JOURNAL OF VIROLOGY, Oct. 1984, p. 266–276
0022-538X/84/100266-11$02.00/0
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Vol. 52, No. 1

Transcription of Minute Virus of Mice, an Autonomous Parvovirus, May Be Regulated by Attenuation

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Received 20 October 1983/Accepted 13 June 1984

To characterize the transcriptional organization and regulation of minute virus of mice, an autonomous parvovirus, viral transcriptional complexes were isolated and cleaved with restriction enzymes. The in vivo preinitiated nascent RNA was elongated in vitro in the presence of [α-^{32}P]UTP to generate runoff transcripts. The lengths of the runoff transcripts were analyzed by gel electrophoresis under denaturing conditions. On the basis of the map locations of the restriction sites and the lengths of the runoff transcripts, the in vivo initiation sites were determined. Two major initiation sites having similar activities were thus identified at residues 201 ± 5 and 2005 ± 5; both of them were preceded by a TATAA sequence. When uncleaved viral transcriptional complexes or isolated nuclei were incubated in vitro in the presence of [α-^{32}P]UTP or [α-^{32}P]CTP, they synthesized labeled RNA that, as determined by polyacrylamide gel electrophoresis, contained a major band of 142 nucleotides. The RNA of the major band was mapped between the initiation site at residue 201 ± 5 and residue 342. We noticed the potential of forming two mutually exclusive stem-and-loop structures in the 142-nucleotide RNA; one of them is followed by a string of uridylic acid residues typical of a procaryotic transcription termination signal. We propose that, as in the transcription of simian virus 40, RNA transcription in minute virus of mice may be regulated by attenuation and may involve eucaryotic polymerase B, which can respond to a transcription termination signal similar to that of the procaryotic polymerase.

The autonomously replicating parvoviruses are the smallest viruses known and have therefore been used as a model system for studies of DNA replication (7, 9, 12, 63) and RNA transcription (26, 60) in animal cells. The infectious virions are icosahedral particles that have diameters of 18 to 30 mm and contain linear single-stranded DNA of about 1.5 × 10^{6} daltons (64). The autonomously replicating parvoviruses package only the minus (coding) strand of the viral DNA (v-strand), in contrast to adeno-associated parvovirus, a defective subgroup which packages both DNA strands (9, 64).

Minute virus of mice (MVM) is one of the group of autonomously replicating parvoviruses of rodents that includes H-1, H-3, and Kilham rat viruses (54). These viruses share a high degree of nucleotide sequence homology and can complement each other in many functions (5, 43, 47, 48).

Initial studies on the transcription of DNA in MVM and H-1 suggested that the genomes of these viruses encode only a single transcription unit with a promoter near map unit (m.u.) 4 (26, 60). However, recent studies have demonstrated that three major transcripts are produced from two overlapping transcription units with separate promoters positioned near the left end (4 m.u.) and the middle (39 m.u.) of both the MVM and H-1 genomes (46, 49). The complete nucleotide sequences of MVM and H-1 have also recently been determined (6, 49).

In eucaryotes, although sequences which constitute promoters have been partially characterized (8, 15, 21, 27, 28), little is known about the sequence specificity of transcription termination (3). In procaryotes, transcription-termination sites are known to be located within as well as at the end of operons (for reviews, see references 1, 11, 13, and 50). The internal termination sites cause premature termination of the transcripts and quantitatively regulate the level of gene expression by selectively reducing the transcription of distal portions of the operon. This mechanism of regulation has been termed attenuation (69). Because pause sites could conceivably be used to set the rate of transcription of a gene or operon below that attainable by polymerase-promoter interaction alone, they could also be considered a mechanism of attenuation (69). The sequences at the termination and attenuation sites share common features. The DNA immediately preceding the site of termination is guanine · cytosine rich and often possesses dyad symmetry. The 3′ terminus of the transcript typically contains a series of uridine residues (1, 11, 13, 50).

In a series of studies with simian virus 40 (SV40) as the experimental system, it has recently been demonstrated that the eucaryotic polymerase B that transcribes SV40 RNA can respond to transcription termination and pausing signals similar to those of the procaryotic polymerase (31, 55, 56) and that a mechanism resembling attenuation in procaryotes regulates late transcription in SV40 (2, 3, 31, 37, 45, 55, 56). Moreover, a model in which attenuation and modulation of mRNA secondary structure in a feedback control mechanism quantitatively regulate SV40 gene expression has been presented (2, 31).

In the present study we mapped the initiation sites of the two overlapping transcription units of MVM and determined the activities of their promoters. In addition, we found that the attenuation mechanism functions in vitro at the left promoter-proximal region, yielding RNA of 142 nucleotides (nt). The DNA sequences at the attenuation site are strikingly similar to those of the termination signal in procaryotes and of the SV40 attenuator. We propose that RNA transcription in MVM may be regulated by attenuation.

MATERIALS AND METHODS

Cells and virus. MVM(T) stock was prepared as described previously (7). For infection, 5 ml of virus stock containing 5 × 10^{7} PFU/ml was added to a subconfluent culture of A9 cells at 10^{7} cells per 15-cm petri dish. After 2 h, the virus inoculum was removed, and the cells were supplemented with fresh medium (Dulbecco modified Eagle medium with 10% calf serum).

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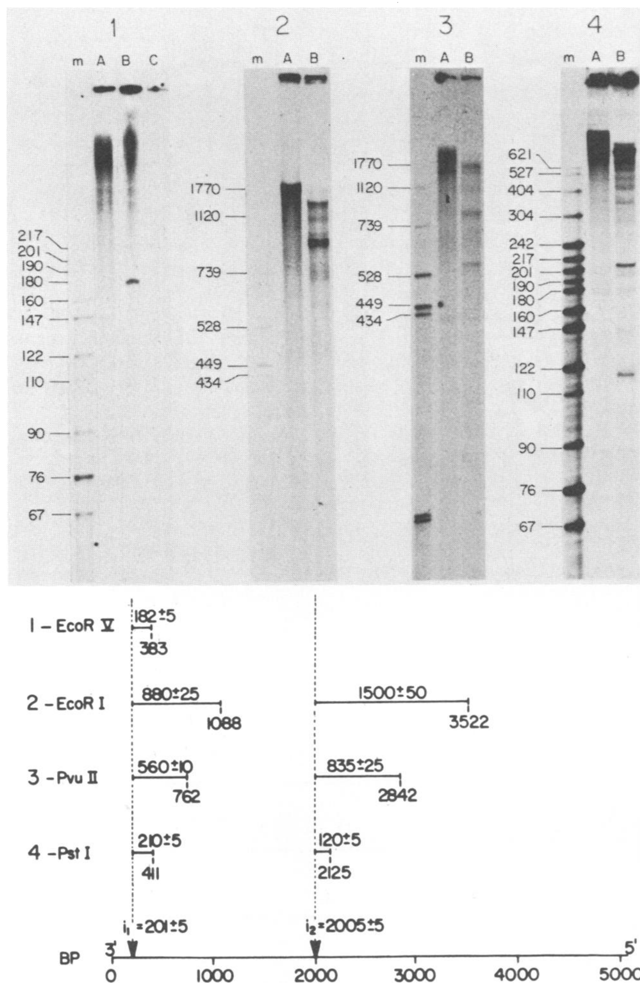

