JOURNAL OF VIROLOGY, Apr. 1988, p. 1448–1451
0022-538X/88/041448-04$02.00/0

Vol. 62, No. 4

The Two Transcription Units of the Autonomous Parvovirus Minute Virus of Mice Are Transcribed in a Temporal Order

KAREN E. CLEMENS AND DAVID J. PINTEL*

Department of Microbiology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

Received 9 November 1987/Accepted 9 December 1987

Using quantitative RNase protection assays, we have monitored the appearance of mRNAs generated during lytic infection of tightly synchronized murine cells by the autonomous parvovirus minute virus of mice. Our results demonstrate that transcripts from the P_4 promoter can be detected prior to those from the P_{39} promoter, providing direct evidence for a temporal order of expression between the two parvovirus promoters.

The parvoviruses are a large family of physically similar viruses that have linear, single-stranded DNA genomes approximately 5 kilobases in length (4, 5, 10, 26). Those that infect vertebrates have traditionally been divided into two genera, *Parvovirus* (autonomous) and *Dependovirus* (nonautonomous), on the basis of their requirement for helper virus. However, these divisions have recently become less distinct (23, 29).

DNA replication and gene expression by the autonomous parvoviruses are entirely dependent on one or more cellular functions expressed transiently during the S phase of the cell cycle (21, 24, 25, 27). Therefore, analysis of the appearance of viral mRNA and proteins and of potential temporal expression between the viral transcription units requires examination of infected cell populations that have been effectively synchronized.

The genome of the autonomous parvovirus minute virus of mice (MVM), like that of all other known parvoviruses, is organized into two major coding regions. These coding regions are expressed from two overlapping transcription units which produce spliced cytoplasmic species of 4.8 kilobases (R1), 3.3 kilobases (R2), and 3.0 kilobases (R3) (1, 20) (Fig. 1A). These mRNAs constitute approximately 15% (R1), 15% (R2), and 70% (R3) of the steady-state viral mRNA detected late in the infection of cultured murine cells (20). Transcripts R1 and R2 are synthesized from a promoter near the left-hand end of the viral genome at map unit (m.u.) 4 (2, 3, 20) and encode the viral nonstructural proteins NS-1 and NS-2, respectively, from open reading frames in the left half of the genome (8, 9). The R3 messages are initiated at a promoter at m.u. 39 (2, 20) and encode the viral capsid proteins from the open reading frame in the right half of the genome (2, 13). All MVM mRNAs are complementary to the genomic DNA strand (20), and they are all polyadenylated near the right-hand end of the genome at approximately m.u. 94 (7). Three splicing patterns are used to excise a small intron at m.u. 44 to 46 common to all three transcript classes, resulting in at least nine stable cytoplasmic mRNA species (2, 12, 18). The R2 transcripts are, in addition, spliced between m.u. 10 and 39 (1, 9, 12, 20).

Recent evidence from transient cotransfection assays has indicated that nonstructural proteins of both the autonomous and nonautonomous parvoviruses can act as transactivators of transcription of the promoters for the capsid-coding genes of those viruses (14, 22, 28). These observations have led to speculation that during infection, the transcripts encoding these transactivating proteins are generated prior to those transcripts which are induced. With porcine parvovirus-infected swine testis cells, the viral large nonstructural protein NS-1, which is the product of the P_4-initiated R1 transcript, can be immunoprecipitated prior to the viral capsid proteins, which are produced from the P_{39}-generated R3 transcript (17).

A previous study (20) of autonomous parvovirus transcription relied on infection of parasynchronized cell populations, which did not allow a precise temporal analysis of the appearance of viral mRNAs. Therefore, we have monitored the appearance of MVM mRNAs at very early times after infection by using a highly efficient double-block synchronization protocol (11, 19) suggested by P. Tattersall, Yale University. In this report, we demonstrate that transcripts from the P_4 promoter can be detected prior to those from the P_{39} promoter, providing direct evidence that there is a temporal order of expression between the two parvoviral transcription units.

The appearance of MVM mRNAs was assayed by analysis of RNase-protected hybrids formed in solution between excess complementary bacteriophage SP6 promoter-generated MVM RNA probes (16, 30) and RNA isolated from synchronized, MVM-infected murine A9 cells. The MVM *Hae*III fragment spanning nucleotide 1854 to 2378 was inserted into the SP6 vector (Fig. 1B). This fragment extends from before the acceptor site of the large splice in the R2 message to within the small intron common to all the viral messages at m.u. 44 to 46 (Fig. 1A). The expected nuclease-protected hybrids between the probe and viral R1, R2, and R3 transcripts are diagrammed in Fig. 1B and can be readily distinguished on a sequencing gel. In addition, mRNA species with either one of the alternate small splice donors can be distinguished. This probe will not distinguish species using alternate small splice acceptor sites, nor will it differentiate between unspliced R1 RNA and viral DNA.

