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Detection of Pseudorabies Virus Transcripts in Trigeminal Ganglia of Latently Infected Swine

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Two pseudorabies virus (PRV) poly(A)$^+$ RNAs, 2.0 and 0.95 kilobases, were detected in the trigeminal ganglia of latently infected pigs. These RNAs were partially colinear at the 3′ end. They were located in the *Bam*HI J fragment (0.706 to 0.737 map units), 3′ of the PRV immediate-early gene. A potential polyadenylation signal was also identified in *Bam*HI-J. The direction of transcription of the latency-associated RNAs was antiparallel (i.e., opposite in polarity) to that of the PRV immediate-early mRNA.

Herpesviruses can establish a latent infection and persist in their host indefinitely after a primary or secondary infection. The mechanism of establishment and maintenance of latency is not known at this time; however, it is clear that viral as well as host cell factors are involved. Recent molecular studies on two alphaherpesviruses, herpes simplex virus type 1 (HSV-1) and bovine herpesvirus 1 (BHV-1), demonstrated that only one region of the viral genome is transcribed during latency (7, 8, 14, 19, 21, 23, 27, 28, 30). For HSV-1 the latency-associated transcripts (LATs) are located at the 3′ end of an immediate-early (IE) gene (ICP0) and are transcribed in the opposite orientation with respect to the ICP0 mRNA.

Pseudorabies virus (PRV) can establish latency in swine (3, 26). Analysis of the tissues of latently infected swine showed that the primary site of PRV latency is the trigeminal ganglia (10, 11, 25). Viral genomes are present in the neurons and are transcriptionally active (22). The latent genomes can exist as linear, circular, or concatemeric molecules (24). PRV is an alphaherpesvirus. Its structure, genome organization, and many biological functions are similar to those of other members of the same family. Therefore, it is logical to hypothesize that transcription of the PRV genome during latency may resemble that of the HSV-1 or BHV-1 genome.

PRV has only one IE gene (IE180), and this gene shares extensive homologous sequences with the HSV-1 ICP4 IE gene. The exact location of the PRV IE gene was mapped (4, 5), and the entire DNA nucleotide sequence has been determined (A. K. Cheung, Nucleic Acids Res., in press). The present work is targeted at the 3′ end of the IE180 gene to search for latency-associated RNAs. In this paper, the detection of two PRV-specific transcripts present in the trigeminal ganglia of latently infected swine is reported.

MATERIALS AND METHODS

Virus and cell culture. The Indiana-Funkhauser (In-Fh) strain of PRV was grown on Madin-Darby bovine kidney (MDBK) cells cultivated in Eagle minimum essential medium supplemented with 10% fetal bovine serum (18).

Trigeminal ganglion tissue of swine. Pigs were exposed to PRV (Becker strain) intranasally. Six weeks postinfection, when virus could not be recovered from the infected animals, the trigeminal ganglia were removed for analysis. These tissues were gifts from K. Platt (Iowa State University). The negative control (normal) pig was obtained from a certified PRV-negative herd.

Preparation of RNAs. PRV-infected and uninfected tissue culture cells or trigeminal ganglion tissues of swine were homogenized with a polytron (Brinkmann Instruments, Inc.) in the presence of guanidinium thiocyanate (6). Total RNAs were isolated from the lysate by centrifugation through a CsCl cushion (9). Poly(A)$^+$ RNAs were selected by oligo(dT)-cellulose chromatography (1). For enrichment of PRV IE mRNA, MDBK cells were treated with 100 μg of cycloheximide per ml before and during PRV infection (5, 13, 20).

