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Analysis of Bovine Herpesvirus 1 Transcripts during a Primary Infection of Trigeminal Ganglia of Cattle

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During an infection of nonneuronal cells, bovine herpesvirus 1 (BHV-1) gene expression proceeds in a well-defined cascade. Products of immediate-early (IE) genes are expressed first, and they activate expression of early (E) and late (L) genes. Although the same cascade is assumed to occur during an infection of neurons in trigeminal ganglia (TG) of cattle, no experimental data is available to support this hypothesis. Consequently, we analyzed BHV-1 gene expression in bovine TG at 1, 2, 4, 7, and 15 days postinfection (dpi). Infectious virus was detected in ocular swabs from 1 to 7 dpi but not 15 dpi. By reverse transcription (RT)-PCR, IE (bICP4), E (thymidine kinase, ribonucleotide reductase [RR]), L (glycoprotein C, and α *trans*-inducing factor), and dual-kinetic (bICP0 and bICP22) transcripts were analyzed. When cDNA synthesis was primed with random hexamers, IE and E transcripts were detected at the same time. However, full-length and poly(A)$^+$ (FL&P) RR or bICP22 RNAs were detected before FL&P IE RNAs. Furthermore, FL&P IE transcripts were not detected until viral DNA increased in TG. IE transcripts were detected before E or L RNAs when rabbit kidney cells were infected with a low multiplicity of infection and the same RT-PCR detection method was used. These studies suggested that expression of full-length and polyadenylated IE transcripts in trigeminal ganglia was not efficient compared to that of RR and bICP22 transcripts.

Bovine herpesvirus 1 (BHV-1) is an important pathogen of cattle that causes respiratory, genital, or occasionally neurological disease (55). BHV-1 is classified together with varicella-zoster virus (VZV) and pseudorabies virus in the varicellovirus group of the *Alphaherpesvirinae* subfamily, *Herpesviridae* family (41). However, BHV-1 has a number of biological properties which are closely related to herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (51). BHV-1 infects cattle through the respiratory or genital tract and replicates in the epithelia at the site of infection (reviewed in reference 55). The virus then invades the peripheral nervous system where it establishes a latent infection (1, 39). Although any sensory ganglia can harbor latent virus, trigeminal ganglia (TG) or lumbosacral ganglia are considered the most important sites of latency. As latent virus can reactivate and be transmitted to other susceptible animals, infection of the peripheral nervous system is essential for BHV-1 perpetuation. Understanding virus-host interactions during an infection in the peripheral nervous system will be important to develop strategies to prevent the spread of BHV-1. No previous study has examined the expression of specific BHV-1 genes in the peripheral nervous system of cattle.

In a similar fashion to that of other members of the *Alphaherpesvirinae* subfamily, BHV-1 genes are classified as immediate early (IE), early (E), or late (L) (53). During a productive infection of cultured cells, IE gene expression is transactivated by a virion protein (28, 29; reviewed in reference 32), does not require previous protein synthesis, and IE proteins regulate viral gene expression. E gene expression is activated by IE gene products but is independent of viral DNA replication. E proteins generally play a role in viral DNA replication and are nonstructural. Late genes are classified as γ_1 or γ_2. Low levels of γ_1 gene expression occur in the absence of viral IE or E gene expression, but maximal γ_1 gene expression requires viral DNA replication and at least one IE protein. γ_2 gene expression requires viral DNA replication. Proteins encoded by γ genes are generally structural components of the virion, some of which are necessary for viral entry into infected cells (51).

Although extensive viral gene expression occurs during a lytic infection, only one transcript, the latency-associated transcript or latency-related transcript, is detected in ganglionic neurons during latency (37–39). Viral gene expression may be restricted during latency because (i) neurons lack cellular factors necessary for expression of IE genes, (ii) viral transactivator proteins are absent in neurons, or (iii) neuron-specific factors repress IE promoter activity (8, 9, 21, 25–27, 37, 38, 52). Viral DNA replication may also be blocked in neurons, resulting in a nonproductive infection (22, 23). HSV-1 promoters can be active in the nervous system in the absence of other viral factors (16, 30), suggesting that viral gene expression in neurons is also repressed after initiating transcription. As with other alphaherpesviruses, the kinetics of viral gene expression during an acute BHV-1 infection of TG neurons has been assumed to be similar to the kinetics of gene expression during a lytic infection of nonneuronal cells (38).

To identify factor(s) that restrict viral gene expression and DNA replication in neurons, it is necessary to know the kinetics of viral gene expression in neurons. We have examined the expression of several BHV-1 genes during a primary infection of bovine TG, the natural host of BHV-1. In bovine TG, full-length and polyadenylated (FL&P) transcripts of RR or bICP22 genes were detected before FL&P transcripts of bICP4 or bICP0. Since FL&P transcripts of IE genes were detected only after the number of viral genomes in TG increased between days 2 and 4 postinfection, these studies are consistent with the hypothesis that establishment of latency occurs because IE gene expression is inefficient in TG neurons. These studies also suggest that polyadenylation of IE transcripts was not efficient in TG neurons.

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TABLE 1. Summary of primers and PCR conditions used for this study

Transcript	PCR conditions				Hybridization probe
	Primers[a]	[$MgCl_2$] (mM)	Gly[b] (%)	*Taq* (U)	
bICP0	+: TTC TCT GGG CTC GGG GCT GC −: AGA GGT CGA CAA ACA CCC GCG GT	1.0	10	0.5	CCG CAA GGG CGG CGC GCT AGC
bICP22	+: GCG CTG GTC CTC CGG CTC C −: CTC GCT GGC GGC GCT TGG	1.5	10	0.5	GGC TCG CCC TCC GGT CGC G
IETU1/E1	+: ACT GCA ACA CCT GCC GCT GCC −: TTC TGC TCC TTC GTC CCC GCT G	1.0	10	0.5	GCG GGG ACG AAG GAG CAG AAA AGC
bICP4	+: GCG CGT GGA GGT GCT CTC CTC −: CCC TCC CTC CCT TCC CGC G	1.0	−	1.0	GTC GGA CGA CGA CGA GGC CGG
RR[c]	+: GAC CGC CTG CTC GCT GCT ATC C −: GCC TGT GTA GTT GGT GCT GCG GC	1.5	−	0.5	TTT CCT TTG GCC CTG ATG ACT GCC GAG
TK	+: GCC GCC GTA CTG GAC ATG CG −: GCC GAG TCC CCG TAA GGC GAT	1.5	10	1.0	CGC GTC AGT TCG CGC TAG AGA TGG C
αTIF	+: CCC AAG CCC CGT TCG CAG C −: TGC CCG CCC GCC CTT AGA A	1.5	10	0.5	CGG CAA ACC CAT TCG GCG GCA
gC[d]	+: GAG CAA AGC CCC GCC GAA GGA −: TAC GAA CAG CAG CAC GGG CGG	1.5	−	0.5	GAA CCT GCC CAC GCG CTG AAA C
BGH[d]	+: TTT CGC CCT GCT CTG CCT GCC −: CCC TTC CTG CCT CCC CAC CCC	1.5	−	1.0	GAC ACC TTC AAA GAG TTT GTA AGC TCC CGA

[a] + and −, the sense of each primer. All primers are 5′ to 3′.
[b] Glycerol concentration in the reaction mix. −, no glycerol.
[c] Amplifies the large and small subunit of RR.
[d] Primers were from Galeota et al. (13). gC primers were used to detect gC transcript and for quantitative DNA PCR. BGH primers were used as the standard for quantitative DNA PCR.

