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RNA polymerase II is capable of pausing and prematurely terminating transcription at a precise location *in vivo* and *in vitro*

(attenuated RNA/minute virus of mice/RNA secondary structure/RNase mapping)

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ABSTRACT **By using the minute virus of mice, we have shown that *in vivo* and *in vitro* RNA polymerase II pauses or prematurely terminates transcription at a specific location 142–147 nucleotides downstream from the P4 promoter. The attenuated RNA was found and mapped *in vivo* in A9 cells late after infection in both the nuclear and cytoplasmic fractions, and the terminal nucleotide was shown to have a 3′ OH group. The 3′ end of the attenuated RNA is capable of forming a hairpin structure that is followed by a stretch of uridines. To distinguish whether the attenuated RNA is formed as a result of processing, pausing, or termination and to dissect structural elements, factors, or mechanisms that are involved in its formation, we used *in vitro* systems: isolated nuclei and cell-free extracts from HeLa cells. The results of the *in vitro* studies show that the attenuated RNA is a result of pausing or termination and not processing. Additionally, a salt-soluble factor and RNA secondary structure were implicated in the process of termination.**

It has been suggested (1) that RNA polymerase II terminates transcription heterogenously at the end of genes downstream from the poly(A) site of the mature mRNA. The poly(A) signal AAUAAA was shown to be required for termination (2). In one case, RNA polymerase II has been shown to terminate at a specific sequence, $A_9T_2A_5TA_4TA_4TA_5$ (3). The actual 3′ end of mRNA is produced by endonucleolytic cleavage and poly(A) addition. The nonpolyadenylylated 3′ ends of histone mRNA are also created by processing (4). All studies of transcription termination are unable to distinguish between termination and a processing mechanism that involves a cleavage of the transcript during transcription and immediate degradation of the downstream product. Thus hereafter all of these activities will be termed termination.

By using run-on transcription in isolated nuclei, it has been shown that RNA polymerase II is also capable of prematurely terminating transcription within a gene. Cellular examples to date in which this type of regulation occurs are the oncogenes c-*myc* (5), c-*fos* (6), and c-*myb* (7). Additional viral examples are the late transcription of simian virus 40 (8), transcription from the major late promoter (MLP) of adenovirus type 2 (Ad2) (9, 10), transcription from the long terminal repeat of human immunodeficiency virus type 1 (11), and transcription from the P4 promoter of the minute virus of mice (MVM) (12).

MVM is a linear single-stranded DNA (5149 base pairs) parvovirus (13). It is an autonomous virus but is dependent upon cellular functions that are expressed during S phase for replication (13). Transcription initiation catalyzed by the host RNA polymerase has been shown to map to two MVM promoters located at 4 (P4) and 39 (P39) map units [1 map unit = 51 nucleotides (nt)] (13). The two promoters have been more precisely mapped to nt 201 ± 5 and nt 2005 ± 4, respectively (12). The site of premature termination was shown to be located 142 nt downstream from the promoter P4, and the proposed secondary structure at the 3′ end of the attenuated RNA is shown in Fig. 1.

In this study, using MVM as a model system, we have shown that RNA polymerase II pauses or prematurely terminates transcription precisely at a specific location 142–147 nt downstream from the promoter P4 *in vivo* and *in vitro*. The attenuated RNA has been identified *in vivo* in both the nuclear and the cytoplasmic fractions. We have investigated the structural features operating at the site, found suggestive evidence for the involvement of factor(s), and reconstructed a system with a HeLa whole-cell extract and a nuclear extract capable of mimicking the *in vivo* results.