Radioactive probes. Single-stranded runoff RNA probes of known specificity were generated from linearized plasmids containing PRV DNA in the presence of [α-^{32}P]UTP (New England Nuclear Corp.) by using T3 or T7 polymerase in accordance with supplier protocol (Stratagene). Probes Ia and Ib were derived from a plasmid containing the rightmost 220 nucleotides (nt) of *Bam*HI-J inserted between the *Eco*RI and *Bam*HI sites of the Bluescript SK$^+$ plasmid. Probes IIa and IIb were generated from a plasmid containing the leftmost 202 nt of *Bam*HI-I inserted between the *Bam*HI and *Xho*I sites of the Bluescript SK$^+$ plasmid. The entire *Bam*HI-J (approximately 4.8 kilobases [kb]) and *Bam*HI-I (4,855 nt) genomic fragments were individually cloned into the *Bam*HI site of the Bluescript SK$^+$ plasmid for the generation of probes IIIa and IIIb and probes IVa and IVb, respectively. The single-stranded probes generated were slightly longer than the PRV DNA insert because the transcripts contained some plasmid sequences at the 5′ and 3′ ends. With respect to the PRV IE180 mRNA, all "a" probes were of the same polarity, while all "b" probes were of the opposite polarity.

S1 nuclease analysis. Hybridization of [^{32}P]UTP-labeled single-stranded RNA probes (10^5 cpm) was carried out with various RNA samples (5 to 10 μg) (15, 33). The hybridization buffer contained 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% formamide. The samples were heated at 85°C for 10 min and cooled to 50°C overnight. The annealed samples were digested with 500 U of S1 nuclease (Pharmacia, Inc.) per ml at 37°C for 30 min (15). The treated samples were analyzed with either a 6% polyacrylamide sequencing gel (16) or a 1% agarose gel after chemical and heat denaturation (5, 17). The polyacrylamide sequencing gels were

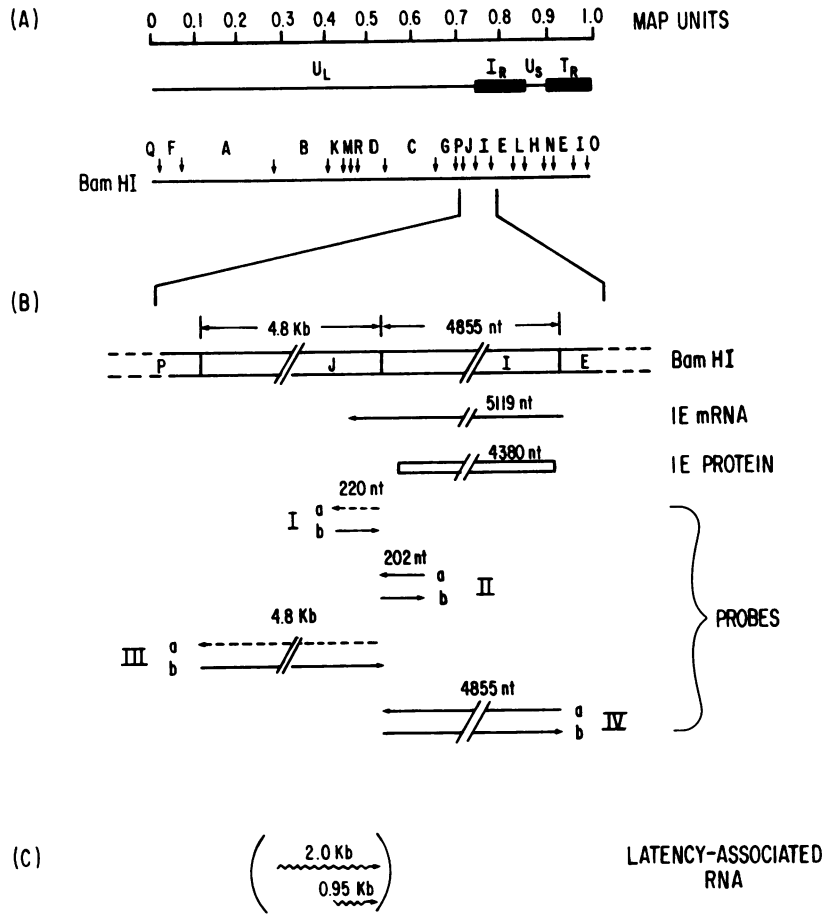

FIG. 1. (A) Organization of the PRV genome and *Bam*HI restriction enzyme map. (B) Locations of transcription and translation of the PRV IE gene; in addition, the size and polarity (arrows) of each probe are also shown. Probes Ia and IIIa (broken arrows) were used to detect LATs. (C) Latency-associated RNAs (not precisely mapped yet).

wet while the agarose gels were dry when exposed to X-ray film.