Initially, this probe was used to analyze total RNA taken 24 h postinfection from murine A9 cells parasynchronized by isoleucine deprivation (15). Total and poly(A)$^+$ RNA were isolated by using guanadinium chloride followed by centrifugation through a 5.7 M CsCl cushion as previously described (7). Poly(A)$^+$ RNA was selected by three passes over an oligo(dT) column. Cytoplasmic RNA was obtained by lysing cells in 0.6% Nonidet P-40 and removing nuclei by centrifugation (6). The probe consisted of SP6 transcripts using the pGEM vector (Promega) containing an MVM_p *Hae*III (nucleotides 1854 to 2378) insert within the polylinker as template. Hybridizations were done overnight at 51°C in

* Corresponding author.

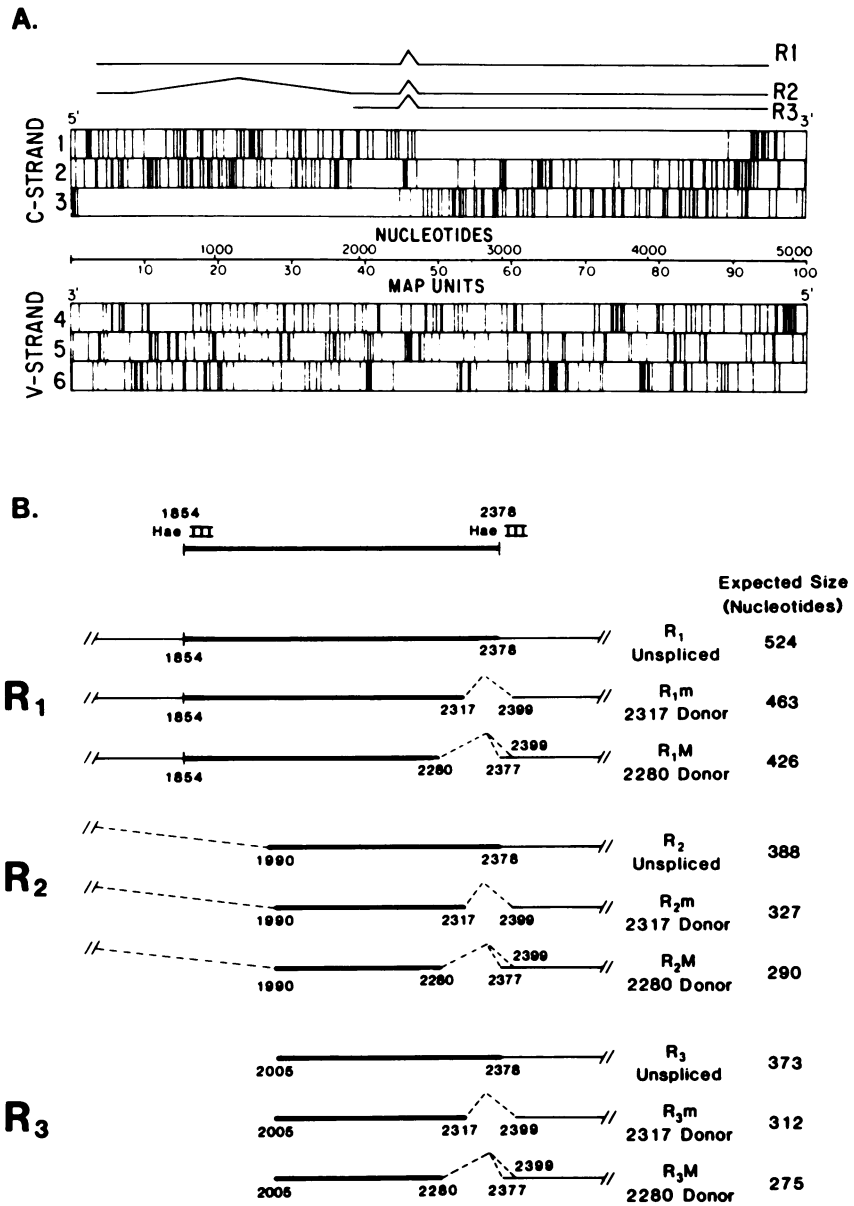

FIG. 1. (A) Map of MVM demonstrating the three major transcripts in relation to the open reading frames deduced from sequence analysis (2). (B) Unspliced and alternative splicing patterns of the R1, R2, and R3 transcripts. Splice donor and acceptor sites are as determined by Morgan and Ward (18). Bold lines indicate the predicted RNase-protected region(s) after hybridization to an SP6-generated MVM-derived RNA probe (*Hae*III fragment, nucleotides 1854 to 2378). The 5' end of the MVM sequence lies upstream of the R3 cap site at approximately nucleotide 2005 (2) and the acceptor site of the large splice within R2 at nucleotide 1990 (12). The 3' end is positioned between donor and acceptor sites of the small splice junction found in all three transcripts. M indicates the major RNA subspecies found in each transcript population, and m indicates those found in less abundance. Discrimination cannot be made between subspecies utilizing common donor but different acceptor sites.

80% formamide–40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 6.4)–400 mM NaCl–1 mM EDTA and combined 5 μg of total or cytoplasmic RNA or approximately 25 ng of poly(A)$^+$ RNA with an approximately 40-fold molar excess of probe (assuming MVM-specific mRNA to be about 0.15% of total cell RNA late in infection). Hybrids were digested at 30°C for 45 min at a final concentration of 40 μg of RNase A and 2 μg of RNase T_1 per ml (30), phenol extracted, and electrophoresed on an 8% polyacrylamide-urea gel (16, 30). The nuclease-protected hybrids detected (Fig. 2A, lane 1) correspond to those predicted in Fig. 1B and are in the same relative ratio as previously determined by Northern (RNA blot) analysis of steady-state MVM RNA (20). RNA from uninfected cells did not protect the complementary SP6-generated probe, and an SP6-generated probe of the same sense as MVM mRNA was not protected by RNA from MVM-infected cells (data not shown).

In addition to the six species protected by spliced MVM mRNAs, two more species were detected (Fig. 2A, bands A and B). These species were the size expected to be protected by unspliced versions of the R1 and R3 messages. They were

