Characterization of microRNAs encoded by the bovine herpesvirus 1 genome

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Bovine herpesvirus 1 (BoHV-1) is a ubiquitous and important pathogen of cattle worldwide. This study reports the identification of 10 microRNA (miRNA) genes, Bhv1-mir-B1–Bhv1-mir-B10, encoded by the BoHV-1 genome that were processed into 12 detectable mature miRNAs as determined by ultra-high throughput sequencing bioinformatics analyses of small RNA libraries and expression studies. We found that four of the miRNA genes were present as two copies in the BoHV-1 genome, resulting in a total of 14 miRNA encoding loci. Unique features of the BoHV-1 miRNAs include evidence of bidirectional transcription and a close association of two miRNA genes with the origin of replication, including one miRNA that is encoded within the origin of replication. The miRNA gene Bhv1-mir-B5 was encoded on the opposite DNA strand to the latency associated transcript, potentially giving rise to antisense transcripts originating from this locus. The association of herpesvirus miRNAs with latency appears to be a common feature in the alphaherpesviruses. Analyses of the BoHV-5 genome for putative miRNA gene orthologues identified a high degree of evolutionary conservation for nine of the BoHV-1 miRNA genes. The possible roles for BoHV-1 miRNAs in the regulation of known BoHV-1 transcription units and the genetics of the BoHV-1 genotypes are also discussed.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is a member of the subfamily Alphaherpesvirinae of the family Herpesviridae of large DNA viruses. The BoHV-1 genome is 135301 bp in length and encodes an estimated 73 open reading frames. BoHV-1 is a major pathogen of cattle throughout the world and is associated with severe and mild respiratory, reproductive, neonatal and dermal diseases in cattle. BoHV-1 has been divided into three genotypes 1.1, 1.2a and 1.2b on the basis of restriction enzyme profiles that appear to correlate with some clinical signs (Engels et al., 1981; Metzler et al., 1985). Subtypes 1.1 and 1.2a have been associated with severe disease and with infection of the foetus, resulting in abortion. To date there has been no specific information to explain the genetic basis of the BoHV-1 genotypes. Nadin-Davis et al. (1996) reported variation in the number of repeat sequences in the 3’ untranslated region of the gene encoding glycoprotein B in a limited number of strains, where genotype 1.1 viruses had fewer C/G repeats compared with type 1.2 viruses. Sequence variations associated with the gene encoding glycoprotein C have also been reported between the BoHV-1 genotypes (Rijsewijk et al., 1999; Esteves et al., 2008).

BoHV-5 is closely related to BoHV-1 and prior to recognition as a separate viral species was classified as genotype BoHV-1.3. The primary clinical distinction between these two viruses is a strong association of BoHV-5 with fatal meningoencephalitis in young cattle (Delhon et al., 2003).

MicroRNAs (miRNAs) are short (20–23 nt) single-stranded RNA molecules that target mRNAs in a sequence-specific manner, leading to either mRNA instability or

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translational repression. Roles for miRNAs have been described in a diverse range of cellular pathways and processes (reviewed by Bushati & Cohen, 2007). In the context of pathogen–host interactions, miRNAs are also implicated in the regulation of the immune system (Lodish et al., 2008; Ghosh et al., 2009).

Since the first identification of miRNAs encoded by Epstein–Barr virus (EBV) (Pfeffer et al., 2004) it has become evident that many viruses utilize miRNA pathways to control viral and host gene expression. To date miRNA genes have been identified in four viral families, herpesviruses, polyomaviruses, adenoviruses and ascoviruses with the majority being identified within the herpesviruses (Umbach & Cullen, 2009; Ghosh et al., 2009).