MATERIALS AND METHODS

Virus and cells. Bovine turbinate (BT) and rabbit kidney (RK) cells were grown in Earle's modified Eagle's medium with 10% fetal bovine serum. The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Viral stocks were prepared by infecting BT cells at a low multiplicity of infection (MOI), typically less than 0.01 tissue culture infectious dose 50% ($TCID_{50}$) per cell, and subsequently titrated on BT cells (35).

Time-course infections. RK cells were split 1:5 in 100-mm-diameter dishes in modified Eagle's medium containing 5% fetal calf serum. The following day medium was removed, 1.5 ml of fresh medium containing the appropriate amount of BHV-1 was added, and adsorption was performed at 4°C. The dishes were rocked every 5 min, and after 30 min of adsorption the inoculum was removed and 12 ml of fresh medium was added to each dish.

RNA preparation. RNA extraction from tissue culture was performed essentially as described by Chomczynski and Sacchi (7). RNA concentrations were determined spectrophotometrically (260 nm), and RNA was reprecipitated with 3 volumes of ethanol. TG from cattle were minced into small pieces, and RNA was extracted as described previously (19). One phenol extraction was performed, and the interface was saved for DNA extraction (see below).

DNA extraction. The interface from the RNA extraction was precipitated with 2 volumes of ethanol at −20°C for at least 16 h. The precipitates were resuspended in TEN buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM EDTA) containing 0.5% of sodium dodecyl sulfate SDS and 0.3 mg of protease K per ml and digested overnight at 55°C. DNA was then extracted once with phenol (Tris-equilibrated, pH > 7)-chloroform-isoamyl alcohol (25:24:1), precipitated with 2 volumes of ethanol, and resuspended in Tris-EDTA to a final concentration of 1 μg/μl.

DNase I treatment and RT. Three micrograms of RNA was treated with 1 U of DNase I (RNase free; Gibco BRL, Gaithersburg, Md.) for 30 min at 20.5°C in the presence of 7 U of RNase inhibitor (RNAsin; Promega, Madison, Wis.). After DNase I treatment, samples were incubated at 65°C for 7.5 min in the presence of 2 mM EDTA to eliminate DNase I activity. One microgram of total RNA (DNase I treated) plus 1 μg of primer, oligo(dT), or random hexamers in 4 μl total volume were incubated at 65°C for 7.5 min and chilled on ice (denaturation). Sixteen microliters of ice-cold RT mix (20 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 0.5 mM each deoxynucleoside triphosphate dNTP, 10 U of RNAsin, and 100 U of RNase H-reverse transcriptase [Stratagene, La Jolla, Calif.]) was added. The reaction mixture was incubated for 10 min at 20.5°C and then for 90 min at 45°C. RT was terminated by incubation at 95°C for 5 min. As a control for DNA contamination in the RNA samples, 0.5 μg of RNA (DNase I treated) was mixed with ice-cold RT mix lacking reverse transcriptase in a final volume of 10 μl.

PCRs. An aliquot (1.5 μl) of the RT reaction mixtures was used for each PCR. PCRs were carried out in 50 μl of 1× commercial PCR buffer, 200 μM each dNTP, 1 μM of each primer, and *Taq* polymerase. Ten percent glycerol was added to the PCR buffer as indicated for each set of primers, and the $MgCl_2$ concentration was as indicated for each set of primers (Table 1). Amplification was carried out for 40 cycles (45 cycles for bovine ICP4 [bICP4]) by denaturing at 95°C for 1 min, annealing at 65°C for 1 min, and extending at 72°C for 2 min. Upon completion of the last cycle, the reaction mixtures were further incubated at 72°C for 7 min to ensure complete extension of the amplified products. All PCRs were "hot started." The use of RNase H-RT, high temperature for reverse transcription, 10% glycerol, and hot start in the PCRs allows amplification through RNA regions of complex secondary structure. Quantitative PCR was performed as previously described (13) except that amplifications were performed in 50.0 μl, of which 20.0 μl was analyzed by agarose gel electrophoresis. The radioactivity which hybridized to bovine growth hormone (BGH)-amplified products or the BHV-1-amplified products was measured with a PhosphorImager. PCR conditions and primers are described in Table 1.

Hybridizations. DNA was electrophoresed in agarose gels and then transferred to nylon membranes by capillary blotting (19, 42). Prehybridization was performed at 63°C for 45 min to 1 h in 1× SSPE (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA; pH 7), 0.2× BP (1× BP is 2% bovine serum albumin, 2% polyvinylpyrrolidone) plus 1% SDS. Hybridizations were performed for 2 to 3 h in 1× SSPE–0.2× BP–1% SDS at 62°C with at least 2×10^6 cpm of the respective probe per ml. After hybridization, membranes were washed 3 times for 5 min each at room temperature in 0.05× SSPE–0.05% SDS. If required, membranes were washed again in the same buffer at hybridization temperature.

Animal experiments. Twelve BHV-1-free crossbred calves (~220 kg) were randomly assigned to six different groups of two calves each. After sedation with xylazine (300 mg, intramuscularly), animals in five of the groups were infected with $10^{8.4}$ $TCID_{50}$ of BHV-1 per nostril and $10^{7.8}$ $TCID_{50}$ per eye, for a total of $10^{8.8}$ $TCID_{50}$ per animal. After euthanasia, the left TG of each calf was collected in a 10-ml plastic tube and immediately frozen in an ethanol-dry ice bath. Trigeminal ganglia were kept at −120°C until RNA was extracted. Eye swabs were obtained from each animal immediately after necropsy. Ocular swabs were frozen at −70°C. After thawing, media from swabs was centrifuged at 2,000 rpm for 10 min, and infectious virus in the supernatant was titrated by 50% endpoint titration in monolayers of BT cells (35).

RESULTS

Selection of genes and PCR primers. To study viral gene expression during an infection of TG, we selected PCR primers to amplify bICP4, IETU1/E1 (IE), RR, TK (E), gC, αTIF (L), bICP22, and bICP0 (dual-kinetics) transcripts. The four BHV-1 IE genes are grouped into two transcription units: IETU1 (bICP4, bICP0, and b*circ*) and IETU2 (bICP22) (12,