MATERIALS AND METHODS

Extraction and Analysis of RNA. A9 cells were grown and infected with MVM as described (12). Cells were labeled with orthophosphate in 5 ml of medium per plate for 4 hr. After infection, cells were extensively washed and collected in isotonic phosphate-buffered saline (PBS). Nuclei and cytoplasm were separated, and RNA was extracted (15). For the RNase mapping and the pCp labeling, the fractionated nuclear and cytoplasmic RNAs were electrophoresed on polyacrylamide gels and RNA from the regions indicated in Fig. 4*A* was electroeluted. pCp labeling was as described (16). RNase mapping was performed as described by Melton *et al.* (17) except that RNase digestion was carried out at 0°C on ice for 30 min. Probes used for the mapping experiments were gel-purified by electroeluting the RNA in dialysis tubing for 6 hr in 0.5× TBE (1× TBE = 90 mM Tris borate/90 mM boric acid/2 mM EDTA, pH 8.0) at 100 V and 4°C. Nuclei were isolated from infected cells (24 hr after infection) and transcription was carried out *in vitro* (18). MVM-specific RNA was selected by hybridization to and elution from nitrocellulose blots on which RI clone DNA was immobilized (12) (for a description of the RI clone, see Fig. 3*C*). For blot hybridization, the labeled RNA was denatured by heating to 90°C in 10 mM Tris·HCl, pH 7.5/1 mM EDTA and was hybridized to filter-bound DNA (15). The whole-cell extract was prepared according to Manley *et al.* (19), the nuclear extract was prepared according to Dignam *et al.* (20), and *in vitro* transcription was performed in 20 and 25 μl, respectively (10). RNA was analyzed by polyacrylamide gel electrophoresis (21).

RESULTS

Accumulation of P4 Promoter-Proximal Transcripts in Isolated Nuclei. We first analyzed viral RNA synthesis in isolated nuclei, a system that elongates RNA chains initiated

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Abbreviations: MVM, minute virus of mice; nt, nucleotide(s); MLP, major late promoter; Ad2, adenovirus type 2.

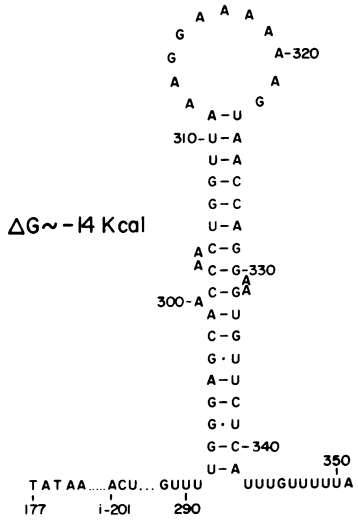

FIG. 1. Proposed secondary structure of the 3′ end of the MVM attenuated RNA (142 nt). Note the stretch of uridines after the stem and loop. The ΔG was calculated as described by Tinoco *et al.* (14).

in vivo and is thus useful for examining the 3′-end formation of RNA (22).

Fig. 2*B* shows the profile of a control in which MVM DNA labeled by nick-translation (23) was hybridized to a blot of the *Pst* I-cleaved MVM DNA. Fig. 2*A* shows a portion of the same blot hybridized to the *in vitro*-elongated RNA. There is a specific enrichment of promoter-proximal transcripts hybridized to fragment E; an analogous enrichment of promoter-proximal transcripts is not seen in the RNA that was initiated from promoter P39 (for the corresponding fragments, see Fig. 2*C*).

To estimate the length of the RNA synthesized in isolated nuclei, the RNA was electrophoresed on a gel after selecting for those transcripts that were initiated from promoter P4 (Fig. 2*D*). In the pulse-labeled sample (lane P) the major RNA species are in a distinct set of bands 142–147 nt long—the predicted size of the attenuated RNA (see Fig. 1). The same set of bands remained after a chase with excess UTP (lane C); however, there was a slight change in the ratio of the individual bands but no overall decrease in the intensity. The labeled RNA hybridized to fragment C (Fig. 2*A*) was not in distinct bands presumably because the RNA was heterogenous in length.

Transcriptional arrest can be caused by either pausing or termination. We have found two ways to eliminate the block in transcriptional elongation in the MVM system that distinguish between pausing and termination: a high-salt wash of the nuclei or the use of an intercalating drug. Salt-soluble factors have been implicated in the termination of RNA polymerase II (18) and RNA polymerase I (24). Fig. 2*E* shows that if nuclei were prewashed with high-ionic-strength buffer and transcription was performed at high ionic strength, although the attenuated RNA was visible in the pulse reaction mixture, most of the RNA was elongated in the chase reaction mixture. This implies that the high-ionic-strength wash either changed the environment in which the RNA polymerase functions or eliminated a factor in the nuclei that allows the elongation complex to pause at this site but does not allow for efficient termination.

