Attenuation of Late Simian Virus 40 mRNA Synthesis Is Enhanced by the Agnoprotein and Is Temporally Regulated in Isolated Nuclear Systems

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Received 21 December 1984/Accepted 22 March 1985

Studies were performed to verify the physiological significance of attenuation in the life cycle of simian virus 40 and the role of agnoprotein in this process. For these purposes, nuclei were isolated at various times after infection and incubated *in vitro* in the presence of [α -³²P]UTP under the standard conditions which lead to attenuation. Attenuation was evident by the production of a 94-nucleotide attenuator RNA, revealed by gel electrophoresis. In parallel, the synthesis of agnoprotein was studied at various times after infection by labeling the cells for 3 h with [¹⁴C]arginine, lysing them, and analyzing the labeled proteins by gel electrophoresis. Both attenuation and the synthesis of agnoprotein were predominant towards the end of the infectious cycle. At earlier times, there was almost no attenuation and no synthesis of agnoprotein. Moreover, there was almost no attenuation even at the latest times after infection in nuclei isolated from cells infected with simian virus 40 deletion mutants that do not synthesize agnoprotein. Finally, analysis by dot blot hybridization showed higher amounts of cytoplasmic viral RNA in cells infected with an agnoprotein gene insertion mutant, Δ 79, that does not produce agnoprotein, compared with cells infected with wild-type virus. The present studies indicate that attenuation is temporally regulated and suggest that agnoprotein enhances attenuation in isolated nuclei and that may also enhance it *in vivo*.

The structural similarity between the major leader of 16S mRNA of simian virus 40 (SV40) and the leader of amino acid operons in bacteria is striking (3, 14, 40). This similarity raised the possibility that the major leader of 16S mRNA participates in an attenuation mechanism that regulates the expression of the downstream structural gene, VP1 (2, 14). Like the leaders of amino acid operons in bacteria, the major leader of 16S mRNA can be folded into two alternative secondary structures. One of the structures consists of two stem-and-loop structures (1 and 2; 3 and 4) and was termed the attenuation conformation (Fig. 1A) (14). The alternative secondary structure contains one stem-and-loop structure (2 and 3) which overlaps with that of the attenuation conformation. This structure was termed the readthrough conformation (Fig. 1B; 14). The 3 and 4 stem and loop, in the attenuation conformation, is followed by a run of uridylic acid residues. This structure acts as a transcription termination signal which prevents transcription from continuing into the structural gene (12, 32). In a further analogy to the leader sequences of the mRNAs from amino acid operons in bacteria, the major leader of 16S mRNA of SV40 codes for a small protein known as agnoprotein (13, 16, 17, 26, 27).

In bacteria, where transcription and translation are coupled, the translation process of the leader peptide regulates the attenuation of transcription (40). We have suggested that in SV40, where transcription and translation are uncoupled, the final product of translation of the leader's codons, i.e., the agnoprotein, regulates the attenuation of transcription (2, 3, 14). According to this suggestion, the agnoprotein is transported from the cytoplasm to the nucleus and has a dual function. In the nucleus it enhances the attenuation of transcription by stabilizing the attenuation conformation in the nascent RNA (Fig. 1A). In the cytoplasm, by stabilizing

the same conformation at the 5' end of the 16S mRNA, it enhances the translation of VP1. In this conformation the AUG start codon of the agnoprotein is sequestered in the 1:2 stem-and-loop structure (Fig. 1A), and the scanning ribosome bypasses it (18-24) and initiates translation from the downstream AUG of VP1. A similar mechanism has been suggested to regulate the synthesis of VP2 and VP3 (2, 3). It has been postulated that attenuation during SV40 late transcription regulates the synthesis of the amounts of the capsid proteins relative to each other and to the copy number of the viral DNA (2, 3, 14). In this way, the efficiency of capsid formation and the process of encapsidation are controlled.

To verify the physiological significance of attenuation in the life cycle of SV40 and to test this hypothesis of the role the agnoprotein plays in enhancing attenuation, we have developed a system with isolated nuclei in which efficient transcription termination occurs at the attenuation site of the SV40 genome. Attenuation is evident by the production of a 94-nucleotide attenuator RNA as revealed by gel electrophoresis (12). Under conditions that lead to attenuation, this isolated nuclear system provides experimental results that indicate that attenuation is temporally regulated, i.e., it is predominant towards the end of the infection cycle, and further suggest that agnoprotein enhances attenuation in isolated nuclei and may enhance it also *in vivo*.

