

produced with the Berkner et al. vector system (3) (AdNP-L200 and AdNP-L33) than with the vectors of Logan and Shenk (26) (AdNP-L172 and AdNP-L0) (Fig. 4a), it was only valid to compare the levels of the NP mRNAs of the two recombinants derived from the same vector system. On the basis both of this Northern analysis and of a quantitation by dot-blot analysis (Table 1), we determined that four- to sixfold more NP mRNA was produced by AdNP-L172 than by AdNP-L0 and that three- to fourfold more NP mRNA was produced by AdNP-L200 than by AdNP-L33. The amount of NP mRNA produced by AdNP-L200 was high (Fig. 4a, lane 4). It was equal to or greater than the level of the L3 mRNAs (Fig. 4b) and also equal to or greater than the level of NP mRNA produced in cells doubly infected with adenovirus and influenza virus (Fig. 4a, lane 6). With all four recombinants, a minor NP-specific poly(A)⁺ RNA of smaller size was observed (most evident in lane 7 of Fig. 4a). Preliminary S1 nuclease protection experiments indicated that this minor species of RNA was generated by splicing between cryptic splice sites within the NP coding region (data not shown).

The reduced amount of NP mRNA produced in cells infected with AdNP-L0 or AdNP-L33 could be caused either by a reduction in the level of transcription or by a posttranscriptional event. To determine whether transcription was reduced, an *in vitro* runoff transcription assay was performed (Table 2). The rate of transcription of the NP sequences of AdNP-L172 was about fourfold greater than that of AdNP-L0, and the rate of transcription of the NP sequences of AdNP-L200 was approximately threefold greater than that of AdNP-L33. These differences in the rates of transcription parallel and thus account for the differences in the steady-state levels of cytoplasmic NP mRNAs observed between the recombinants. These results indicate that transcription from the MLP is enhanced by the

TABLE 1. Effect of tripartite leader sequences on the steady-state levels of NP mRNA produced by the recombinant adenoviruses^a

Fold dilution	RNA hybridized (cpm) ^b		Ratio of AdNP-L172 to AdNP-L0	RNA hybridized (cpm) ^b		Ratio of AdNP-L200 to AdNP-L33
	AdNP-L172	AdNP-L0		AdNP-L200	AdNP-L33	
0	4,324	708	6:1	30,568	10,200	3:1
2	2,180	338	6:1	18,552	6,220	3:1
4	1,034	216	5:1	9,650	2,918	3:1
8	570	148	4:1	6,310	1,614	4:1

^a Twofold dilutions (from 1 to 0.125 μ g) of poly(A)⁺ RNA extracted from recombinant virus-infected 293 cells were dot blotted onto nitrocellulose filters and probed with ³²P-labeled NP probes as described in Materials and Methods. The amount of RNA hybridized was quantitated by liquid scintillation counting of dots.

^b The values obtained were after subtraction of background counts per minute for each sample.

presence of tripartite leader sequences immediately downstream (see Discussion).

The amount of NP protein synthesized by the recombinant was directly proportional to the amount of NP mRNA synthesized. To determine the efficiency of translation of the recombinant NP mRNA *in vivo*, cells infected with each of the recombinants were labeled for 1 h with [³⁵S]methionine at 20 h postinfection and cell-equivalent aliquots of the labeled proteins were analyzed by gel electrophoresis (Fig. 5a). The NP protein synthesized by the recombinants (lanes 2 to 5) comigrated with the NP protein synthesized in influenza virus-infected cells (lanes 6 and 7). In addition, a minor amount of a smaller protein was synthesized by the recombinants but not by wild-type adenovirus. This species, which migrated between NP and M1 (most evident in lanes 2 and 4), was most likely generated by the minor spliced NP mRNA. Different amounts of the full-sized NP protein were synthesized by the different recombinants. The least amount was synthesized by AdNP-L0 (lane 3). In fact, with this recombinant, detection of the NP protein required longer exposure of the gel (not shown) or immunoprecipitation with NP-specific antiserum (Fig. 5b).