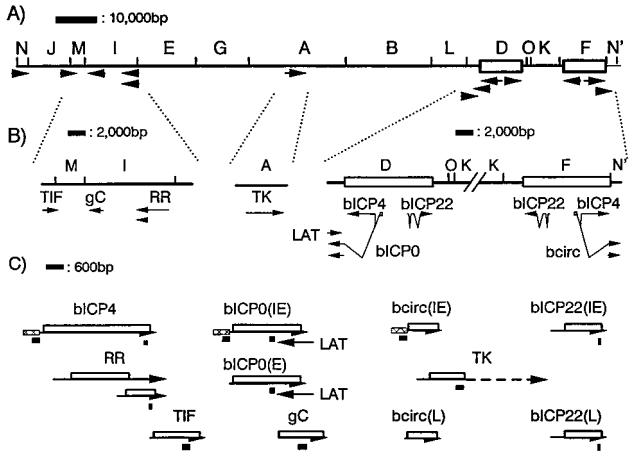

FIG. 1. Location of genes and PCR primers used for study of viral gene expression during reactivation. (A) Schematic representation (drawn to scale) of the BHV-1 genome. Rectangles indicate internal and terminal repeats flanking the unique short genomic region (US). Letters on top of the genome denote *Hin*dIII fragments. *Hin*dIII fragment N′ is found in the circularized form of the genome in infected cells. Arrows below the genome indicate locations and orientations of the transcripts from the different viral genes used in this study (approximately to scale). (B) Genomic location of the genes analyzed. Arrows below the genome denote the positions of the transcripts analyzed in this study (drawn to scale). Approximate location of the exons and introns of the respective RNAs is also given. The two diagonal lines in *Hin*dIII fragment K indicate that the scale is interrupted between the lines. Note that IETU1/E1 is common to bICP4 and bICP0 (in the internal repeat) or bICP4 and b*circ* (in the terminal repeat) and bICP22 and bICP4 are diploid genes. (C) Schematic representation of the transcripts analyzed in this study (drawn to scale). bICP0(IE) is the IE transcript from which bICP0 is translated. bICP0(E) is the early transcript from which bICP0 is translated and is colinear with the second exon of bICP0(IE). bICP22(IE) is the IE transcript from which bICP22 is translated. bICP22(L) is the late transcript from which bICP22 is translated, and it is identical to bICP22(IE). For clarity, most transcripts are presented in the same orientation. The exception is BHV-1 latency-associated transcript RNA, which is drawn in the opposite orientation to show its overlap with bICP0(IE) and bICP0(IE) transcripts. Arrows represent the transcripts. Open boxes on top of the transcripts represent ORFs. Dark black rectangles at the bottom of each transcript represent the regions of the respective RNAs amplified in the PCRs. (Top) IE transcripts. The cross-hatched rectangles at the left end of bICP4, bICP0(IE), bICP0(IE), and b*circ* indicate the untranslated exon that is shared by the three IE transcripts (IETU1/E1). Note that IE/1 primers amplify the transcripts of bICP4, bICP0, and b*circ*. bICP0 and bICP4 are also amplified by a second set of primers used in this study. (Center) E transcripts. The dashed line at the end of the TK transcript indicates the long 3′ end of this transcript (3). Note that RR primers amplify the transcripts from both RR subunits. (Bottom) L transcripts. Note that bICP22 (L) is identical to bICP22 (IE) and that no primers amplify b*circ* (L).

44, 45, 54) (Fig. 1). bICP4 is a major regulator of viral gene expression during a productive infection and is exclusively expressed as an IE transcript (45, 54). bICP22 and b*circ* are expressed under IE and L conditions, whereas bICP0 is expressed under IE and E conditions (12, 44, 45, 54). When transcribed at IE times, bICP4, bICP0, and b*circ* all share a common leader exon (IETU1/E1) (12, 44, 54). A set of primers were selected, IE/1, that amplify the 3′ end of IETU1/E1 (Fig. 1). Since IETU1/E1 is transcribed exclusively under IE conditions, IE/1 primers detect only IE transcripts but cannot distinguish between bICP0, bICP4, or b*circ* transcripts. Downstream from IETU1/E1, each of the three transcripts (bICP0, bICP4, and b*circ*) is spliced to a different second exon (12, 44, 54). Primers were selected that amplify the 3′ end of the bICP4 transcript or the 3′ end of bICP0 (Fig. 1). bICP0 primers detect IE and E transcripts but do not amplify BHV-1 LAT (19) (Fig. 1).

Both E genes analyzed, thymidine kinase (TK) and ribonucleotide reductase (RR), are important for synthesis of dNTPs. As neurons have low dNTP levels, TK and RR are important for replication of alphaherpesviruses in neurons (20, 34; reviewed in reference 40). Each of the subunits of BHV-1 RR (R1 and R2) is translated from a different transcript (49, 50), but the transcripts are 3′ coterminal. Primers were selected to amplify the 3′ end of both transcripts. To detect the TK transcript, primers were selected to amplify sequences immediately upstream of the TK stop codon (Fig. 1) (3, 31).

Both L genes studied (gC and α *trans*-inducing factor [αTIF]) encode major structural proteins and are located in the unique long region (UL) (6, 10, 28, 53). BHV-1 gC is expressed at low levels prior to DNA synthesis and thus is expressed as a γ_1 transcript during a productive infection (46). To detect gC transcripts, we used primers previously described (13) (Fig. 1). Although the transcript of BHV-1 αTIF has not been precisely mapped, genomic sequences flanking its open reading frame (ORF) contain a consensus TAATA box and polyadenylation signals (6). Wirth et al. (53) described a RNA expressed under γ_1 conditions which is consistent with the predicted αTIF transcript in size, genomic location, and kinetics of expression. Thus, we selected a set of primers that amplify sequences upstream of the stop codon of αTIF ORF (Fig. 1).

The sensitivity of all primers was assessed by using logarithmic dilutions of BHV-1 DNA. All of the primers amplified approximately 40 copies of BHV-1 DNA (Fig. 2A and B), indicating they have similar sensitivities.

PCR analysis of gene expression during an infection of cultured cells. Although all primers detected BHV-1 DNA with similar sensitivity, it was essential to determine whether the primers could detect viral RNA from the same number of infected cells and yield the expected kinetics. It is known that BHV-1 IE transcripts are expressed prior to E or L RNAs in productively infected cultured cells (53). Thus, experiments were performed to determine if RT-PCR could be used to examine the kinetics of viral gene expression. To address this issue, RK cells ($\sim 3 \times 10^6$) were infected with BHV-1 at different MOIs. RNA was extracted at different times postinfection, treated with DNase I, reverse transcribed, and PCR amplified with the respective sets of primers. When the RT reaction was primed with random primers [$N_{(6)}$], all primers except TK could detect viral RNA at an MOI of 0.002 $TCID_{50}$/cell ($10^{3.8}$ $TCID_{50}$/100 mM dish; Fig. 3 through 6). When performing the PCRs at the limit of sensitivity (0.002 $TCID_{50}$/cell), amplification occasionally did not occur, as expected. Assuming that each infectious particle infected a different cell and knowing that approximately 50 to 70 μg of RNA were obtained per infected dish, each microgram of RNA should represent approximately 100 infected cells. Three micrograms of RNA was used for the DNase I reaction, 30% of the DNase I reaction volume for RT and 10% of the RT reaction for the PCR. Therefore, it was estimated that all primers were capable of detecting viral RNAs from approximately 10 infected cells. When RT was primed with an oligo(dT) primer [$T_{(12-18)}$], the sensitivity of all primers decreased but IE gene transcripts were routinely detected before E or L RNAs (Fig. 3 to 6). bICP4 and bICP22 primers, but not RR, detected viral RNA at an MOI of 0.002 $TCID_{50}$/cell when cDNA synthesis was primed with oligo(dT). When reverse transcriptase was omitted from the RT reactions, no amplification was observed after the PCR (data not shown). At a higher MOI ($\sim$10 $TCID_{50}$/cell), all transcripts were detected as soon as 30 min after infection and for as long as 5 h postinfection (pi) (data not shown). In conclusion, these studies demonstrated that all primers could detect their respective cDNAs from an equivalent number of

