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Detection of Latency-Associated Transcripts of Equid Herpesvirus 1 in Equine Leukocytes but Not in Trigeminal Ganglia

P. M. CHESTERS, R. ALLSOP, A. PUREWAL, AND N. EDINGTON*

Department of Pathology and Infectious Diseases, The Royal Veterinary College, London NW1 OTU, United Kingdom

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Results from Southern hybridization and PCR amplification experiments using a randomly synthesized reverse transcription-PCR product showed that peripheral blood leukocytes from horses showing no clinical signs of disease expressed a putative latency-associated transcript antisense to and overlapping the 3′ end of the equid herpesvirus 1 (EHV-1) immediate-early gene (gene 64). A PCR product derived from this transcript has ≥96% identity with the published EHV-1 sequence. In situ hybridization studies of equine bronchial lymph nodes corroborated these findings and are consistent with reactivation data (D. A. Smith, A. Hamblin, and N. Edington, unpublished data), indicating that EHV-1 latency is established predominantly in $CD5^+/CD8^+$ leukocytes.

Equid herpesvirus 1 (EHV-1; equine abortion virus) is an alphaherpesvirus that is an important pathogen of horses. Infection may cause respiratory disease but can also cause paresis or abortion in susceptible mares (1). Latency, during which the entire viral genome is present in the infected cell but only a limited part undergoes transcription, is a characteristic of herpesvirus infection. Subsequent reactivation may occur to produce infectious virus (27).

Latent infection with EHV-1 and EHV-4 has been shown in at least 60% of horses (9). The prototype of the alphaherpesvirus subgroup that includes EHV-1, herpes simplex virus (HSV), establishes latency in neuronal tissue (32, 33). In contrast, EHV-1 was found by us (9, 10, 36) to establish latency in lymphoid tissues, particularly those draining the respiratory tract. Latent virus has been detected in experimentally infected ponies by PCR and by cocultivation of lymphoid tissue (36), while reactivation of latent virus in horses has been demonstrated following corticosteroid treatment (4, 9, 24). Although EHV-1 reactivation has not been observed from neuronal tissue by cocultivation in vitro, EHV-1 DNA has been detected at low frequency in such tissue by direct PCR (10, 11, 36). In addition, a recent paper reports the presence of EHV-1 sequences in sections of trigeminal ganglia (TG) (3).

Latency has been studied in the greatest detail in HSV type 1 (HSV-1). In the latent phase the major detectable transcript lies within an 8.6-kb sequence antisense to and overlapping the immediate-early (IE) gene IE-1 (ICP0). This latency-associated transcript (LAT) is a 2.0-kb transcript lacking polyadenylation and found largely in the nuclei of latently infected neurons. Evidence that the major LAT is a stable intron derived from a larger precursor exists (reviewed in reference 14).

In this work we have investigated the presence of EHV-1 LATs in total RNA derived from equine peripheral blood leukocytes (PBLs) by constructing a cDNA library by a random PCR technique (15). Using this random technique, we have localized a transcript from PBLs taken from a pony that had been experimentally infected with EHV-1 6 months previously but showed neither clinical signs of infection nor direct recovery of virus at the time of sampling. This transcript was antisense to and overlapping the EHV-1 IE gene (gene 64 [34]). Corroborative data are provided both from in situ hybridization studies described in this report and from studies carried out by Smith et al. (29) of the reactivation of EHV-1 from equine T lymphocytes by cytokines and hormones. Our findings are discussed in the context of other alphaherpesviruses and work (3) published during the course of this investigation in which a transcript located in ganglionic neurons was found to overlap EHV-1 gene 63 (homologous to HSV-1 IE-1 [ICP0] [34]).

Initial work focused on the IE region of EHV-1 in the light of current understanding of latency in other alphaherpesviruses (see Discussion).

MATERIALS AND METHODS

Standard molecular biology procedures were used as described by Sambrook et al. (28) except where otherwise specified.

Tissues. Equine blood, bronchial lymph nodes (BLNs), and TG obtained from an abattoir and from experimental ponies were transported to the laboratory at ambient temperature. Solid tissues were transported in cell culture medium.

RNA extraction. Rouletting and sedimentation of equine erythrocytes allowed leukocytes to be recovered from supernatant plasma after standing for 30 min. After this supernatant was centrifuged for 15 min at 750 × *g*, leukocytes were resuspended in Ultraspec reagent (AMS Biotechnology) for extraction of total RNA (8). Tissue from BLNs was homogenized in Ultraspec reagent with a Dounce homogenizer. Extracted RNA was stored in aqueous ethanol at −70°C. Before use in reverse transcription (RT)-PCR experiments, RNA was subjected to direct PCR to detect the presence of viral DNA. DNA was removed by digestion with RNase-free DNase (Promega) for 30 min at 37°C. The process was repeated as necessary until no DNA amplification could be detected.