The intercalating agent proflavine prevents processing of RNA (25) and prevents transcription termination (26), presumably by interfering with the formation of RNA secondary structure. To test whether proflavine affected the production of the attenuated RNA of MVM, it was added to the infected cells 5 min before preparation of the nuclei. This treatment led to an increase in the read-through transcripts and a

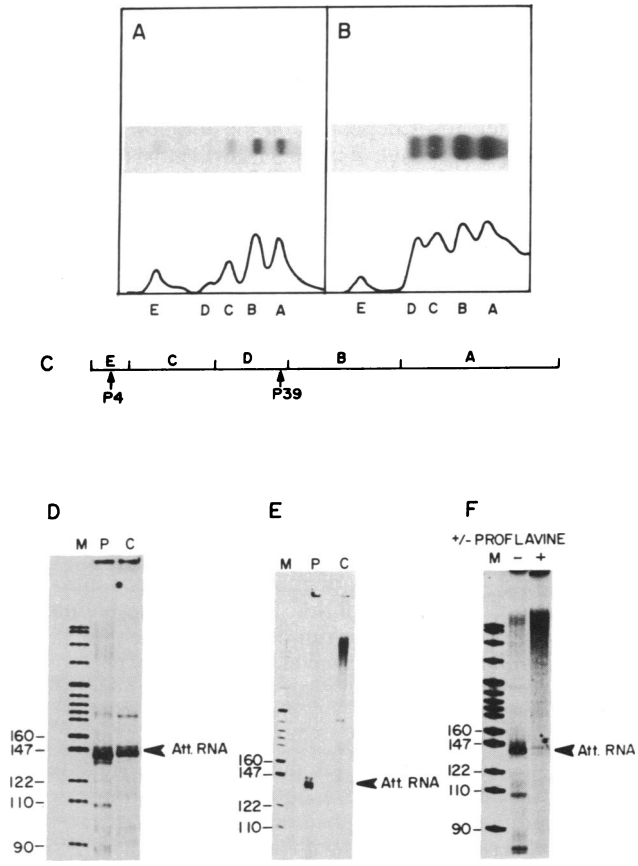

FIG. 2. (*A*) Hybridization of ^{32}P-labeled MVM RNA from a pulse–chase experiment with 3×10^7 nuclei to *Pst* I fragments of MVM DNA. The pulse was 7 min; the chase was 30 min (100 μM UTP). A densitometric tracing of the hybridization pattern is shown below the blot. The letters refer to the DNA fragments shown in *C*. Note that the 142- to 147-nt attenuated RNA is encoded by fragment E. (*B*) Hybridization of ^{32}P-labeled MVM DNA (labeled by nick-translation) to *Pst* I fragments of MVM DNA. Densitometric tracing of the hybridization pattern is shown below the blot. (*C*) Restriction map of MVM DNA digested with *Pst* I. The two viral promoters are indicated with arrows. (*D*) Size analysis of labeled viral RNA synthesized in isolated nuclei. Att. RNA, attenuated RNA. The numbers on the left are the sizes of the DNA markers in nt. The same marker, pBR322 DNA digested with *Hpa* II and end-labeled, was used in all experiments. Lanes: M, molecular size markers; P, pulse; C, chase. (*E*) Size analysis of viral RNA produced in a pulse–chase experiment of high-ionic-strength washed nuclei. Nuclei were washed by resuspending them in a solution of 50 mM Hepes, (pH 7.9), 15% (vol/vol) glycerol, 5 mM KCl, and 100 mM $(NH_4)_2SO_4$ and centrifuging them at $1500 \times g$ for 1 min. Nuclei were then resuspended in the high-ionic-strength transcription buffer and transcription was allowed to proceed. The pulse was 7 min; the chase was 30 min. Lanes: M, molecular size markers; P, pulse; C, chase. (*F*) Size analysis of viral RNA produced in isolated nuclei of proflavine-treated cells. Five minutes prior to isolation, nuclei were exposed to 80 μM proflavine. Transcription was carried out by using pulse conditions for 7 min. Lanes: M, molecular size markers; −, no proflavin; +, 80 μM proflavine.

significant decrease in the attenuated RNA (Fig. 2*F*). This occurred without the use of chase conditions, indicating that proflavine blocks the transcriptional pause signal.