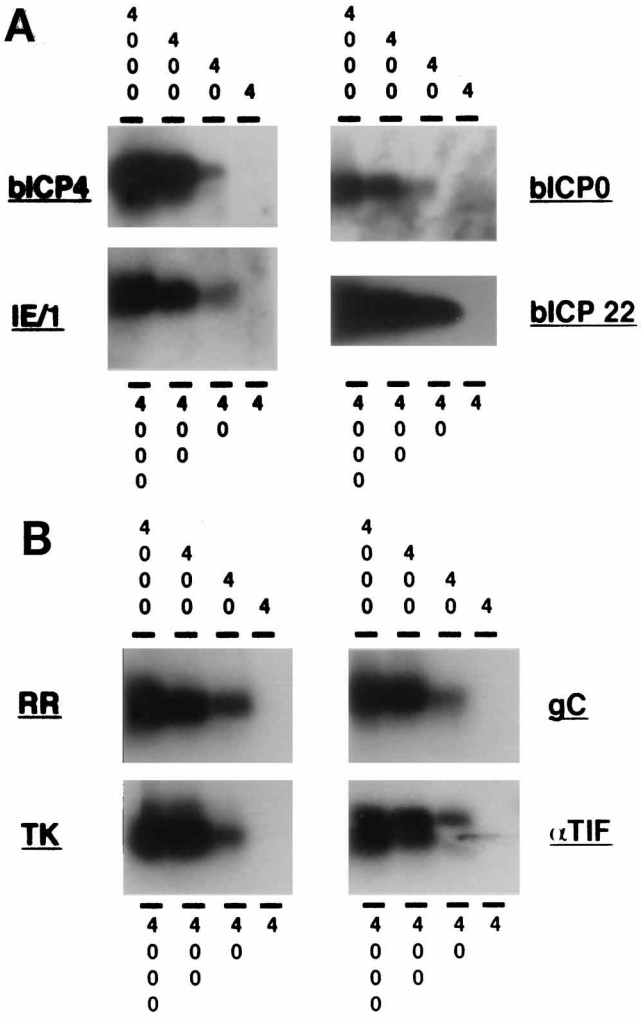

FIG. 2. Analysis of sensitivity of primers directed against BHV-1 genes. Logarithmic dilutions of purified BHV-1 DNA (13) were PCR amplified in independent experiments, using the primers described in Table 1. Amplified products were electrophoresed in agarose gels, blotted to a nylon membrane, and hybridized with the respective probes. For each set of primers, the last three positive dilutions and the first negative dilution of BHV-1 DNA are presented. Numbers on the top and bottom of each blot indicate the estimated number of viral genome copies used as a template for each reaction. (A) Amplification of bICP4 and IE/1 (IE genes) or bICP0 and bICP22 (dual-kinetic genes). (B) Amplification of RR and TK (E genes) or gC and αTIF (L genes).

infected cells and that RT-PCR can yield the expected cascade of viral gene expression in productively infected cells.

Experimental infection of calves. Ten calves were infected with BHV-1, and 9 were euthanized at different times postinfection. One infected calf died as a consequence of a secondary bacterial infection. Two uninfected calves were used as controls. Infectious virus was detected in swabs taken from the eyes of all infected calves from 1 to 7 days pi. All infected animals showed clinical symptoms, including dyspnea, keratitis, conjunctivitis, and apathy. The severity of symptoms peaked between 4 and 6 days pi. The animal necropsied on day 15 pi had completely recovered from clinical disease by the time of necropsy, and infectious virus was not detected in eye swabs. All calves were BHV-1 seronegative at the beginning of the experiment, and only the animal euthanized at day 15 pi seroconverted, confirming the calves were not previously exposed to BHV-1. In summary, the clinical data are consistent with those of previous studies which examined the experimental infection of calves with BHV-1 (11).

PCR analysis of gene expression during an in vivo acute infection of TG. At the time of necropsy, TG were collected and immediately frozen. RNA was then extracted from the TG, DNase I treated, reverse transcribed, and PCR amplified with the different primers. None of the primers amplified cDNAs from noninfected calves or from infected animals necropsied at 1 day pi (Fig. 7 to 10, lanes MI and 1, respectively). When expression of viral transcripts was analyzed in the $N_{(6)}$-primed cDNAs, most primers detected viral RNA from 2 to 7 days pi. Surprisingly, bICP4 was detected at 2 days pi in only 2 of 3 PCRs from one calf (no. 73), 1 of 3 PCRs from the other calf (no. 100), 3 of 4 PCRs at 4 days pi from one calf (no. 89), and 2 of 4 PCRs from the other calf (no. 90), suggesting the levels of bICP4 were low (Fig. 6). gC was detected at 2 and 4 days pi in 1 of 2 PCRs from one calf (no. 73 and 89, respectively; Fig. 10).

When cDNA synthesis was primed with $T_{(12–18)}$, RR and bICP22 were the only viral transcripts detected at 2 days pi (Fig. 8 and 9). RR and bICP22 were also consistently detected at 4 or 7 days pi but not at 15 days pi. Transcripts from bICP4 and IETU1/E1 (IE) were not detected until 4 days pi (Fig. 7). Furthermore, transcripts of bICP0 (IE and E), TK (E), αTIF (L), and gC (L) were not detected until 7 days pi (Fig. 8 to 10). Only IE/1 and bICP22 primers amplified $T_{(12–18)}$-primed cDNA from the calve euthanized at 15 days pi (Fig. 7 to 10). RR or TK transcripts were not detected at 15 days pi (Fig. 9). If reverse transcriptase was omitted from the RT reactions, amplified products were not observed after the PCR (data not shown). In summary, FL&P IE transcripts in TG were first detected at the peak of the acute infection (Fig. 7). Surprisingly, FL&P RR or bICP22 transcripts were consistently detected before bICP4 or bICP0 transcripts (Fig. 7 to 9).

PCR quantitation of viral DNA in TG during an in vivo acute infection. To examine the levels of viral DNA in TG of infected cattle, quantitative PCR was performed as described previously (13). This assay relies on coamplification of a viral gene, gC, and a bovine gene, BGH. Viral DNA was not detected at day 1 pi by the quantitative technique (Fig. 11) or by a standard DNA PCR (data not shown). At 2 days pi, approximately $10^{-4.05}$ copies of viral DNA/cell equivalent were present (Fig. 11B). The gC/BGH signal ratio (which is proportional to the logarithm of the relative amount of viral DNA) increased 1.5-fold between days 2 to 4 and 2-fold between days 2 to 7 (Fig. 11). At 7 days pi, the levels of viral DNA in TG was approximately $10^{-2.51}$ copies of viral DNA/cell equivalent. At 15 days pi, the levels of viral DNA in TG were similar to those at 4 days pi. A similar result was obtained with HSV-1 during a primary infection of mouse TG (15). In summary, these studies indicated that the levels of viral DNA increased in TG during an acute infection.