FIG. 1. Size analyses of runoff transcripts synthesized on truncated VTC. VTC were extracted from infected cells by the Sarkosyl method (23, 35). Sarkosyl was then removed by centrifugation through a sucrose gradient (see text), and portions of the purified VTC were subjected to restriction enzyme digestion followed by in vitro transcription in the presence of [α-^{32}P]UTP. RNAs produced were purified and analyzed by acrylamide gel electrophoresis under denaturing conditions (40). The labeled RNAs were detected by autoradiography. The gels in panels 1 and 4 contained 10% acrylamide; the gels in panels 2 and 3 contained 4% acrylamide. Size markers (m) in panels 1 and 4, pBR322 DNA digested with *Hpa*II; m in panels 2 and 3, SV40 DNA digested with *Hin*dIII and Taq I. Lanes A, RNA produced on noncut templates; lanes B, RNA produced by VTC cut with *Eco*RV (panel 1), *Eco*RI (panel 2), *Pvu*II (panel 3), and Pst I (panel 4); lane C, RNA of VTC incubated in the presence of 0.5 μg of α-amantin per ml. The scheme at the bottom of the figure shows the lengths of the transcripts revealed in the gel (the numbers above the lines) and the restriction enzyme cleavage sites of the v-strand (the numbers below the lines) (6). BP, Base pairs of replicative-form DNA. The residue numbers pertain to the v-strand (6). i_1, Left initiation site; i_2, right initiation site.

Preparation of VTC for a runoff reaction. Cells were labeled with [*methyl*-^{3}H]thymidine (38.4 Ci/mmol; Nuclear Research Center, Beer-Sheva, Negev, Israel) from 22 to 26 h postinfection. At the end of the labeling period, viral transcriptional complexes (VTC) were prepared by Sarkosyl lysis of nuclei as described by Laub and Aloni (35). The Sarkosyl extract (0.5 to 1.0 ml) was layered on a 5 to 30% (wt/wt) linear sucrose gradient in a buffer containing 0.5 M NaCl 40 mM Tris-hydrochloride (pH 7.9), and 5 mM β-mercaptoethanol. Sedimentation was carried out in a Beckman SW41 rotor for 18 h at 35,000 rpm and 4°C. Fractions (0.5 ml) were collected from the bottom of the tube, and 0.02-ml samples were assayed for radioactivity. Peak fractions containing VTC (16S to 18S) were pooled and concentrated under vacuum dialysis against a buffer containing 40 mM Tris-hydrochloride (pH 7.9), 50 to 150 mM NaCl (depending on the restriction enzyme used), and 5 mM β-mercaptoethanol. The concentrated material from 10 plates was recovered in 1 ml. Fractions (0.3 ml) were taken for further reactions. Intact templates were taken as such; truncated templates were obtained by cutting with restriction enzymes. Digestion with restriction enzymes was performed by adding $MgCl_2$ to a final concentration of 10 mM, followed by 50 U of enzyme. Digestion proceeded for 30 min at 28°C, after which the reaction mixture was cooled on ice and adjusted for the in vitro transcription reaction.

In vitro transcription reaction. In vitro elongation of the nascent RNA for generating runoff transcripts was carried out after adjusting the dialyzed VTC mixture to 500 mM NaCl–2 mM $MnCl_2$–1 mM dithiothreitol–0.5 mM concentration each of ATP, GTP, and CTP. A 10-min pulse of [α-^{32}P]UTP (100 to 400 Ci/mmol; Amersham Corp., Amersham, England) was then provided at a concentration of 1 to 2 μM, after which UTP was added to a final concentration of 200 μM and the reaction was allowed to proceed for 50 min at 28°C. At the end of the incubation period, sodium dodecyl sulfate was added to a final concentration of 0.5%, and the [^{32}P]RNA was extracted as described previously (30, 36).

For the production of attenuated RNA, transcription was carried out in the Sarkosyl extract [0.15 M $(NH_4)_2SO_4$, 5 mM KCl, 0.3% Sarkosyl, 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)–NaOH (pH 7.5)] after it was adjusted to a final concentration of 2 mM $MnCl_2$–1 mM dithiothreitol–a 0.5 mM concentration each of ATP, GTP, and CTP–a 1 to 10 μM concentration of [α-^{32}P]UTP as indicated in the figure legends. Transcription proceeded at 28°C for a short time (5 to 10 min), and [^{32}P]RNA was then extracted (31, 36).

For transcription in isolated nuclei prepared as described previously (7), the nuclei (ca. 10^8) were suspended in the transcription reaction mixture containing 5 mM KCl, 1.5 mM $MnCl_2$, 1 mM dithiothreitol, 12.5% glycerol, 150 mM $(NH_4)_2SO_4$, 30 mM HEPES–NaOH (pH 7.9), a 400 μM concentration each of ATP, GTP, CTP, and UTP as indicated in the legend to Fig. 4, and 300 to 500 μCi of [α-^{32}P]UTP (400 mCi/mmol; Amersham) in a final volume of 1.0 ml. Transcription was carried out at 28°C for 5 min and stopped by the addition of 100 μg of RNase-free DNase per ml for 1 min at 25°C, followed by extraction with phenol-chloroform (30, 36).

Purification of virus-specific RNA. [^{32}P]RNA was hybridized to a nitrocellulose blot (58) of an *Eco*RI cut of a clone of pBR322 into which the MVM genome had been inserted at the *Pst*I site (a gift of D. Ward). (In the figures only the MVM DNA fragments are represented.) Hybridization was carried out in a buffer containing 70% formamide, 0.3 M NaCl, 0.2% sodium dodecyl sulfate, 2 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.4) at 37°C for 36 h. Elution was carried out in a buffer containing 90% formamide, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.4) at 37°C for 1 h (29). The eluted RNA was collected by precipitation with ethanol.

Analysis of the RNA by gel electrophoresis. For analysis of

the RNA by gel electrophoresis, the labeled RNA was denatured in 98% formamide for 2 min at 80°C and subjected to electrophoresis on acrylamide gels in 7 M urea (40). Electroelution of the RNA from the gel was carried out as described previously (57).