MATERIALS AND METHODS

Preparation of isolated nuclei. BSC-1 cells were infected with SV40 (30 to 50 PFU per cell) as described elsewhere (25). At times postinfection (p.i.) as indicated in the figure legends, the cells were washed and collected in cold hypotonic buffer (50 mM Tris-hydrochloride [pH 7.9], 1.5 mM MgCl₂, 1 mM dithiothreitol) by centrifugation for 1 min at 1,000 $\times$ g. Nuclei were isolated by suspending 5 $\times$ 10⁷ cells in 10 ml of hypotonic buffer, pipetting the solution up and

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mamide, 0.01 M NaCl, 1 mM EDTA and 10 mM HEPES-NaOH (pH 7.5) at 37°C for 30 min, and then washed three times with 0.5× SSC. The bound RNA was eluted by incubating the filter in 90% formamide and 10 mM HEPES-NaOH (pH 7.9) for 1 h at 37°C. The [³²P]RNA was collected by ethanol precipitation.

RNA analysis by gel electrophoresis. For analysis of the RNA by gel electrophoresis, the RNA was denatured in 5 μl of 90% formamide and 10 mM HEPES-NaOH (pH 7.5) for 1 min at 90°C and subjected to electrophoresis on a 12% polyacrylamide (bis:acrylamide, 1:29) gel containing 7 M urea in TBE (89 mM Tris-hydrochloride [pH 8.3], 89 mM boric acid, 2.5 mM EDTA). Electrophoresis was at a constant current of 20 mA for 4 to 5 h.

Dot blot hybridization. A nitrocellulose sheet was equilibrated with 20× SSC and dried. Samples were applied in 5 μl, and the filter was baked at 80°C for 3 h. Before hybridization, the filter was incubated in 4× SSC containing 2× Denhardt buffer (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone) for 4 to 12 h at 68°C. ³²P-labeled DNA probe was denatured (100°C for 10 min in 0.01× SSC), and hybridization was carried out at 68°C for 24 h in 4× SSC, 0.2% SDS, and 1× Denhardt buffer. At the end of the incubation, the membrane was washed once with 2× SSC and then three times with 0.1× SSC at 55°C for 45 min, air dried, and subjected to autoradiography with Kodak X-ray film (XAR-5).

Analysis of proteins by gel electrophoresis. BSC-1 cells (0.3 × 10⁶) grown in 3-cm plates were infected with SV40. At various times p.i., the cells were labeled with [¹⁴C]arginine in arginine-free medium. Cells were lysed and collected in a sample buffer containing 0.01 M sodium phosphate (pH 7.2), 7 M urea, 1% SDS, 1% 2-mercaptoethanol, and 0.01% bromophenol blue. Before application, the samples were boiled for 2 min. The samples were loaded on 15% polyacrylamide gel (bis:acrylamide, 0.8:30) containing 0.1 M sodium phosphate (pH 7.2), 0.1% SDS, and 6 M urea. On the top of the gel, an upper gel containing 3.5% acrylamide was layered. The running was carried out in 0.1 M sodium phosphate (pH 7.2) and 0.1% SDS at 5 to 6 V/cm at room temperature for 20 to 24 h. At the end of the run, the gel was dried and exposed to Kodak X-ray film (XAR-5).

RESULTS

Increased attenuation directly correlated with the progress of infection. To examine the physiological significance of attenuation in the life cycle of SV40, we have determined when attenuation is predominant during the SV40 infection cycle. For this work, nuclei were isolated from the infected cells at different times during the late phase of the SV40 life cycle, and incubated *in vitro* under the standard conditions (100 mM NaCl, 10 μM [α-³²P]UTP) that lead to the production of the 94-nucleotide attenuator RNA (12). ³²P-labeled RNA was extracted, and viral RNA was purified by hybridization to and elution from SV40 DNA immobilized on nitrocellulose filters. The ³²P-labeled viral RNA was then analyzed by gel electrophoresis under denaturing conditions. Only minor quantities of the attenuator RNA are synthesized at the beginning of the late phase of the SV40 infection cycle (Fig. 2), while long viral transcripts accumulate (Fig. 2A and B). The long RNA represents transcripts of the viral DNA fragments that are remote from the major transcription initiation site. The fact is evident from their hybridization, after elution from the gel, with the late DNA fragments b (0.76 to 0.0 map units [m.u.]) and d (0.0 to 0.17