Recombinant DNA. All subgenomic clones of EHV-1 were derived from a 17-kb *Eco*RI C fragment (Fig. 1b) cloned into the *Eco*RI site of plasmid pSPT18 (Boehringer Mannheim). Restriction endonuclease-digested DNA fragments were separated by agarose gel electrophoresis and extracted from excised gel slices by the freeze-squeeze method (30), involving two cycles of freezing and thawing followed by centrifugation at full speed (approximately 12,000 × *g*) for 15 min at 4°C in an MSE Microcentaur microcentrifuge. The purified DNA fragments, consisting of deleted virus-plasmid DNA recombinants and viral DNA subfragments, were respectively recircularized or recloned into plasmid DNA linearized with the appropriate restriction endonuclease(s). These clones are represented diagramatically in Fig. 1c. Recombinant DNA was used to transform competent *Escherichia coli* (DH5; GIBCO-BRL) according to the supplier's instructions.

* Corresponding author. Mailing address: Department of Pathology and Infectious Diseases, The Royal Veterinary College, Royal College St., London NW1 OTU, United Kingdom. Phone: 44 171 468 5299. Fax: 44 171 383 4670. E-mail: nedingto@rvc.ac.uk.

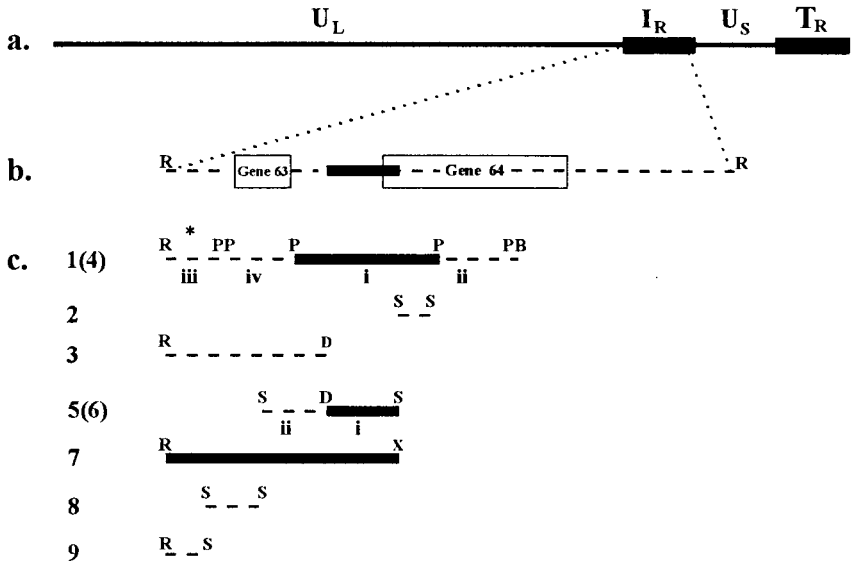

FIG. 1. EHV-1 DNA constructs and summary of Southern hybridization results. (a) Representation of the entire EHV-1 genome showing the unique regions (long [U_L] and short [U_S]) and the repeated sequences (internal [I_R] and terminal [T_R]). (b) *Eco*RI C region of the genome including the homologs of the HSV-1 IE genes 1 (ICP0) and 3 (ICP4) (EHV-1 genes 63 and 64, respectively), shown as open boxes. The solid line partially overlapping gene 64 represents the maximum limits of the extent of the putative LAT as deduced from the pattern of hybridization shown in panel c. (c) Relative positions of the EHV-1 DNA fragments. The fragments were made from the *Eco*RI C fragment of EHV-1 by digestion with the restriction endonucleases indicated followed by religation or subcloning into vector pSPT18 as described in Materials and Methods. The numbers in parentheses refer to sets of subfragments (described below) made for Southern hybridizations by further digestion with restriction endonucleases recognizing the internal sites indicated. Restriction endonuclease sites are indicated as follows: B, *Bam*HI; D, *Dra*III; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I. Constructs 1, 7, 3, and 9 are coterminal nested deletions, and constructs 2, 5, and 8 are subclones. Constructs 4i through iv are derived from construct 1 by digestion with restriction endonuclease *Pst*I. Constructs 6i and ii are derived from construct 5 by digestion with restriction endonuclease *Dra*III. The size of each fragment is as follows: **1**, 8.4 kb, *Eco*RI-*Bam*HI; 2, 0.7 kb, *Sal*I-*Sal*I; 3, 3.5 kb, *Eco*RI-*Dra*III; **4i**, 3.6 kb, *Pst*I-*Pst*I; 4ii, 1.7 kb, *Pst*I-*Bam*HI; 4iii, 4.5 kb, *Pst*I-*Pst*I EHV-1–plasmid recombinant; 4iv, 1.4 kb, *Pst*I-*Pst*I; **5**, 3.5 kb, *Sal*I-*Sal*I; **6i**, 1.9 kb, *Dra*III-*Sal*I; 6ii, 1.6 kb, *Sal*I-*Dra*III; **7**, 5.3 kb, *Eco*RI-*Xho*I; 8, 1.2 kb, *Sal*I-*Sal*I; 9, 0.8 kb, *Eco*RI-*Sal*I. (Fragments in boldface are smaller than expected from the published sequence [34]. This is due to a deletion of approximately 200 bp in the EHV-1 DNA used to prepare these constructs. This does not affect the interpretation of these hybridization results, since none of the nonhybridizing fragments overlaps the deletion, which is situated within the repeating sequences indicated in Fig. 5b.) The solid lines represent the DNA fragments hybridizing to PCR-amplified cDNA-65 (see Fig. 3). Dashed lines represent sequences not hybridizing to cDNA-65. *, fragment 4iii is covalently linked to the plasmid to give a hybrid of 4.5 kb in size.