DISCUSSION

In this report, we examined the temporal pattern of BHV-1 transcription in bovine TG. Detection of IE transcripts (FL&P) was after detection of RR and bICP22 transcripts (FL&P) and followed an increase of viral DNA in TG. It was not possible to determine whether the increase of viral DNA in TG was due to replication in neurons versus new viral particles entering the TG. One of the most abundant viral RNAs in lytically infected cells, gC (10, 46, 53), was difficult to detect in TG. Based on immunocytochemistry, it appears that most viral gene expression in TG occurred in neurons (data not shown)

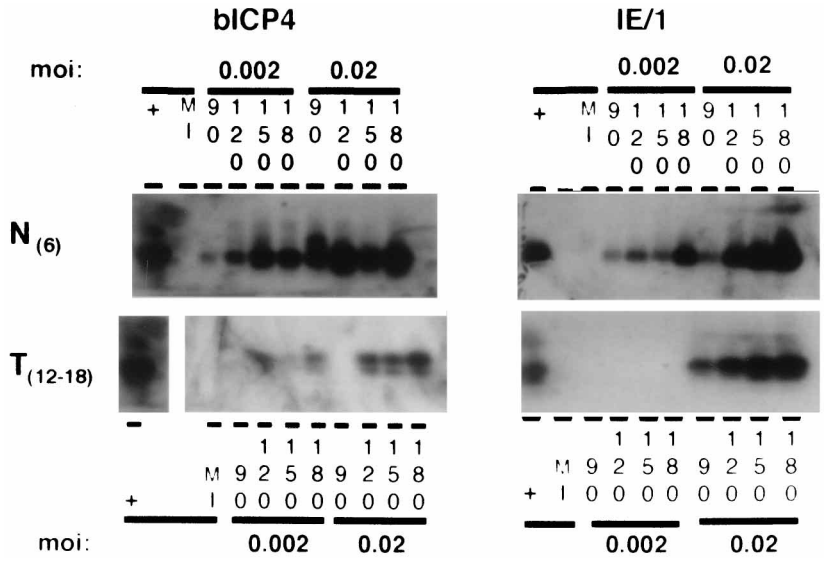

FIG. 3. Detection of IE transcripts during an in vitro lytic infection. cDNAs from RK cells infected with 0.002 or 0.02 $TCID_{50}$ of BHV-1 per 100-mm-diameter dish (0.002 and 0.02, respectively), and positive and negative controls were PCR amplified, blotted to a nylon membrane, and hybridized with the respective probes. RNA was extracted at 90, 120, 150, or 180 min after infection as indicated. RNA was treated with DNase I, reverse transcribed with the indicated primer, and PCR amplified with either bICP4 (left panels) or IE/1 (right panels) primer. As a positive control, we used BHV-1 DNA (+). As negative controls we used RNA from mock-infected cells (MI) and also amplifications in reaction tubes to which no template was added (not shown). The + lane in $N_{(6)}$ and $T_{(12–18)}$ of the bICP4 panels is the same positive control, as bICP4-amplified products from both bICP4 PCRs, $N_{(6)}$ and $T_{(12–18)}$-primed cDNAs were run in the same gel. PCRs from both $N_{(6)}$ and $T_{(12–18)}$ cDNAs were run at the same time with the same PCR mix. (Top panels) Amplification from bICP4 or IETU1/E1 RNA in random-primed cDNA [$N_{(6)}$]. (Bottom panels) Amplification from bICP4 or IETU1/E1 RNA in oligo(dT)-primed cDNA [$T_{(12–18)}$].

which is consistent with results of a previous study (18). These results suggested that the cascade of viral gene expression was altered in TG, and consequently we hypothesized this may favor the establishment of latency.

Genes that should be important for a productive infection of neurons were chosen for amplification. The sequences of some BHV-1 E genes (such as DNA polymerase) were not available to the public at the time these experiments were performed.

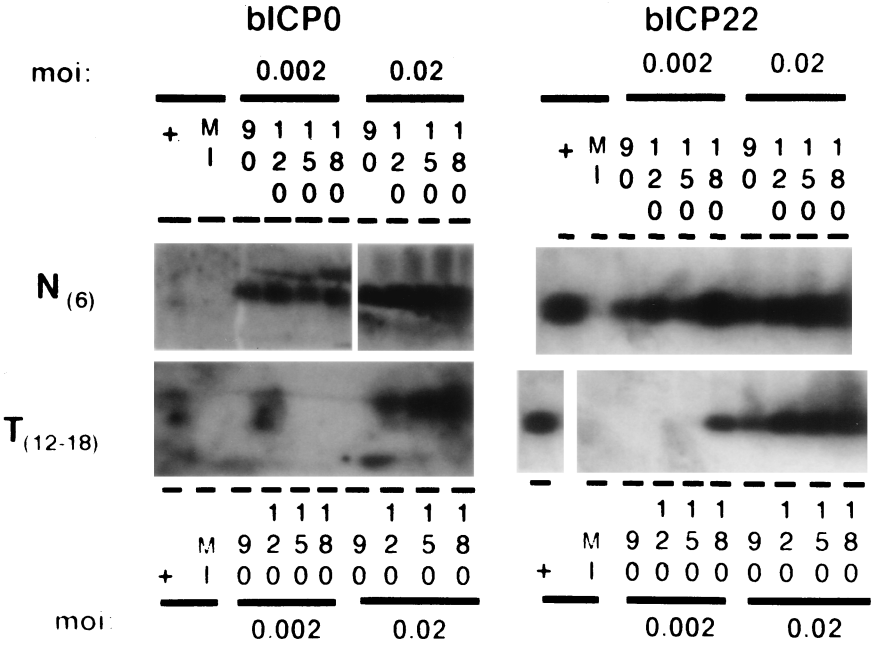

FIG. 4. Detection of dual-kinetics transcripts during an in vitro lytic infection. Infections, RNA extraction and processing, and controls were the same as described in the legend to Fig. 2. cDNAs were PCR amplified with either bICP0 (left panels) or bICP22 (right panels) primer. The + lane in $N_{(6)}$ and $T_{(12–18)}$ bICP22 panels is the same positive control, as bICP22-amplified products from $N_{(6)}$ and $T_{(12–18)}$-primed cDNAs were run in the same gel. PCRs from both $N_{(6)}$ and $T_{(12–18)}$ cDNAs were run at the same time with the same PCR mix. (Top panels) Amplification from bICP0 or bICP22 RNA in random-primed cDNA [$N_{(6)}$]. (Bottom panels) Amplification from bICP0 or bICP22 RNA in oligo(dT)-primed cDNA [$T_{(12–18)}$].

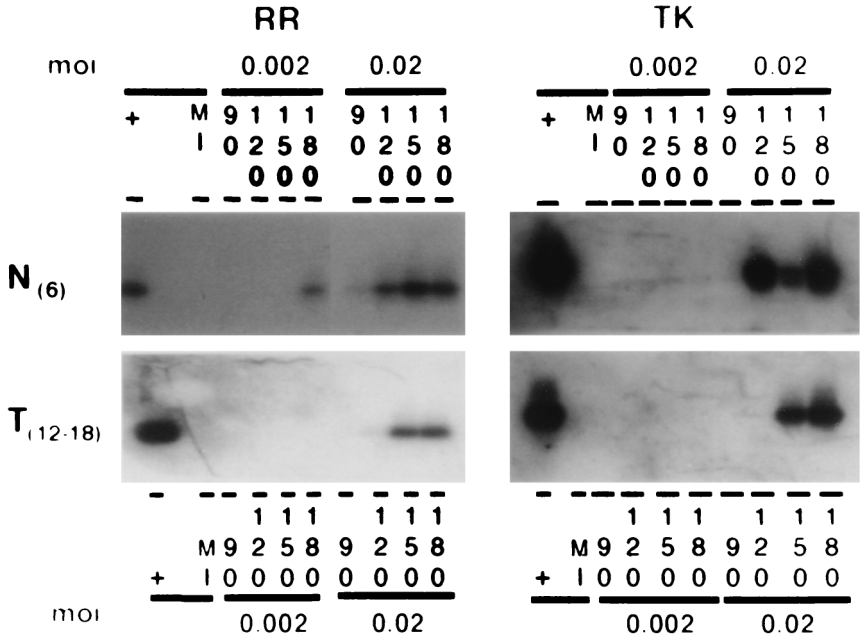

FIG. 5. Detection of E transcripts during an in vitro lytic infection. Infections, RNA preparation, and controls were the same as described in the legend to Fig. 2. cDNAs were PCR amplified with either RR (left panels) or TK (right panels) primer. (Top panels) Amplification from RR or TK RNA in random-primed cDNA [$N_{(6)}$]. The RR panel is a composite figure. (Bottom panels) Amplification from RR or TK RNA in oligo(dT)-primed cDNA [$T_{(12-18)}$].