RESULTS

Mapping of the in vivo initiation sites of transcription and analysis of the activities of the corresponding promoters. The experimental approach was developed by Kessler and Aloni (32), and we used it in the present study as follows. VTC were purified and cleaved with restriction enzymes, and the in vivo preinitiated nascent RNA was then elongated in vitro. It is noteworthy that no in vitro initiation occurs in the VTC system (19, 29, 35). This was confirmed by incubating the VTC with or without heparin (2 mg/ml). This concentration of heparin is known to inhibit transcription initiation but not RNA elongation (35). Similar rates of [^{32}P]UMP incorporation into RNA were observed in both cases (data not shown). Runoff transcripts were generated, and their lengths were determined by gel electrophoresis under denaturing conditions. The in vivo initiation sites were then determined on the basis of the map locations of the restriction sites and the lengths of the runoff transcripts. A similar approach has been used successfully in in vitro initiating systems (41, 46). When there was more than one in vivo initiation site of transcription, the activities of the corresponding promoters could also be compared. For this, in vitro elongation was performed with a limited concentration of the labeled nucleotide for a short period of time to allow elongation of the nascent RNA by only a few nucleotides. The runoff transcripts were then generated after a chase with a high concentration of the same, but unlabeled, nucleotide for a long period of time. Under these pulse-chase conditions the relative intensities of the corresponding runoff bands depended only on the activities of the promoters and not on the lengths of the RNA transcripts. With this protocol it was preferable to compare short runoff transcripts rather than long ones to avoid the possibility of some RNA polymerase molecules failing to reach the ends of long DNA fragments.

When in vitro transcription was performed with uncleaved VTC, no discrete reproducible bands corresponding to large transcripts were revealed under the present conditions of analysis (Fig. 1, lanes A). However, after digestion of the template DNA with *Eco*RV, which has a single recognition site on the MVM genome at nt 383 of the v-strand (6), a single discrete reproducible band with an estimated length of 182 ± 5 nt was obtained (Fig. 1, panel 1, lane B). The 5′ end of this transcript is calculated to be at residue 201 ± 5 [= 383 − (182 ± 5)] (see scheme in Fig. 1). A low concentration of α-amanitin (0.5 μg/ml) abolished transcription completely (Fig. 1, panel 1, lane C), indicating that the runoff transcripts are all produced by polymerase B.

These results are consistent with the occurrence of one transcriptional unit initiating close to the 3′ end of the genome (26, 60). However, the existence of additional promoters is not excluded as a possibility. For verification of the latter possibility, VTC were digested with *Eco*RI, and runoff transcripts were generated. Lane B in panel 2 of Fig. 1 shows two major bands with estimated lengths of 880 ± 25 and 1,500 ± 50 nt. The map locations of the two initiation sites were determined as follows. Assuming that the major template is a monomer duplex DNA (replicative form), *Eco*RI cleaves the MVM DNA template at two sites, residues 1088 and 3522. The 1,500 ± 25-nt runoff transcript is longer than the 1,088-nt fragment, and its 5′ end therefore

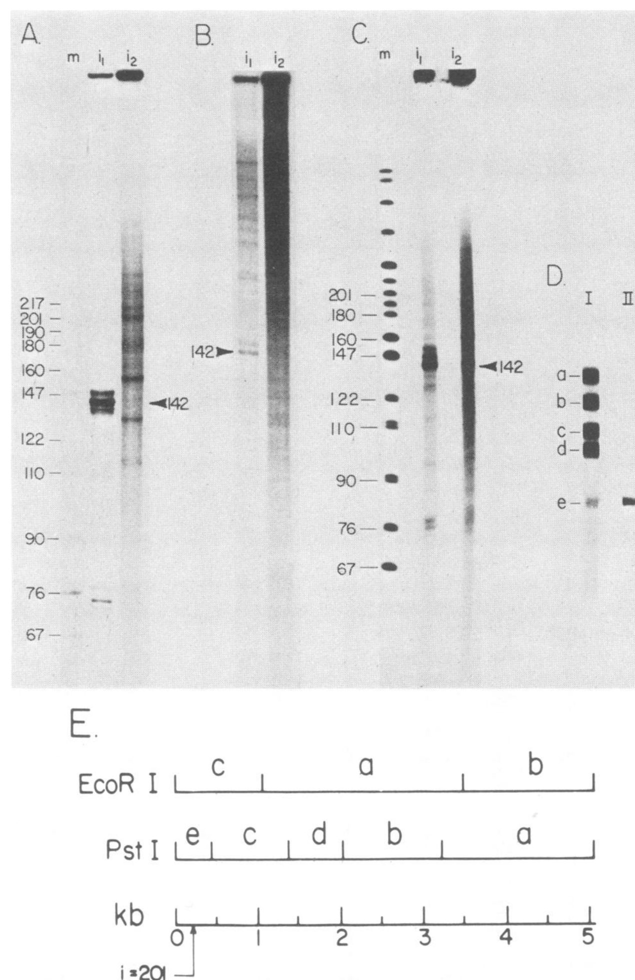

FIG. 2. Size analysis of viral RNA synthesized from the two promoters. (A) In vitro transcription of VTC in the presence of 1 μM [α-^{32}P]UTP allowed to proceed for 5 min. (B) In vitro transcription of VTC in the presence of 10 μM [α-^{32}P]UTP allowed to proceed for 10 min. (C) In vitro transcription of VTC in the presence of 10 μM UTP and 1 μM [α-^{32}P]CTP allowed to proceed for 5 min. RNAs were extracted, and viral RNAs were selected by hybridization to and elution from *Eco*RI-cleaved MVM DNA blotted on nitrocellulose (see text). Lanes i_1, RNA transcripts eluted from the *Eco*RI c fragment; lanes i_2 RNA transcripts eluted from the *Eco*RI a and b fragments. The ^{32}P-labeled viral RNAs were subjected to electrophoresis on 10% acrylamide gels (40). Size markers (m) are as described in the legend to Fig. 1 for panels 1 and 4. (D) Lane I, hybridization of ^{32}P-MVM DNA labeled by nick translation with *Pst*I fragments of MVM DNA; lane II, the RNA in the 142-nt band shown in panel A, lane i_1, eluted (57) and hybridized to *Pst*I-cleaved MVM DNA. (E) Restriction map of MVM DNA cut with *Eco*RI and *Pst*I (6). Note that MVM DNA inserted in pBR322 was cleaved with *Eco*RI, and replicative-form DNA was cleaved with *Pst*I. Only the viral DNA fragments are represented. kb, Kilobases. i, Left initiation site.

cannot reside within this fragment. It could, however, be a runoff transcript at the cleavage site at residue 3522. In this case, there is a second initiation site at residue 2022 ± 50 [= 3522 − (1500 ± 50)]. The 880 ± 25-nt runoff transcript fits well with being initiated at the left initiation site (201 ± 5) (see scheme in Fig. 1). The appearance of the 880 ± 25-nt runoff band as a doublet is apparently an artifact of the gel electrophoresis. This assumption is based on the following