To determine the relative amounts of the NP protein that were synthesized by the recombinants, the cell extracts were immunoprecipitated and the precipitate was subjected to gel electrophoresis. The NP protein band was excised from the gel, and the radioactivity in this band was quantitated by liquid scintillation counting. To ensure complete recovery, the supernatant from the first immunoprecipitation was subjected to a second immunoprecipitation. As was already evident from the gel analyses shown in Fig. 5, the amount of NP protein made in cells infected with AdNP-L172 was sevenfold more than that made with AdNP-L0 and the amount made with AdNP-L200 was threefold more than that made with AdNP-L33 (Table 3). Thus, the relative amounts of the NP protein correlated with the amounts of the NP mRNA synthesized by the recombinant viruses. This indicated that the efficiency of translation of the NP mRNAs was not affected by the lengths of their 5' tripartite leader sequences.

The tripartite leader did not facilitate initiation of NP mRNA translation. If the initiation of translation of an mRNA is more efficient than that of another mRNA with a coding region of identical size, then the more efficient mRNA should be associated with more ribosomes, i.e., with faster-sedimenting polysomes (24, 25). For this reason, we compared the polysomal pattern of the NP mRNAs of the recombinants with each other and with the polysomal pattern of the NP mRNA synthesized in cells infected by both influenza virus and adenovirus.

The absorbance profile of a sucrose gradient analysis of the cytoplasmic extract from doubly infected cells is shown

(Fig. 6a). The cytoplasmic extract was treated with detergents to release the polysomes associated with the cytoskeleton, and 500 mM KCl was included in the sucrose gradients to minimize any artifactual sticking of RNA to polysomes (22, 24). As indicated, the gradient fractions containing the polysomes were pooled into eight samples, with sample 1 containing the largest polysomes and sample 8 containing the smallest polysomes along with any residual monosomes. Sample 9 contained the ribosomal subunits, and sample 10 contained the material sedimenting slower than ribosomal subunits. The poly(A)⁺ RNA from each of the 10 samples was analyzed by Northern analysis, using labeled NP plasmid as probe. Most of the polysomes containing NP mRNA in the doubly infected cells sedimented as a peak in samples 2 through 5 (Fig. 6b). On the basis of the positions of β actin and glyceraldehyde-3-phosphate dehydrogenase mRNAs in these gradients (14), the polysomes in samples 2 through 5 most likely ranged in size from about 5 to 12 ribosomes (25). The largest amount of NP mRNA was found in sample 4 in this experiment, but in other experiments the largest amount was found in sample 3. Some of the NP mRNA was also found in slower-sedimenting polysomes (samples 6 to 8) and in sample 9, containing the ribosomal subunits. In this particular experiment only a small amount of NP mRNA was found in sample 10, but much larger amounts were seen in other experiments. This variability probably reflected the variable action of nucleases on those NP mRNAs which were not associated with ribosomes or ribosomal subunits. Consequently, the relative amounts of polysomal and non-polysomal NP mRNAs showed large variations among experiments, whereas the sizes of the polysomes containing NP mRNA were reproducible. This was also the case for each of the NP mRNAs encoded by the recombinant adenoviruses.

The analysis of the polysomes containing NP mRNA from cells infected by either the AdNP-L172 recombinant or the

TABLE 2. Effect of tripartite leader sequences on the transcription of the NP gene in the recombinant adenoviruses^a

Recombinant adenovirus	Rate of NP gene transcription (cpm) ^b	Ratio
AdNP-L172	1,337	4:1
AdNP-L0	308	
AdNP-L200	5,808	3:1
AdNP-L33	1,905	

^a Nuclei were prepared from 293 cells infected for 20 h with the indicated recombinant adenovirus. *In vitro* transcription in the presence of (α -³²P)UTP was carried out as described in Materials and Methods. Labeled nuclear RNA was hybridized to filters containing excess amounts of either L3 or NP probes followed by quantitation of RNase A-resistant counts by liquid scintillation counting.