FIG. 2. Size analyses of viral RNAs produced in nuclei isolated from SV40-infected cells at various times after infection. Nuclei were isolated from SV40-infected cells and incubated in a standard transcription reaction mixture as described in the text. The labeled viral RNAs produced were purified and analyzed by gel electrophoresis. Each lane was loaded with equal amounts of viral RNA. Lane A, Nuclei isolated at 24 h p.i.; lane B, nuclei isolated at 36 h p.i.; lane C, nuclei isolated at 48 h p.i.; lanes m, labeled DNA fragments used as size markers. Labeled RNA was eluted from lane A as indicated and hybridized to a blot of SV40 DNA restriction fragments obtained by cleavage of SV40 DNA with *Eco*RI, *Bgl*II, and *Hpa*I restriction enzymes (14). The arrow points to the position of the 94-nucleotide attenuator RNA.

m.u.) that are far removed from the major initiation site at residue 325. Residue 325 is located on fragment e. Hybridization with the early fragments a (0.67 to 0.37 m.u.) and c (0.37 to 0.17 m.u.) was also noted (Fig. 2; 14). The hybridization with the early fragments could represent either early or late transcripts. Almost no hybridization occurred with the promoter proximal DNA fragment e. In contrast, the viral RNA synthesized at a later time in the infection cycle produced a major 94-nucleotide RNA (Fig. 2C). This RNA was previously characterized and was termed the attenuator RNA (14, 33, 34). It hybridized to fragment e (0.67 to 0.76 m.u.), and it mapped between residue 419 and the major initiation site of late transcription at residue 325. Almost no long viral RNA was synthesized at this time p.i. The RNA of about 125 nucleotides is not a transcript of the leader region because it is synthesized also in cells infected with SV40 mutants that contain deletions in the leader region. We also found that this RNA does not hybridize to SV40 DNA fragments. We assume that the 125-nucleotide RNA is induced after infection, and it is trapped on the SV40 DNA during hybridization. It is worth noting that by using hybrid

FIG. 3. Time course of synthesis of agnoprotein. At various times after infection, 0.5×10^6 cells were labeled for 3 h with [^{14}C]arginine, collected in 0.1 ml of sample buffer (10 mM sodium phosphate [pH 7.2], 7 M urea, 1% SDS, 1% β -mercaptoethanol, and 0.01% bromophenol blue), lysed, and analyzed on a 15% polyacrylamide gel containing 0.1% SDS in 6 M urea. The gel was fluorographed and dried. The autoradiogram was obtained by exposing the dried gel to Kodak XR-2 film at -80°C . (A) Exposure for 6 h; (B) exposure for 24 h; m, molecular weight markers.

selection technique, Alwine observed RNA of a similar size and concluded that it is of cellular origin (4). In conclusion, the isolated nuclear system reveals that while readthrough occurs at the beginning of the late phase of infection, attenuation is predominant at the end of the infection cycle. These results imply that regulation at the attenuation site has a temporal and physiological significance.

Time course of synthesis of agnoprotein. In our original model, we postulated that agnoprotein has a *trans*-acting negative effect on late transcription (2, 3, 13, 14). To test this hypothesis we have first studied when during the infection cycle agnoprotein is synthesized. For this study, at various times after infection cells were labeled for 3 h with [^{14}C]arginine, and the synthesized proteins were analyzed by gel electrophoresis. We chose to label the cells for 3 h because this time has previously been shown to be the half-life of agnoprotein (13, 17). The gel was exposed to X-ray film for 6 h for quantitating the major capsid proteins, i.e., VP1, VP2, and VP3, and for 24 h for quantitating the agnoprotein. As can be seen in Fig. 3A, the synthesis of the capsid proteins increases up to 42 h p.i. At 50 h p.i., there is no further increase in the rate of their synthesis, and there is even some decrease. In contrast, the agnoprotein is synthesized primarily at the latest time after infection (Fig. 3B). These results, with the observation that attenuation is predominant towards the end of the late phase (Fig. 2), demonstrate a direct correlation between the amount of agnoprotein in the cell and the level of attenuation.