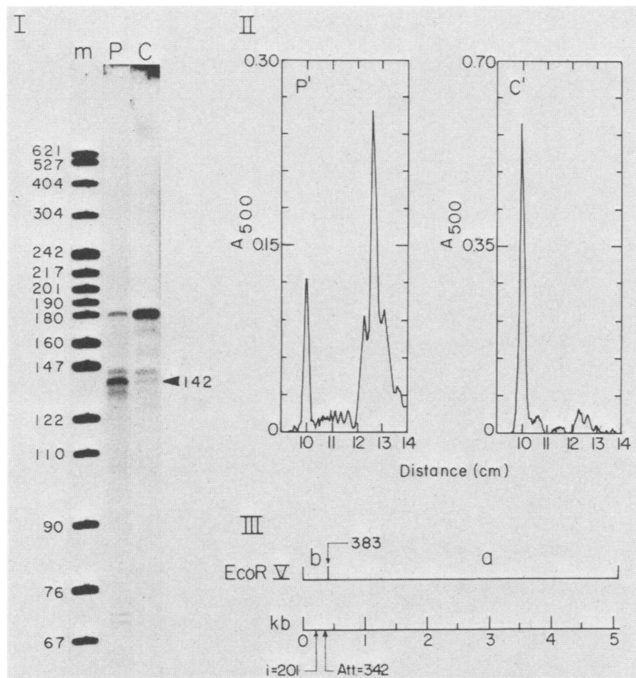

FIG. 3. Mapping of the 5' end of the 142-nt RNA. (Panel I) *Eco*RV-digested VTC incubated in vitro in the presence of 1 μM [α-^{32}P]UTP for 5 min (lane P) or incubated as in (P) followed by 30 min of incubation in the presence of 200 μM UTP (lane C). [^{32}P]RNA was selected by hybridization to and elution from *Eco*RI fragment c (see Fig. 2). The viral RNA was subjected to gel electrophoresis as described in the legend to Fig. 1. Size markers (m) are as described in the legend to Fig. 1 for panels 1 and 4. (Panel II) Phototracing of the gel in (I) by a scanner (Beckman DU-8). The absorbance at wavelength 500 nm (A_{500}) versus the distance from the origin of the gel was plotted. P' and C' correspond to P and C in (I). (Panel III) Restriction map of *Eco*RV-cut MVM DNA indicating the residue numbers (v-strand) of the *Eco*RV cleavage site (6) and the left initiation (i) and attenuation (Att.) sites. kb, Kilobases.

observations: the appearance of the runoff band as a doublet was not reproducible, and the corresponding band produced after cleavage of VTC with other restriction enzymes was unique. Other minor bands that appeared in these analyses seem to be artifactual, resulting from pausing of the polymerase or from premature termination of transcription. We make this conclusion because no corresponding bands were produced after cleavage of VTC with other restriction enzymes but new bands appeared (see below).

The two experiments described above indicated the existence of two major promoters on the MVM genome. Indeed, when VTC were cleaved with *Pvu*II, which also cleaves the MVM genome at two sites (residues 762 and 2842) (6), and when in vitro transcription was performed, two major bands were reproducibly produced and revealed by gel electrophoresis (Fig. 1, panel 3, lane B). The estimated lengths of 560 ± 10 and 835 ± 25 nt fit well with the existence of two initiation sites at residues 201 ± 5 and 2005 ± 5, respectively (see scheme in Fig. 1). Finally, to further corroborate the above finding, to map the initiation sites more precisely, and to compare the activities of the two promoters, we used *Pst*I, which cleaves the MVM genome at two sites close to the identified initiation sites, to truncate the template before generating runoff transcripts. Lane B in panel 4 of Fig. 1 shows the expected two promoter-proximal transcripts, 201 ± 5 and 120 ± 5 nt long. The lengths of these runoff transcripts fit well with their being initiated at the above-identified initiation sites at residues 201 ± 5 and 2005 ± 5, respectively (see scheme in Fig. 1). *Pst*I cleavage of MVM resulted in an additional three fragments, each of which gave rise to a runoff band. These bands were longer than the two promoter-proximal transcripts. In support of the existence of these two major initiation sites is the occurrence of TATAA sequences, 24 and 29 nt upstream of residues 201 and 2005, respectively (6), which are known to specify transcription initiation (for a review, see reference 53). These findings are in agreement with the map locations of the 5' ends of the major MVM mRNAs. These have recently been mapped by the S1 nuclease method at residue 2003 (a major start site) for the mRNAs of VP_2 and VP_3 and at residues 205 to 225 for the mRNA of VP_1 and for a nonstructural protein (46). The present study also substantiates the assumption that the major viral template for transcription is a monomer duplex DNA (replicative form).

It is noteworthy that transcription could initiate at alternative nucleotides within the identified initiation sites and that the existence of other minor initiation sites is not excluded as a possibility by the present study. The upper band (RNA initiated at residue 201 ± 5) was at least as intense as the lower band (RNA initiated at residue 2005 ± 5) (Fig. 1, panel 4, lane B). This indicates the existence of a similar number of active RNA polymerase molecules close to each of the initiation sites. In other words, the two promoters seem to have similar activities, at least at the time after infection at which they were extracted from the infected cells. It is possible that the relative usage of the two promoters depends upon virus-cell interaction and the progress of the infection. In this regard, it is interesting to note that the RNA transcripts initiated at the right promoter (39 m.u.) in vivo were found to be more abundant than those initiated at the left promoter (4 m.u.) (data not shown) (46). This difference may result from the particular time after infection at which each analysis was done or may be a consequence of several control mechanisms which operate after transcription initiation, leading to the production of mature RNA (14).

Regulation of transcription of MVM by attenuation. Attenuation is a mechanism whereby a site within the operon causes pausing or premature termination of transcription. This site, which acts conditionally to allow either pausing and termination or transcriptional readthrough, is termed attenuator site (69). If attenuation regulates MVM transcription, one would expect to find in vivo short RNA of a discrete length. Unfortunately, in eucaryotes primary RNA transcripts and nonfunctional RNAs are extremely unstable, and it is difficult to identify them. Thus, to date, termination sites for any pre-mRNA have not been identified (14). As a way to overcome this difficulty, isolated nuclei and various transcriptional systems are being used. One of them is the VTC system, which has been shown in SV40 to identify at least 90% of the VTC that generate viral RNA in intact nuclei and presumably in vivo (10, 23, 24, 35, 52). We therefore assume that the VTC system can represent the pattern of in vivo transcription. Thus, as a result of attenuation, we expected to find in VTC a sharp decrease in the number of nascent RNAs beyond an attenuation site. To verify this possibility, VTC were extracted from infected cells at 24 h postinfection and the in vivo preinitiated nascent RNA was elongated in vitro for 5 min in the presence of 1 μM [α-^{32}P]UTP. The nascent RNAs are elongated by about 20 nt under these conditions (35, 52, 55), allowing determination of their in vivo distribution along the DNA template (36). The [^{32}P]RNA was then purified and hybridized to a blot of