Several studies have demonstrated that virally encoded miRNAs play important roles in regulating the expression of genes from viruses and their respective hosts (reviewed by He et al., 2008; Ghosh et al., 2009). For example, miRNAs from Kaposi sarcoma-associated herpesvirus (miRNA K12-11) and gallid herpesvirus 2 (GaHV-2) (MDV-miR-M4) are homologues of the human miRNA (miRNA K12-11) and gallid herpesvirus 2 (GaHV-2) (MDV-miR-M4) are homologues of the human miRNA mir-155, which is involved in B- and T-cell regulation. Further, miRNAs encoded by EBV, human cytomegalovirus and human herpesvirus 1 (HHV-1) have been implicated in regulating the immediate-early (IE) phase of the viral gene expression cascade and the characteristic herpesvirus processes of latency (Grey et al., 2007; Grey & Nelson, 2008; Murphy et al., 2008; Lu et al., 2008; Umbach et al., 2008). Two recent studies of GaHV-2 have also demonstrated a role for virus-encoded miRNAs in the molecular basis of herpesvirus pathogenicity (Morgan et al., 2008; Xu et al., 2008).

In this study, we hypothesized that similarly to other members of the family Herpesviridae, BoHV-1 would encode and express miRNAs. Given the close genetic relationship between BoHV-1 and BoHV-5, we also hypothesized that at least some miRNAs identified in BoHV-1 would also be present in the BoHV-5 genome. To validate these hypotheses, we constructed small RNA libraries derived from bovine kidney cells inoculated with BoHV-1 and sequenced them using Illumina’s ultra-high throughput sequencing (UTS) technology. Bioinformatics analyses of the deep sequencing data identified 10 BoHV-1 genes that encode miRNAs. Comparative sequence analyses identified that nine of the BoHV-1 miRNAs were evolutionarily conserved in BoHV-5. The unique features and association of these miRNAs with known BoHV-1 transcription units are also discussed.

RESULTS

Identification of BoHV-1-encoded miRNAs in the deep sequencing data

The Illumina’s UTS analyses produced approximately 5 million high quality sequence reads for each small RNA library, which represented approximately 600 000 unique sequences derived from each sample. To simplify the sequencing data, all sequence reads representing ligated adaptors with inserts shorter than 16 bp were excluded from further analyses. Then identical sequence reads in each small RNA library were grouped and converted into a single sequence tag with an associated number of counts representing the number of individual sequence reads from the library. These steps resulted in the identification of over 300 000 high quality sequence tags for each library. To identify BoHV-1 genomic loci that gave rise to these small RNAs, the sequence tags were mapped onto the BoHV-1 genome sequence using the BLAT software (Kent, 2002). The analyses of sequence tags that did not align to the BoHV-1 genome are reported elsewhere as these represent known and novel bovine miRNAs along with other non-coding RNAs (Glazov et al., 2009).

The BoHV-1-encoded miRNAs represented in the sequencing data were identified using the following criteria: (i) to be considered for further analysis an individual locus had to be supported by at least two independent identical sequence reads originating from one or both of the virus-infected samples; (ii) the loci lacking predicted hairpin-like RNA secondary structures encompassing the sequence tags were eliminated; (iii) predicted miRNA precursor structure and the position of the small RNA sequence had to meet the requirements of a genuine pre-miRNA as described by Ambros et al. (2003).

Following the alignment of the sequence tags to the putative RNA hairpin-like structures encoded within the BoHV-1 genome, 14 loci were identified that were supported by sequence tags and were selected for further analyses. Eight of these loci were encoded with the internal and terminal repeat sequences and therefore represent four unique sequences. The final dataset comprised 10 unique sequences identified as encoding new viral miRNA (Table 1). Two loci had small RNAs originating from both genomic strands, suggesting transcription and processing from both strands giving rise to two mature miRNAs with complementary sequences and potentially different functions. The BoHV-1 mature miRNAs were named bhv1-miR-B1–bhv1-miR-B10 using conventional nomenclature (Ambros et al., 2003). The hairpin structures encoding the mature miRNAs detected by UTS were deemed miRNA genes and are subsequently referred to as Bhv1-miR-B1–Bhv1-miR-B10 (Fig. 1). For Bhv1-miR-B6, we detected sequence tags originating from both arms of the miRNA hairpin precursor, these were named bhv1-miR-B6 and bhv1-miR-B6*, respectively, according to their relative abundance (Table 1). A similar pattern was also observed for Bhv1-mir-B8, except there were similar numbers of small RNA tags derived from both arms of the miRNA hairpin precursor and therefore these were designated bhv1-miR-B8-5p and bhv1-miR-B8-3p, respectively (Table 1), as suggested by Griffiths-Jones et al. (2006). BoHV-1 miRNAs with copies in the internal and terminal repeat sequences were designated 1 and 2, respectively.
As a result of the 100% sequence identity of the miRNA gene copies encoded in the repeat regions the sequencing data does not permit discrimination of the transcription from the individual loci. The secondary structure of the BoHV-1 miRNA precursors and the location of the mature miRNAs identified by UTS are illustrated in Fig. 1(a). A schematic representation of the genomic locations of the BoHV-1 miRNA genes, Bhv1-mir-B1–Bhv1-mir-B10, is illustrated in Fig. 2.