^b Values were normalized with respect to the amount of L3 transcription.

FIG. 5. Amount of NP protein synthesized in recombinant adenovirus-infected cells. (a) At 20 h postinfection, 293 cells infected with *d/309* (lane 1), AdNP-L172 (lane 2), AdNP-L0 (lane 3), AdNP-L200 (lane 4), AdNP-L33 (lane 5), *d/309* for 16 h and an additional 4 h with influenza virus (lane 6), or influenza virus alone for 4 h (lane 7) were labeled with [35 S]methionine for 1 h. Extracts of these cells were analyzed on a 14% polyacrylamide gel as described in Materials and Methods. The positions of some late adenoviral proteins are indicated on the left; influenza virus proteins are indicated on the right. (b) [35 S]methionine-labeled extracts from 293 cells infected with either AdNP-L172 (lane 1), AdNP-L0 (lane 2), AdNP-L200 (lane 3), or AdNP-L33 (lane 4) were immunoprecipitated with pooled anti-NP monoclonal antibodies, and the immunoprecipitates were electrophoresed on a 14% polyacrylamide gel. Lanes 1 and 2 were exposed eight times longer than lanes 3 and 4.

AdNP-L0 recombinant is shown (Fig. 7). With the AdNP-L0 recombinant (panel b), there was a distinct polysome peak containing NP mRNA in samples 2 through 5. Some NP mRNA was also found in slower-sedimenting polysomes and in nonpolysomal material. Thus, the sizes of the polysomes containing NP mRNA were similar to those found in doubly infected cells, indicating that the efficient initiation of trans-

lation of NP mRNA was independent of the tripartite leader and of other influenza virus gene products. With AdNP-L172 (panel a), the polysome peak was also in samples 2 through 5, but this peak was consistently skewed toward sample 5. Clearly, the NP mRNA did not need 172 nucleotides of the tripartite leader to shift it to larger polysomes. In fact, the NP mRNA containing these tripartite leader sequences (AdNP-L172) was on slightly smaller polysomes and hence probably initiated translation slightly less efficiently than the NP mRNA with no tripartite leader sequences (AdNP-L0).

As shown in Fig. 8, the polysomal pattern of the NP mRNA in cells infected with either AdNP-L200 or AdNP-L33 was similar to that of AdNP-L0. The polysome peak was in samples 2 through 5. The slight difference in the shape of the polysome peaks of AdNP-L200 and AdNP-L33 shown in Fig. 8 was not observed in other experiments. Thus, the initiation of translation of NP mRNA in adenovirus-infected cells at late times was not significantly enhanced by the presence of the tripartite leader.

DISCUSSION

We evaluated the effect of the tripartite leader on the translation of mRNAs in adenovirus-infected cells at late times by constructing four recombinant adenoviruses con-

TABLE 3. Relative amounts of NP protein synthesized by the recombinant adenoviruses^a

Recombinant adenovirus	NP protein (cpm)	Ratio
AdNP-L172	24,924	7:1
AdNP-L0	3,346	
AdNP-L200	199,962	3:1
AdNP-L33	74,994	

^a Extracts from infected cells (labeled with [35 S]methionine for 1 h at 20 h postinfection) were immunoprecipitated with anti-NP monoclonal antibodies, and the precipitate was analyzed by gel electrophoresis as described in Materials and Methods. The NP protein band excised from the gel was counted in a liquid scintillation counter. The supernatant from the first immunoprecipitation was subjected to a second immunoprecipitation, and the amount of label in the resulting NP band was added to that obtained in the first immunoprecipitation.