Oligonucleotides. Oligonucleotide primers used for cDNA synthesis, PCR, and sequencing reactions were synthesized by Pharmacia Biotech.

cDNA synthesis and PCR amplification. PCR was carried out using AmpliTaq DNA polymerase (Applied Biosystems Division of Perkin-Elmer) according to the manufacturer's instructions. A hot-start procedure using TaqStart antibody (Clontech Laboratories Inc.) was employed. PCR conditions for each set of specific primers were optimized with purified EHV-1 DNA. Synthesis of cDNA was carried out with a proprietary kit (InVitrogen Corp.) in conjunction with a random primer system. All conditions specifically applying to the random primer system were as described elsewhere (15).

In this method, two primers were used. The first strand of cDNA was synthesized by incubation at 37°C with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) by using a primer oligonucleotide (universal primer dN_6) that consists of a defined sequence contiguous with a random hexamer at the 3′ end: 5′-GCCGGAGCTCTGCAGAATTCNNNNNN-3′, where N is G, A, T, or C. The second strand was made by the addition of Klenow enzyme (Boehringer Mannheim) and further incubation at 37°C. Unreacted primer oligonucleotides were then removed by filtration through a microcentrifuge filter having a molecular weight cutoff of 10^5 (Ultrafree-MC 100,000 NMWL; Millipore). The cDNA was amplified by PCR using a universal primer, the sequence of which (5′-GCCGGAGCTCTGCAGAATTC-3′) is identical to that of universal primer dN_6 without the random hexanucleotide sequence at the 3′ end. This primer shows no significant complementarity with EHV-1 sequences.

Filter hybridization. Filter hybridizations were carried out at 68°C using Hy-Bond membranes (Amersham International) in a volume of 10 ml containing DNA radiolabelled with ^{32}P by oligolabelling (13) to a specific activity of 1×10^8 to 2×10^8 dpm/μg in sixfold-concentrated standard saline citrate (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) (28) containing 0.25% low-fat dried milk (BLOTTO [21]). The filters were washed as described elsewhere (21) with the high-stringency wash carried at 10 to 15°C below melting temperature. Membranes were exposed to X-ray film (Fuji) at −70°C without preexposure.

In situ hybridization. BLNs and TG were snap frozen in liquid nitrogen as soon as possible after excision. Sections (12 μm thick) were cut on a cryostat, fixed on glass slides by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) (28), and then dehydrated by immersion in graded alcohols for storage at −20°C.

In situ hybridization was carried out by standard procedures (2, 35). Briefly, preparations were rehydrated by immersion for 15 min in 0.2% Triton in PBS and washed in PBS. Proteinase K digestion was carried out for 15 to 20 min at 37°C with 1 μg of enzyme per ml in 0.1 M Tris HCl and 50 mM EDTA at pH 8. Enzyme digestion was ended by immersion for 5 min in 0.1 M glycine in PBS. Postfixation was carried out for 3 min with 4% paraformaldehyde in PBS. Preparations were then treated for 10 min in a solution containing 0.25% acetic anhydride and 0.1 M triethanolamine at pH 8, rinsed briefly in distilled water, and dried at 37 to 40°C. Hybridizations were carried out with single-stranded RNA probes generated with a commercial kit (Boehringer Mannheim) using either ^{35}S or digoxigenin as a label. EHV-1 DNA construct 7 (Fig. 1c) was used to synthesize the labelled RNA probes used in these hybridizations. The template was linearized with either *Eco*RI or *Hin*dIII and transcribed with SP6 or T7 polymerase to give transcripts from each DNA strand. The transcripts were treated with RNase-free DNase (Boehringer Mannheim) for 30 min at 37°C to remove DNA templates, extracted with phenol-chloroform, and partially hydrolyzed with a solution of sodium bicarbonate (40 mM) and sodium carbonate (60 mM) at 60°C to give a mean RNA size of approximately 500 bp.

For hybridization, labelled riboprobes were diluted to a concentration of 0.5 ng/μl in buffer (6× SSC; 10% dextran sulfate; 5× Denhardt's solution [28]; 2% sodium dodecyl sulfate; 100 μg of denatured, sheared, herring sperm DNA per ml; 50% deionized formamide). Diluted probe was applied to sections in 10-μl quantities, covered with a siliconized glass coverslip, and incubated for 16 to 20 h at 42°C in a sealed humidity chamber. After washing at high stringency, sections were treated with RNase to remove residual labelled probe. Preparations treated with radioactively labelled probes were dehydrated through graded alcohols and dried in air. Autoradiography was carried out by dipping slides in liquid photographic emulsion (Kodak). Preparations treated with digoxigenin-labelled probes were treated with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) and Nitro Blue Tetrazolium as specified by the labelling kit suppliers (Boehringer Mannheim) to make visible sites of hybridization. Cells were counterstained with hematoxylin.