an *Eco*RI-restricted clone of MVM in pBR322. Just before elution of the RNA, the blot was cut (Fig. 2E) so that the fragment carrying the left promoter at 4 m.u. (fragment c, - spanning residues 1 to 1088) was separated from the other two fragments, a and b, carrying the rest of the MVM genome, including the right promoter at 39 m.u. Thus, nascent RNAs of up to about 900 nt initiated at the left promoter were separated from nascent RNA molecules initiated at the right promoter. The eluted RNA was analyzed by gel electrophoresis under denaturing conditions. A discrete major band with an apparent length of 142 nt was eluted from the fragment containing the left promoter (Fig. 2A, lane i_1). Two minor bands above and below the major band were also recognizable. However, almost no longer viral RNA transcripts were revealed, as indicated by the low level of radioactivity above the major band. This observation is consistent with a mechanism of in vivo pausing or premature termination at a site located 142 nt downstream from the left initiation site. The low level of long nascent RNA was not an artifact caused by the loss of long RNA molecules during preparation of VTC because heterologously sized RNA was eluted from the fragments containing the right promoter, as indicated by the radioactivity along the gel as well as at the origin of the gel (Fig. 2A, lane i_2). Moreover, when in vitro incubation was carried out for 10 min in the presence of 10 μM [α-^{32}P]UTP, the 142-nt band remained major, at least on a molar basis, but long RNA hybridizing to *Eco*RI fragment c appeared (Fig. 2B, lane i_1). It is noteworthy that at this UTP concentration, the rate of synthesis was about 100 nt per min (unpublished data). The level of the 142-nt band depended therefore both on the UTP concentration and on the in vitro incubation time. Under these conditions more and longer RNA hybridized to *Eco*RI fragments a and b carrying the right promoter (Fig. 2B, lane i_2).

Several groups have reported that RNA polymerase transcribing certain templates in vitro pauses transiently at particular sites on the DNA, resulting in a nonuniform rate of chain elongation and thereby generating discrete size classes of RNAs detectable as bands on acrylamide gels (38, 39). At least in some cases, such pauses in transit are apparently related to the use of low concentrations of nucleoside triphosphates. We therefore tested whether the transcription block which leads to the production of the 142-nt band is alleviated and if other bands appear when the concentration of CTP is reduced to 1 μM and the concentration of UTP is increased to 10 μM. (Fig. 2C, lane i_1). It is apparent that two bands, the 142-nt band and the minor band above it, remained (cf. Fig. 2A, lane i_1). However, the minor band below the 142-nt band in Fig. 2A, lane i_1, disappeared, and a new minor band of ca. 150 nt appeared. Furthermore, the minor bands of ca. 70 nt which appeared in Fig. 2A, lane i_1, disappeared, and new minor bands of about 80 nt appeared. On the basis of these results we conclude that the appearance of the 142-nt band was not caused by a limited UTP concentration in the reaction mixture, but the production of the minor bands depended at least in part on a limited concentration of a particular nt.

To delineate the region of the genome which serves as the template for the 142-nt band, we eluted the RNA from the 142-nt band shown in Fig. 2A, lane i_1, and hybridized it to a blot of *Pst*I-cut MVM DNA (Fig. 2E), followed by RNase treatment of the blot. Whereas uniformly labeled ^{32}P-MVM DNA hybridized extensively to fragments a through d, less ^{32}P-MVM DNA hybridized to fragment e because of its smaller size (Fig. 2D, lane I). The RNA of the major band hybridized exclusively to fragment e (Fig. 2D, lane II).

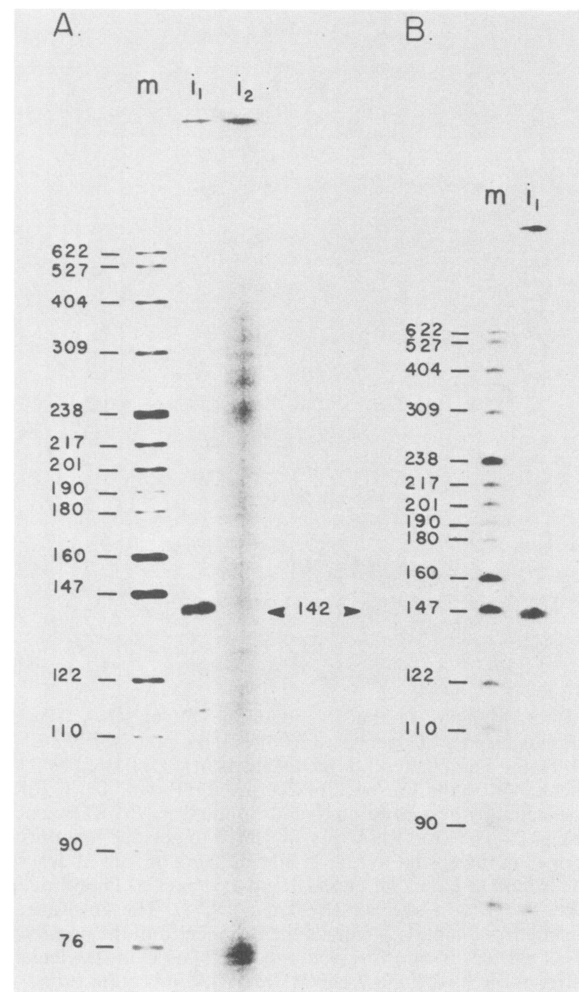

FIG. 4. Size analysis of viral RNA synthesized in isolated nuclei. In vitro transcription in isolated nuclei in the presence of 10 μM [α-^{32}P]UTP (A) or 100 μM [α-^{32}P]UTP (B) was allowed to proceed for 5 min. RNAs were extracted, and viral RNAs were selected by hybridization to and elution from a blot of *Eco*RI-cleaved MVM DNA as described in the legend to Fig. 2. Lanes i_1, RNA transcripts eluted from the *Eco*RI c fragment; lane i_2, RNA transcripts eluted from the *Eco*RI a and b fragments. Size markers (m) are as described in the legend to Fig. 1 for panels 1 and 4. The ^{32}P-labeled viral RNAs were subjected to gel electrophoresis as described in the legend to Fig. 2.

These results establish that the RNA of the major band is transcribed from a region of the genome spanning residues 201 to 411 (6).