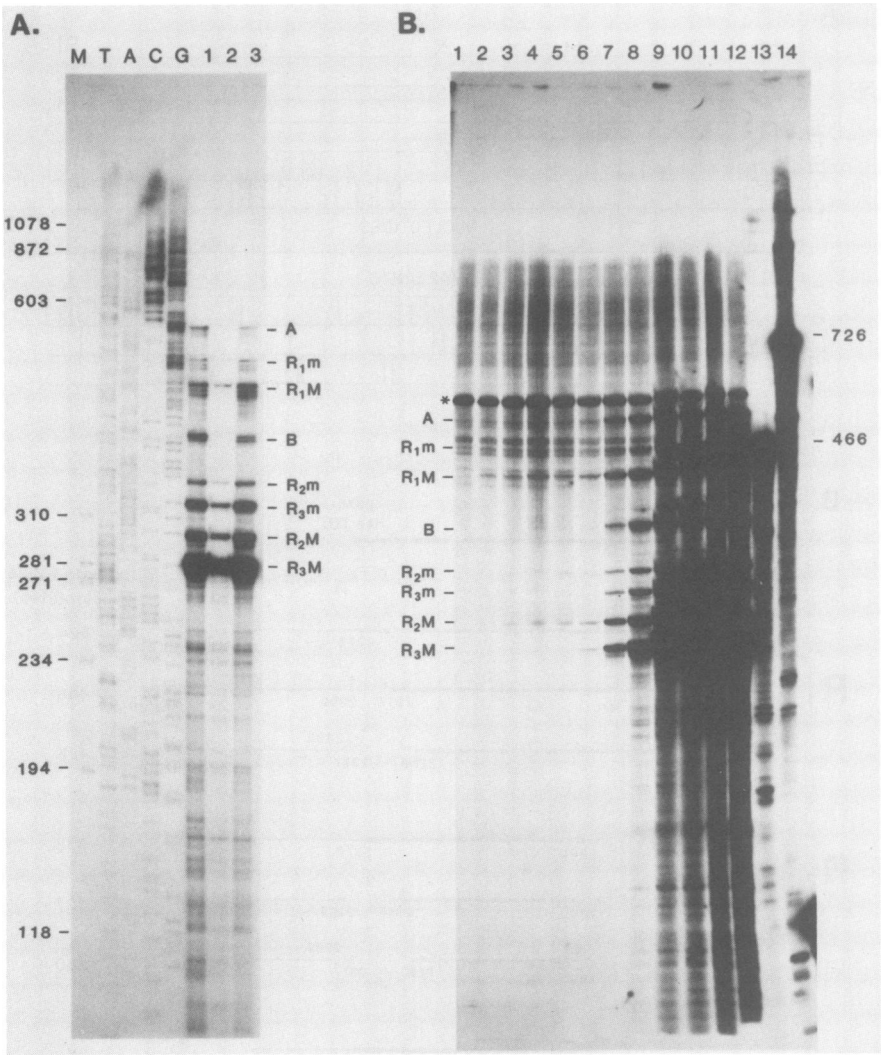

FIG. 2. (A) RNase protection assay using total, cytoplasmic, and poly(A)$^+$ RNA taken 24 h postinfection from MVM-infected A9 cells parasynchronized by isoleucine deprivation. Lane M is bacteriophage φX174 digested with *Hae*III. Sequencing lanes T, A, C, and G are dideoxy sequencing reactions with M13-cloned MVM sequence as a template. Lanes 1, 2, and 3 display RNase-protected hybrids with total, cytoplasmic, and poly(A)$^+$ RNA from MVM-infected A9 cells, respectively. Bands A and B are the sizes (approximately 524 and 373 nucleotides, respectively) expected to be protected by unspliced R1 and R3. See the text for details. The identities of the bands are as explained in the legend to Fig. 1 and agree well with the predicted sizes. (B) Time course of RNA appearance as monitored by RNase protection assay. MVM-infected A9 cells were deprived of isoleucine for 48 h and transferred to complete media with 5 μg of aphidicolin per ml for 20 h. At 10 h prior to aphidicolin release, the cells were infected with MVM_p at a multiplicity of infection of 10. RNA was isolated at hourly intervals. RNA (5 μg) from each time point was combined with excess RNA probe, hybridized, and treated with RNase as discussed previously. Lanes 1 through 12 are analyses with RNA from uninfected cells (lane 1), infected but unreleased cells (lane 2), cells at the time of release (lane 3), cells at 1 to 8 h postrelease (lanes 4 through 11, respectively), and cells at 24 h postrelease (lane 12). Lanes 13 and 14 are SP6 transcripts of 466 and 726 nucleotides, respectively. Bands A and B are as described in the legend to panel A above. The band designated by an asterisk is undigested probe.

drastically reduced in cytoplasmic RNA preparations (Fig. 2A, lane 2); however, they seemed to be predominantly polyadenylated. Their presence was essentially undiminished after three cycles of poly(A) selection (Fig. 2A, lane 3), which enriched at least 100-fold for poly(A)-containing RNA as monitored by ethidium bromide staining of rRNA followed by Northern analysis of MVM RNA.