Cocultivation. Cocultivation was carried out essentially as described previously (11, 36). Preparations of tissue were cultivated separately with rabbit kidney (RK13) cells and with equine embryonic kidney cells, which act as detectors of reactivated virus. Duplicate preparations were made of ground tissue, to detect the presence of infective virus particles in the tissue preparations. All cocultivations were kept for 10 days in RPMI 1640 medium supplemented with 10% fetal

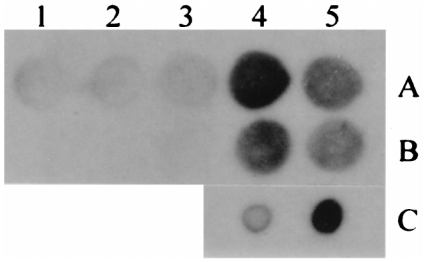

FIG. 2. Dot hybridization of equine leukocyte random RT-PCR products with ^{32}P-labelled subgenomic EHV-1 DNA. A dot blot of randomly synthesized RT-PCR products obtained with RNA from PBLs from three ponies and BLNs from two other ponies 6 months after experimental infection with EHV-1 (strain Ab4) is shown. The hybridization probe was a ^{32}P-labelled 8.4-kb subfragment of EHV-1 *Eco*RI C DNA spanning genes 63 and 64 (Fig. 1c, construct 1). Row A dots: 1, pony A BLN; 2, pony B BLN; 3, pony C BLN; 4 and 5, EHV-1-infected cells. Row B dots: 1 and 2, water; 3, bacteriophage MS2 RNA; 4 and 5, PBLs from ponies D and E. Row C dots: 4 and 5, EHV-1 DNA (1 and 10 pg, respectively, applied directly to the filter).

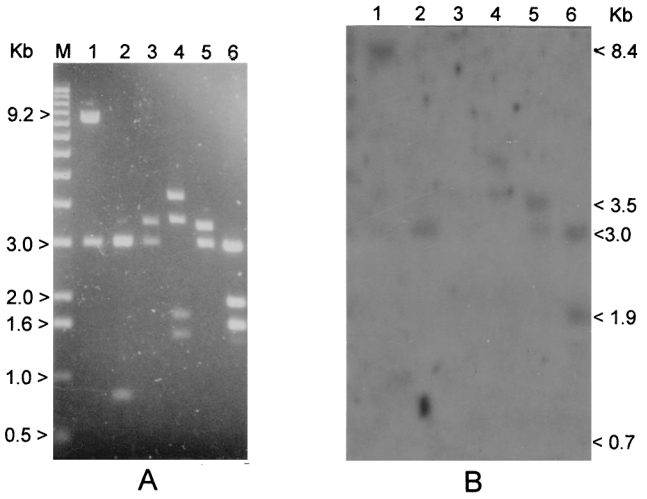

FIG. 3. Hybridization analysis of subgenomic DNA fragments of EHV-1. (A) A 1% agarose gel, with a wedge-shaped longitudinal cross section for enhanced resolution (4), stained with ethidium bromide to show the following EHV-1 *Eco*RI C DNA subfragments: lane 1, 8.4 kb (Fig. 1c, construct 1); lane 2, a 0.7-kb EHV-1 fragment (Fig. 1c, construct 2); lane 3, 3.5 kb (Fig. 1c, construct 3); lane 4, subfragments derived from the 8.4-kb fragment shown in lane 1, digested with *Pst*I to give four bands (a 4.5-kb plasmid–EHV-1 recombinant DNA fragment [iii]; also 3.6-kb [i], 1.7-kb [ii], and 1.4-kb [iv] EHV-1 fragments [Fig. 1c, constructs 4i through iv]); lane 5, 3.5 kb (Fig. 1c, construct 5); lane 6, 1.9-kb (i) and 1.6-kb (ii) subfragments derived from the 3.5-kb fragment in lane 5 by *Dra*III digestion (Fig. 1c, constructs 6i and ii). Lanes 1 to 3, 5, and 6 show 3.1-kb plasmid DNA in addition to EHV-1 DNA. Lane M shows a 1-kb DNA marker ladder (GIBCO-BRL). (B) A Southern blot of the agarose gel shown in panel A, hybridized with amplified random cDNA-65 radiolabelled with ^{32}P as described in Materials and Methods. Hybridization of EHV-1 DNA is shown as follows: lane 1, 8.4 kb (Fig. 1c, construct 1); lane 4, 3.6 kb (Fig. 1c, construct 4i); lane 5, 3.5 kb (Fig. 1c, construct 5); lane 6, 1.9 kb (Fig. 1c, construct 5i). The 1.9- and 1.6-kb bands in lane 6 (panel A) were distinguished on the autoradiograph by measuring the migration of the bands with a ruler photographed with the ethidium bromide-stained gel (not shown). Hybridization is seen with plasmid DNA (3.1 kb) in panel B, lanes 1, 2, 5, and 6, indicating the presence of bacterial sequences in the cDNA library. Hybridization is also seen with a plasmid–EHV-1 hybrid band (4.5 kb) in lane 4.

bovine serum and trypsinized and passaged twice if no cytopathic effects were seen.

Sequencing. Sequencing was carried out with an ABI 373A automated DNA sequencer with fluorescently labelled dideoxynucleotide chain terminators.

RESULTS

Initial work was carried out with cDNA libraries prepared by a random PCR technique (15) from RNA obtained from equine PBLs and BLNs.