Mapping of the 142-nt RNA. To localize the initiation site of the 142-nt RNA, we prepared *Eco*RV-cleaved VTC, divided them into two fractions, and incubated them in vitro under pulse and pulse-chase conditions. Labeled RNAs complementary to *Eco*RI fragment c (see Fig. 2) were purified from the two fractions and analyzed by gel electrophoresis under denaturing conditions. After the pulse, a major band of 142 nt and two minor bands, similar to those shown in Fig. 2A, lane i_1, were produced (Fig. 3, panel I, lane P). However, a new band of 182 nt now appeared (cf. Fig. 2A, lane i_1). This band contained runoff transcripts at the *Eco*RV cleavage site (Fig. 1, panel 1, lane B). After the chase, the 182-nt band became major, and the intensity of the 142-nt band was considerably reduced (Fig. 3, panel I,

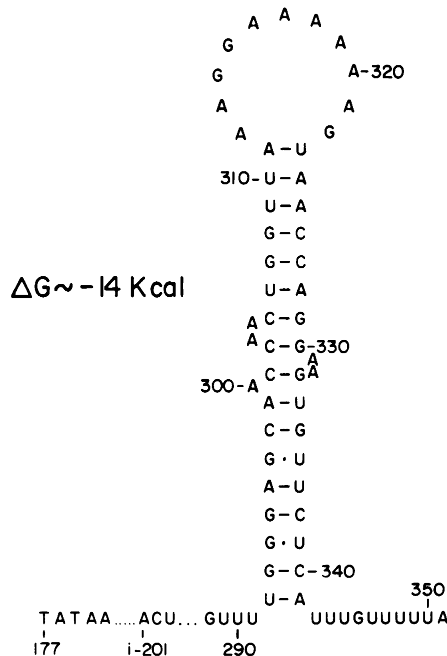

FIG. 5. Secondary structure at the 3′ end of the attenuated RNA. ΔG was calculated as described previously (66). Nucleotide residue numbers are from Astell et al. (6). i, Left initiation site.

lane C). These results indicate that after the chase, the radioactivity in the 142-nt band ran off at the *Eco*RV cleavage site. As *Eco*RV cuts MVM DNA at residue 383 (see Fig. 3, panel III), we conclude that the initiation site of the RNA of the 142-nt band is at residue 201 (= 383 − 182). On the basis of these calculations, in vitro pausing or premature termination occurs 142 nt downstream from the left initiation site, namely at residue 342.

A scan of the autoradiogram revealed that although most of the radioactivity in the 142-nt band was chased, about 10% of it was unchaseable (Fig. 3, panel II, C′). In vitro termination of transcription at this specific site may simply involve the cessation of chain elongation by the enzyme-DNA complex, namely, pausing, or it may be accompanied by the actual release of the RNA chain. It is worth noting that in the *trp* attenuator, the termination event in vitro does not include release of the transcript from the template (38).

Production of the 142-nt RNA in isolated nuclei. To exclude the possibility that the 142-nt RNA was synthesized only by a special class of VTC, we analyzed the viral RNA synthesized in isolated nuclei. As for RNA synthesis in VTC, RNA synthesis in isolated nuclei depends on nucleoside triphosphates, and thus nuclear RNA can be highly labeled with high-specific-activity nucleoside triphosphates. Several studies have suggested that in vitro chain elongation in isolated nuclei is an accurate reflection of a brief label inside cells (14). Therefore, isolated nuclei are widely used to prepare nascent RNA for the measurement of differential transcripts of specific genes and transcription rates. In isolated nuclei, previously initiated RNA polymerase B molecules elongate growing chains by less than 500 nt in 10 to 20 min (14).

In the present experiment, nuclei were prepared with a detergent-free hypotonic buffer (7) from uninfected and MVM-infected cells at 24 h postinfection. The in vivo preinitiated nascent RNA was elongated in vitro for 5 min in the presence of either 10 or 100 μM [α-^{32}P]UTP. The [^{32}P]RNA was then purified, and the viral RNA synthesized from the two promoters was selected by hybridization to restriction fragments as described in the legend to Fig. 2. The eluted RNA was analyzed by gel electrophoresis under denaturing conditions. A discrete major band with an apparent length of 142 nt was eluted from the fragment containing the left promoter when the nuclei were incubated with either 10 (Fig. 4A, lane i_1) or 100 (Fig. 4B, lane i_1) μM [α-^{32}P]UTP. This band was absent in RNA hybridizing to the fragment carrying the right promoter (Fig. 4A, lanes i_2). No radioactivity hybridized to either fragment when RNA from uninfected cells was analyzed (data not shown). The synthesis of the 142-nt RNA in isolated nuclei may indicate that it is also synthesized in infected cells. The 142-nt RNA could be produced as a result of either pausing of the RNA polymerase or transcription termination. It is noteworthy that in nuclei prepared from SV40-infected cells, a cellular factor and RNA secondary structure were found to be essential elements of transcription termination at the SV40 attenuator (30).

Pause and termination sites within the operon are used to set the rate of transcription of a gene or an operon below that attainable by polymerase-promoter interaction alone. Both of these mechanisms are considered to be mechanisms of attenuation (69). We therefore designated the 142-nt RNA as attenuated RNA.

Termination of the attenuated RNA by a transcription termination signal. Examination of the attenuated RNA for the presence of a potential secondary structure that might relate to the transcription termination event revealed that the DNA region 142 nt downstream from the left initiation site is adenine-thymine rich and is immediately preceded by a region that exhibits dyad symmetry. Figure 5 shows a proposed secondary structure of the RNA transcribed from this region. A stem-and-loop structure followed by uridylic acid residues is evident. This secondary structure resembles the transcription termination signal in procaryotes (1, 11, 13, 50) and the attenuator in SV40 (31, 55, 56).

It is noteworthy that the RNA transcribed from the attenuator region of MVM can be folded into two alternative conformations which are mutually exclusive. These are designated as attenuation and readthrough in Fig. 6A and B, respectively. It seems that the presence of such unique structures at the promoter-proximal region allows one at least to speculate that it functions in vivo as the recognition site for protein or proteins which influence the frequency of transcription. We suggest that in the nucleus, the attenuation conformation serves as a typical transcription termination structure that has been implicated in the process of premature termination (2, 31, 56). Conversely, the readthrough conformation leads to readthrough and to the production of mRNA. It is also interesting to note that in the cytoplasm, the same alternative conformations can be formed at the 5′ end of the viral mRNA. Consequently, in the attenuation conformation, the AUG initiating codon of VP_1 or of another, still unidentified protein at residues 261 to 263 (6) is free for ribosome binding. Conversely, in the readthrough conformation, the same AUG is sequestered in the stem (Fig. 6B) and apparently is not free for ribosome binding (34, 44). As for our model for SV40 (2, 31, 45, 55, 56), we speculate that modulation between the two conformations could be a fundamental element in a feedback control mechanism that regulates in the nucleus the amount of mRNA synthesized and in the cytoplasm the amount of protein produced. The details of the model should await the characterization of the

