

Two Parvoviruses That Cause Different Diseases in Mink Have Different Transcription Patterns: Transcription Analysis of Mink Enteritis Virus and Aleutian Mink Disease Parvovirus in the Same Cell Line

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The two parvoviruses of mink cause very different diseases. Mink enteritis virus (MEV) is associated with rapid, high-level viral replication and acute disease. In contrast, infection with Aleutian mink disease parvovirus (ADV) is associated with persistent, low-level viral replication and chronic severe immune dysregulation. In the present report, we have compared viral transcription in synchronized CRFK cells infected with either MEV or ADV using a nonradioactive RNase protection assay. The overall level of viral transcription was 20-fold higher in MEV- than in ADV-infected cells. Furthermore, MEV mRNA encoding structural proteins (MEV mRNA R3) was dominant throughout the infectious cycle, comprising approximately 80% of the total viral transcription products. In marked contrast, in ADV-infected cells, transcripts encoding nonstructural proteins (ADV mRNA R1 and R2) comprised more than 84% of the total transcripts at all times after infection, whereas ADV mRNA R3 comprised less than 16%. Thus, the ADV mRNA coding for structural proteins (ADV mRNA R3) was present at a level at least 100-fold lower than the corresponding MEV mRNA R3. These findings paralleled previous biochemical studies analyzing *in vitro* activities of the ADV and MEV promoters (J. Christensen, T. Storgaard, B. Viuff, B. Aasted, and S. Alexandersen, *J. Virol.* 67:1877–1886, 1993). The overall low levels of ADV mRNA and the paucity of the mRNA coding for ADV structural proteins may reflect an adaptation of the virus for low-level restricted infection.

The two autonomous parvoviruses of mink cause very different diseases. Mink enteritis virus (MEV) causes an acute disease in which the virus replicates to high titers within the mesenteric lymph nodes and in epithelial cells of the small intestine (46, 55). This results in lymphopenia, loss of intestinal mucosa, and diarrhea in susceptible animals (45, 46, 55). Virus replication peaks 4 days after infection, with a viral load of 250,000 genomes per infected cell (39, 55). A strong neutralizing antibody response follows, which reduces virus load to undetectable levels by 8 days after infection (55).

In contrast, Aleutian mink disease parvovirus (ADV) causes a chronic disease associated with low-level viral replication. The replication of ADV peaks 10 days after infection, with a viral load that is 25-fold lower than that seen for MEV (1, 5, 11). In addition, ADV replication is not associated with any acute histopathology or disease (42). Although ADV rapidly induces a strong antibody response, virus persists and a chronic disease ensues, associated with plasmacytosis, hypergammaglobulinemia, and immune complex-mediated disease (18, 23–25, 43).

Under special circumstances, ADV infection can cause acute disease. Neonatal seronegative mink kits develop an acute fulminant interstitial pneumonia when infected with ADV (2, 28, 56) and have a viral load comparable to that in MEV infection (6, 55). The kinetics of ADV replication are

still slow compared to those of MEV, but the immaturity of the immune system allows the viral load sufficient time to accumulate to the high levels associated with acute disease (3, 6, 7). Mink kits from seropositive dams or mink kits passively given anti-ADV antibodies do not develop acute disease but instead progress to develop the persistent form of the disease (3, 7). Somehow, anti-ADV antibodies down regulate the viral DNA replication and transcription in infected cells in these mink kits (7, 8).

It is tempting to speculate that the slowness of ADV replication might allow a competent immune system the time necessary to damp viral expression, preventing acute disease. Thus, the inherently low level of replication might be crucial for the absence of acute disease and potentially also for the persistence of ADV (3, 14, 51, 55).

The replication of the cell culture-adapted strain of ADV is significantly slower than that of MEV in CRFK cells (14, 55). When considered with the *in vivo* observations, it has led us to propose that low-level replication is an intrinsic property of ADV (8, 55). Because of its potential involvement in the unique pathogenesis of ADV, we have been interested in exploring the molecular mechanisms governing this low-level replication. Comparative studies with MEV are attractive in this respect since both viruses replicate permissively in CRFK cells, with relative kinetics that resemble their *in vivo* kinetics (14, 41, 55).

One aspect of the viral life cycle that has been implicated in the slow ADV replication is viral transcription (3, 4, 8, 14, 51). In order to analyze the mRNA transcription, it is necessary

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briefly to consider the molecular organization of the viral genomes. ADV and MEV both have a single-stranded, negative-sense DNA genome of approximately 5,000 nucleotides (nt) (10, 27). The cellular DNA replication machinery active during the S phase of the cell cycle is necessary for conversion of the single-stranded genome into a double-stranded replicative form (RF DNA). RF DNA then functions both as a template for viral DNA replication and for viral mRNA transcription (16). The genome is essentially divided into two large open reading frames. A left open reading frame encodes the two nonstructural proteins (NS1 and NS2) by differential splicing of an mRNA initiated from a promoter located at either map unit 3 (ADV P3) or map unit 4 (MEV P4). The right open reading frame encodes the two structural proteins VP1 and VP2 through an mRNA initiated from a viral promoter located at either map unit 36 (ADV P36) or map unit 38 (MEV P38) (4, 10, 13, 27, 31, 40, 44).

Comparison of the viral promoters from ADV, MEV, and the prototypic parvovirus minute virus of mice (MVM) revealed that the slowness of ADV replication correlates with weakness of the viral promoters in *in vitro* promoter studies (14, 51). As such, both the constitutive activity of the ADV promoters and the level of NS1-mediated transactivation were very low compared to the corresponding promoters of MEV and MVM (14).

In the present studies, we investigated if the activity of the ADV and MEV promoters reflected the actual amount of viral RNA in infected cell cultures. We developed an RNase protection assay (RPA) to compare the viral mRNA levels and kinetics of accumulation in synchronized CRFK cells infected with either MEV or ADV. We found the total amount of viral transcripts in MEV-infected cells was 20-fold higher than that in ADV-infected cells. Furthermore, transcripts encoding the viral structural proteins were the predominant mRNAs at all times after infection with MEV. In contrast, transcripts encoding the viral nonstructural proteins were the predominant mRNAs at all times after infection with ADV.

MATERIALS AND METHODS

Cell synchronization and infection. CRFK cells (17) were synchronized at 37°C by a combination of topoinhibition and hydroxyurea (HU) blocking as described previously (35). This protocol results in more than 90% of the cells with G₀/G₁ DNA content at the time of release (35). After release from HU, the cells transverse the S phase for 12 to 14 h and then enter mitosis between 16 to 21 h (35).