We adopted a random approach for two reasons. First, we were uncertain of the level of EHV-1 transcription in latently infected cells, and so we chose a PCR method as the most sensitive available. Second, because major LATs known to exist in some other herpesviruses are not polyadenylated, it was considered prudent to construct the library from total RNA. This precluded the use of specific primers annealing to the poly(A) tail. The random library was used first in filter hybridization studies to broadly localize a region within EHV-1 subgenomic DNA fragments encoding the putative LAT. In order to more closely define the region encoding the LAT, pairs of PCR primers annealing to sequences within this positively hybridizing region of the EHV-1 genome were then used to amplify putative LAT sequences within the random cDNA library. In order to identify the strand of EHV-1 DNA encoding the putative LAT, RT-PCR was carried out on total RNA from equine PBLs with one of the pairs of primers that gave an amplified product with the cDNA library.

Identification of LATs by random RT-PCR and Southern hybridization. Initially, random RT-PCR was carried out using RNA obtained from PBLs from two ponies and BLNs from three other ponies, all of which had been experimentally infected with EHV-1 (strain Ab4) (36) but showed neither clinical signs of infection nor the presence of actively replicating virus. Aliquots of the amplified cDNA products were spotted onto hybridization membranes and hybridized with a radiolabelled, plasmid-purified 8.4-kb EHV-1 DNA fragment (Fig. 1c, construct 1) spanning the region of the genome including gene 63 and most of gene 64. This region was presumed to include the LAT by comparison with genomes of other alphaherpesviruses.

The DNA-free RNA preparations obtained from all five ponies hybridized with the probe, whereas negative controls all failed to give a detectable signal (Fig. 2), indicating the presence of EHV-1 viral transcripts. One of these amplified random cDNA products (designated cDNA-65) was used for further experiments. EHV-1 DNA had been previously detected in DNA extracted from PBLs from this pony by PCR using primers specific for the region of the viral genome encoding glycoprotein gB (36).

Figure 3B shows a Southern blot (31) of EHV-1 DNA fragments (Fig. 1c) hybridized with amplified random cDNA-65 radiolabelled with ^{32}P. Viral DNA was detected in all lanes except 2 and 3 (the corresponding ethidium bromide-stained gel is shown in panel A).

A summary of all the hybridization results representing four separate experiments is shown in Fig. 1c. Hybridizations of constructs 8 (1.2 kb) and 9 (0.8 kb) (Fig. 1c), both of which are negative in these experiments, are not shown here. A comparison of the hybridization pattern of overlapping DNA fragments delimits the putative LAT to a 2.1-kb region (Fig. 1b) partially overlapping the 3′ end of the IE gene 64.

Localization by PCR. Further localization of the putative LAT was obtained by means of a series of PCR amplification experiments. Figure 4 shows typical results of agarose gel electrophoresis of PCR products obtained with a set of PCR primer pairs (A to G [Fig. 5b]) that together span the region of the EHV-1 genome that hybridizes with cDNA-65. The putative LAT region delimited by hybridization with cDNA-65 is shown in Fig. 5a. This is the region investigated in more detail by PCR. Figure 4 shows that amplified product of the expected size is obtained from cDNA-65 with primer pairs E and F (Fig.

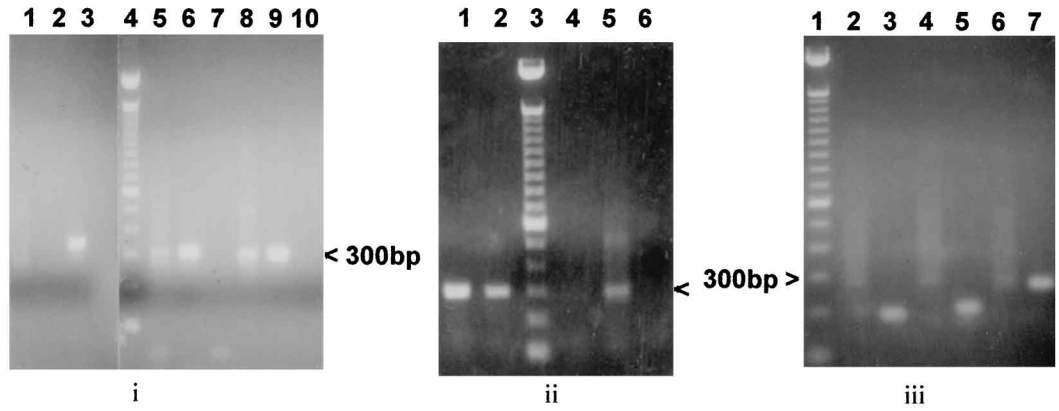

FIG. 4. PCR amplification of cDNA-65. (i) Results obtained with primer pair H (lanes 1, 2, and 3), pair E (lanes 5, 6, and 7), and pair F (lanes 8, 9, and 10) (see Fig. 5b) by using as a template cDNA-65 (lanes 1, 5, and 8), EHV-1 DNA (lanes 3, 6, and 9), and water (lanes 2, 7, and 10). Lane 4 shows a 100-bp DNA marker ladder (GIBCO-BRL). With cDNA-65 as a template, a PCR product comigrating with the positive controls in lanes 6 and 9 is obtained with primer pairs E and F in lanes 5 and 8, respectively, but no product is obtained with primer pair H in lane 1. (ii) Results obtained with primer pair G (see Fig. 5b) by using as a template EHV-1 DNA (lanes 1 and 2), water (lanes 4 and 6), and cDNA-65 (lane 5). Lane 3 shows a 100-bp DNA marker ladder. Using cDNA-65 as a template with primer pair G (lane 5) gives a product similar in size to the positive controls (lanes 1 and 2). (iii) Results obtained with primer pairs A (lanes 2 and 3), B (lanes 4 and 5), and C (lanes 6 and 7) (see Fig. 5b) by using as a template cDNA-65 (lanes 2, 4, and 6) and EHV-1 DNA (lanes 3, 5, and 7). Lane 1 shows a 100-bp ladder (GIBCO-BRL). No product of the correct size is obtained following PCR treatment of cDNA-65 (lanes 2, 4, and 6).