Reduced synthesis of attenuator RNA in nuclei isolated from cells infected with SV40 mutants that do not produce agnoprotein. To directly examine whether agnoprotein enhances the synthesis of the attenuator RNA in isolated nuclei, cells were infected with several mutants of SV40 that lack the coding reading frame of agnoprotein (frameshift mutants). Nuclei were isolated, and the production of the attenuator RNA was analyzed and compared with that of RNA pro-

duced in nuclei isolated from wild-type (WT) infected cells. The mutants used were as follows. (i) *dl1626* is a mutant in which the entire coding region of agnoprotein is deleted (35). (ii) *dl1811* is a mutant in which nucleotides 305 to 345 are deleted. This deletion removes the AUG start codon of agnoprotein, the major initiation site at residue 325, and the first stem-and-loop structure (1;2) of the attenuation conformation of the WT attenuator RNA (Fig. 1). In this mutant the major initiation site of late transcription is shifted upstream to residue 290 (11). As a result of the deletion, an alternative, more stable RNA secondary structure can be formed upstream of the termination signal ($\Delta G = -24$ kcal [ca. 10^5 J]). In comparison with that of WT, the readthrough conformation is unchanged (Fig. 4, *dl1811*, and for comparison, see Fig. 1). (iii) The mutant $\Delta 79$ is an insertion mutant in which 2 nucleotides, C and G, were inserted at the *Hpa*II site. This insertion has almost no effect on the stability of the secondary structure of the attenuation and readthrough conformations. However, as a result of the insertion, there is a shift in the coding frame of the agnoprotein. The new coding frame codes for a 31-amino-acid protein. The new stop codon, UAG, is at residues 432 to 434 (Fig. 4, WT/ $\Delta 79$). This protein, if translated, is probably very unstable. We were unable to detect it in infected cells even after short pulse-labeling procedures and exposures of the X-ray film for a long duration (Hay and Aloni, unpublished results; 13).

Nuclei were prepared from the various infected cells and incubated *in vitro* in the presence of [α - ^{32}P]UTP for 15 min in a standard transcription reaction mixture which led to premature termination and the production of the 94-nucleotide attenuator RNA (12). These mutants were previously used, under conditions which led to pausing of the RNA polymerase, to map the attenuator RNA. [^{32}P]RNA was extracted, and viral RNA was selected by hybridization to and elution from filters bearing SV40 DNA. The ^{32}P -labeled viral RNA was then analyzed by gel electrophoresis under denaturing conditions. Figure 5 shows a significant reduction in the amount of the attenuator RNA and an increase of long RNA transcripts in all the mutants used. With *dl1626*, there is a complete disappearance of the attenuator RNA, because in this virus the entire coding region of the attenuator RNA is deleted (35). With *dl1811*, there is a faint band of about 90 nucleotides which could result from reduced termination at the termination signal (residue 419 in the attenuation conformation). In a short pulse with limited UTP concentrations, there is a pause at this site (14). With $\Delta 79$, there is a faint band corresponding to an RNA longer by two nucleotides compared with the WT attenuator RNA.

The experiment with $\Delta 79$ was repeated by isolating the nuclei at later times of the infection cycle to exclude the possibility that the reduced amount of the attenuator RNA is a consequence of a slower progression of the infection cycle rather than of the absence of the agnoprotein in the infected cells. In none of these experiments, even at later times than 56 h p.i., has the level of the attenuator RNA ever reached the level of the attenuator RNA produced in nuclei isolated at 48 h p.i. from cells infected with WT virus.

These experiments provide more direct evidence for the function of agnoprotein in enhancing attenuation in isolated nuclei. However, the unlikely possibility that the effect of the two nucleotides inserted into $\Delta 79$ is in *cis* (a conformational effect) cannot be excluded.

Increased amount of viral RNA in the cytoplasm of cells infected with $\Delta 79$. In our original model, we postulated that in the cytoplasm, during translation, agnoprotein can reduce

WT/ Δ79

FIG. 4. Schemes of alternative conformations of the attenuator RNA region in mutants of SV40. *dl1811* was obtained from W. Fiers, and $\Delta 79$ was obtained from G. Khoury. Note that the alternative conformations can exist both in the attenuator RNA and at the 5' end of the major 16S mRNA. (A) Attenuation conformation showing stem-and-loop structure in which the 3;4 structure ($\Delta G = -12$ kcal [ca. 50 kJ]) signals transcription termination (14). (B) Readthrough conformation. For details see Hay et al. (14). The ΔG was calculated as described by Tinoco et al. (37). The residue number is from Tooze (38). It is worth noting that the putative termination site could be at any of the U residues that follow the 3;4 stem-and-loop structure (32).