Northern (RNA) blot analysis. RNA samples (15 μg) were denatured with glyoxal and dimethyl sulfoxide and electrophoresed in a 1% agarose gel (17). The RNAs were transferred to and immobilized on Gene-screen membranes (Du Pont Co.) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization was carried out at 65°C in the presence of 50% formamide–5× SSC–5× Denhardt solution (1× Denhardt solution is 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone). Blots were washed at 65°C with SSC solutions (2× SSC, 1× SSC, and 0.1× SSC) for 1 h each. Filters were dried and exposed to X-ray film.

Plaque assay. Viruses were titrated on a porcine kidney cell culture (MVPK) (29) with a 0.5% agarose overlay. The cells were fixed in 100% methanol and stained with 0.1% crystal violet.

RESULTS

PRV-specific RNAs in the trigeminal ganglia of swine. Total RNAs from cycloheximide-treated, PRV-infected MDBK cells and from the trigeminal ganglia of PRV-infected swine were examined by the S1 nuclease technique. This technique is specific for identifying nucleotide sequences homologous to the nucleic acid probes used. Single-stranded, PRV-specific probes (Ia and Ib) generated in vitro from linearized plasmids (Fig. 1) were hybridized with various RNA samples, treated with S1 nuclease, and analyzed on a 6% polyacrylamide sequencing gel (Fig. 2). A portion of probe Ib was protected from digestion in the presence of RNA samples from PRV-infected but not mock-infected, cycloheximide-treated MDBK cells (Fig. 2, lanes 3 and 2, respectively). The size of the protected fragment corresponded to the expected value of 215 nt. When probe Ia was used, common bands were observed with both the mock (lane 5)- and PRV (lane 6)-infected MDBK RNAs; however, no additional bands could be attributed to the presence of PRV-specific nucleic acids. These background bands were possibly the results of homologous cellular sequences. In any event, these data clearly demonstrated the polarity of the probes and their ability to distinguish PRV-specific sequences from background sequences.

In the same experiment, total RNAs from the trigeminal ganglia of an unexposed pig and four PRV-exposed pigs were also examined with probe Ia (Fig. 2). The size of the protected fragment was expected to be 220 nt. Although similar background bands were observed in all five samples, additional PRV-specific sequences (217 and 222 nt) were detected in PRV-exposed pig 2 (lane 9) and, upon longer film exposure, pig 3 (lane 10). No PRV-specific sequences were