Five additional BoHV-1 miRNA candidates were supported by a single sequence read in the sequencing data (Supplementary Fig. S1, available in JGV Online). Although these miRNA candidates satisfy the general requirement of genuine miRNAs they did not confirm to the filtering criteria used in this study that required at least two independent sequence reads to be present in a dataset and as such were not analysed further.

Conservation within the genus Varicellovirus

To investigate evolutionary conservation of the BoHV-1-encoded miRNAs, the complete herpesvirus genomes from the genus Varicellovirus that infect bovine, horse, pig and human were analysed for highly similar sequences. Viral sequences identified by the similarity searches were then confirmed as putative miRNA candidates by analysis of the predicted RNA structures. The analysis of the genome of BoHV-5 identified nine miRNA candidates that showed conservation in the primary sequence, predicted RNA structure and genomic location compared to BoHV-1, and thus can be considered as potential BoHV-1 miRNA orthologues (Fig. 1b). The percentage identity of these candidate miRNAs sequences varied from 73 to 97% when comparing BoHV-1 miRNA genes to BoHV-5. No homologue for Bhv1-mir-B10 was identified in BoHV-5. The analyses of the herpesvirus genomes from other host species did not return any sequences of sufficient identity according to the applied criteria.

Detection of BoHV-1 miRNAs by Northern blot analysis

To complement UTS-based miRNA detection, the expression of the selected BoHV-1 miRNAs was further investigated by using Northern blot analyses on the small (Table 1). As a result of the 100% sequence identity of the miRNA gene copies encoded in the repeat regions the sequencing data does not permit discrimination of the transcription from the individual loci. The secondary structure of the BoHV-1 miRNA precursors and the location of the mature miRNAs identified by UTS are illustrated in Fig. 1(a). A schematic representation of the genomic locations of the BoHV-1 miRNA genes, Bhv1-mir-B1–Bhv1-mir-B10, is illustrated in Fig. 2.

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**Table 1. Summary of results of the experimental validation of the miRNAs expressed by BoHV-1**