FIG. 5. Size analyses of viral RNAs produced in nuclei isolated from cells infected with various mutants of SV40. At 48 h p.i., nuclei were isolated from cells infected with various mutants of SV40, as indicated (A). Nuclei were isolated from $\Delta 79$ -infected cells at 56 h p.i. also (B). The nuclei were incubated for 15 min at 30°C in a standard transcription reaction mixture. The labeled viral RNAs produced were purified and analyzed by gel electrophoresis. Each lane was loaded with equal amounts of viral RNA. m, Labeled DNA fragments used as size markers. The arrow points to the position of the 94-nucleotide attenuator RNA.

its own synthesis by stabilizing the RNA conformation at the 5' end of the 16S mRNA by sequestering its own AUG start codon (Fig. 1A). Consequently, the downstream AUG start codon of VP1 becomes functional, and VP1 is synthesized (14). In $\Delta 79$ -infected cells, this regulation cannot operate because of the lack of functional agnoprotein in the infected cells, and VP1 is synthesized at high levels (13). We argue that the synthesis of high levels of VP1 in $\Delta 79$ -infected cells can be accomplished because of reduced attenuation of SV40 late transcription (Fig. 5) and a resultant increase in the synthesis of viral RNA and its accumulation in the cytoplasm of infected cells. The increased amount of viral mRNA should be able to compensate for the less efficient translation of VP1 in $\Delta 79$ -infected cells.

To test this argument, we compared the levels of viral RNA in the cytoplasm of cells infected with WT and $\Delta 79$. At 48 h p.i., nuclear and cytoplasmic fractions were prepared, RNAs were extracted from the cytoplasm, and DNAs were extracted by the Hirt procedure (15) from the nuclear fractions. Twofold dilutions of RNAs and DNAs were dot blotted onto nitrocellulose paper and hybridized with a probe of SV40 labeled DNA. Figure 6 shows that for an equal amount of viral DNA, the cytoplasm of cells infected with the $\Delta 79$ mutant contained approximately twice the amount of viral RNA present in the cytoplasm of WT-infected cells.

DISCUSSION

The present studies have shown that attenuation of late SV40 mRNA is temporally regulated; i.e., with the progress of SV40 infection, there is increased attenuation. At 24 and 36 h p.i., most of the transcribed viral RNA chains are long. At 50 h p.i., there is a sharp decrease of long viral transcripts, and there is synthesis of a 94-nucleotide attenuator RNA in parallel. These results imply that regulation at the attenuation site has a physiological significance. At the latest time in infection, the DNA encapsidation process is most extensive. Attenuation could control the efficiency of encapsidation by adjusting the amounts of the capsid proteins relative to each other and also to the copy number of the viral DNA. In this respect it is worth noting that although mutants with deletions in the leader region are viable, they are all somewhat defective in the rate of production of infectious virions in established cell lines. This defectiveness is indicated by both the delayed appearance and smaller sizes of the plaques these mutants produce and the lower rates of production of PFU during a single growth cycle (6, 27).

Incorporation of amino acid precursors during the growth cycle of SV40 showed an increased synthesis of agnoprotein as the infection cycle progressed (Fig. 3). Similar observations have been reported previously (30). This result, with the observation that attenuation is also increased with the progress of infection, demonstrates a direct correlation between the amount of agnoprotein in the cell and the level of attenuation in isolated nuclei, suggesting a possible involvement of agnoprotein in attenuation. The observation that SV40 mutants that do not produce agnoprotein failed to synthesize the 94-nucleotide attenuator RNA, especially mutant $\Delta 79$, with its 2-nucleotide insertion, in which the stability of the secondary structure of nascent RNA is almost unchanged, provides direct evidence for the function of agnoprotein in enhancing attenuation at least in an isolated

FIG. 6. Quantitation by dot blot hybridization of viral RNA present in the cytoplasm of WT- and $\Delta 79$ -infected cells. At 48 h p.i., cells were infected with WT SV40 or $\Delta 79$, or they were mock infected. Cytoplasmic and nuclear fractions were prepared by lysing the cells with 0.5% NP-40 (31). RNAs were extracted from the cytoplasmic fractions, and viral DNAs were extracted by the Hirt procedure (15) from the pelleted nuclei. The RNA fractions were treated with DNase, and the DNA fractions were treated with RNase. Twofold dilutions of DNA and RNA were dot blotted on nitrocellulose paper and hybridized with nick-translated SV40 DNA. The paper was dried and exposed to X-ray film.