FIG. 6. Polysomal distribution of influenza virus NP mRNA in adenovirus-infected 293 cells superinfected with influenza virus. (a) Sedimentation profile of polyribosomes from doubly infected cells. A cytoplasmic extract from approximately 2×10^8 cells was subjected to centrifugation on a 10 to 50% sucrose gradient as described in the text. Fractions were collected, and the A_{260} was determined. The gradient fractions were pooled as indicated in the text, yielding samples 1 through 10. (b) Northern blot analysis of samples 1 to 10 from the gradient shown in panel a, using ^{32}P -labeled pAPR502 to probe NP mRNAs. The positions of 18S and 28S rRNAs are shown on the left.

taining the influenza virus NP gene expressed under the control of the adenovirus MLP. We chose the influenza virus NP mRNA because previous results showed that influenza virus NP mRNA was an extremely effective initiator of translation in cells infected with both adenovirus and influenza virus (13, 15). The influenza virus mRNAs in these doubly infected cells contain 10 to 13 5' terminal nucleotides stolen either from late adenovirus mRNAs (containing the tripartite leader) or from other viral and cellular capped RNAs (13). The four adenovirus recombinants that we constructed differed with respect to the absence or presence of various portions of the tripartite leader at the 5' ends of the encoded NP mRNAs. The NP mRNA either lacked any tripartite leader sequence or contained 33, 172, or all 200

nucleotides of the tripartite leader. Two lines of evidence indicated that the translation of NP mRNA was independent of the presence of tripartite leader sequences. First, the relative amounts of the NP protein synthesized in cells infected by the recombinant adenoviruses were directly proportional to the amounts of the NP mRNA made, indicating that the presence of 5' tripartite leader sequences did not enhance the translation of NP mRNA. In addition, the sizes of the polysomes containing NP mRNA were not increased by the presence of tripartite leader sequences, indicating that the initiation of translation was not enhanced by these sequences. The sizes of the polysomes containing the NP mRNA with no tripartite leader sequences were similar to those found for the NP mRNA synthesized in cells infected with both influenza virus and wild-type adenovirus. We can thus conclude that the structure of influenza virus NP mRNA per se allows it to initiate translation efficiently in competition with late adenovirus mRNAs.

Previous results from others concerning the effect of the tripartite leader on the translation of marker mRNAs encoded in recombinant adenovirus genomes under the control of the MLP have given conflicting results. With three marker mRNAs, adenovirus E1A mRNAs (26), hepatitis B surface antigen mRNA (6), and mouse dihydrofolate reductase mRNA (4), the tripartite leader was reported to enhance translation. Specifically, the marker mRNA lacking any tripartite leader sequences or containing only the first segment of the leader at its 5' end was translated with much less efficiency than the same mRNA containing the entire, or almost entire, tripartite leader. In contrast, with the SV40 T antigen mRNA, it was found that the tripartite leader did not enhance translation (28). Little or no difference in the efficiency of translation of this mRNA was seen whether it contained only a portion or the entirety of the tripartite leader. Consequently, SV40 T antigen mRNA behaved like influenza virus NP mRNA.

These results actually may not be in conflict but rather may indicate that the effect of the tripartite leader on translation is dependent on the nature of the marker mRNA encoded in the recombinant adenovirus genome. On the basis of our results, we would predict that if the marker mRNA itself already is a good initiator of translation, it should be able to compete effectively for ribosome-binding sites with the massive amounts of adenovirus mRNAs that are present at late times of adenovirus infection. Addition of the tripartite leader to the 5' end of such a marker mRNA would not be expected to enhance its translation. In contrast, if the marker mRNA is intrinsically not a good initiator of protein synthesis, then the translation of this mRNA might be enhanced by the tripartite leader in late adenovirus-infected cells. However, the mechanism by which this enhancement occurs is not clear. In any case, the observations that two marker mRNAs, influenza virus NP mRNA (present study) and SV40 T antigen mRNA (28), and at least one adenovirus mRNA, the pIX mRNA (20), do not require the tripartite leader for efficient translation in adenovirus-infected cells at late times of infection clearly establish that a 5' tripartite leader is not a general requirement for efficient translation of mRNAs in these cells. An important issue that remains is whether the tripartite leader is actually required for translation of those late adenovirus mRNAs that contain this leader. For example, it is not known whether the adenovirus hexon mRNA that naturally contains the tripartite leader is translated more efficiently than a hexon mRNA that either lacks any tripartite leader sequences or contains only a portion of these sequences. Conversely, it is also not