To monitor the appearance of MVM mRNAs during the course of infection, we chose to examine infected cells that had been blocked by isoleucine deprivation (15) followed by release into the αDNA polymerase inhibitor aphidicolin (19). Cells so treated (11) begin DNA synthesis rapidly upon removal of the inhibitor and exhibit a biphasic S phase lasting 8 to 10 h (9; K. E. Clemens and D. J. Pintel, unpublished data). MVM virions were added to the cells 10 h prior to removal of the inhibitor, and RNA samples were isolated and assayed as described above at hourly intervals after removal.

The R1 and R2 mRNA products of the P_4 promoter were detected even before release into complete medium without aphidicolin, and they began to accumulate prior to detection of the P_{39}-generated R3 messages (Fig. 2B). R3 was detected about 3 h postrelease and became predominant, reaching steady-state ratios at about 7 h postinfection. The ratio of the

different spliced versions of each RNA class remained constant throughout infection. These results indicate that the products of the P_4 promoter appear before the products of the P_{39} promoter and provide direct evidence for a temporal order of expression between the two viral promoters. These results also suggest that initial P_4 transcription comes from the original covalently closed monomer replicative-form molecule.

Southern blot analysis monitoring the appearance of viral replicative forms in these experiments showed that double-stranded replicative-form molecules became detectable at about 3 h postrelease and accumulated rapidly in parallel with the accumulation of the viral R3 message (data not shown). Whether the phasing detected between the two viral transcription units is mediated by one of the viral nonstructural proteins or is merely due to the dramatic increase in the double-stranded transcription template is not addressed by the experiments presented here. However, the observation that the large nonstructural proteins of many parvoviruses transactivate the viral capsid-coding genes in transient cotransfection assays (14, 22, 28) sets a precedent for their involvement in the phasing between the two promoters. Analyses similar to those presented here, with a recently isolated temperature-sensitive mutant of the MVM NS-1 gene (G. E. Tullis and D. J. Pintel, manuscript in preparation), may resolve this question.

