The latency-related gene of bovine herpesvirus-1 can inhibit the ability of bICP0 to activate productive infection

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Transfection of bovine cells with bovine herpesvirus-1 genomic DNA yields low levels of infectious virus. Cotransfection with the bICP0 gene enhances productive infection and virus yield because bICP0 can activate viral gene expression. Since the latency-related (LR) gene overlaps and is antisense to bICP0, the effects of LR gene products on productive infection were tested. The intact LR gene inhibited productive infection in a dose-dependent fashion but LR protein expression was not required. Further studies indicated that LR gene sequences near the 3' terminus of the LR RNA are necessary for inhibiting productive infection. When cotransfected with the bICP0 gene, the LR gene inhibited bICP0 RNA and protein expression in transiently transfected cells. Taken together, these results suggest that abundant LR RNA expression in sensory neurons is one factor that has the potential to inhibit productive infection and consequently promote the establishment and maintenance of latency.

Bovine herpesvirus-1 (BHV-1) infections cause a significant economic loss to the cattle industry. In addition to clinical symptoms associated with acute infection, abortions, encephalitis, conjunctivitis, respiratory disease, genital disease, pneumonia and immunosuppression can result from infection (Bowland & Shewen, 2000; Jones, 1998; Tikoo et al., 1995). BHV-1-induced immunosuppression leads frequently to secondary bacterial infections, resulting in bronchopneumonia and even death. Increased susceptibility to secondary infection correlates with depressed cell-mediated immunity after infection (Carter et al., 1989; Griebel et al., 1987a, b, 1990). CD8+ T cell recognition of infected cells is impaired by downregulation of major histocompatibility complex class I expression and the transporter associated with antigen presentation (Hariharan et al., 1993; Hinkley et al., 1998; Nataraj et al., 1997). CD4+ T cell function is impaired during acute infection of calves because BHV-1 has the ability to infect these cells and induces apoptosis (Winkler et al., 1999, 2000). Thus, BHV-1 infections have a profound effect on the health of cattle.

Infection in cattle is initiated by productive infection of mucosal epithelium. During productive infection, 70–80 viral genes are expressed temporally. Like other members of the Alphaherpesvirinae subfamily, BHV-1 establishes and maintains a lifelong latent infection in the sensory ganglionic neurons of its host. The only known viral gene that is expressed abundantly in latently infected neurons is the latency-related (LR) transcript (Rock et al., 1987). LR RNA is antisense to bICP0, suggesting that bICP0 expression can be regulated by the antisense nature of the LR RNA. bICP0 is a promiscuous transactivator that can activate all classes of viral genes and is believed to play an important role in the reactivation from latency.

A fraction of LR RNA is polyadenylated and alternatively spliced in bovine trigeminal ganglia, indicating that a subset of LR transcripts is translated into a protein (Devireddy & Jones, 1998; Hossain et al., 1995). A LR protein has been identified (Hossain et al., 1995) that associates with cyclin-dependent kinase 2 (cdk2)–cyclin complexes (liang et al., 1998). LR gene products inhibit S phase entry (Schang et al., 1996) and interfere with chemically induced apoptosis in transiently transfected cells (Ciacci-Zanella et al., 1999). A mutation in the LR gene interferes with virus shedding from the eye during acute infection of calves and inhibits dexamethasone-induced reactivation from latency (Inman et al., 2001a, 2002). Taken together, these studies indicate that LR gene products play an important role during the infection process of cattle. Our long-term goals are to understand the mechanisms by which LR gene products regulate the latency reactivation cycle in cattle.