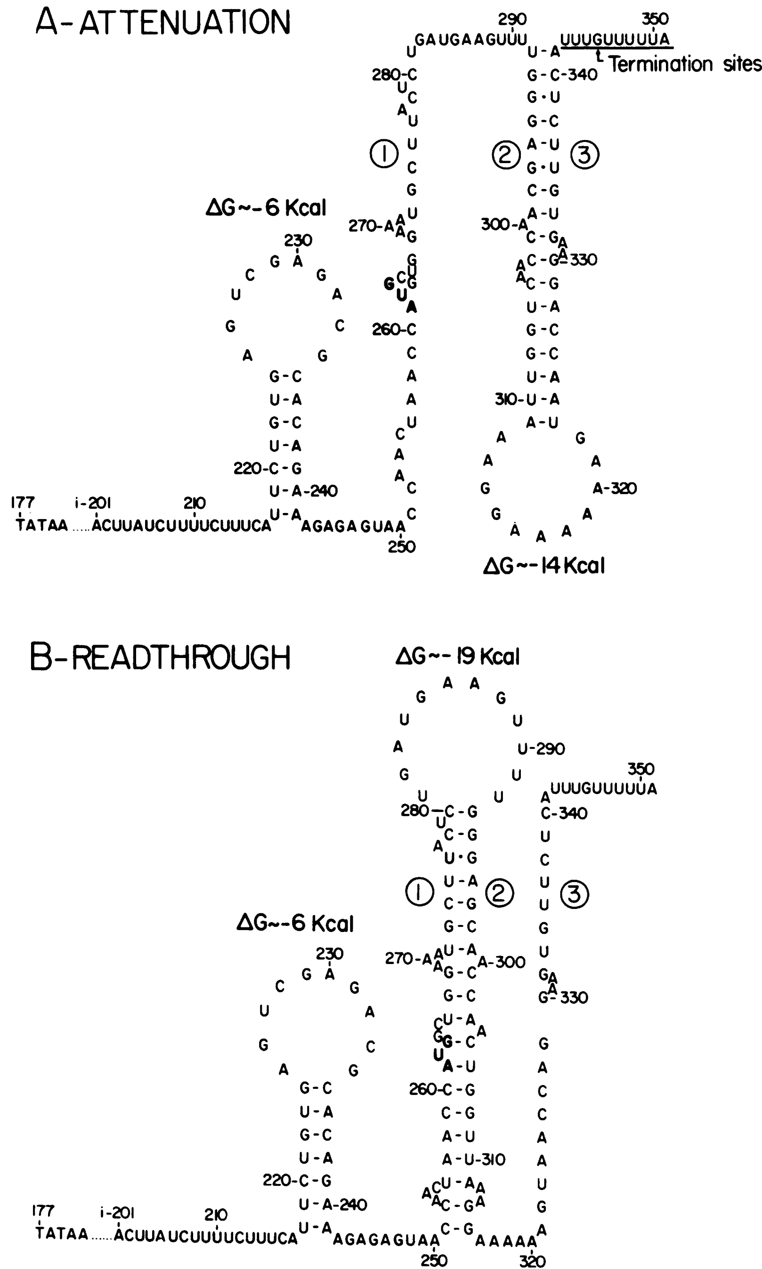

FIG. 6. Schema of alternative conformations in the attenuated RNA and the 5′ end of the viral mRNA initiated at residue 201 ± 5. i, Left initiation site. (A) Attenuation conformation showing a typical termination signal (2 and 3). The initiation codon (boldface letters) at residues 261 to 263 of the mRNA (6) is free for ribosome binding. (B) Readthrough conformation showing sequestration of the initiation codon at residues 261 to 263 of the mRNA. Residue numbers are from Astell et al. (6). ΔG was calculated as described by Tinoco et al. (66).

various proteins encoded by the MVM genome and the location of their initiating codons.

Is attenuation a prevalent control mechanism in animal viruses? As an approach to answering this question, we scrutinized the DNA sequences of promoter-proximal regions of five animal viruses: SV40 and BK virus of the papovavirus group and MVM, H-1 virus, and Kilham rat virus of the parvovirus group. These analyses revealed the existence of dyad symmetries which, in the resulting RNAs, have the potential of forming stem-and-loop structures that are immediately followed by uridylic acid residues. Figure 7 represents our proposed transcription termination signals in these viruses (Fig. 7B), as compared with the attenuation sites of the amino acid biosynthetic operons in bacteria (Fig. 7A) (33). It is apparent that in comparison to the procaryotic termination signals (Fig. 7A), the stem-and-loop structures of the animal viruses (Fig. 7B) are less stable, have an adenine-rich loop, and include unpaired bases. Also, it is interesting to note that all the stems contain the tetranucleotide CCAG in the proximity of the unpaired bases (Fig. 7B, shadowed regions). It is conceivable that all these features provide the recognition specificity for viral attenuator or