All primers were selected to anneal at the same temperature and when possible to amplify the 3′ end of the respective transcript. Therefore, the primers used in this study should not detect transcripts which prematurely terminate. Computer analysis did not predict complex secondary structures between any of the primer sets and the 3′ end of the respective transcript. There are no adenine-rich sequences in any of the primer sets and the 3′ end of the respective transcripts, making it unlikely that the oligo(dT) primer would anneal nonspecifically. The strategy for designing primers was successful, because the respective primer pairs had similar efficiency (Fig. 2), and they detected IE RNA expression prior to E or L RNAs in nonneural cells (Fig. 3 to 6).

It is likely that viral proteins are primarily translated from

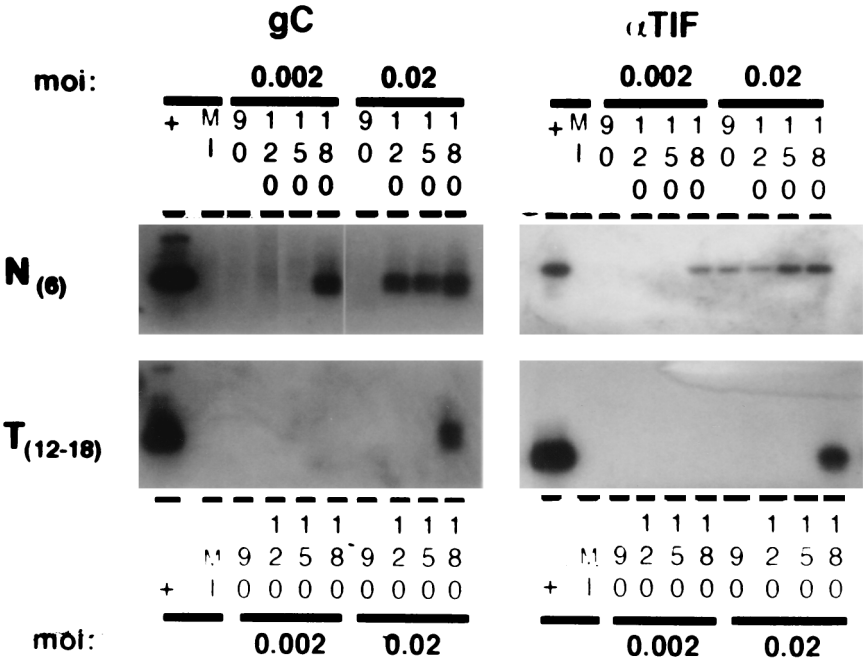

FIG. 6. Detection of L transcripts during an in vitro lytic infection. Infections, RNA preparation, and controls were the same as described in the legend to Fig. 2. cDNAs were PCR amplified with either gC (left panels) or αTIF (right panels) primer. (Top panels) Amplification from gC or αTIF RNA in random-primed cDNA [$N_{(6)}$]. The gC panel is a composite figure. (Bottom panels) Amplification from gC or αTIF RNA in oligo(dT)-primed cDNA [$T_{(12-18)}$].

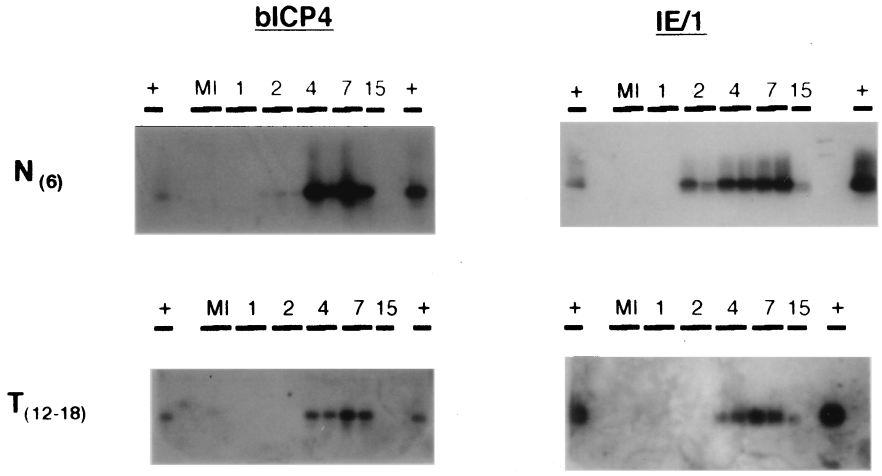

FIG. 7. Detection of IE transcripts during an in vivo infection of TG. cDNAs from TG of BHV-1-infected or control calves and a positive control were PCR amplified, blotted to a Nylon membrane, and hybridized with the respective probes. RNA was extracted from TG of calves euthanized at 1, 2, 4, 7, or 15 days pi as indicated, DNase I-treated, reverse transcribed with the indicated primer, and PCR amplified with either bICP4 (left panels) or IE/1 (right panels) primer. As a positive control, we used BHV-1 DNA (+). As negative controls we used RNA from mock-infected animals (MI) and amplifications in reaction tubes to which no template was added (not shown). In this and all subsequent figures, the order of the samples from the calves, from left to right, is: 21, 41 (MI); 24, 50 (1 dpi); 73, 100 (2 dpi); 89, 90 (4 dpi), 25, 93 (7 dpi), and 200 (15 dpi). A molecular weight marker (phi X, *Hae*III digested) was run in the lanes between the positive control at each side of the gel and the first or last sample. In both $N_{(6)}$ panels, the positive control in the leftmost lane was a 1/10 dilution of the positive control in the rightmost lane. (Top panels) Amplification from bICP4 or IETU1/E1 RNA in random-primed cDNA [$N_{(6)}$].

FL&P transcripts, and thus their expression has more biological relevance. Since cDNA synthesis which is primed by poly(dT) primers leads primarily to amplification of poly(A)$^+$ RNA, we believe this procedure reflects FL&P RNA. In contrast to a productive infection in rabbit kidney cells, bICP22 (IE and L) and RR (E) transcripts (FL&P) were detected before IE transcripts (FL&P), during the acute phase of a TG infection (Fig. 7 to 10). It is not clear whether RR proteins were expressed prior to IE proteins, because RR protein expression is regulated by posttranscriptional mechanisms (49, 50). Although RR primers detected two transcripts, bICP4 and bICP22 primers also detected two transcripts because these IE genes are located in the genomic repeats (44, 45) (Fig. 1). Furthermore, the IE/1 primers detected three transcripts, (bICP4, bICP0, and b*circ*), and one of them (bICP4) is diploid (Fig. 1). Therefore, the ability of the RR primers to detect the transcripts of the large and small subunits of RR was not the sole reason for their early detection. It is also possible that FL&P IE RNAs were expressed before E transcripts, but their levels were too low to be detected by RT-PCR.