4i, lanes 5 and 8, respectively) and G (Fig. 4ii, lane 5). No amplified product is obtained with primer pairs A, B, and C (Fig. 4iii, lanes 2, 4, and 6, respectively) or primer pair H (Fig. 4i, lane 1).

The results of all the PCR experiments are summarized in Fig. 5b.

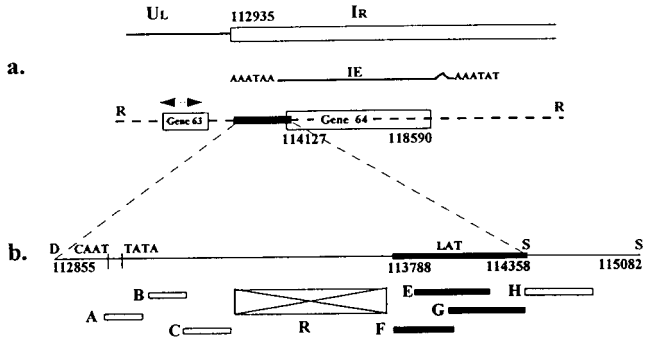

FIG. 5. Summary of the results of the PCR LAT analysis. (a) Representation of the *Eco*RI C region of the EHV-1 genome in relation to the unique long region (U_L) and the internal repeated sequences (I_R) beginning at nucleotide 112935. Genes 63 and 64, the homologs of the HSV-1 IE genes 1 (ICP0) and 3 (ICP4), respectively, are represented as open boxes, with the translational start and stop signals at coordinates 118590 and 114127, respectively. The IE transcript is also shown, indicating the positions of the TATAAA motif (first nucleotide at 119244) and the polyadenylation signal AATAAA (first nucleotide at 113843) (18, 19). The double-headed arrow above the box representing gene 63 denotes the range of the primers (nucleotides 110153 to 112009) used to test equine RNA preparations for the presence of LATs in this region, as described in Results. The putative LAT region defined by hybridization with cDNA-65 is shown as a solid box. This was investigated by PCR and is expanded in panel b to show the positions of the PCR primer pairs. (b) The crossed diagonal lines labelled R represent a region of repeating sequences not susceptible to this PCR analysis. The range of each of the PCR primer pairs is shown as a solid box, denoting a positive result, or an open box, denoting a negative result. The coordinates of the primers are as follows: A, 112487 to 112670; B, 112708 to 112904; C, 112872 to 113078; E, 113894 to 114204; F, 113788 to 114097; G, 114053 to 114358; and H, 114336 to 114669. The region of repeating sequences (R) extends from nucleotides 113092 to 113774. The putative LAT region is indicated, as are potential promoter sequences (CAAT at 112539 and two possible TATA boxes at 112648 and 112650). Restriction endonuclease sites are denoted as follows: D, *Dra*III; R, *Eco*RI; S, *Sal*I.

Both sets of data, obtained by hybridization and by PCR analysis, are consistent and together indicate that part of the putative LAT region lies in a 570-bp region within the internal repeat region and partially overlapping the 3′ end of EHV-1 IE gene 64. Potential promoter elements for the putative LAT are shown about 1 kb upstream of upstream primer F (Fig. 5b). No open reading frames (>10 codons) are present in the putative LAT.

Strand-specific RT-PCR amplification of RNA from equine leukocytes. As a final confirmation, primer pair E was used in an RT-PCR analysis of RNA obtained from PBLs from two slaughtered horses. From previous studies (9) such horses would be expected to have a high probability of harboring latent virus. Preliminary experiments have shown the presence of an interfering 200-bp band in such PCR products. While the nature of this band is unclear, sequence data show no significant similarity with the published EHV-1 sequence (34). Furthermore, it contained a recognition site for restriction endonuclease *Bpm*I that is not present in the region of the EHV-1 genome under investigation. Use was made of this site to maximize the PCR amplification of the EHV-1 300-bp product by treating the first-round PCR product with *Bpm*I. The object of this procedure was to prevent reamplification by subsequent PCR of the extraneous 200-bp product that may be hindering amplification of the expected 300-bp product. Figure 6 shows the result of reamplification of the initial PCR product (40 cycles for each round) either with *Bpm*I as described (panel ii) or without it (panel i). PCR products obtained with cDNA made from the primer complementary to the putative LAT sense strand show a 300-bp band (Fig. 6i, lanes 1 and 3, and ii, lane 4). No PCR products are visible in Fig. 6i, lanes 2 and 4, which show parallel reactions carried out in the absence of reverse transcriptase, indicating that the PCR product derives from RNA.

Using the primer complementary to the IE sense strand (the strand of opposite sense to the putative LAT strand) in the presence of reverse transcriptase (Fig. 6i, lane 5) produces no visible band at the 300-bp level, indicating the absence of IE sequences in cDNA-65.