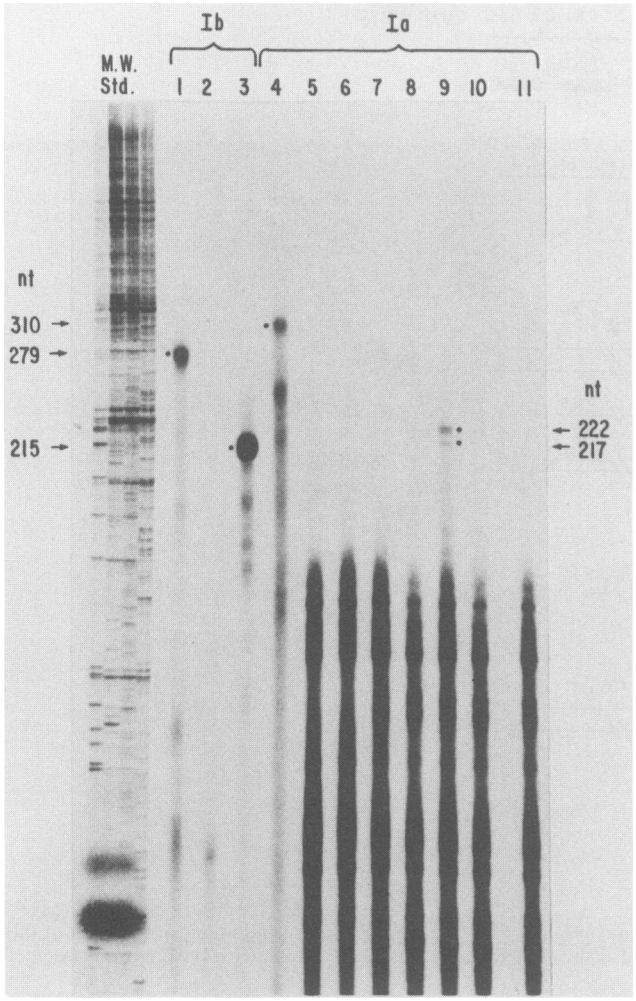

FIG. 2. Detection of PRV-specific sequences in latently infected swine by S1 nuclease analysis on a 6% sequencing gel. A known sequencing ladder (M.W. Std.) was electrophoresed to provide size standards (sizes on the left in nucleotides [nt]). Probe Ib (lane 1) was used to analyze RNAs from mock-infected (lane 2) and PRV-infected (lane 3) MDBK cells. Probe Ia (lane 4) was used to analyze RNAs from mock-infected MDBK cells (lane 5), PRV-infected MDBK cells (lane 6), trigeminal ganglia of a normal pig (lane 7), and trigeminal ganglia of four PRV-exposed pigs (pigs 1 to 4, lanes 8 to 11, respectively). The sizes of the pertinent bands are indicated on both sides in nucleotides (nt).

detected in the RNA samples from the other two PRV-exposed pigs (pigs 1 and 4, lanes 8 and 11, respectively) or in the RNA sample from the unexposed control animal (lane 7). These results demonstrated that PRV-specific RNAs were present in at least two of the latently infected pigs. Furthermore, these RNAs were transcribed in the opposite polarity (rightward) to that of the IE180 mRNA (leftward). Since the RNA sample from pig 2 (lane 9) gave the strongest signal, all further experiments were performed with RNAs isolated from the trigeminal ganglia of pig 2 (sample 2).

Analysis of the RNAs from latently infected pig 2. Detailed analysis of the total RNAs from the trigeminal ganglia of the above-described pig 2 (sample 2) as well as an unexposed control pig was carried out (Fig. 3). Four sets of single-stranded probes were used (Fig. 1), each set consisting of two probes of complementary polarity. Experiments with probe Ia confirmed the earlier observation that two protected sequences of 217 and 222 nt were obtained with sample 2 (lane E) and not with the normal pig sample (lane N). Probe Ib did not detect any PRV-specific sequences, although background bands were seen with both samples. Probes IIa and IIb contain 202 bases of PRV-specific sequences and are derived from the leftmost *Xho*I subfragment of *Bam*HI-I. Only background bands were observed with these two probes.

Much larger probes (IIIa, IIIb, IVa, and IVb), approximately 4.8 kb, were used to analyze the same unexposed pig RNA sample as well as the pig 2 RNA sample. In the same type of S1 nuclease experiment, virus-specific sequences were detected only in sample 2 with probe IIIa (lane E), which has the same polarity as probe Ia and the IE180 gene. In other words, the PRV RNA(s) present in the pig 2 sample is of opposite polarity (transcribed rightward) to the IE180 gene (transcribed leftward). Although the sizes of the protected bands could not be determined precisely from this sequencing gel, they were estimated to be greater than 350 nt. No PRV-specific RNAs were detected in the normal pig sample or the pig 2 sample when probes IIIb, IVa, and IVb were used, although various amounts of background bands were observed.

Since the above-described sequencing gel was not adequate for resolving high-molecular-weight nucleic acids, the same samples (analyzed with probes IIIa, IVb, IVa, and IVb) were treated with glyoxal, boiled for 5 min, and electrophoresed on an agarose gel to detect larger protected fragments. Probes IIIb, IVa, and IVb did not detect any PRV-specific RNAs in any of the samples. With probe IIIa, a cluster of protected bands ranging from 350 to 550 nt was obtained with the pig 2 RNA sample (data not shown).