The UTS data were summarized as the number of unique sequencing tags detected at varying m.o.i. at 6 h p.i. The representative sequence for each BoHV-1 miRNA is shown (5’–3’) along with the genomic position in GenBank accession no. AJ004801 sequence. The direction of transcription is shown in parentheses. Northern blot analyses were carried out using the small RNA fractions from bovine cells infected with BoHV-1 (m.o.i. of 10). The RNA molecules detected for the selected BoHV-1 miRNAs are shown as either detected (+), not detected (−) or not done (ND). The qPCR results are also shown for each BoHV-1 miRNAs as either detected (+) or not detected (−) at 12 h p.i. (m.o.i. of 10).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sequence (5’–3’)</th>
<th>Genomic position</th>
<th>Sequence tags</th>
<th>Northern blot analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>m.o.i. 1</td>
<td>m.o.i. 10</td>
</tr>
<tr>
<td>bhv1-miR-B1</td>
<td>ACACGAGAUCCUGGAGACGAG</td>
<td>47722–47743 (+)</td>
<td>0 2</td>
<td>ND ND +</td>
</tr>
<tr>
<td>bhv1-miR-B2</td>
<td>GCCGGCGCAUUGUGGAGACG</td>
<td>67463–67482 (−)</td>
<td>0 4</td>
<td>− − +</td>
</tr>
<tr>
<td>bhv1-miR-B3</td>
<td>CCACGGGUGUGUGUGAGUUGAG</td>
<td>72544–72563 (+)</td>
<td>0 3</td>
<td>− − −</td>
</tr>
<tr>
<td>bhv1-miR-B4</td>
<td>CGCGUACUCAUGGAGGUGUGACG</td>
<td>78653–78673 (−)</td>
<td>0 3</td>
<td>− − −</td>
</tr>
<tr>
<td>bhv1-miR-B5</td>
<td>CUCCGGGGCCUCCGGGACGUGACU</td>
<td>101811–101834 (−)</td>
<td>0 3</td>
<td>ND ND +</td>
</tr>
<tr>
<td>bhv1-miR-B6</td>
<td>AAGUCCGACCGGGGAGACUAGUG</td>
<td>103452–103474 (+)</td>
<td>19 46</td>
<td>+ + +</td>
</tr>
<tr>
<td>bhv1-miR-B6-2</td>
<td>134,744 – 134,766 (−)</td>
<td>134,744–134,766 (−)</td>
<td>3 4</td>
<td>ND ND +</td>
</tr>
<tr>
<td>bhv1-miR-B6-3</td>
<td>134,702–134,724 (−)</td>
<td>134,702–134,724 (−)</td>
<td>2 3</td>
<td>ND ND +</td>
</tr>
<tr>
<td>bhv1-miR-B7</td>
<td>CACGUCCCAGCGGGGAGACU</td>
<td>103452–103474 (−)</td>
<td>134,744–134,766 (−)</td>
<td>47 252</td>
</tr>
<tr>
<td>bhv1-miR-B7-2</td>
<td>CGCGGCGAAAAAUCACAAUUGC</td>
<td>110945–110966 (−)</td>
<td>42 131</td>
<td>+ + +</td>
</tr>
<tr>
<td>bhv1-miR-B8</td>
<td>UAUUGCAUGUUUUGGCGCAAGCGG</td>
<td>110981–111003 (−)</td>
<td>127252–127273 (−)</td>
<td>23 63</td>
</tr>
<tr>
<td>bhv1-miR-B8-3</td>
<td>UUGGGCCAGUGCCAGGAGCCCUG</td>
<td>127056–127076 (−)</td>
<td>11142–11162 (−)</td>
<td>+ + +</td>
</tr>
<tr>
<td>bhv1-miR-B9</td>
<td>ACAUGGCGGACCGUGGUGUGACG</td>
<td>117572–117593 (−)</td>
<td>3 2</td>
<td>− − +</td>
</tr>
</tbody>
</table>

†Duplicated miRNAs in the BoHV-1 genome, as both copies have identical sequences it is not possible to determine from which copy the sequence tags are derived from.
RNA fractions from BoHV-1-infected MDBK cells at 6, 12 and 24 h post-infection (p.i.) and uninfected cells as a control. Hybridizations with a probe specific for bovine U6 small non-coding RNA confirmed uniform loading of RNA extracts for each sample on all Northern blots (data not shown).
Northern blot analyses identified the pre-miRNAs (60 nt) for bhv1-miR-B6, bhv1-miR-B8-5p, bhv1-miR-B8-3p and bhv1-miR-B9 at the time points examined (Fig. 3). The reduced signal observed for bhv1-miR-B8-3p compared with bhv1-miR-B8-5p resulted from lower than expected loading of small RNA on the gel as indicated by U6 hybridization (data not shown). The expression level of the pre-miRNA, however, varied between time points as well as candidate miRNAs. The observed increase in expression of these pre-miRNAs from 6 to 24 h p.i. maybe due to either elevated expression during infection or due to an increased proportion of infected cells. Mature miRNAs (approx. 23 nt) were detected for bhv1-miR-B6 and bhv1-miR-B9-1 (Fig. 3a and d). No hybridization was evident in the RNA extracts from mock-infected cells in all the Northern blots (Fig. 3).

Detection of BoHV-1 miRNAs by quantitative real-time PCR (qPCR)

The expression of the mature miRNAs identified by UTS was also investigated by using qPCR analyses, which validated the expression of all the BoHV-1 miRNAs except for bhv1-miR-B4. While qPCR confirmed the expression of the majority of the mature miRNA, the use of $2^{-\Delta\Delta C_{T}}$ methodology and normalization to uninfected cells prevents comparative analyses being made for the miRNA expression levels and therefore the qPCR results are presented as positive or negative (Table 1).