Herpes simplex virus type 1 (HSV-1) encodes a latency-associated transcript (LAT), which is transcribed abundantly during latency and is antisense to bICP0 (Jones, 1998; Wagner & Bloom, 1997). LAT expression promotes establishment and reactivation from latency in mouse and rabbit models (Maggiojcalda et al., 1996; Perng et al., 1994, 1996, 2000a; Sawtell, 1997; Sawtell & Thompson, 1992; Thompson &
Fig. 1. The LR gene inhibits productive infection in a dose-dependent manner. (A) Partial restriction map of the 2 kb LR gene. The position of bICP0 (Wirth et al., 1989) and the LR RNA that is transcribed during productive and latent infection is indicated (Bratanich et al., 1992; Delhon & Jones, 1997; Schang et al., 1996). The construction and characteristics of pCMV-bICP0 (bICP0), pCMV-LRT (LRTwt) (Inman et al., 2001c), pCMV-LRTstop (LRTstop), pCMV-LRTΔSmal (LRTΔSmal), pCMV-LRTΔHX (LRTΔHX) (Ciacci-Zanella et al., 1999), pCMV-LRTΔPstI (LRTΔPstI) (Jones et al., 1990) and pCMV-LRTΔSphI (LRTΔSphI) (Schang et al., 1996) were described previously. LRTstop is a plasmid that has a deletion between nt 778 and 809 (SphI sites), an oligonucleotide containing three stop codons and a unique EcoRI site inserted between the two SphI sites (the position of the stop codons are denoted by the arrowhead). pCMV-LRTSacI (LRTSacI) was constructed by cloning the 3′ terminal 564 bp region of the LR gene (1373–1937 bp) into the pcDNA3.1− vector (Invitrogen) by standard molecular cloning techniques. All plasmids were purified using caesium chloride purification. (B) Bovine cells (9.1.3) were cotransfected using Superfect (Qiagen), the designated amount of LRTwt, viral DNA prepared from BHV-1 blue virus (1 µg DNA) and the bICP0 plasmid (15–62 ng DNA). Because of the size difference in the plasmid and the BHV-1 genome, this ratio is an approximate 1:1 molecular ratio between BHV-1 DNA and the bICP0 plasmid. This ratio of viral DNA to bICP0 plasmid was optimal for increasing plaque formation and the number cells expressing β-Gal (Inman et al., 2001c). Increasing concentrations of LR plasmids (4:1, 16:1 and 64:1 concentrations of LR plasmid to bICP0) were added to test the effects of LR gene products on bICP0-induced transactivation. A blank expression vector (pcDNA3.1−) was used to maintain equivalent amounts of DNA. At 24 h after transfection, cells were fixed, stained and the number of blue cells counted. The number of cells expressing β-Gal in cultures cotransfected with bICP0 and BHV-1 DNA was set at 100 per cent productive infection efficiency (black bar). This minimized the differences in cell confluence, Superfect lot variation and transfection efficiency. The results are the average of at least three independent experiments.
Sawtell, 1997, 2001). LAT inhibits apoptosis (Ahmed et al., 2002; Inman et al., 2001b; Perng et al., 2000a) and productive infection (Mador et al., 1998), suggesting that LAT and LR gene products have similar functions. In keeping with this observation, a recent study demonstrated that LR gene products can restore spontaneous reactivation to a HSV-1 LAT\(^-\) mutant (Perng et al., 2002).

Since the LR gene and LAT appear to have certain functional similarities, we hypothesized that cells expressing LR gene products would inhibit productive BHV-1 infection. To test this hypothesis, increasing concentrations of a plasmid containing the entire LR gene (LRT\(^{wt}\)) (Fig. 1A) were cotransfected with bICP0 and BHV-1 DNA into bovine epidermal cells (9.1.3). A BHV-1 recombinant that contains the \(\beta\)-galactosidase (\(\beta\)-Gal) gene inserted downstream of the gC promoter in place of the gC ORF (BHV-1 blue virus) was used for this study. BHV-1 blue virus grows to similar titres as wild-type BHV-1. \(\beta\)-Gal expression correlates directly with virus replication because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. A time-point of 24 h after transfection was used to count cells expressing \(\beta\)-Gal to minimize the number of virus-infected cells that resulted from virus spread (data not shown). As reported previously (Inman et al., 2001c), the bICP0 gene alone strongly induced
productive infection when cotransfected with genomic DNA (Fig. 1B). The ability of bICP0 to activate gene expression correlates with stimulating productive infection.

The intact LR gene (LRT\textsuperscript{wt}) inhibited the ability of bICP0 to stimulate productive infection in a dose-dependent fashion and at the highest concentration of LRT\textsuperscript{wt}, a threefold reduction in productive infection was observed (Fig. 1B). Insertion of three in-frame stop codons at the amino terminus of the first ORF (LRT\textsuperscript{stop}) or deletion of the \textit{Smal} site that encompasses splicing sites within the LR gene (LRT\textsubscript{∆}\textit{Smal}) did not relieve repression (Fig. 2A), indicating that expression of a LR protein was not required for repression.