FIG. 7. Polysomal distribution of AdNP-L172 and AdNP-L0 mRNAs. Northern blot analyses of samples 1 to 10 from sucrose gradients of cytoplasmic extracts from 293 cells infected with either AdNP-L172 or AdNP-L0, using ^{32}P -labeled pAPR502 to probe NP mRNAs. The positions of 18S and 28S rRNA markers are shown on the left. Panel b was exposed five times longer than panel a.

known whether the translation of the late adenovirus mRNA that lacks tripartite leader sequences, the pIX mRNA, is enhanced by the addition of these sequences.

The rate of transcription of the NP sequence differed for the four adenovirus recombinants that we constructed. Two different effects were observed. First, a greater rate of transcription of the NP sequences was seen by using the Berkner et al. (3) vector system than by using the Logan and Shenk (26) vector system. This can be readily seen by comparing the rate of transcription of the NP sequences of AdNP-L172 with that of AdNP-L200. The most significant difference between the adenovirus recombinants generated by the two vector systems is that the recombinants generated with the Logan and Shenk vectors lack the sequence from 3.8 to 7.9 map units in the early region of the adenovirus genome. Consequently, the gene products specified by this region need to be supplied by the 293 cells, whereas with the recombinants generated with the Berkner et al. vectors,

these gene products are specified by multiple copies of the viral genome. This may cause the difference in transcription rates of NP sequences between the two sets of recombinants.

The second effect on transcription of the NP gene was due to the presence of tripartite leader sequences immediately downstream of the MLP. Initially, we observed that NP transcription was lower with AdNP-L0 than with AdNP-L172 and reasoned that this difference reflected the requirement of sequences in the first segment of the tripartite leader for optimal transcription from the MLP. Transient transfection assays in 293 cells have demonstrated that nucleotides immediately downstream of the cap site are required for good transcription from the MLP (23). Consistent with this, one of the transcription factors required for transcription from the MLP has been shown to bind to a region extending from 45 nucleotides upstream (−45) to 35 nucleotides downstream (+35) of the cap site (32). Based on this reasoning, we

FIG. 8. Polysomal distribution of AdNP-L200 and AdNP-L33 mRNAs. Northern blot analysis of samples 1 to 10 from sucrose gradients of cytoplasmic extracts from 293 cells infected with AdNP-L200 or AdNP-L33. The probe used was ^{32}P -labeled pAPR502 to locate NP mRNAs. The 18S and 28S rRNA markers are indicated on the left. Panel b was exposed three times longer than panel a.

constructed the AdNP-L33 recombinant containing most of the first leader segment (33 of 41 nucleotides) immediately downstream of the MLP and expected that NP transcription with this recombinant would not be reduced relative to that occurring with the AdNP-L200 recombinant containing the entire tripartite leader. This was not the case. The rate of transcription of the NP gene of AdNP-L200 was found to be threefold greater than that of AdNP-L33. Another study has also found that sequences downstream of +33 are needed for optimal transcription from the MLP (28). In this study, the adenovirus recombinants contained not only one or more of the three leader segments but also part or all of the two introns between the three leaders. It was found that optimal transcription was obtained with a construct containing all 41 nucleotides of the first leader segment plus 149 nucleotides of the adjacent intron, whereas a lower transcription rate was observed with a construct containing only 33 nucleotides of the first leader segment. These and our data indicate that the entire 41 nucleotides of the first leader segment are required for optimal transcription from the MLP.

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