**DISCUSSION**

This study has confirmed that the BoHV-1 genome encodes and expresses at least 10 miRNA genes as demonstrated by using UTS, computational analyses, Northern blot analysis and qPCR. Four of these genes are encoded in the internal and terminal repeat sequences and
are therefore duplicated, resulting in a total of 14 loci that express miRNAs (Figs 1 and 2).

The Northern blot analyses illustrated the complexity of miRNA expression with three differing profiles being observed in regard to the ratio of the amount of pre-miRNAs to the amount of mature miRNAs detected (Fig. 3). Such expression patterns originating from differential processing and/or stabilization of miRNAs are becoming evident in many systems (reviewed by Winter et al., 2009).

Northern blot and qPCR analyses confirmed the expression of mature miRNAs for bhv1-miR-B6 and bhv1-miR-B9 (Fig. 3a and d). While qPCR also confirmed expression of bhv1-miR-B8-5p and bhv1-miR-B8-3p these miRNAs could not be detected by Northern blot analysis, indicating that these miRNAs maybe overrepresented in the UTS data (Table 1, Fig. 3b and c). Recently, Linsen et al. (2009) have demonstrated bias detection of specific miRNAs when comparing various UTS technologies. Moreover the authors determined that these biases are consistent within a UTS technology, therefore concluding that UTS technologies are still the best method for discovering unknown miRNAs. Though bhv1-miR-B8-5p and bhv1-miR-B8-3p maybe overrepresented in our UTS datasets, the 5p/3p nomenclature has been retained as it is reasonable to conclude that while the number of sequence tags maybe altered if a different UTS technology was used, the ratio of sequence tags would remain the same. These differences in the detection efficiencies highlight the need to investigate miRNA expression using a variety of different technologies.

As described above four of the BoHV-1 miRNA genes are encoded within the repeat regions (Fig. 2), a distribution pattern which has been observed in other herpesviruses and is suggestive of this being a common feature of the subfamily (Yao et al., 2008). To date there have been no studies examining the effects of deleting the miRNAs repeated copies hence the significance of this redundancy, if any, is unknown. For other elements in these repeat regions, such as origins of replication (oriS) and the key viral transcription regulators such as the infected cell protein 0 (ICP0) and the infected cell protein 4 (ICP4) redundancy appears to be important as it is common in many of the herpesviruses.

A unique feature of the BoHV-1 miRNAs was the identification of two highly expressed miRNA genes, Bhv-miR-B8 and Bhv-miR-B9, proximal to the BoHV-1 oriS (Table 1, Fig. 2c). The gene Bhv-miR-B8 was located 74 nt upstream of the oriS, while Bhv-miR-B9 was located within the oriS (Fig. 2c). It is well recognized that the regions surrounding the oriS of herpesviruses are transcriptionally active with multiple-binding sites for transcription factors (Wong & Schaffer, 1991; Nguyen-Huyhn & Schaffer, 1998). It has also been demonstrated that sequential deletion of the putative promoter regions both upstream and downstream of the origin can reduce the level of HHV-1 replication (Isler & Schaffer, 2001). The significance of the proximity of these miRNAs genes to the BoHV-1 oriS remains to be determined. Given the degree of conservation with respect to sequence, function and structure of herpesvirus origins of replication it is considered likely this feature is not unique to BoHV-1. For example, the comparison to BoHV-5 indicates conservation of this miRNA within the oriS between these viruses (Fig. 3). Surprisingly, however, Bhv1-miR-B9 was
the least conserved of the miRNAs that are conserved between BoHV-1 and BoHV-5 with 73% identity (Fig. 1).

This is the first study to experimentally identify mature viral miRNAs, bhv1-miR-B6 and bhv1-miR-B7, which are 100% complementary (Fig. 2c). Previously, partially complementary miRNAs have been described for HHV-1 and murine cytomegalovirus (Buck et al., 2007; Umbach et al., 2008). Based on these examples it would appear that similar to the common property of overlapping coding sequences on sense and antisense strands in herpesvirus genomes also extends to non-coding RNA transcripts as well.