Production of the Attenuated RNA in Cell-Free Systems. To define the elements involved in the transcriptional block by using a simpler system, we turned to whole-cell and nuclear extracts from HeLa cells. In preliminary experiments using the RI template truncated with *Eco*RI (for a diagram of the expected RNA lengths, see Fig. 3*C*), we found that if Sarkosyl was added to the HeLa whole-cell or nuclear extract

reaction mixtures after the initiation of transcription, an RNA band of 142–147 nt, corresponding in size to the attenuated RNA in isolated nuclei (Fig. 2*D*), appeared and the run-off band (883 nt) correspondingly disappeared. The appearance of the attenuated RNA depended on the addition and on the concentration of Sarkosyl; at 0.2% Sarkosyl most of the RNA was attenuated. Both the attenuated RNA and the run-off RNA were α-amanitin-sensitive (0.5 μg/ml), indicating that they are transcribed by RNA polymerase II (results not shown).

To examine whether the attenuated RNA was produced by processing, pausing, or termination and to determine whether the production of the attenuated RNA was dependent on the labeled nucleotide, we performed a kinetic analysis of the cell-free reaction using either [α-^{32}P]UTP or [α-^{32}P]CTP as the labeled ribonucleotide. The RI clone truncated with *Pst* I was transcribed in a nuclear extract in the presence of [α-^{32}P]UTP with the addition of Sarkosyl after initiation. Samples were analyzed throughout the 45-min reaction period (Fig. 3*A*). If the attenuated RNA was a result of processing from the run-off RNA during transcription, one would expect to see the attenuated RNA only after the appearance of the run-off RNA. However, the attenuated RNA was visible before the run-off RNA, indicating that it was most probably formed by pausing and not by processing. If a chase of 500 μM UTP began 2 min into the elongation reaction (lane 2 + C), the attenuated RNA did not accumulate; whereas if the chase began 45 min into the elongation reaction, the attenuated RNA did accumulate (results not shown). Similar results were obtained when transcription was carried out in the presence of [α-^{32}P]CTP.

Would this signal function with another promoter? For this analysis we used the MLP of Ad2, a promoter that has been well characterized in cell-free systems. The region downstream of promoter P4 was inserted after MLP (for a diagram of the construct, see Fig. 3*D*), and transcription was carried out in the presence of [α-^{32}P]CTP. As can be seen in Fig. 3*B*, transcription of the MLP-MVM clone under the same conditions also produced attenuated RNA, and the kinetic analysis showed that exchange of promoters did not alter the mechanism of its formation. This construct confirmed that the 3′ end of the attenuated RNA mapped to 342–347 nt (MVM numbering). In addition this construct showed that the necessary signal for the formation of attenuated RNA is not contained within nt 1–259 but is contained within nt 260–416. The results shown were also obtained with [α-^{32}P]UTP. We have noticed a difference between the intensity of the two attenuated RNA transcripts that depends on the labeled nucleotide and not the promoter.

Attenuated RNA Identified *in Vivo* in the Nuclear and Cytoplasmic Fractions. The observation that transcription from promoter P4 was regulated by pausing and premature termination *in vitro* clearly needed to be substantiated *in vivo* to ascertain its significance. To assay this directly, we extracted total RNA 27 hr after infection from MVM-infected A9 cells that had been labeled with orthophosphate (23–27 hr after infection with 15 mCi per plate; 1 Ci = 37 GBq). A portion of the RNA was hybridized to and eluted from filters containing a pBR322 clone carrying the *Eco*RI fragment C (1–1083 nt) of MVM. This procedure selects for those transcripts that initiated at promoter P4. The viral-selected and not-selected RNA were analyzed on a polyacrylamide gel. Fig. 4*A* shows that there is a specific enrichment of an RNA of ≈145 nt in the viral-selected RNA.