MEV and ADV-G (41) inocula were adjusted to produce approximately 50% virus-positive cells as previously described (35). After release from HU, the cells were incubated at 31.8°C.

In order to determine the relative stability of the different viral mRNAs in infected cells, transcription was blocked by actinomycin D (40 µg/ml) 5.5 h after HU release. Cells were subsequently lysed in 4 M guanidine thiocyanate (GuSCN) at 1 and 3 h after the block of transcription. In a parallel experiment, transcription was blocked at 11 h after HU release and the cells were lysed in GuSCN 9 h later.

Nonradioactive RPA. The cells from each 60-mm-diameter dish were lysed in 1 ml of 4 M GuSCN. Viral mRNAs in this lysate were stable at -20°C.

In selected instances cytoplasmic RNA was prepared by lysing the cells from a 60-mm-diameter dish in 200 µl of 0.15 M NaCl-1.5 mM MgCl₂-0.65% Nonidet P-40 (21). Nuclei were removed by centrifugation for 5 min at 1,500 × g at 4°C, and the cytoplasmic fraction was transferred to another tube containing 1 ml of 5 M GuSCN.

Hybridizations with the various probes were performed using a modified RPA that involved direct hybridization in GuSCN cell lysate (19, 22, 52) with biotinylated RNA probes (54). Ten microliters of cell lysate was hybridized overnight to an excess of biotinylated RNA probe in a total of 25 µl of 4 M GuSCN at 37°C. The hybridization solution was diluted by adding 195 µl of H₂O and 50 µl of RNase dilution buffer (250 mM Tris-HCl [pH 7.5], 2 M NaCl, 50 mM EDTA), followed by digestion with 5 µl of RNase A/T₁ mix (0.5 U/µl of RNase A and 20 U/µl of RNase T₁) (Ambion, Austin, Tex.) for 30 min at 37°C. Subsequently, RNase and cellular proteins were digested by adding 10 µl of 10% Sarkosyl and 5 µl of proteinase K (20 mg/ml) followed by an additional 30-min incubation at

37°C. The protected hybrids were precipitated by adding 250 µl of isopropanol and centrifuging at 10,000 × g at 4°C for 15 min.

The protected hybrids were recovered in 5 µl of sequencing gel loading buffer (47), denatured at 90°C for 5 to 10 min, and separated on a 5% denaturing acrylamide gel (Long Ranger; FMC, Rockland, Maine) (8 M urea, 1 × TBE [47]) at 35 V/cm. The separated RNA fragments were transferred to positively charged nylon membranes (Hybond-N⁺; Amersham Life Science, Arlington Heights, Ill.) in 0.5 × TBE for 30 min at 10 V, using a Trans-Blot SD transfer cell (Bio-Rad Laboratories, Hercules, Calif.), and subsequently cross-linked to the membranes by 5 min of incubation in 0.05 M NaOH (9).

The membranes were rinsed twice for 5 min in wash buffer (1 × phosphate-buffered saline [pH 6.9], 0.5% sodium dodecyl sulfate, 100 ng of total yeast RNA per ml), blocked three times for 10 min each in blocking solution (Ambion), and incubated for 30 min with streptavidin-alkaline phosphatase (Avidin-AP; Tropix, Bedford, Mass.) diluted 1:3,000 in blocking solution. Next, the membranes were washed for 10 min in blocking solution and four times for 10 min each in wash buffer and rinsed briefly four times in assay buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 250 ng of yeast RNA per ml). Finally, the membranes were incubated for 5 min in CDP-Star substrate (Tropix) and exposed to X-Omat film (Eastman Kodak, Rochester, N.Y.).

The intensities of the specific bands were measured by laser scanning (Ultra-Scan; LKB, Bromma, Sweden), using GelScan 2.1 software (Pharmacia). Linearity of the data was assured by comparing the ratio of bands from several different exposure times. Finally, the signals were normalized to the number of cytosine molecules in the different protected fragments and these normalized numbers were used in constructing Fig. 2, 3, and 4.

Once bound to membranes, the RNA probes (single stranded) were very sensitive to degradation by RNase activity in washing and blocking buffer. Therefore, all reagents were treated with diethylpyrocarbonate (DEPC) when possible and all blocking and washing steps were done using a sterile technique. Using these precautions, we could routinely detect less than one copy of mRNA per cell in the total cell lysate.

Probe design. (i) MEV probe. The probe for detection of MEV transcription was designed based on transcription data from canine parvovirus (CPV), a host range variant. These data have kindly been made available to us by Colin Parrish, James A. Baker Institute for Animal Health, New York State College of Veterinary Medicine, Cornell University, Ithaca, N.Y., and have in part been referenced previously (14, 40, 51). Figure 1B depicts a schematic transcription map.

To discriminate between mRNA species, we constructed a minus-sense MEV RNA probe (MEV₂₂₆₄₋₁₉₁₁) spanning nt 1912 to 2264 (36) by subcloning the relevant DNA fragment of CPV into pGEM-3Z (Promega, Madison, Wis.). The single-stranded RNA probe complementary to mRNA was subsequently transcribed by using SP6 RNA polymerase. The full-length probe generated is 390 nt long, including plasmid sequences. MEV R1, encoding NS1, will protect a 353-nt fragment of this RNA probe, MEV R2, encoding NS2, will protect a 240-nt fragment, and R3, encoding the structural proteins, will protect a 240-nt fragment.

(ii) ADV probes. Probes for detection of ADV transcription were designed based on the published transcription map of ADV (4). Figure 1A depicts a schematic transcription map.

To discriminate between mRNA species, we constructed a minus-sense ADV probe (ADV_{2321-1701-B}) spanning ADV nt 1701 to 1960 and nt 2043 to 2321 (Fig. 1A). Since this probe does not contain the B intron (nt 1961 to 2042), unspliced pre-mRNA from the ADV P3 promoter (pre-P3) protects a 540-nt fragment from RNase digestion after hybridization (Fig. 1A). If splice C is made in pre-P3, the precursor pre-P3C is generated. Pre-P3C and R1 protect the same 432-nt fragment of ADV_{2321-1701-B}. Pre-P3C can be spliced to either R1 or R2, depending on whether the B intron or the A intron is removed (Fig. 1A). R2 mRNA protects a 170-nt fragment. As such four RNA species initiate from the ADV P3 promoter. R3 mRNA, initiated from the ADV P36 promoter, protects a 389-nt fragment (Fig. 1A) (4). The probe was constructed by subcloning the relevant ADV template in pZERO-1 (Invitrogen, San Diego, Calif.) and transcribing the single-stranded RNA probe using SP6 RNA polymerase. This generated a 555-nt full-length probe including plasmid sequences.