Data from a single sequencing run with purified 300-bp PCR

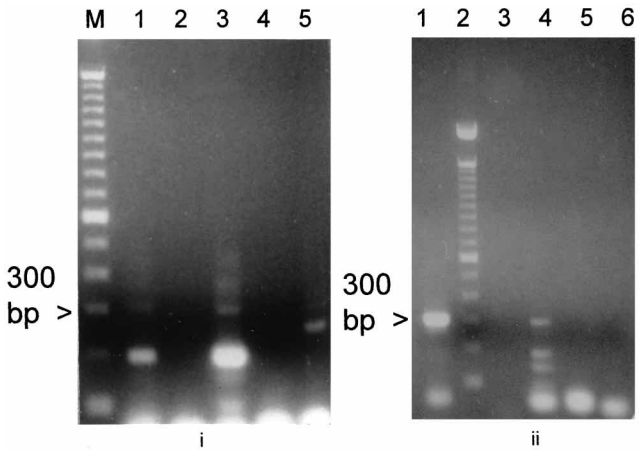

FIG. 6. Strand-specific RT-PCR amplification of RNA from equine leukocytes using primer pair E (see Fig. 5b) and reamplification of the *Bpm*I-digested product. (i) Lane M, 100-bp DNA marker ladder (GIBCO-BRL); lanes 1 and 3, RT-PCR products obtained with the primer complementary to the putative LAT DNA strand by using RNA from two different horses (A and B, respectively); lanes 2 and 4, parallel RT-PCRs in the absence of RT enzyme using RNA from horses A and B, respectively; lane 5, RT-PCR products obtained with the primer complementary to the DNA strand of the opposite polarity to the putative LAT DNA strand by using RNA from horse A. In both horses there is amplification of fragment E but also of a 200-bp fragment. (ii) Lane 1, EHV-1 DNA; lane 2, 100-bp DNA ladder (GIBCO-BRL); lane 3, water; lane 4, reamplified, *Bpm*I-digested RT-PCR product; lane 5, no RNA template in the first-strand cDNA synthesis; lane 6, no cDNA template in first PCR amplification. While the 200-bp fragment has not been eliminated, the expected 300-bp product is clearly visible.

product showed a minimum of 96% identity with EHV-1 DNA in the expected region of the genome.

In view of a recent report locating a putative LAT in a region overlapping the 3′ end of gene 63 (3), we decided to examine cDNA-65 by PCR using primers specific for this region (Fig. 5a). We failed to obtain amplification using cDNA-65 as a template, while purified EHV-1 DNA gave products of the expected size.

In situ hybridization. In order to confirm our findings and to localize the site of latency within lymphoid tissue, in situ hybridization studies were carried out with frozen sections of BLNs removed from two horses randomly selected at an abattoir and three ponies that had been experimentally infected 18 months previously. Frozen sections of TG from the same animals were also examined in this way.

Virus was detected by cocultivation (see Materials and Methods) in BLN from both of the abattoir horses and from two of the three experimental ponies. No virus was detected in TG. No infectious virus was recovered after direct inoculation of indicator cells with cell extracts of BLN or TG, indicating that cocultivation was detecting reactivation of latent virus.

Labelled riboprobes were transcribed from each strand of a 5.3-kb template spanning genes 63 and 64 (construct 7, Fig. 1c) and hybridized to tissue sections as described in Materials and Methods. All BLN sections examined clearly showed the presence of transcripts hybridizing to the riboprobe antisense to the putative LAT strand of viral DNA at a frequency of approximately 10 to 20 foci of hybridization/section. Since each BLN section (Fig. 7) typically contained 7×10^5 cells, this implies an approximate mean frequency of latently infected cells of 2×10^{-5}. Positive cells are predominantly in the parafollicular areas. Negative controls consisting of sections hybridized in parallel with labelled riboprobes in the opposite sense were consistently negative. Similar results were obtained with digoxigenin-labelled riboprobes.

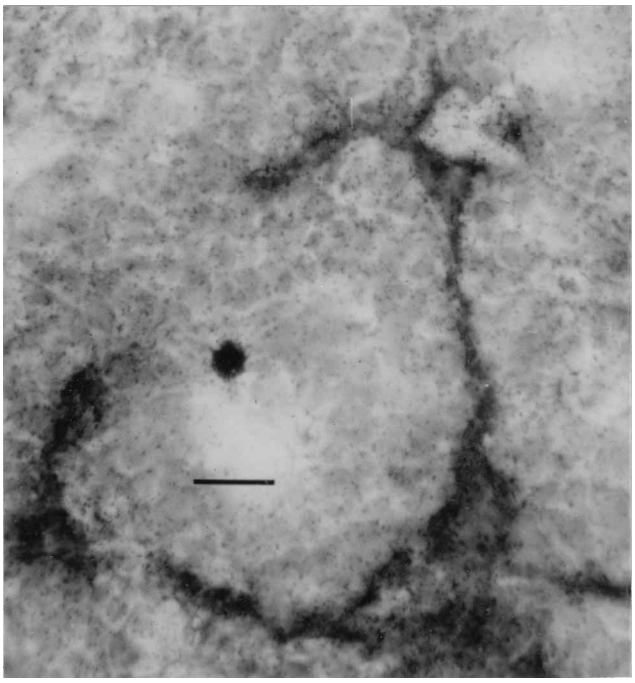

FIG. 7. In situ hybridization of a section of equine BLN. A frozen section of a BLN from a pony experimentally infected with EHV-1 18 months previously is shown. A paracortical lymphocyte shows in situ hybridization with a 5.3-kb EHV-1 subgenomic antisense riboprobe (transcribed from construct 7, Fig. 1c) labelled with ^{35}S. Cocultivation of 10^6 BLN mononuclear cells with RK-13 cells yielded infectious EHV-1 virus particles after three passages. The scale bar represents 10 μm.