To map this RNA precisely, RNase protection was performed (17). Unlabeled nuclear and cytoplasmic RNA was extracted 26 hr after infection. To eliminate the possible artifacts created by RNase digestion of uridine-rich sites

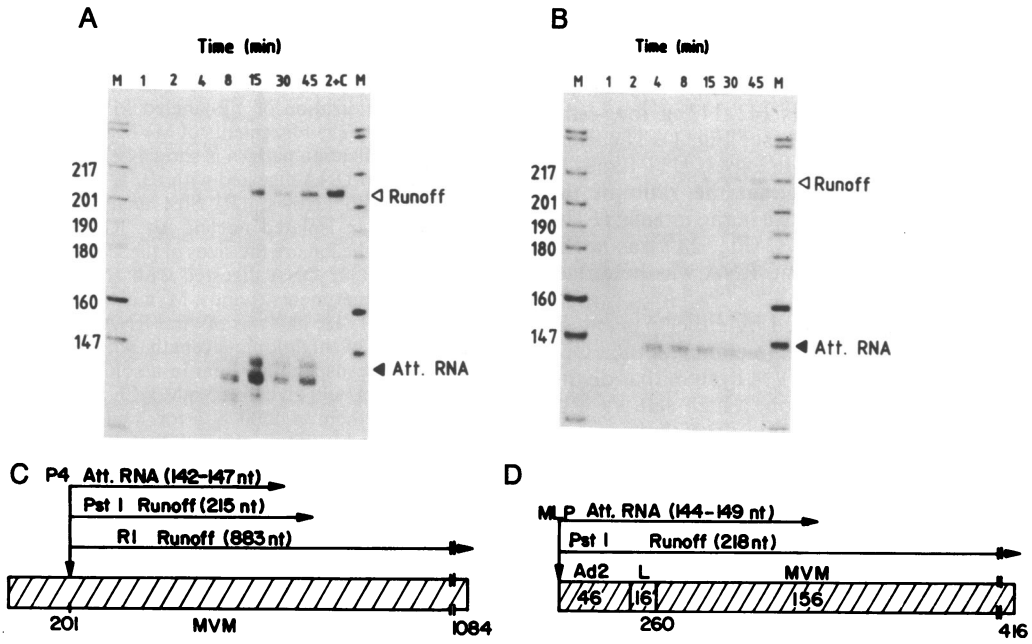

FIG. 3. Size analyses of RNA synthesized *in vitro* directed by promoter MVM P4 or Ad2 MLP with a HeLa nuclear extract. (*A*) Transcription in the nuclear extract in the presence of 0.5% Sarkosyl directed by the MVM promoter P4 with the RI plasmid digested with *Pst* I (see *C*). Transcription was initiated with [α-^{32}P]UTP and samples were taken at times indicated. Transcription was stopped by freezing the reaction mixtures in liquid N_2. In lane 2+C, a chase (500 μM UTP) began 2 min after the initiation of transcription and transcription was allowed to proceed for 45 min. Lanes M have molecular size markers; other lane labels indicate incubation time. (*B*) Transcription in the nuclear extract in the presence of 0.5% Sarkosyl directed by the Ad2 promoter MLP with the MLP-MVM plasmid digested with *Pst* I (see *D*). The nucleotide used as a label was CTP. Samples were processed as in *A*. Lanes M have molecular size markers; other lane labels indicate incubation time. (*C*) Schematic drawing of the RI construct and the lengths (in nt) of the run-off and attenuated RNA transcripts. Only the MVM sequences are shown. Promoter P4 and the *Pst* I and *Eco*RI sites are indicated. (*D*) Schematic drawing of the MLP-MVM construct and the lengths (in nt) of the run-off and attenuated RNA transcripts. The construct is composed of an Ad2 fragment that contains the MLP sequences (nt 5634–6083) followed by a linker of 16 base pairs and MVM sequences (nt 260–416).