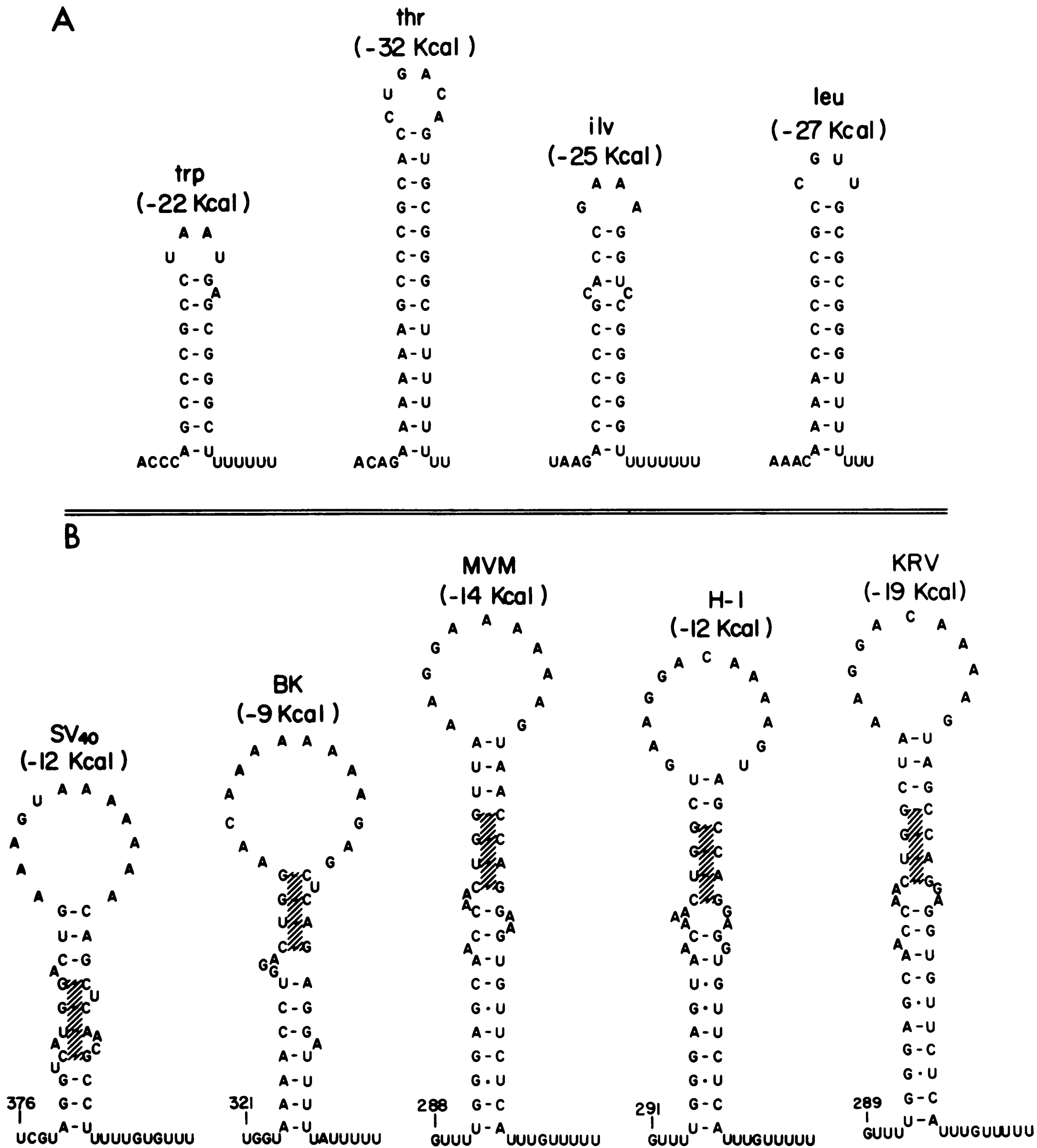

FIG. 7. Comparison between proposed secondary structures of the attenuators of the amino acid biosynthetic operons of bacteria (A) and those of animal viruses (B). Those of bacteria were adopted from Kolter and Yanofsky (33). The structures of the attenuators of animal viruses suggested here are based on the nucleotide sequences of SV40 and BK virus (67), MVM (6), H-1 virus (49), and Kilham rat virus (KRV) (5). In (B) the shadowed regions represent the tetranucleotide CCAG present in the stems of the attenuators of animal viruses.

antiattenuator factors or both or for a cellular termination factor which takes part in the transcription termination process (30, 59).

DISCUSSION

We identified and mapped two in vivo major initiation sites on the MVM genome at residues 201 ± 5 and 2005 ± 5, respectively. For this, we used a runoff protocol (41, 46) on VTC (35) truncated with various restriction enzymes. The VTC can elongate the in vivo preinitiated nascent RNA in vitro, but no in vitro initiation occurs (19, 23, 24, 29, 35). Thus, on the basis of the known map location of the restriction site and the length of the runoff transcript, the in vivo initiation site could be determined. On the basis of reversed runoff cDNA copying and S1 nuclease digestion data, the same two start sites were previously identified (46, 49). In the present study, the two sites were demonstrated to exist in the VTC, thus making it much more likely that the assigned sites really do represent initiation sites for transcription, rather than simply 5′ termini in mature mRNAs that are the result of posttranscriptional processing. Moreover, by comparing the intensities of the runoff bands, we could estimate the relative activity of each promoter. With this procedure we found that at late times after infection, the two promoters of MVM have similar activities in vivo.

In the present study, we also demonstrated that RNA chains initiated in vivo at nt 201 ± 5 and then transcribed under pulse conditions in vitro are synthesized to a length of

142 nt and are terminated at a region that has a secondary structure previously described in procaryotes (1, 11, 33, 50, 69) and in SV40 (2, 31, 45, 55, 56) as a signal for transcription termination. The fingerprint of the T_1 digest of the RNA and the nearest-neighbor analyses of each spot indicate that termination occurs primarily at the first and second uridylic acid residues (nt 342 and 343, respectively) (unpublished data). It is noteworthy that in the VTC system, the UTP concentration in the transcription mixture (typically 1 to 2 μM) could be a condition which favors termination at these two uridylic acid residues. Indeed, under pulse-chase conditions (chased at 200 μM), a 145 to 147-nt band became major (see Fig. 3), indicating in vitro termination at another uridylic acid residue. It is therefore apparent that, depending on the UTP concentration, transcription termination can occur at various sites along the stretch of uridylic acid residues spanning nt 342 to 350. In isolated nuclei, the effect of UTP concentration on the exact termination site is not as valid as in the VTC system, presumably because of the involvement of a termination factor (30). If the mechanism of premature termination is similar to the mechanism of transcription termination in procaryotes, we can predict that transcription slows down at the stem-and-loop structure (see Fig. 5), leading to the accumulation of the 142-nt band. The ribouridylate-deoxyadenylate duplex that is subsequently formed is exceptionally unstable. The unstable interaction could then provide the driving force for termination and release of the transcript (18, 25, 42). As a low UTP concentration reduces the rate of transcription, the pausing of the polymerase at the stem-and-loop structure is enhanced and leads to efficient termination. Conversely, a high UTP concentration stimulates the rate of synthesis, and the enzyme crosses the critical region at an increased rate, resulting in a shorter pause and leading to less efficient termination. Indeed, it has been noticed in procaryotic systems (18) and in SV40 (30, 45) that the concentration of ribonucleotides may influence the extent of termination at any given site. Moreover, at the end of the *trp* operon, only when the in vitro transcription system contains a low UTP concentration does it yield termination efficiencies approaching those seen in vivo (17). The present study thus supports and substantiates previous conclusions that transcription termination signals in procaryotes and for RNA polymerase B in eucaryotes are similar (2, 31, 56).

We believe that at the time that the VTC or nuclei were prepared (24 h postinfection), a large proportion of RNA polymerase molecules in vivo terminated transcription at the attenuation site, whereas other RNA polymerase molecules paused at a site located upstream of the attenuation site. This is based on the observation that radioactivity decreased sharply at the region of the gel where molecules larger than the attenuator RNA should migrate (see Fig. 2 and 4).

As far as the physiological significance of the attenuation mechanism in MVM is concerned, we suggest that this mechanism enables a communication between the two major promoters which is essential for coordinated synthesis of viral proteins. Thus, we speculate that a protein encoded by the right transcription unit (VP_2 or another protein) acts as an attenuator or antiattenuator factor, most likely by a mechanism that stabilizes one of the alternative RNA conformations (see Fig. 6). The present study thus supports the previous suggestion for SV40 that attenuation and modulation of RNA secondary structures in a feedback mechanism regulate viral gene expression (2, 31). That this mechanism could be widespread in animal viruses is indicated by the occurrence of similar termination signals in promoter-proximal regions of several viruses (see Fig. 7) and by the occurrence of abundant populations of promoter-proximal RNA in adenovirus-infected cells (16, 20, 62) and in vesicular stomatitis virus transcribed in vitro (65). Moreover, we also believe that a premature termination process resembling attenuation in animal viruses operates in the cell itself. This belief is based on the following observations: (i) there is an abundant population of promoter-proximal RNA chains in whole nuclear RNA (51, 61); (ii) there is a clustering of RNA polymerase B molecules in the 5′ moiety of the adult β-globin gene (22, 68); and (iii) a mechanism of attenuation may conceivably operate in the control of leucine synthesis in yeasts (4). The present report further supports the idea that similar basic mechanisms regulate gene expression in viruses and cells.

ACKNOWLEDGMENTS

We thank Ruchama Leiserowitz for technical assistance, D. Ward for his generous gift of MVM cloned in pBR322, and Florette Ungar for comments on the manuscript.

This research was supported by U.S. Public Health Service research grant CA 14995 and in part by the Norman V. Wechsler Endowment Fund.

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