EHV-1 transcripts could not be detected in 30 TG sections (approximately 5.5×10^3 neurons).

DISCUSSION

In this paper we have presented three lines of evidence for the occurrence of LATs in equine lymphoid tissue. Analysis of a cDNA library derived from equine leukocyte RNA by both filter hybridization and PCR techniques has localized a putative LAT to a region of the EHV-1 genome antisense to and partially overlapping the EHV-1 IE gene 64 (HSV-1 IE-3 [ICP4] homolog). A PCR product derived from this transcript has been sequenced and found to have ≥96% identity to the published EHV-1 sequence (34). This conforms to the pattern exhibited by other alphaherpesviruses, as discussed below. Using in situ hybridization, we have obtained evidence that EHV-1 establishes latent infection at a frequency of approximately 2×10^{-5} within BLNs. That this is latent infection being detected and not residual low-level expression in lytically infected cells is borne out by the failure to detect specific transcripts in the same transcriptional sense as the IE gene. This is also evidence that genomic viral DNA is not being hybridized or amplified. We failed to detect EHV-1 transcripts in TG neurons. Latent infection with alphaherpesviruses is usually associated with the transcription of a single region of the genome, overlapping and antisense to an IE gene. This is known to be true of bovine herpesvirus 1 (reviewed in reference 26), pseudorabies virus (PRV; suid herpesvirus) (reviewed in reference 12), and feline herpesvirus 1 (FHV-1) (25). Marek's disease virus (MDV) also shows this pattern. Although MDV has been classified as a gammaherpesvirus, its genomic organization is more similar to that of the alphaher-

pesviruses (5, 16, 27); MDV, in common with EHV-1, infects and establishes latency in lymphoid cells (7, 22). In contrast, latent infection with varicella-zoster virus is associated with transcription of large regulatory genes also active in the lytic cycle (reviewed in reference 20). Our characterization of the putative LAT is consistent with the usual alphaherpesvirus pattern in that it overlaps and is antisense to the single EHV-1 IE gene (gene 64), although we cannot rule out the possibility that latency is also associated with transcription from other parts of the genome not investigated here. Where EHV-1 appears to differ from most other alphaherpesviruses is in its ability to establish latent infection in lymphoid tissue, whereas the majority of alphaherpesviruses studied to date are characterized by the capacity to establish latency in neuronal tissue (27, 33). However, it is known that PRV can be routinely isolated from tonsils and lymphoid tissue as well as from sensory ganglia in animals that survive a primary infection (12). In addition, PRV can replicate in a wide variety of nonneuronal cells in culture. More controversially, it is also thought that the virus may be able to establish latent or persistent infections in lymphocytes and their precursors (12). Evidence, albeit inconclusive, that HSV (33) and varicella-zoster virus (20) can establish latency in nonneuronal tissue also exists. In contrast FHV-1 (25) and bovine herpesvirus 1 (26) appear to be exclusively neurotropic. At the other extreme, MDV seems to establish latency solely in lymphoid tissue, latency being associated with malignant transformation of T lymphocytes (7, 22, 23).

Our finding that EHV-1 appears to establish latency in lymphoid tissue is supported by cocultivation studies (6, 10, 11) and current complementary work (29) showing that EHV-1 can be reactivated predominantly from $CD5^+/CD8^+$ leukocytes from both PBLs and BLNs. However, EHV-1 DNA has been detected at a low frequency in neuronal tissue by direct PCR (10, 36). Furthermore, a recently published paper (3) shows that EHV-1 sequences overlapping gene 63 but not gene 64 are present at a low frequency in sections of TG. It is difficult to make a meaningful comparison between the cited study (3) and our work in view of the fact that those authors detect both a different region of the viral genome and a different host cell. This difficulty is compounded by the low frequency of positive neurons: 1 in the 40 sections examined by these workers, a low frequency reflected by in situ hybridization studies reported here and reactivation studies carried out in vitro showing that only approximately 2×10^{-5} leukocytes is latently infected (29). However, the sensitivity of PCR is such that our detection procedure is not appreciably constrained by the low frequency of occurrence of infected cells. Thus, our negative results are probably more meaningful than those in the cited study (3).

Another unusual aspect of the cited study (3) is that EHV-1 gene 63 is not an IE gene (17, 19), as has been shown for HSV-1 (14), PRV (12), FHV-1 (25), and MDV (22, 23). However, this finding is not entirely unprecedented, since the BHV-1 LAT overlaps a gene that is expressed, differentially spliced, at both IE and early times postinfection (26).

An alternative explanation for the discrepancy between the two sets of results is that latency is marked by differential transcription in different tissues. However, if this is so, it would be novel in herpesvirus latency.

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