nuclear system. It should be mentioned that it has recently been suggested that agnoprotein interacts in a specific way with VP1 and plays a role in virion assembly. These suggestions were based on an observation that a VP1-defective mutant could be suppressed by a point mutation in the agnoprotein (26). We believe that a direct involvement in virion assembly as a major role of agnoprotein, is unlikely, because the assembly process occurs in the nucleus (10), and the agnoprotein is found predominantly in the cytoplasm (3, 30). In any case, interaction between VP1 and agnoprotein would not exclude the regulatory role of agnoprotein as suggested in our original model (2, 3, 14). Moreover, because VP1 is known to be transported from the cytoplasm to the nucleus (10), interaction between VP1 and agnoprotein could facilitate the transport of the agnoprotein to the nucleus, where it can function in enhancing attenuation.

In our original model, we also suggested the existence of an antiattenuation factor that competes with the agnoprotein either by stabilizing the readthrough conformation in the nascent RNA or by interacting with the RNA polymerase, allowing it to transcribe through the attenuation site (2, 3, 14). The results with the mutant *dl1811*, that does not synthesize agnoprotein, tend to support the existence of an antiattenuation factor, because in this virus the attenuation conformation of the nascent RNA is more stable than in the WT virus. Nevertheless, there is almost no synthesis of the attenuator RNA under these conditions. This fact may indicate that antitermination factor stabilizes the readthrough conformation. For the same reason, we suggest that there is almost no attenuation at early times in the infection when the low amount of agnoprotein in the cell cannot compete with the hypothetical antiattenuation factor and readthrough conformation.

In the present experiments, attenuation was analyzed in isolated nuclei. That agnoprotein may enhance attenuation also *in vivo* could be suggested by the experiments showing higher levels of viral cytoplasmic RNA in cells infected with $\Delta 79$, a mutant that does not synthesize agnoprotein, compared with cells infected with WT virus (Fig. 6).

Furthermore, we have previously shown that *in vivo* the RNA polymerase pauses at sites close to the 1;2 stem-and-loop structure (Fig. 1; 34). It is assumed that the formation of the 1;2 stem-and-loop structure prevents the formation of the competing 2;3 readthrough conformation, and consequently, the transcription termination signal 3;4 is formed (2, 3, 14). We further suggest that at the latest time after infection, the agnoprotein enhances the formation of the 1;2 stem-and-loop structure, leading to an increased number of polymerase molecules at the pause sites (33). During *in vitro* elongation in isolated nuclei, the termination signal 3;4 is formed, and the attenuator RNA is synthesized (1, 2, 12, 14, 32–34). In $\Delta 79$ -infected cells, because of the absence of functional agnoprotein (13), the stability of the 1;2 stem-and-loop structure is reduced, and the proportion of newly synthesized molecules having the competing 2;3 readthrough conformation is increased. Consequently, during *in vitro* elongation, the synthesis of the attenuator RNA is reduced, and there is an increased synthesis of long RNA molecules. Further experiments are needed to establish the *in vivo* role of agnoprotein in enhancing attenuation.

With regard to the generality of the attenuation mechanism in both animal viruses and cells, attenuation of transcription has recently been shown to exist in the autonomous parvovirus minute virus of mice (7), and it has been thought to exist in polyoma virus (29), vesicular stomatitis virus (36), and retroviruses (8). In Ad-2 and Ad-5, premature

termination at specific sites was found to be temporally regulated, preferentially observed, at the late times of the infection cycle (28; M. Sieberg, A. J. Levine, and Y. Aloni, manuscript in preparation). Attenuation with specific eucaryotic genes has also been documented (5, 9, 39). It is clear then that the attenuation mechanism described here will continue to serve as a model for many general cases of gene regulation.

ACKNOWLEDGMENTS

We thank Ruchama Leiserowitz for technical assistance, A. J. Levine and E. B. Jakobovits for comments on the manuscript, and Noël Mann for typing the manuscript. Y.A. thanks A. J. Levine for the kind hospitality in his laboratory, where this work was completed.

This research was supported by U.S. Public Health Service research grant CA 14995 and in part by a grant from the Israel Academy of Sciences. A part of the work reported in this paper was undertaken during the tenure of an American Cancer Society Eleanor Roosevelt International Cancer Fellowship awarded to Y.A. by the International Union Against Cancer.

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