Since the ADV_{2321-1701-B} probe cannot discriminate between R1 and pre-P3C, we made another probe, ADV₂₀₆₃₋₁₇₀₁ (Fig. 1A) by subcloning the relevant fragment of ADV into pZERO-1 and transcribing the single-stranded RNA probe using SP6 RNA polymerase. This generated a 364-nt full-length probe including plasmid sequences. Pre-mRNA initiated from ADV P3 (pre-P3 and pre-P3C) will protect a 362-nt long fragment from this probe, R1 will protect a 260-nt fragment, and ADV R3 will protect a 217-nt fragment. Although the ADV₂₀₆₃₋₁₇₀₂ probe by itself will not detect R2 (Fig. 1A), by combining the results from both ADV probes, quantification of all the ADV mRNAs becomes possible.

Probe and RNA size marker labeling. All RNA probes were transcribed with biotin-14-CTP using the BrightStar BIOTINscript kit essentially as described by the manufacturer (Ambion). We routinely obtained enough probe for 1,000 RPAs from a single transcription reaction. Probes were stable at -20°C when resuspended in DEPC-treated water with 500 ng of sheared yeast RNA (Ambion) per µl.

Biotinylated RNA markers were made with the Century Marker Template (Ambion) according to the manufacturer's instructions and diluted 1:500 in

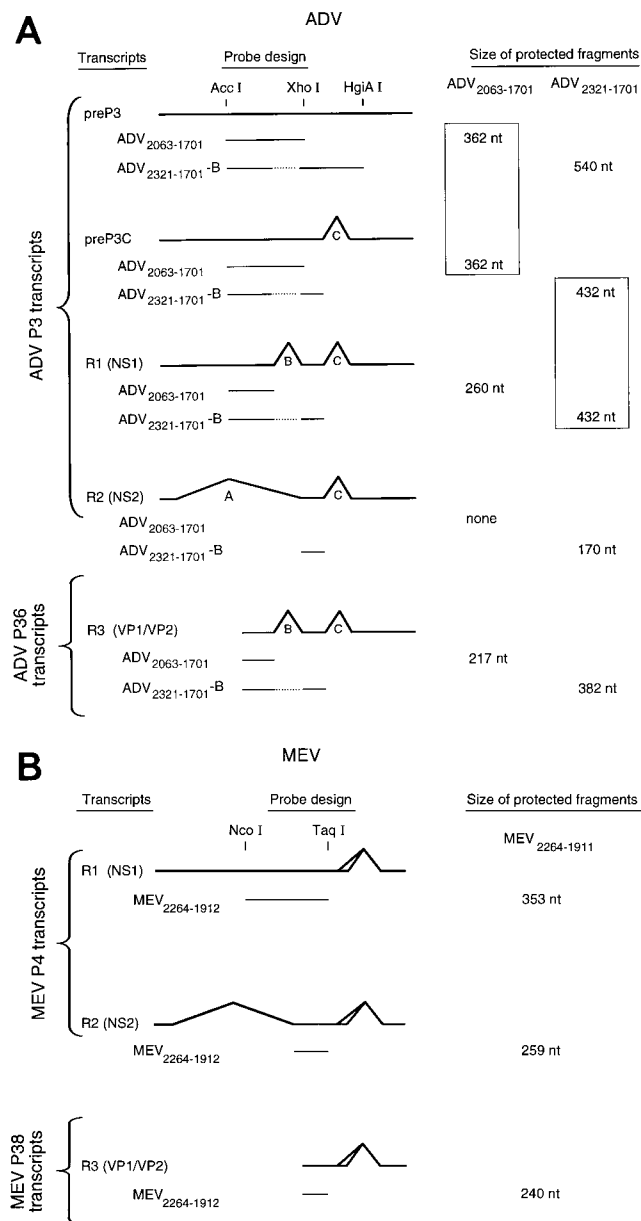


FIG. 1. Schematic transcription maps of ADV and MEV, showing segments of each probe protected by the different transcripts. (A) Schematic of ADV transcripts with splices A, B, and C indicated. In parentheses are shown the proteins encoded by the different transcripts. The probe segments that each transcript is protecting are indicated by thin lines. The ADV₂₃₂₁₋₁₇₀₁-B probe lacks the B intron (stippled) and does not discriminate whether the B intron is present or not. Therefore, both pre-P3C and R1 will protect the same 432-nt fragment of ADV₂₃₂₁₋₁₇₀₁-B. The other probe, ADV₂₀₆₃₋₁₇₀₁, does not detect the presence of the C intron. Therefore, both pre-P3 and pre-P3C will protect the same 362-nt fragment. The drawing is not to scale. (B) Schematic of MEV transcripts with the large and small introns indicated. In parentheses are shown the proteins encoded by the different transcripts. The probe segment that each transcript is protecting from the MEV₂₂₆₄₋₁₉₁₁ probe is indicated by thin lines. The drawing is based on data kindly provided by C. Parrish and is not to scale.

sequencing gel loading buffer (47). The biotinylated RNA markers were stored -20°C .

Special consideration. Under the conditions used for cell lysis and hybridization, encapsidated single-stranded genomic DNA may be released. Because the bulk of the single-stranded ADV genome is of minus sense (11), it could potentially hybridize to the viral mRNA, preventing hybridization of viral mRNA to the minus-sense probe, thus leading to an artificially low level of detection of

mRNA. To evaluate this possibility, we denatured the reactions at 65°C for 5 min before the hybridization. The amount of detected mRNA did not increase, showing that single-stranded DNA was not interfering with the assay.

RESULTS

Kinetic analysis of MEV transcription in synchronized CRFK cells. MEV is considered a more typical parvovirus than ADV. Consequently, we will describe the MEV results first. To study the kinetics of transcription, MEV-infected CRFK cells were lysed in GuSCN at different times after HU release and hybridized with the MEV probe. In the following, all time points refer to hours after release from HU block. Low levels of both R3 encoding the structural proteins and R1 encoding NS1 could be detected before release, reflecting the low number of cells evading the HU block (35). Initial levels of R1 and R3 remained low until 2.5 h after release, when they increased sharply, reaching a maximum at 11 h, corresponding to the end of the S phase (Fig. 2). R2 encoding NS2 was not detected until 5.5 h after release, and its level gradually increased over the next 20 h, at which time it was twofold higher than R1 (Fig. 2). MEV R3 encoding the structural proteins was the most abundant mRNA at all times, comprising approximately 80% of the total viral mRNA.