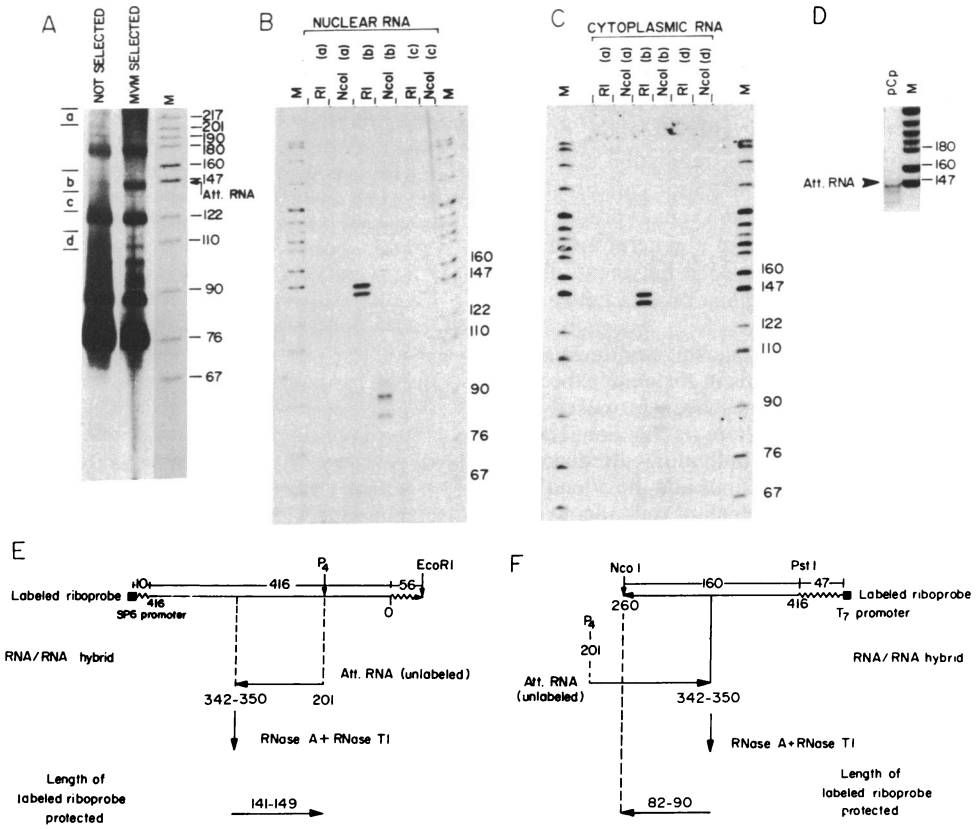

FIG. 4. *In vivo* identification, mapping, and characterization of the MVM-attenuated RNA. (*A*) Identification of the attenuated RNA by orthophosphate labeling of MVM-infected A9 cells. Approximately 10^7 cells were labeled with 15 mCi for 4 hr (23–27 hr after infection). Total RNA was extracted; viral RNA was selected and analyzed by gel electrophoresis. Lane M, molecular size markers. (*B*) Riboprobe mapping of unlabeled nuclear RNA extracted from MVM-infected A9 cells. RNA from 4×10^7 cells was extracted at 26 hr after infection. In each lane the equivalent of 5×10^6 nuclei were used in the riboprobe analysis. The probes used are diagrammed in *E* (RI probe) and *F* (Nco probe); 50,000 cpm of each probe was used per reaction mixture. RNase digestion was done at 0°C for 30 min. In *B*, a, b, and c are the regions of the gel in *A* from which the RNA used for mapping was eluted. M, molecular size markers. (*C*) Riboprobe mapping of unlabeled cytoplasmic RNA extracted from MVM-infected A9 cells. In each lane the cytoplasmic equivalent of 5×10^5 cells was used for the riboprobe analysis. The probes used are the same as those used in *B*. In *C*, a, b, and d are the regions of the gel in *A* from which the RNA used for mapping was eluted. M, molecular size markers. (*D*) pCp-end-labeling of unlabeled nuclear RNA from region b of *A*. Eluted RNA from region b in *A* was pCp-labeled, viral RNA was selected by hybridization to filters containing MVM DNA, and the eluted RNA was electrophoresed on a polyacrylamide gel. Lane M, molecular size markers. (*E* and *F*) Schematic representation of the riboprobe mapping experiment with the RI and Nco probes, respectively.