Determination of the sizes of latent PRV transcripts. Northern blots were prepared from total RNAs of the trigeminal ganglia of pig 2 and a control animal. Hybridization was carried out on duplicate blots with single-stranded probes Ia, Ib, IIIa, and IIIb. Probes Ib and IIIb did not hybridize to any RNA species (data not shown). Probes Ia and IIIa hybridized to background RNAs in both samples; however, two additional bands of 2.0 and 0.95 kb were detected in the pig 2 sample but not in the unexposed pig sample (Fig. 4).

Polyadenylated latent PRV transcripts. For determination of whether the latency-associated RNAs were polyadenylated, sample 2 RNAs were fractionated into poly(A)$^-$ and poly(A)$^+$ portions by oligo(dT)-cellulose chromatography. S1 nuclease experiments were performed on both RNA fractions with probe Ia. PRV-specific sequences were present only in the poly(A)$^+$ RNA fraction (Fig. 5).

Are the PRV transcripts in pig 2 associated with latency or reactivation? The above data clearly demonstrated that at least two PRV-specific RNAs were present in the trigeminal ganglia of pig 2; however, it was not clear whether those transcripts were present during latency or were the result of reactivation, with the virus being in the process of a productive infection. The productive infection cycle of PRV (In-Fh) was therefore examined.

Cultured MDBK cells were infected with PRV (In-Fh), and at various times postinfection growth medium was collected and total RNAs were isolated from the infected cells. The one-step growth curve for PRV (In-Fh) on MDBK cells (about 12 h) (Fig. 6A) was established by determining the number of PFU in each sample of growth medium collected. Total RNAs that covered the entire replicative