Part of what is detected as R1 in the total cell lysate might be unspliced P4 transcript and therefore restricted to the nucleus (Fig. 1B). In addition, R1 in the nucleus might be further spliced to R2 and as such might represent pre-R2. In order to determine whether there would be relatively less R1 in the cytoplasm we studied cytoplasmic RNA at 24 h. Although the ratio of P4 versus P38 transcript was unaltered, the R2/R1 ratio increased twofold, indicating that part of the R1 detected in the total cell lysate gets retained in the nucleus for further splicing to R2 or is in fact unspliced pre-mRNA from the P4 promoter.

We wanted to know if apparent mRNA ratios were affected by different stabilities. Therefore, the amount of viral transcripts was measured at different times after inhibition of de novo transcription by actinomycin B. This had no significant effect on the ratio of the different MEV transcripts, showing that the dominance of R3 was not a function of differential mRNA stability.

These results suggested that MEV transcription in cell culture was similar to that of parvovirus MVM in that the mRNA encoding the structural proteins was predominant. However, we could not identify a temporal shift from early transcription encoding predominantly nonstructural proteins to late transcription encoding predominantly structural proteins as has been reported for MVM (15, 48).

Kinetic analysis of ADV transcription in synchronized CRFK cells. The kinetics of ADV transcription were also examined in the same synchronized cell line. Using the ADV₂₃₂₁₋₁₇₀₁-B probe, ADV P3 transcripts encoding the nonstructural proteins were detected at 30 min and remained at a low level until 2.5 h. Then, they accumulated steadily until 11 h, corresponding to the end of the S phase (Fig. 3). ADV P36 transcript (R3), encoding the structural proteins, showed a different pattern of transcription. It was first detected at 2.5 h and remained at low levels until 5.5 h, after which time it increased over the remaining period studied (Fig. 3). However, R3 levels never became higher than 16% of the total viral mRNA (Fig. 3). Thus, ADV was very different from MEV in that transcripts encoding the nonstructural proteins dominated at all times in ADV-infected CRFK cells. As such there was no shift to dominance of transcripts encoding structural proteins.

The first ADV P3 transcript, detected at low levels at 30 min,

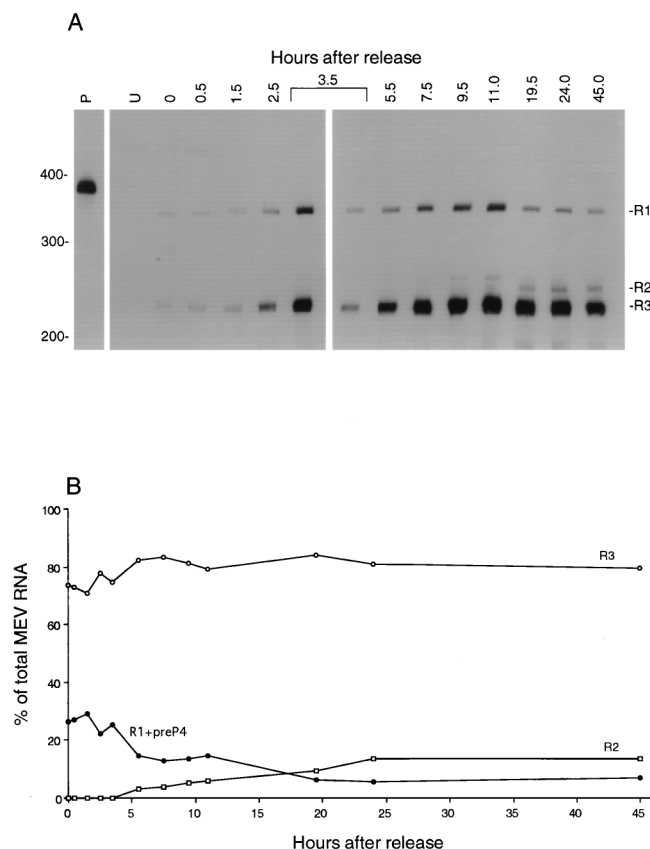


FIG. 2. Quantitative time course of MEV transcription. (A) Synchronized CRFK cells infected with MEV were lysed at the indicated times after release from HU. Ten microliters of cell lysate was hybridized to an excess of MEV₂₂₆₄₋₁₉₁₁ probe as described in Materials and Methods. Lane P contains undigested probe, lane U contains uninfected CRFK cells. To visualize early transcription levels, lanes U to 3.5 h represent a long exposure. Lanes 3.5 to 45 h represent a short exposure. For comparison the 3.5-h lane is shown in both exposures. The identity of each protected fragment is indicated on the right, using the nomenclature introduced in the legend to Fig. 1. The molecular mass in nucleotides is indicated at the left. (B) The protected bands from panel A were quantitated by densitometry, and the intensity was normalized to the number of cytosine molecules in the different protected fragments, plotted as a percentage of total MEV transcripts seen as a function of time after HU release. The MEV₂₂₆₄₋₁₉₁₁ probe does not discriminate between unspliced mRNA from the MEV P4 promoter (pre-P4) and R1, encoding MEV NS1. Furthermore, R1 in the nucleus may be further spliced to R2 encoding NS2. R3 encodes the structural proteins VP1 and VP2.

was unspliced mRNA (pre-P3). At 90 min, R2, encoding NS2, was detected. It comprised approximately 70% of the viral mRNA, and its relative quantity remained almost constant (Fig. 3B). The absolute amount of R2 increased more than 50-fold from 2.5 to 11 h and then remained almost constant (Fig. 3A and C). The same pattern, although at a lower level, was seen for the 432-nt protected fragment representing both R1 encoding NS1 and pre-mRNA with splice C (pre-P3C) (Fig. 1 and 3).

By using the other probe, ADV₂₀₆₃₋₁₇₀₁, on the same sample set, these data were confirmed; ADV P3 transcripts encoding the nonstructural proteins appeared first and reached a plateau at 11 h, while the P36 transcript (R3) encoding the structural proteins was evident later but continued to increase. Use of the ADV₂₀₆₃₋₁₇₀₁-B probe gave a much higher signal for P3 pre-mRNA than was found with the ADV₂₃₂₁₋₁₇₀₁-B probe (data not shown). This resulted because pre-P3C colocalized with R1

when using probe ADV₂₃₂₁₋₁₇₀₁-B whereas it colocalized with pre-P3 when using probe ADV₂₀₆₃₋₁₇₀₁ (Fig. 1A). Apparently, most of the P3 pre-mRNA is found as pre-P3C and not as pre-P3, suggesting that splice C is made immediately after transcription, while splice B or A is made later. No unspliced P36 transcripts were detected at any time point, showing that transcript initiated from the ADV P36 is spliced to the final R3 immediately after transcription.