We thank Peter Tattersall for suggesting the aphidicolin cell synchronization procedure and Ram Guntaka for critically reading the manuscript.

This work was supported in part by Public Health Service grants RO1 AI-21302 and T32 AI07276 from the National Institutes of Health and by the Life and Health Insurance Medical Research Fund.

LITERATURE CITED

1. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, $MVM_{(i)}$, and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–669.
2. **Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
3. **Ben-Asher, E., and Y. Aloni.** 1984. Transcription of minute virus of mice, an autonomous parvovirus, may be regulated by attenuation. J. Virol. **52**:266–276.
4. **Berns, K. I. (ed.).** 1984. The parvoviruses. Plenum Publishing Corp., New York.
5. **Berns, K. I., and M. A. Labow.** 19874. Parvovirus gene regulation. J. Gen. Virol. **68**:601–614.
6. **Carter, B. V., and V. A. Rose.** 1974. Transcription *in vivo* of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. Virology **61**:182–199.
7. **Clemens, K. E., and D. Pintel.** 1987. Minute virus of mice (MVM) mRNAs predominantly polyadenylate at a single site. Virology **160**:511–514.
8. **Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two non-structural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
9. **Cotmore, S. F., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
10. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**:91–174.
11. **Heintz, N. H., J. D. Milbrandt, K. S. Greisen, and J. L. Hamlin.** 1983. Cloning of the initiation region of a mammalian chromosomal replicon. Nature (London) **302**:439–441.
12. **Jongeneel, C. V., R. Sahli, G. K. McMaster, and B. Hirt.** 1986. A precise map of splice junctions in the mRNAs of the minute virus of mice, an autonomous parvovirus. J. Virol. **59**:564–573.
13. **Labieniec-Pintel, L., and D. Pintel.** 1986. The minute virus of mice P_{39} transcription unit can encode both capsid proteins. J. Virol. **57**:1163–1167.
14. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
15. **Ley, K. D., and R. A. Tobey.** 1970. Regulation of initiation of DNA synthesis in Chinese hamster cells. J. Cell Biol. **47**:453–459.
16. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
17. **Molitor, T. W., H. S. Joo, and M. S. Collett.** 1985. Identification and characterization of a porcine parvovirus nonstructural polypeptide. J. Virol. **55**:554–559.
18. **Morgan, W. R., and D. C. Ward.** 1986. Three splicing patterns are used to excise the small intron common to all minute virus of mice RNAs. J. Virol. **60**:1170–1174.
19. **Pedrali-Noy, G., S. Spadari, A. Miller-Faures, A. O. A. Miller, J. Kruppa, and G. Koch.** 1980. Synchronization of HeLa cell cultures by inhibition of DNA polymerase alpha with aphidicolin. Nucleic Acids Res. **8**:377–387.
20. **Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward.** 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. **11**:1019–1038.
21. **Rhode, S. L., III.** 1973. Replication process of the parvovirus H-1. I. Kinetics in a parasynchronous cell system. J. Virol. **11**:856–861.
22. **Rhode, S. L., III.** 1985. *trans*-Activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
23. **Schlehofer, J. R., R. Heilbronn, B. Georg-Fries, and H. Zur Hausen.** 1983. Inhibition of initiator-induced SV40 gene amplification in SV40-transformed Chinese hamster cells by infection with a defective parvovirus. Int. J. Cancer **32**:591–595.
24. **Siegl, G., and M. Gautschi.** 1973. The multiplication of parvovirus LuIII in a synchronized culture system. I. Optimum conditions for virus replication. Arch. Gesamte Virusforsch. **40**:105–118.
25. **Tattersall, P.** 1972. Replication of the parvovirus MVM. I. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. **10**:586–590.
26. **Tattersall, P., and D. Ward (ed.).** 1978. Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Tennant, R. W., K. R. Layman, and R. E. Hand, Jr.** 1969. Effect of cell physiological state on infection by rat virus. J. Virol. **4**:872–878.
28. **Tratschin, J. D., J. Tal, and B. J. Carter.** 1986. Negative and positive regulation in *trans* gene expression from adeno-associated virus vectors in mammalian cells by a viral rep gene product. Mol. Cell. Biol. **6**:2884–2894.
29. **Yakobson, B., T. Koch, and E. Winocour.** 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J. Virol. **61**:972–981.
30. **Zinn, K., D. MiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34**:865–879.