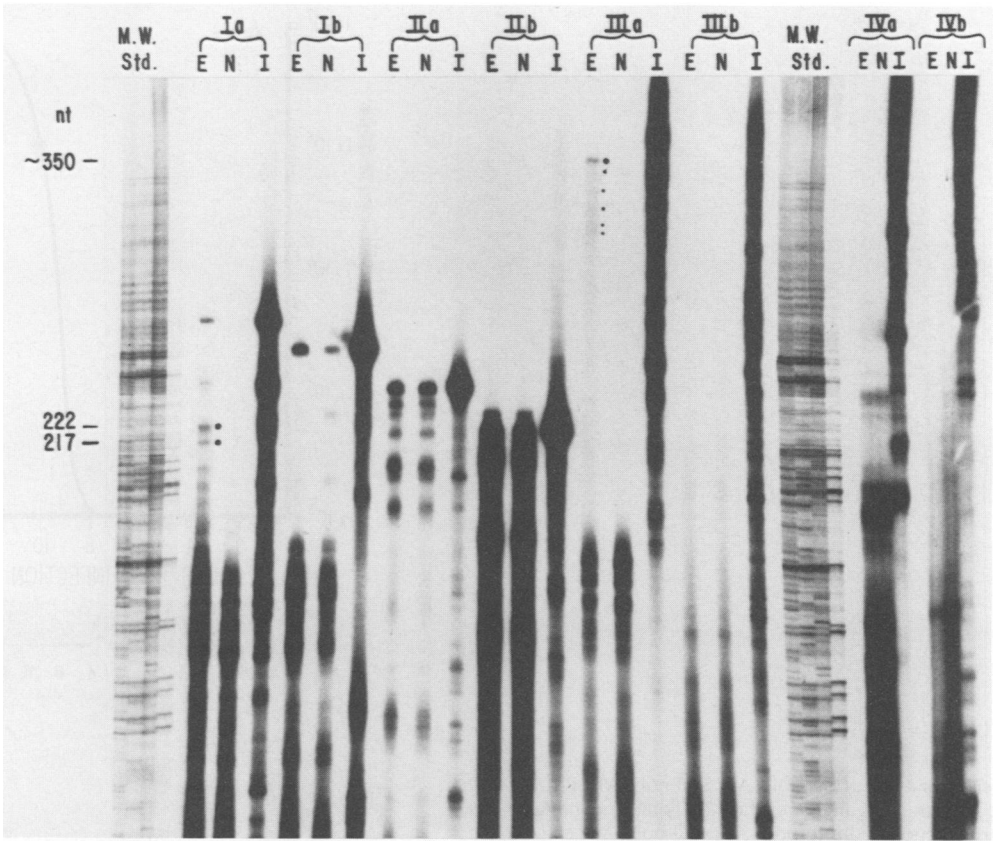

FIG. 3. Detailed analysis on a 6% sequencing gel of the PRV-specific RNAs in the trigeminal ganglia of pig 2. S1 nuclease experiments were carried out with four sets of probes (see Materials and Methods and Fig. 1), and known sequencing ladders (M.W. Std.) were also run. All lanes E contained pig 2 RNA samples, all lanes N contained normal pig RNA samples, and all lanes I contained the input probes for each set of experiments. The size of probes IIIa, IIIb, IVa, and IVb is approximately 4.8 kb; however, non-full-length runoff RNAs were made (as shown). Routinely, greater than 80% of the probes were full-length transcripts.

cycle were examined by the S1 nuclease technique with probe Ib. Probe Ib was chosen because it is specific for the IE180 mRNA (Fig. 2, lane 3). The IE180 gene is the first PRV gene expressed (5, 13, 19) and is absolutely required for a productive infection. During the replicative cycle of PRV on tissue culture cells (probably the same process after reactivation), the IE180 mRNA was present at all times (Fig. 6B). The fact that the pig 2 RNA sample did not contain the PRV IE180 mRNA (Fig. 3, probe Ib) suggested that viral reactivation probably had not occurred.

DISCUSSION

Although latency-associated RNAs encoded in *Bam*HI-J (the DNA fragment located 3′ of the PRV IE gene) were detected, the presence of additional LATs encoded by other regions of the genome cannot be excluded. If RNA transcription of PRV during latency is similar to that of HSV-1 or BHV-1, *Bam*HI-J may be the only region expressed.

The data obtained demonstrated that two poly(A)$^+$ RNAs (2.0 and 0.95 kb) were present in the trigeminal ganglia of pig 2 during latency. These two transcripts were partially colinear and were of opposite polarity to the IE gene (Fig. 1C). The 3′ ends of the transcripts were very close to the *Bam*HI-J–*Bam*HI-I junction, since the entire Ia probe was protected, while no protected bands were detected with

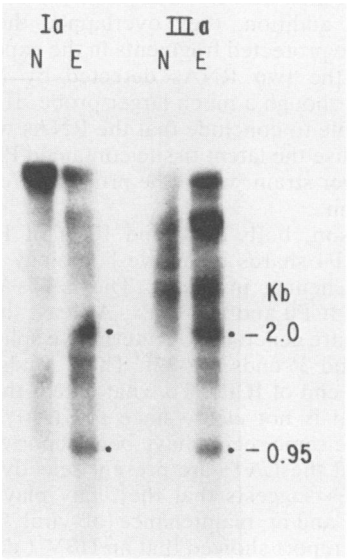

FIG. 4. Northern blot analysis. Total trigeminal ganglion RNAs from a normal pig (lanes N) and pig 2 (lanes E) were electrophoresed and blotted on nylon membranes. Hybridization was carried out with probes Ia and IIIa.

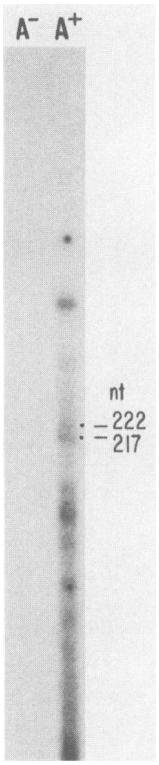

FIG. 5. S1 nuclease analysis of the poly(A)$^+$ and poly(A)$^-$ RNA fractions from the trigeminal ganglia of pig 2. Total trigeminal ganglion RNAs were fractionated into poly(A)$^+$ and poly(A)$^-$ samples by oligo(dT)-cellulose chromatography. The fractionated samples were analyzed with probe Ia on a 6% sequencing gel.

probe IIa. In fact, a consensus polyadenylation signal (AATAAA) is located in *Bam*HI-J, 177 nt from the junction (5). Thus, these RNAs were located in an area (0.706 to 0.737 map units) in which no PRV transcripts have been reported before (2); in addition, they overlapped the PRV IE180 mRNA. All the protected fragments in the experiments were smaller than the two RNAs detected by Northern blot analysis (even though a much larger probe, IIIa, was used). It is not possible to conclude that the RNAs were spliced at this time because the latent tissue contained PRV sequences from the Becker strain, while the probes were derived from the In-Fh strain.