Since the B and C introns are present on both R1 and R3, these findings suggested that additional features of ADV P3 transcripts delayed final splicing. One possibility would be that the presence of the splice A acceptor on the ADV P3 transcripts interfered with the splicing, since splices A and B (Fig. 1A) utilize the same splice donor (4).

We also examined mRNA stabilities in ADV-infected CRFK cells. The amount of viral transcripts was measured at different times after inhibition of de novo transcription using actinomycin B (data not shown). There was no significant effect on the ratio of the different ADV transcripts, showing that the dominance of ADV R2, encoding NS2, was not the result of differential mRNA stability. In addition, the stabilities of the ADV transcripts were not significantly different from those of the MEV transcripts.

These results for ADV showed major differences from those for MEV. ADV P3 transcripts encoding the nonstructural proteins dominated at all times, and this result appeared to be a function of transcription rate and not of mRNA stability. As for MEV, we did not detect any temporal shift in ADV transcription.

Comparison of overall transcription levels between MEV and ADV. When comparing the overall level of accumulated MEV P4 and ADV P3 transcripts, the former accumulated to a fivefold higher level than ADV P3 transcripts (Fig. 4). The difference was particularly pronounced when comparing the levels of R1, in that a higher proportion of MEV P4 transcript is processed to R1 than is seen for ADV P3 transcript (Fig. 2 and 3).

Even more conspicuous was the difference observed between MEV and ADV R3 initiated from MEV P38 and ADV P36, respectively. MEV R3 reached a level almost 300 times higher than the ADV R3 transcript at 11 h (Fig. 4) and, although the level of ADV R3 slowly increased, it was always at least 100 times lower than the peak for MEV R3 (Fig. 4). So not only were the MEV mRNAs encoding the viral structural proteins produced much faster, but they also attained much higher levels than the corresponding ADV mRNA.

DISCUSSION

We have compared the transcription of two mink parvoviruses, MEV and ADV, in the same cell line. The overall level of viral transcription for MEV was 20-fold higher than for ADV. Moreover, in MEV-infected CRFK cells, approximately 80% of the viral transcripts derived from the P38 promoter (Fig. 2), while the corresponding value for ADV P36 was less than 16% (Fig. 3). This difference resulted in 100- to 300-fold higher amounts of mRNAs encoding structural proteins in MEV- than in ADV-infected cells (Fig. 4). This ratio is remarkably similar to the 100-fold higher activity of the MEV P38 promoter than of the ADV P36 promoter found using a reporter assay after transient transfection of CRFK cells (14).

The observed dominance of ADV P3 transcripts, encoding nonstructural proteins, is also supported by our previous promoter studies in which we found that the constitutive activity of the ADV P3 promoter was 12 times higher than the activity of the ADV P36 promoter (14). In the presence of ADV NS1, the

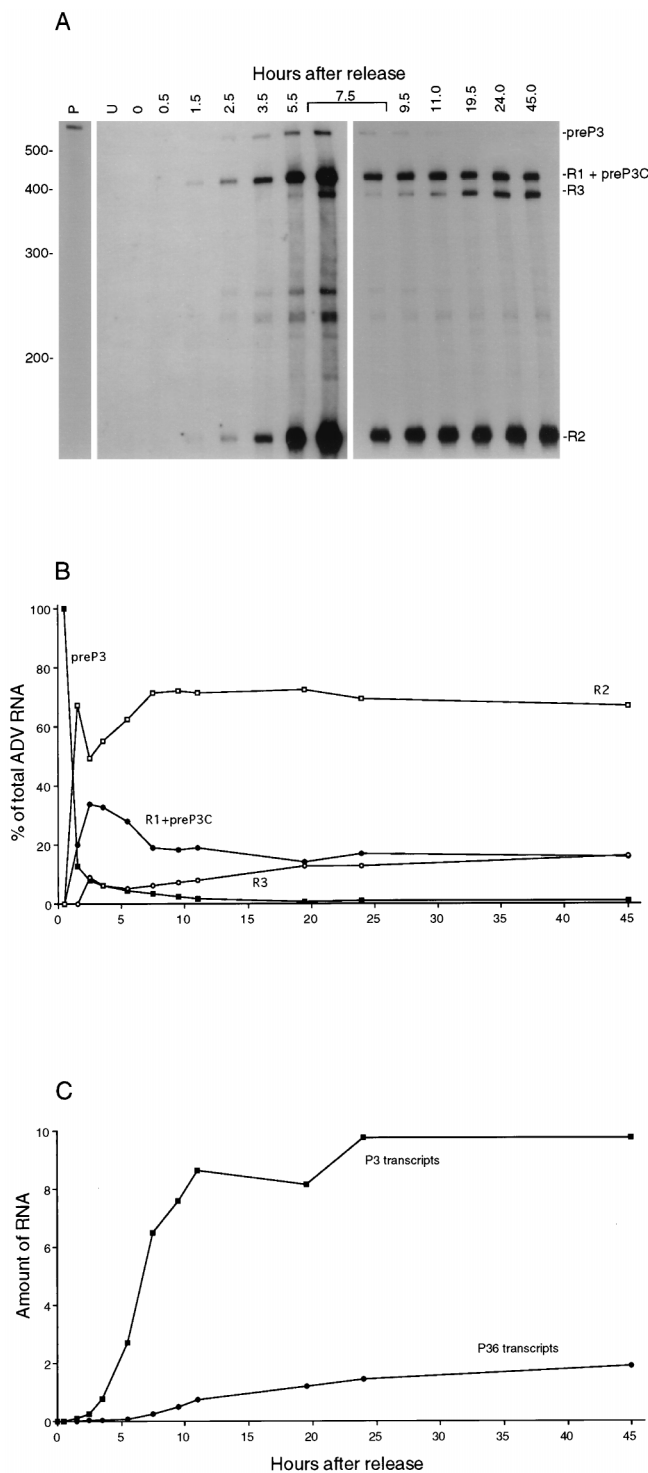


FIG. 3. Quantitative time course of ADV transcription. (A) Synchronized CRFK cells infected with ADV were lysed at the indicated times after release from HU. Ten microliters of cell lysate from each time point was hybridized to an excess of the $ADV_{2321-1701}$ -B probe as described in Materials and Methods. Lane P contains undigested $ADV_{2321-1701}$ -B probe, and lane U contains uninfected CRFK cells. To visualize early transcription levels, lanes U to 7.5 h represent a long exposure, while lanes 7.5 to 45 h represent a short exposure. For comparison the 7.5-h lane is shown in both exposures. The identity of the different protected fragments is indicated on the right, using the nomenclature introduced in the legend to Fig. 1. The molecular mass in nucleotides is indicated on the left. (B) The protected bands from panel A were quantitated by densitometry, and the intensity was normalized to the number of cytosine molecules in the different protected fragments, plotted as percentage of total ADV