within the RNA·RNA hybrid and artifacts that result from using a mixed-length RNA population, an additional step was added to the RNase protection assay (21, 27). For this purpose, the unlabeled nuclear and cytoplasmic RNAs were electrophoresed on polyacrylamide gels and RNAs with the expected size of the attenuated RNA were eluted from the gels and analyzed with the two labeled RNA probes diagrammed in Fig. 4*E* (RI probe) and *F* (Nco probe). RNAs from above and below the size of the attenuated RNA were also eluted and analyzed as a control for degradation of the RNA. The sizes of the eluted RNA corresponded to the areas of the gel denoted a–d in Fig. 4*A*. The results of the analysis of the nuclear RNA are seen in Fig. 4*B*. When the RI probe was used, which is expected to give full-length protection of the attenuated RNA, two distinct bands at 142–147 nt were seen. These bands were correspondingly shortened with the use of the Nco probe. RNA from the regions above and below the attenuated RNA showed no hybridization. The same results were obtained with the cytoplasmic RNA (Fig. 4*C*). These results confirm that the enriched band of 147 nt in Fig. 4*A* was the attenuated RNA and indicate that it was initiated at promoter P4 and terminated 142–147 nt downstream. We were able to label the eluted RNA with pCp (Fig. 4*D*), which indicates that the *in vivo*-isolated attenuated RNA had a 3′ OH group and not a 3′ phosphate group.

DISCUSSION

In this study we have shown that RNA polymerase II is capable of pausing or prematurely terminating transcription at a precise location *in vivo* and *in vitro* 142–147 nt downstream from the MVM promoter P4 in a stretch of uridines (Fig. 1). The terminal nucleotide has a 3′ OH group, and we propose that a salt-soluble factor and the secondary structure are involved in the process of termination.

The attenuated RNA of MVM was found, by using orthophosphate labeling and RNase mapping, to be present *in vivo* in MVM-infected A9 cells late after infection in both the nuclear and cytoplasmic fractions. Since the RNA is small, perhaps a portion of the attenuated RNA leaked to the cytoplasmic fraction during preparation of the nuclei or, alternatively, perhaps the attenuated RNA was transported to the cytoplasm prior to separation from the nuclei. It is apparent that at late times after infection a significant proportion of the transcription from P4 is attenuated and in some way is protected from degradation—possibly it is folded into a protective secondary structure, it is hybridized to another RNA, or it is complexed with protein(s).

In MVM promoter P4 directs the transcription of two viral RNAs that are translated into the two nonstructural proteins, NS-1 and NS-2, while promoter P39 directs the transcription

of the RNA for the capsid proteins (13). In H-1, an MVM-related parvovirus, promoter P38 was shown to be activated by the NS-1 protein (13). The presence of the NS-1 protein greatly enhanced the expression of promoter P38, as assessed by superinfection with the virus or cotransfection of a plasmid encoding the protein. Therefore, regulation of promoter P4 by a block in transcriptional elongation can directly affect promoter P39 and its subsequent gene products. Alternatively, this may be an example of a general mechanism to prevent transcriptional interference between adjacent genes by placing a regulated termination site between them (28).

Increasingly, evidence suggests that pausing and termination of transcription are regulatory steps in the gene expression of eukaryotes. Such regulation has been proposed in viral models (8–12) and, although there is no sequence homology between these sites, the individual attenuated RNAs terminate within a stretch of uridines and the 3′ end of the RNA can be folded into a hairpin element (refs. 8 and 10 and Fig. 1). A block in transcription elongation has been described in the c-*myc* gene (5), in the c-*fos* gene (6), and in the c-*myb* gene (7) in differentiating systems. These observations suggest that a mechanism of modulation for these attenuation-like sites that would include termination and read-through factors (29) is likely to be an important part of eukaryotic gene regulation.

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