While the identification of BoHV-1 miRNA targets and functions was beyond the scope of this investigation, two miRNAs were closely associated with key viral transcriptional units. The miRNA genes Bhv1-mir-B6-1 and Bhv1-mir-B7 were identified as being antisense and sense to intron 3 of the gene encoding BoHV-1 ICP0 (bICP0) (Fig. 1b). During the IE phase of BoHV-1 gene expression, transcripts encoding bICP0 and BoHV-1 ICP4 (bICP4) are generated through differential splicing to produce IE transcripts IER4.2 and IER2.9 (Wirth et al., 1992). As the BoHV-1 gene expression cascade moves from IE to the early (E) phase expression of the primary transcript is downregulated by an unknown mechanism. The level of bICP0 is maintained by the transcription of the E transcript, ER2.6, for which the promoter resides within intron 3. This example of transcript/promoter switching is unique to BoHV-1 (Wirth et al., 1992). Given the complex transcriptional switching that occurs in this region of the BoHV-1 genome, future studies are required to determine if bhv1-miR-B6 and or bhv1-miR-B7 are involved in the regulation of these transcriptional units.

It has recently been demonstrated that at least three miRNAs play critical roles in gene regulation during latent infections of HHV-1 and HHV-2 (Umbach et al., 2008; Tang et al., 2009). The present study identified a close association of bhv1-miR-B5 with the BoHV-1 latency associated transcript (LAT) (Table 1, Fig. 2b). While LAT and open reading frame E are expressed constitutively during in vitro growth they are the only transcripts expressed during latency (reviewed by Jones et al., 2006). As bhv1-miR-B5 is encoded antisense to the 3' untranslated region of the BoHV-1 LAT this is considered strong evidence that this miRNA is involved in BoHV-1 latency.

Nine of the BoHV-1 miRNA genes demonstrated high conservation with respect to sequence, structure and genomic arrangement when compared to the genome of BoHV-5 (Fig. 1b). Conservation of the genomic arrangements of miRNA genes between herpesviruses sharing the same host have previously been reported, but conservation of miRNA sequences had not been reported (Waidner et al., 2009). The level of conservation between BoHV-1 and BoHV-5 was not unexpected as BoHV-5 was initially considered a subspecies of BoHV-1 and the two viruses exhibit a high degree of nucleotide sequence conservation (Delhon et al., 2003). When comparing the putative BoHV-5 miRNA genes to the BoHV-1 miRNA genes, eight demonstrated over 80% sequence identity (Fig. 1b). In contrast to this, the least conserved miRNA gene was Bhv1-mir-B9 with 73% sequence identity. Such high degrees of sequence conservation, and as a result structural conservation, have not been identified for any other closely related herpesviruses.

BoHV-1 and BoHV-5 share the same host and the primary clinical distinction between these two viruses is the stronger association of BoHV-5 with fatal meningoencephalitis in younger cattle. Given the high genetic similarity between the two viruses it is tempting to speculate that there may be as yet undiscovered BoHV-5-specific miRNAs that are associated with this altered cell and tissue tropism in vivo. Further work is required to determine if the putative BoHV-5 miRNAs identified in this study are expressed and if the genome expresses novel miRNAs.

The current study has utilized virus derived from an infectious clone of a virus strain belonging to the BoHV-1.2b subtype (Mahony et al., 2002). The analyses of the molecular data in this study have utilized the BoHV-1 genomic sequence, which is derived from five BoHV-1 strains of varying genotypes. Morgan et al. (2008) have recently demonstrated that differential expression of specific GaHV-2 miRNAs could be correlated with the in vivo virulence of the infecting virus strain. This indicates that regulatory sequences such as miRNA gene promoters are also important in determining influence of miRNAs in host–pathogen interactions. Thus, future studies on the BoHV-1 miRNAs should also include characterization of those sequence elements that regulate the expression of miRNAs genes, as this could provide insight into the underlying genetics of the BoHV-1 subtypes.