ADV P3 promoter was still threefold stronger than the ADV P36 promoter (14). This was in contrast to the MVM P38 promoter which was transactivated 100-fold by NS1 to a much higher level than the MVM P4 promoter (14). As such, the efficiency of NS1-mediated transactivation may explain the shift from early to late transcription in MVM infection (15, 48) and the lack of such a shift in ADV infection. However, we were surprised not to find any shift in MEV-infected cells from the early dominance of transcripts encoding nonstructural proteins to the later dominance of transcripts encoding structural proteins. In addition to MVM, such a shift has been shown for the distantly related B19 parvovirus, regulated at the level of splicing (49), and for the porcine parvovirus (PPV) at the protein level (32). This conservation of early and late transcription patterns among distantly related parvoviruses may point to an important biological function. One such function might be to delay capsid formation until sufficient amounts of viral DNA have accumulated. This would reduce the amount of empty capsids and by that an unnecessary energy load on the infected cell. At the molecular level the observed dominance of MEV transcripts encoding structural proteins at all times might be a function of the high constitutive activity of the P38 promoter in CRFK cells (14). It would be of interest to study the MEV transcription in other permissive cell lines to evaluate whether the dominance of transcripts encoding structural proteins even early in infection is a unique function of the CRFK cells or a special property of MEV.

R2 was the predominant mRNA at all times after ADV infection of CRFK cells, comprising approximately 70% of the total viral transcripts (Fig. 3). R2 encodes ADV NS2. For parvoviruses MVM and H1, NS2 increases the level of viral proteins posttranscriptionally, an effect most pronounced for the structural proteins (29, 30, 33). ADV NS2 might have similar functions so that the low level of R3 could be partly compensated for by efficient translation mediated by NS2. Alternatively, NS2 predominance can be seen as a selection to minimize acute cell damage because splicing of the P3 transcript to predominantly R2 will reduce the amount of cytotoxic NS1.

For both ADV and MEV the accumulation of viral transcripts was restricted to the period from 2.5 to 11 h after release, which corresponds to the migration of the CRFK cells through the S phase of the cell cycle (35). ADV infection arrests the cells in the late S or G₂ phase of the cell cycle so that viral DNA replication can continue for the full time period studied (35). Therefore, the stall in transcription at 11 h cannot be interpreted as a coupling of viral transcription to viral DNA replication in the S phase. Similarly, the stall of transcription does not seem to be an effect of saturation of the transcription machinery or viral cytotoxic effect, since it happened for both MEV and ADV despite dramatic differences in the amount of viral transcripts. Instead, it might indicate that host factors present in the S phase of the cell cycle facilitate viral transcription.

No direct evidence is available linking the expression level of the viral structural proteins to the level of viral replication. Nevertheless, such a relationship could directly explain the lower viral load observed in ADV versus MEV infection. As

transcripts seen as a function of time after HU release. The $ADV_{2321-1701}$ -B probe does not discriminate between R1 encoding NS1 and pre-P3C. The latter can get spliced to R2 encoding NS2 or to R1. R3 encodes the structural proteins VP1 and VP2. (C) Transcripts initiated from the ADV P3 and P36 promoters were quantitated by totaling their respective mRNAs, and the results were plotted on an arbitrary scale seen as a function of time after HU release.

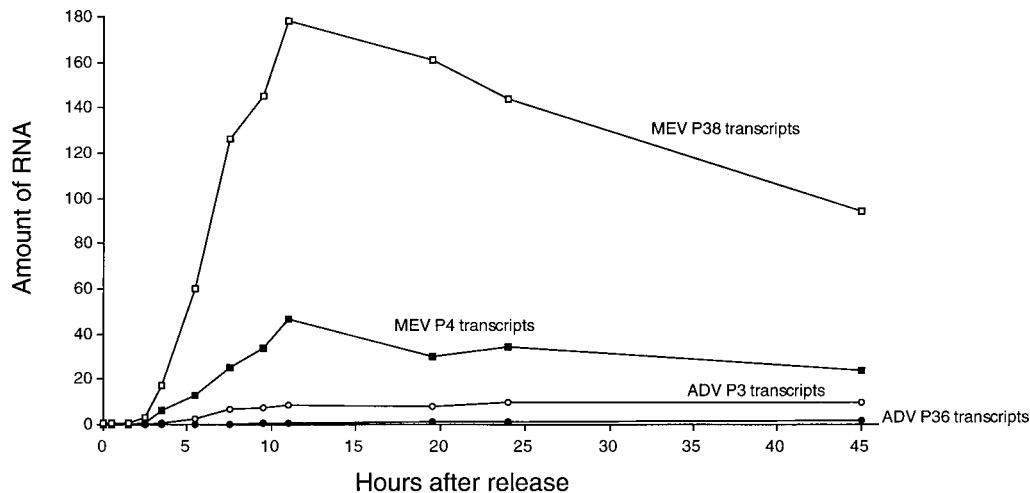


FIG. 4. Quantitative comparison of MEV and ADV transcription. Synchronized CRFK cells infected with either ADV or MEV were lysed at the indicated times after release from HU. The total amount of viral transcripts initiated from the different viral promoters was quantified by RPA and plotted on an arbitrary scale as a function of time after HU release. Transcripts initiated from the MEV P4 and ADV P3 promoters encode the nonstructural proteins, while transcripts initiated from the MEV P38 and ADV P36 promoters encode the structural proteins.

such, low-level viral transcription might be seen as another adaptation of ADV to minimize acute cell damage. We have previously noted how limited mutations in the ADV P36 promoter region seem to have adapted ADV to low-level expression of capsid protein (51).

Apparently the two different mink parvoviruses have evolved completely different strategies of expression. In this respect it may be worth noting that both MEV and CPV are host variants of feline panleukopenia virus (FPV) (50), which adapted to their hosts relatively recently in evolutionary terms (37, 53). This group of viruses is shed at very high levels during the acute phase of the disease (12, 55), which may have played a significant role in the spread of infection. When FPV adapted to canines, it spread worldwide within a short time span, indicating rapid and very efficient spread (38). Similarly, different isolates of FPV, CPV, or MEV have very high homology levels, indicating efficient spread (53). In contrast, different isolates of ADV have significantly lower degrees of homology but, nevertheless, seem to be stable isolates (10, 20, 34). This suggests that ADV spread may be more localized and less efficient. Perhaps the solitary lifestyle of mink limits the efficiency of virus spread and has selected for a virus that does not cause acute cell damage and short-term, high levels of virus excretion but rather a persistent infection in which the animal can survive and continue breeding. As such the virus is very efficient in that 55% of wild mink have been reported to be infected with ADV (26).