BHV-1 IE promoters are activated by αTIF (28, 29), suggesting that if αTIF protein is transported into the nucleus of a neuron with the rest of the tegument it does not stimulate IE promoter activity. There is also evidence that HSV-1 IE promoter activity is repressed by neuron-specific transcription factors (21, 25, 26). IE RNA expression may also be repressed because of premature termination or improper splicing/processing. This hypothesis is based on three findings: (i) IE RNA

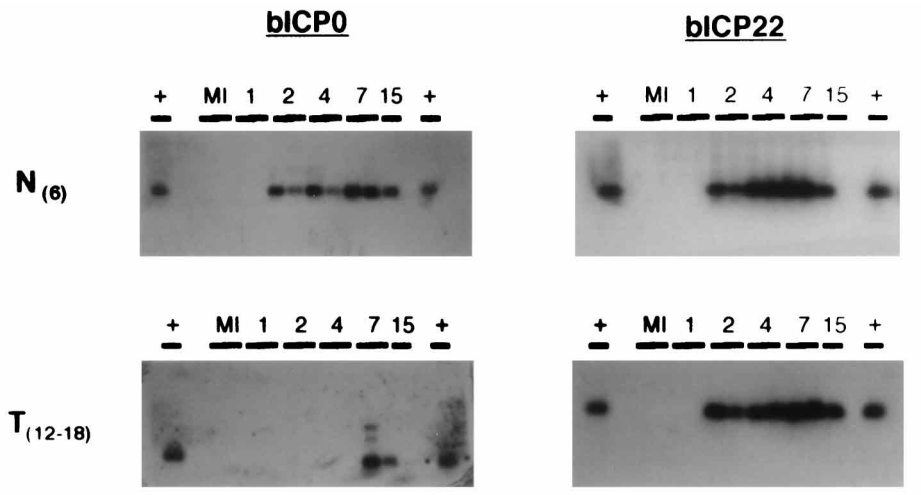

FIG. 8. Detection of dual-kinetics transcripts during an in vivo infection of TG. Infection of calves, RNA preparation, and controls were the same as described in the legend to Fig. 6. cDNAs were PCR amplified with either bICP0 (left panels) or bICP22 (right panels) primer. A molecular weight marker (phi X, *Hae*III digested) was run in the lanes between the positive control at each side of the gel and the first or last sample. (Top panels) Amplification from bICP0 or bICP22 RNA in random-primed cDNA [$N_{(6)}$]. (Bottom panels) Amplification from bICP0 or bICP22 RNA in oligo(dT)-primed cDNA [$T_{(12–18)}$].

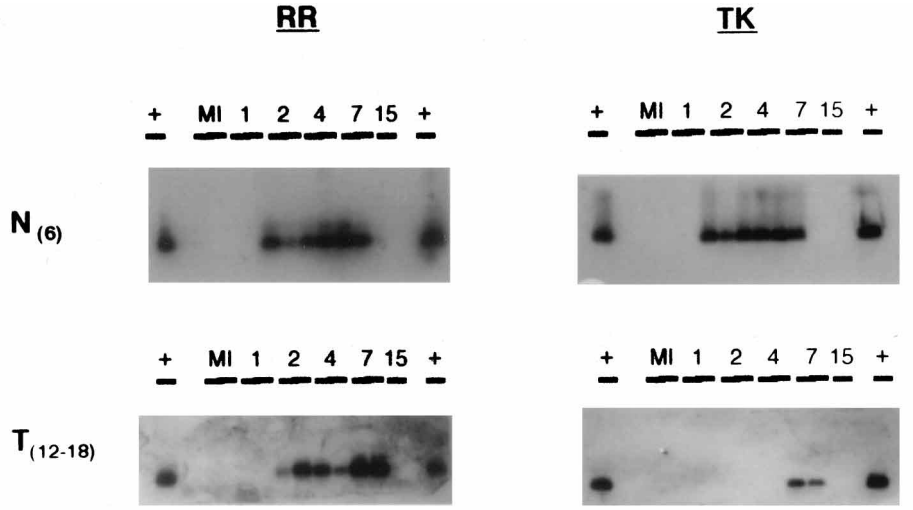

FIG. 9. Detection of E transcripts during an in vivo infection of TG. Infection of calves, RNA preparation and controls were the same as described in the legend to Fig. 6. cDNAs were PCR amplified with either RR (left panels) or TK (right panels) primer. A molecular weight marker (phi X, *Hae*III digested) was run in the lanes between the positive control at each side of the gel and the first or last sample. (Top panels) Amplification from RR or TK RNA in random-primed cDNA [$N_{(6)}$]. (Bottom panels) Amplification from RR or TK RNA in oligo(dT)-primed cDNA [$T_{(12-18)}$].

was detected in TG after E RNA (FL&P), (ii) random priming of cDNA allowed detection of IE transcripts before poly(dT) priming of cDNA, and (iii) random priming or poly(dT) priming of cDNA led to simultaneous detection of RR transcripts. The delay in detecting (FL&P) IE RNA can be explained by at least four different mechanisms: (i) polyadenylation does not readily occur in infected TG, (ii) the 3′ end of IE RNAs is prone to degradation in neurons, (iii) transcription prematurely terminates, and/or (iv) splicing of IE RNAs does not readily occur in neurons. Conversely, it is possible that FL&P IE transcripts were only transiently expressed in a small subset of neurons and thus not detected. The fact that IE genes are present in regions which are high in GC content may make it more difficult to detect FL&P IE transcripts. To rule out the possibility that FL&P IE RNA is not the first RNA expressed in a few infected neurons or that our approach was not sensitive enough to measure the initial expression of IE RNA, it will be necessary to develop procedures which can accurately measure viral gene expression from single neurons or by in situ techniques.

The finding that polyadenylated bICP22 and RR transcripts were detected before IE RNA suggested their respective promoters were activated by cellular factors, the transcripts were stable in infected neurons, and/or the transcripts were more efficiently polyadenylated. Since bICP0 represses the bICP22 promoter (54), this may explain in part the relative abundance