In comparison, both ICP0 and ICP4 of HSV-1 are IE genes, and ICP4 shares extensive homology with the PRV IE180 gene (Cheung, in press). The LATs of HSV-1 are antiparallel to ICP0 and not ICP4. At least three LATs are present. They are generated by alternative splicing and share the same 5′ and 3′ ends (30–32). The 3′ ends of the LATs overlap the 3′ end of ICP0. To what extent these RNAs are polyadenylated is not clear, since poly(A)$^-$ and poly(A)$^+$ LATs from the same region have been reported (19, 27, 30).

The fact that the LATs are present selectively in latently infected tissues suggests that they may play a role in the establishment and/or maintenance of viral latency. However, a recent report showed that an HSV-1 deletion mutant lacking the ability to express LATs was capable of establishing latency in the trigeminal ganglia of mice (12). Thus, the functions of the LATs and their involvement in herpesvirus latency are not clear and require further investigation.

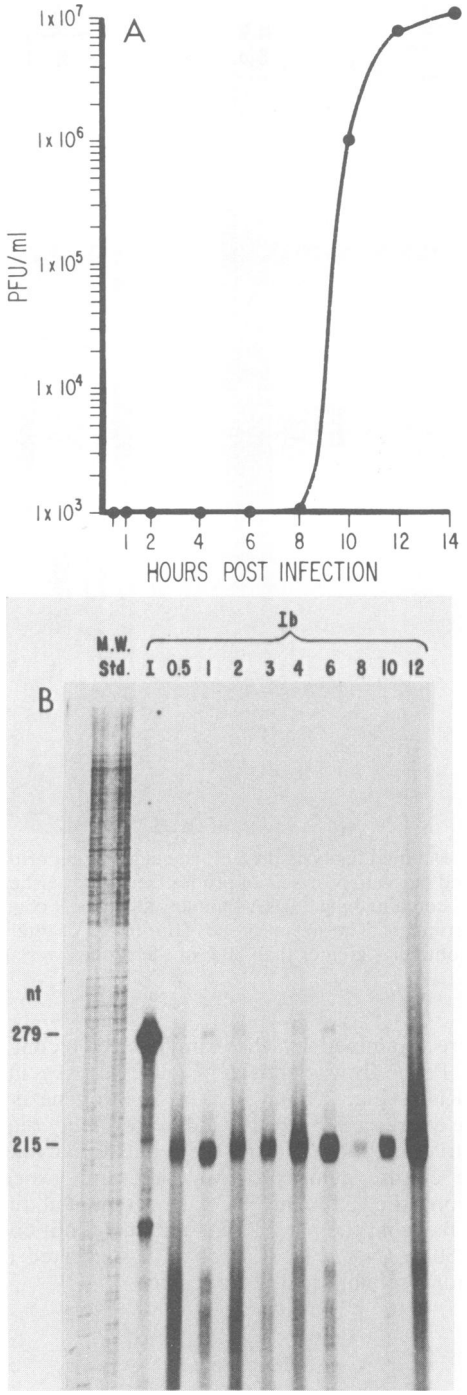

FIG. 6. Presence of the PRV IE180 mRNA throughout the reproductive cycle. (A) A one-step growth curve for strain In-Fh on MDBK cells was determined by a plaque assay. (B) Probe Ib (lane I) was used to detect the IE180 mRNA present at different times postinfection (0.5 to 12 h). The results were analyzed on a 6% sequencing gel. M.W. Std., Size standards.

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