In summary, we have compared the viral infection of ADV and MEV in the same synchronized cell line and found major differences both in the level and the pattern of viral transcription. ADV had a very low level of transcription, and mRNA encoding the nonstructural proteins dominated, even late in infection. In contrast, MEV had a high level of transcription and mRNA encoding the structural proteins dominated even early in infection.

Experiments are in progress to determine whether the high sensitivity of the RPA may allow us to examine the *in vivo* transcription pattern of ADV not only in permissive infections of newborn mink kits but also in the restricted infection of adult mink. This would make it possible to relate the *in vitro* findings of this report to the *in vivo* infection of mink.

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REFERENCES

1. Aasted, B., S. Alexandersen, and M. Hansen. 1988. Treatment of neonatally Aleutian disease virus (ADV) infected mink kits with gammaglobulin containing antibodies to ADV reduces the death rate of mink kits. *Acta Vet. Scand.* **29**:323-330.
2. Alexandersen, S. 1986. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. *Vet. Pathol.* **23**:579-588.
3. Alexandersen, S. 1990. Studies on the pathogenesis of disease caused by the Aleutian mink disease parvovirus. *Acta Pathol. Microbiol. Immunol. Scand. Sect. A* **98**(Suppl. 14):1-32.
4. Alexandersen, S., M. E. Bloom, and S. Perryman. 1988. Detailed transcription map of Aleutian mink disease parvovirus. *J. Virol.* **62**:3684-3694.
5. Alexandersen, S., M. E. Bloom, and J. Wolfenbarger. 1988. Evidence of restricted viral replication in adult mink infected with Aleutian disease of mink parvovirus. *J. Virol.* **62**:1495-1507.
6. Alexandersen, S., M. E. Bloom, J. Wolfenbarger, and R. E. Race. 1987. *In situ* molecular hybridization for detection of Aleutian mink disease parvovirus DNA by using strand-specific probes: identification of target cells for viral replication in cell cultures and in mink kits with virus-induced interstitial pneumonia. *J. Virol.* **61**:2407-2419.
7. Alexandersen, S., S. Larsen, A. Cohn, Å. Utenthal, R. E. Race, B. Aasted, M. Hansen, and M. E. Bloom. 1989. Passive transfer of antiviral antibodies restricts replication of Aleutian mink disease parvovirus *in vivo*. *J. Virol.* **63**:9-17.
8. Alexandersen, S., T. Storgaard, N. Kamstrup, B. Aasted, and D. D. Porter. 1994. Pathogenesis of Aleutian mink disease parvovirus infection: effects of suppression of antibody response on viral mRNA levels and on development of acute disease. *J. Virol.* **68**:738-749.
9. Beckers, T., P. Schmidt, and P. Hilgard. 1994. Highly sensitive Northern hybridization of rare mRNA using a positively charged nylon membrane. *BioTechniques* **16**:1074-1078.
10. Bloom, M. E., S. Alexandersen, S. Perryman, D. Lechner, and J. B. Wolfenbarger. 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a non-pathogenic and a pathogenic strain of ADV. *J. Virol.* **62**:2903-2915.
11. Bloom, M. E., R. E. Race, and J. B. Wolfenbarger. 1987. Analysis of Aleutian disease of mink parvovirus infection using strand specific hybridization probes. *Intervirology* **27**:102-111.
12. Carmichael, L. E., J. C. Joubert, and R. V. H. Pollock. 1980. Hemaggluti-