Deletion of the first 523 bp of the LR promoter (LRT\textsubscript{∆HX}) only reduced the level of repression slightly (Fig. 2A). A LR promoter construct that contained approximately 200 nt of LR-coding RNA sequences (Bratanich \textit{et al.}, 1992) (LRT\textsubscript{∆PstI}) did not interfere with bICP0-activated productive infection. Deletion of the \textit{SalI} fragment (986–1777 bp) (LRT\textsubscript{∆SalI}) repressed productive infection, but not as efficiently as LRT\textsuperscript{wt}, LRT\textsuperscript{∆Hx} or LRT\textsubscript{∆Smal}. This suggested that the 163 bp \textit{SalI–SacI} fragment played a role in repressing productive infection. Attempts to clone the 163 bp fragment failed, indicating that the fragment was unstable in \textit{Escherichia coli} (data not shown). A larger construct containing 564 bp of the 3' terminus of LR-coding RNA sequences was cloned (LRT\textsubscript{SacI}) and this construct interfered consistently with productive infection, but not as efficiently as LRT\textsuperscript{wt} (Fig. 2A). In summary, these studies demonstrated that LR RNA sequences located near the 3' terminus of the LR gene played an important role in inhibiting productive BHV-1 infection.

The 9.1.3 bovine cell line is an immortalized bovine epidermal cell line that expresses the simian virus 40 large T antigen (Hegde \textit{et al.}, 1998), a gene that can regulate the cell cycle and apoptosis (Hardwick, 1998). To rule out the possibility that the T antigen influenced the conclusions from Fig. 2A, the ability of the LR gene to interfere with productive infection was examined in bovine foetal lung cells, a low passage cell type. As observed in 9.1.3 cells, LRT\textsuperscript{wt}, LRT\textsubscript{∆SalI}
and LRT5aδ1 repressed bICP0-induced activation of productive infection (Fig. 2B). In contrast, LRT5aδf did not repress bICP0-induced productive infection.

Additional experiments were performed to test whether the LR gene could interfere with bICP0 expression. Cotransfection of 9.13 cells with bICP0 and BHV-1 DNA clearly led to higher levels of bICP0 RNA (Fig. 3A, lane 2) compared to cultures that were cotransfected with BHV-1 DNA and LRT5aδf (Fig. 3A, lane 1). When a plasmid expressing LR gene products (LRT5wt) was cotransfected with the bICP0 plasmid and BHV-1 DNA, lower levels of bICP0 RNA were detected at 24 h after transfection (Fig. 3A, lane 3). As expected, abundant bICP0 RNA was detected in 9.1.3 cells infected with BHV-1 (Fig. 3A, lane 4) but not in mock-infected cells (Fig. 3A, lane 5).

Increasing levels of LRT5wt were cotransfected with BHV-1 DNA to test whether there was a dose-dependent reduction in bICP0 expression (Fig. 3B). At all concentrations of the LRT5aδf plasmid, higher levels of the bICP0 transcript were observed relative to cultures that were cotransfected with LRT5wt and BHV-1 DNA. As expected, cells transfected with the bICP0 plasmid but not LRT5wt expressed a transcript that hybridized with the bICP0 probe.

The effects of LRT5wt on bICP0 protein expression were examined in transiently transfected cells, in the absence of other viral genes. Human 293 cells were used for this study because they can be transfected very efficiently and high levels of bICP0 are expressed in transiently transfected 293 cells (Fig. 3C). A plasmid expressing LRT5wt cotransfected with a bICP0 plasmid expressed lower levels of the bICP0 protein when compared to cultures cotransfected with the bICP0 plasmid and LRT5aδf (Fig. 3C). However, LRT5wt had no effect on the steady-state levels of cdk2. In summary, the studies in Fig. 3 indicated that the LR gene has the potential to inhibit bICP0 RNA and protein expression.

Taken together, these studies indicated that LR RNA repressed productive infection by reducing bICP0 RNA and protein steady-state levels. Furthermore, it was also clear that LR gene products did not completely block productive infection in cultured bovine cells. Since proteins encoded by the LR gene interfere with apoptosis in transiently transfected cells (Ciacci-Zanella et al., 1999) and the latency reactivation cycle in cattle (Inman et al., 2001a, 2002), the LR gene appears to have more than one function. We propose that in the context of neuronal latency, LR gene products and neuronal-specific cellular factors inhibit productive infection and promote neuronal survival by inhibiting apoptosis.

LR RNA and bICP0 have complementary nucleic acid sequences; therefore, the two mRNAs could hybridize causing a reduction in bICP0 expression. Double-stranded RNA (dsRNA) formed from the hybridized mRNAs may be cleaved and degraded through the stimulation of interferon-regulated, dsRNA-activated pathways. The first pathway involves a protein kinase (PKR), which phosphorylates and inactivates the translation factor eIF2, leading to a generalized suppression of protein synthesis and cell death (reviewed by Clemens & Elia, 1997). A second dsRNA-response pathway involves the synthesis of 2′-5′ polyadenylic acid followed by activation of a sequence-non-specific RNase (RNaseL) (Player & Torrence, 1998). Additional antisense effects may result in the reduction in translation initiation or premature termination of transcription.

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References


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