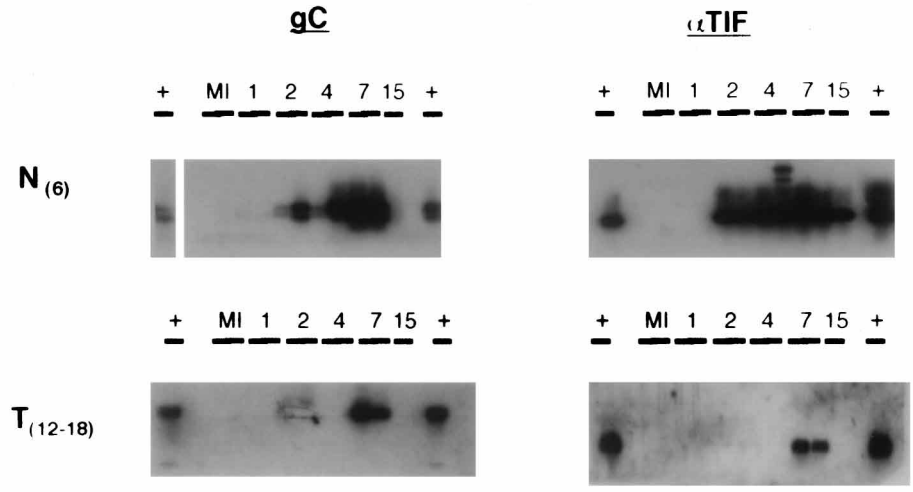

FIG. 10. Detection of L transcripts during an in vivo infection of TG. Infection of calves, RNA preparation, and controls were the same as described in the legend to Fig. 6. cDNAs were PCR amplified with either gC (left panels) or αTIF (right panels) primer. A molecular weight marker (phi X 174, *Hae*III digested) was run in the lanes between the positive control at each side of the gel and the first or last sample. (Top panels) Amplification from gC or αTIF RNA in random-primed cDNA [$N_{(6)}$]. In the gC panel, variability in the intensity of the signal among different calves is representative. Both positive control lanes were run at the left of the gel. The double band observed in all calves is usually seen in PCRs performed with these primers. In the αTIF panel, the bands of higher molecular weight are occasionally observed in amplifications of $N_{(6)}$-primed cDNAs. They may be produced by nonspecific annealing of the primers. (Bottom panels) Amplification from gC or αTIF RNA in oligo(dT)-primed cDNA [$T_{(12-18)}$].

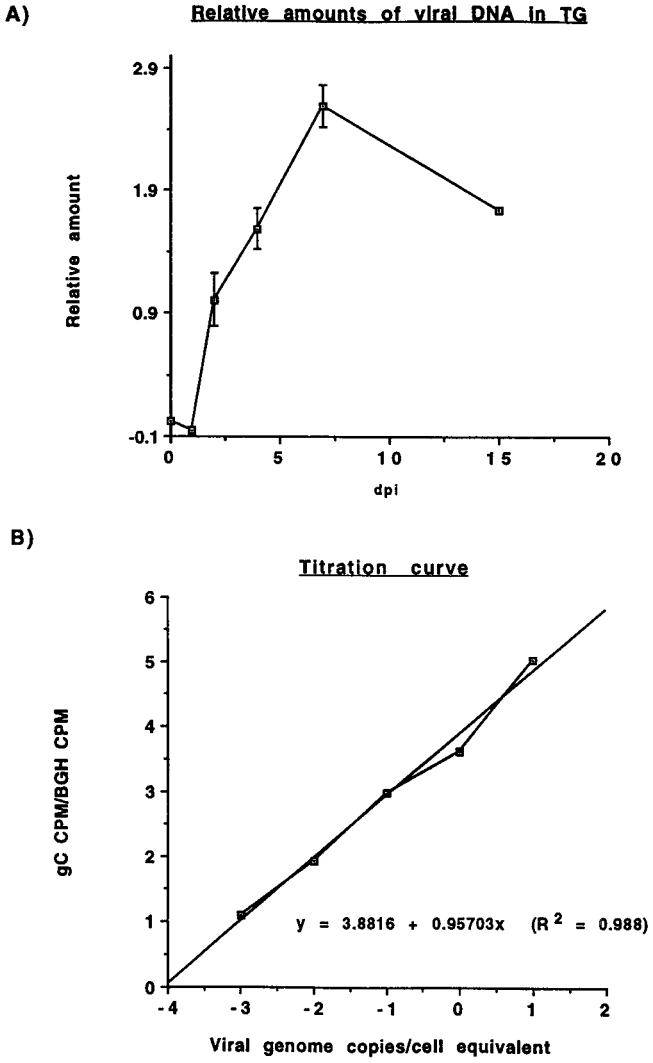

FIG. 11. Quantification of BHV-1 DNA in TG during an in vivo primary infection. (A) DNAs from TGs of BHV-1-infected or control calves were PCR amplified with both gC and BGH primers. Amplified products were blotted to a nylon membrane, hybridized to the gC probe, stripped, and hybridized to the BGH probe. The ratio (counts per minute hybridized to gC probe/counts per minute hybridized to BGH probe) from all animals was standardized by assigning the value 1 to the average ratio of the animals necropsied at 2 d pi (first day in which viral DNA was detected). All calves were quantitated twice, and the value of both measurements was averaged to obtain the relative ratio for each animal. The relative ratio was then plotted against the time of necropsy. Each point represents the mean relative ratio of the two animals necropsied each day (except for day 15 pi, in which only one animal was necropsied). Error bars indicate the range at each time point. There is no error bar at day 15 pi because only one animal was necropsied at this time. The relative ratio is approximately directly proportional to the logarithm of the actual viral burden per cell. (B) The titration curve from the experiment in which the total amount of viral DNA per cell equivalent was estimated. The ratio (radioactivity [counts per minute] hybridized to the gC amplified products/radioactivity [counts per minute] hybridized to the BGH-amplified products) was plotted against the number of viral copies/cell equivalent in each tube of the titration curve. The same ratio obtained from the samples was interpolated or extrapolated in the titration curve to estimate the total amount of viral DNA in the TG of the different animals. The linear regression function is expressed; note the high coefficient of correlation (r^2).

of bICP22. Cellular factors associated with proliferation (c-*jun* or c-*myc*) activate the HSV-2 large subunit of the RR promoter (14, 56). A transcription factor that regulates cell cycle progression, E2F, activates the HSV-1 TK promoter through GC-rich DNA sequences which contain consensus Sp1 binding sites (48). Cellular factors in certain cell types, including a mouse neuroblastoma cell line, can replace ICP0 for activation of E promoters and support an HSV-1 infection (5, 33, 57). These factors are active when cells are released from growth arrest, suggesting a factor involved with the cell cycle is responsible for this activity. Interestingly, our recent results determined that cyclin A, a protein required for S phase, was induced in neurons of rabbit TG during the acute phase of a BHV-1 infection and reactivation (43). It is also possible that novel BHV-1 gene products may be expressed in neurons and stimulate expression of certain viral genes in the absence of IE gene expression. This hypothesis is supported by the finding that in the absence of ICP4, a novel viral transcript, L/ST or ORF P, is expressed by HSV-1 (4, 24, 58).

ICP22 is a conserved gene which is present in HSV-1, HSV-2, pseudorabies virus, VZV, equine herpesvirus 1 and 4, and Marek's disease virus (17, 45, 59). Since HSV-1 ICP22 induces an infection-specific phosphorylation of the carboxy-terminal domain of RNA PolII (36) and is essential for viral growth in certain cell lines (47), it is likely that bICP22 plays a regulatory role in a BHV-1 infection. Its prolonged expression during an acute infection in TG also suggested that it plays a role in establishing a latent BHV-1 infection.

Two independent studies with other alphaherpesviruses have concluded that tissue-specific viral gene expression occurs. When human neurons or Schwann cells are infected with VZV, limited E gene expression was detected before IE gene expression (2). As expected, VZV IE genes were detected before E genes in human fibroblasts or astrocytes, supporting the hypothesis that viral gene expression is regulated by cell-specific factors. Inhibitors of viral TK, viral DNA polymerase, or TK deletion mutants reduced HSV-1 IE gene expression in TG neurons (22, 23). Furthermore, extensive IE expression only occurs after viral DNA replication in neurons, suggesting that an inhibitor of IE expression is titrated out after an increase in genome copies. Our studies support these conclusions and provide evidence that cell-type-specific expression of BHV-1 genes occurred during an in vivo infection of bovine TG.

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