- nation by canine parvovirus: serologic studies and diagnostic applications. *Am. J. Vet. Res.* **41**:784-791.
13. Christensen, J., S. Alexandersen, B. Bloch, B. Aasted, and Å. Uttenthal. 1994. Production of mink enteritis parvovirus empty capsids by expression in a baculovirus vector system: a recombinant vaccine for mink enteritis parvovirus in mink. *J. Gen. Virol.* **75**:149-155.
 14. Christensen, J., T. Storgaard, B. Viuff, B. Aasted, and S. Alexandersen. 1993. Comparison of promoter activity in Aleutian mink disease parvovirus, minute virus of mice, and canine parvovirus: possible role of weak promoters in the pathogenesis of Aleutian mink disease parvovirus infection. *J. Virol.* **67**:1877-1886.
 15. Clemens, K. E., and D. J. Pintel. 1988. The two transcription units of the autonomous parvovirus minute virus of mice are transcribed in a temporal order. *J. Virol.* **62**:1448-1451.
 16. Cotmore, S. F., and P. Tattersall. 1987. The autonomously replicating parvoviruses. *Adv. Virus Res.* **33**:91-174.
 17. Crandell, R. A., C. G. Fabricant, and W. A. Nelson-Rees. 1973. Development, characterization, and viral susceptibility of a feline (*Felis catus*) renal cell line (CRFK). *In Vitro (Rockville)* **9**:176-185.
 18. Eklund, C. M., W. J. Hadlow, R. C. Kennedy, C. C. Boyle, and T. A. Jackson. 1968. Aleutian disease of mink: properties of the etiologic agent and the host responses. *J. Infect. Dis.* **118**:510-516.
 19. Firestein, G. S., S. M. Gardner, and W. D. Roeder. 1987. Quantitative molecular hybridization with unfractionated, solubilized cells using RNA probes and polyacrylamide gel electrophoresis. *Anal. Biochem.* **167**:381-386.
 20. Gottschalk, E., S. Alexandersen, T. Storgaard, M. E. Bloom, and B. Aasted. 1994. Sequence comparison of the non-structural genes of four different types of Aleutian mink disease parvovirus indicates an unusual degree of variability. *Arch. Virol.* **138**:213-231.
 21. Gough, N. M. 1983. Core and E antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than genome length viral messenger RNAs. *J. Mol. Biol.* **165**:683-699.
 22. Haines, D. S., and D. H. Gillespie. 1992. RNA abundance measured by a lysate RNase protection assay. *BioTechniques* **12**:736-741.
 23. Helmboldt, C. F., and E. L. Jungherr. 1958. The pathology of Aleutian disease in mink. *Am. J. Vet. Res.* **19**:212-222.
 24. Henson, J. B., J. R. Gorham, G. A. Padgett, and W. C. Davis. 1969. Pathogenesis of the glomerular lesions in Aleutian disease of mink. *Arch. Pathol. Lab. Med.* **87**:21-28.
 25. Henson, J. B., R. W. Leader, and J. R. Gorham. 1961. Hypergammaglobulinemia in mink. *Proc. Soc. Exp. Biol. Med.* **107**:919-920.
 26. Ingram, D. G., and H. J. Cho. 1974. Aleutian disease in mink: virology, immunology and pathogenesis. *J. Rheumatol.* **1**:74-92.
 27. Kariatsumari, T., M. Horiuchi, E. Hama, K. Yaguchi, N. Ishiguro, H. Goto, and M. Shinagawa. 1991. Construction and nucleotide sequence analysis of an infectious DNA clone of the autonomous parvovirus, mink enteritis virus. *J. Gen. Virol.* **72**:867-875.
 28. Larsen, S., S. Alexandersen, E. Lund, P. Have, and M. Hansen. 1984. Acute interstitial pneumonitis caused by Aleutian disease virus in mink kits. *Acta Path. Microbiol. Immunol. Scand. Sect. A* **92**:391-393.
 29. Li, X., and S. L. Rhode III. 1991. Nonstructural protein NS2 of parvovirus H-1 is required for efficient viral protein synthesis and virus production in rat cells *in vivo* and *in vitro*. *Virology* **184**:117-130.
 30. Li, X., and S. L. Rhode III. 1993. The parvovirus H-1 NS2 protein affects viral gene expression through sequences in the 3' untranslated region. *Virology* **194**:10-19.
 31. Martyn, J. C., B. E. Davidson, and M. J. Studdert. 1990. Nucleotide sequence of feline panleukopenia virus: comparison with canine parvovirus identifies host-specific differences. *J. Gen. Virol.* **71**:2747-2753.
 32. 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.
 33. Naeger, L. K., N. Salome, and D. J. Pintel. 1993. NS2 is required for efficient translation of viral mRNA in minute virus of mice-infected murine cells. *J. Virol.* **67**:1034-1043.
 34. Oie, K. L., G. Durrant, J. B. Wolfenbarger, D. Martin, F. Costello, S. Perryman, D. Hogan, W. J. Hadlow, and M. E. Bloom. 1996. The relationship between capsid protein (VP2) sequence and pathogenicity of Aleutian mink disease parvovirus (ADV): a possible role for raccoons in the transmission of ADV infections. *J. Virol.* **70**:852-861.
 35. Oleksiewicz, M. B., and S. Alexandersen. 1997. S-phase dependent cell cycle disturbances caused by Aleutian mink disease parvovirus. *J. Virol.* **71**:1386-1396.
 36. Parrish, C. R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. *Virology* **183**:195-205.
 37. Parrish, C. R. 1994. The emergence and evolution of canine parvovirus—an example of recent host range mutation. *Semin. Virol.* **5**:121-132.
 38. Parrish, C. R., P. Have, W. J. Foreyt, J. F. Everman, M. Senda, and L. E. Carmichael. 1988. The global spread and replacement of canine parvovirus strains. *J. Gen. Virol.* **69**:1111-1116.
 39. Parrish, C. R., C. W. Leathers, R. Pearson, and J. R. Gorham. 1987. Comparisons of feline panleukopenia virus, canine parvovirus, raccoon parvovirus, and mink enteritis virus and their pathogenicity for mink and ferrets. *Am. J. Vet. Res.* **48**:1429-1435.
 40. Pintel, D. J., A. Gersappe, A. Haut, and J. Pearson. 1995. Determinants that govern alternative splicing of parvovirus pre-mRNAs. *Semin. Virol.* **6**:283-290.
 41. Porter, D. D., A. E. Larsen, N. A. Cox, H. G. Porter, and S. L. Suffin. 1977. Isolation of Aleutian disease virus of mink in cell culture. *Intervirology* **8**:129-144.
 42. Porter, D. D., A. E. Larsen, and H. G. Porter. 1969. The pathogenesis of Aleutian disease of mink. I. *In vivo* viral replication and the host antibody response to viral antigen. *J. Exp. Med.* **130**:575-589.
 43. Porter, D. D., A. E. Larsen, and H. G. Porter. 1973. The pathogenesis of Aleutian disease of mink. III. Immune complex arteritis. *Am. J. Pathol.* **71**:331-344.
 44. Reed, A. P., E. V. Jones, and T. J. Miller. 1988. Nucleotide sequence and genome organization of canine parvovirus. *J. Virol.* **62**:266-276.
 45. Reynolds, H. A. 1969. Some clinical and hematological features of virus enteritis of mink. *Can. J. Comp. Med.* **33**:155-159.
 46. Reynolds, H. A. 1970. Pathological changes in virus enteritis of mink. *Can. J. Comp. Med.* **34**:155-163.
 47. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 48. Schoborg, R. V., and D. J. Pintel. 1991. Accumulation of MVM gene products is differentially regulated by transcription initiation, RNA processing and protein stability. *Virology* **181**:22-34.
 49. Shimomura, S., S. Wong, K. E. Brown, N. Komatsu, S. Kajigaya, and N. S. Young. 1993. Early and late gene expression in UT-7 cells infected with B19 parvovirus. *Virology* **194**:149-156.
 50. Siegl, G., R. C. Bates, K. I. Berns, B. C. Carter, D. C. Kelly, E. Kurstak, and P. Tattersall. 1985. Characteristics and taxonomy of Parvoviridae. *Intervirology* **23**:61-73.
 51. Storgaard, T., J. Christensen, B. Aasted, and S. Alexandersen. 1993. *cis*-acting sequences in the Aleutian disease mink parvovirus late promoter important for transcription: comparison to the canine parvovirus and minute virus of mice. *J. Virol.* **67**:1887-1895.
 52. Thompson, J., and D. Gillespie. 1987. Molecular hybridization with RNA probes in concentrated solutions of guanidine thiocyanate. *Anal. Biochem.* **163**:281-291.
 53. Truyen, U., A. Gruenberg, S.-F. Chang, B. Obermaier, P. Veijalainen, and C. R. Parrish. 1995. Evolution of the feline-subgroup parvoviruses and the control of canine host range *in vivo*. *J. Virol.* **69**:4702-4710.
 54. Turnbow, M. A., and C. W. Garner. 1993. Ribonuclease protection assay: use of biotinylated probes for the detection of two messenger RNAs. *BioTechniques* **15**:267-270.
 55. Uttenthal, Å., S. Larsen, E. Lund, M. E. Bloom, T. Storgård, and S. Alexandersen. 1990. Analysis of experimental mink enteritis virus infection in mink: *in situ* hybridization, serology, and histopathology. *J. Virol.* **64**:2768-2779.
 56. Viuff, B., B. Aasted, and S. Alexandersen. 1994. Role of alveolar type II cells and of surfactant-associated protein C mRNA levels in the pathogenesis of respiratory distress in mink kits infected with Aleutian mink disease parvovirus. *J. Virol.* **68**:2720-2725.