In conclusion this study has demonstrated that BoHV-1 encodes at least 10 miRNA genes. Expression analyses detected 11 mature miRNAs and one miRNA* originating from 14 loci. This study provides the basis for future investigations of the role of these miRNAs in the post-transcriptional regulation of genes encoded by BoHV-1 and its host. Critical to these studies will be the identification of the viral and host targets of these miRNA that will provide insight into understanding the virulence and pathogenesis of this important livestock pathogen.

**METHODS**

**Virus and cells.** The BoHV-1 virus utilized for this study was recovered using the infection clone, pBACBH-37 (Mahony et al., 2002), of the BoHV-1 strain V155 (Snowdon, 1964) as previously described in Madin–Darby bovine kidney (MDBK) cells (ATCC number CCL-22) (Madin & Darby, 1958). The MDBK cells were maintained in Earle's minimal essential medium containing non-essential amino acids, glutamax, 25 mM HEPES and 5% (v/v) fetal calf serum at 37°C in a 5% CO2 atmosphere. All reagents utilized for cell and virus propagation were obtained from Invitrogen.
Preparation of RNA samples. MDBK cells were seeded into flasks 24 h prior to use and infected at approximately 80% confluency with BoHV-1 and incubated at 37 °C. The MDBK monolayers were infected at either low (0.75–1) or high (7.5–10) m.o.i. At 6, 12 or 24 h p.i. monolayers were harvested and total RNA recovered using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was recovered from mock-infected MDBK monolayers for control purposes. The total RNA was resuspended in DEPC-treated water. The RNA was quantified using a NanoDrop spectrophotometer and it was either used immediately or stored at −70 °C until required.

The small RNA fraction (<400 nt) was recovered from total RNA using previously described methods (Mitter et al., 2003). Briefly, polyethylene glycol 8000 and NaCl were added to the total RNA solution and the volume adjusted to give final concentrations of 10% (w/v) and 500 mM, respectively, followed by incubation on ice for 30 min. The samples were centrifuged at 10 000 u for 10 min at 4 °C. The supernatant was transferred to a new microfuge tube and 3 vols of 100% ethanol was added. The small RNA fraction was allowed to precipitate by incubation overnight at −20 °C prior to recovery by centrifugation at 10 000 u for 30 min. The resultant RNA pellet was washed once with 75% ethanol and air-dried. The small RNA pellet was resuspended in de-ionized formamide for electrophoresis prior to Northern blot analyses. The concentration and quality of the small RNA fractions were estimated using a NanoDrop Spectrophotometer and gel electrophoresis.

Sequencing of small RNA libraries. Total RNA (20 μg) isolated 6 h p.i. from MDBK cells either infected (m.o.i. of 1 and 10) or mock infected with BoHV-1 was supplied to GeneWorks Pty Ltd for small RNA libraries construction and subsequent sequence analyses on the Illumina Genome Analyser G1 according to the manufacturer’s instructions. Initial data quality assessment and base calling was done using Illumina’s Data Analysis Pipeline Software v1.0.

Deep sequence data analysis. Data analyses for each library were carried out as described by Glazov et al. (2008) with some modifications. In brief, all identical sequences in the primary dataset were counted and compiled to generate a set of unique sequences with associated read counts, which are referred to as sequence tags in subsequent analyses. After trimming the 3’ adaptor sequence and elimination of sequence reads shorter that 16 bp from the datasets, the sequence tags were mapped onto the BoHV-1 genome sequence (GenBank accession no. AJ004801) using the BLAT software (Kent, 2002). The resulting files containing the genomic coordinates of sequence tags for each small RNA library were converted into .gff (general feature format) files. The annotated BoHV-1 genome sequence and individual .gff files were uploaded into the Argo Genome Browser (V1.0.28 http://www.broad.mit.edu/annotation/argol/) for visualization and further analyses.

To identify putative virus-encoded precursor miRNA (pre-miRNA) all hairpin-like RNA structures that encompassed the small RNA sequence tags were identified using RNAfold (Hofacker, 2003). The sequence and structural features of the predicted hairpin-like structures were further analysed to distinguish genuine miRNA precursors from similar RNA structures encoded in the viral genome. Hairpin-like RNA structures were viewed using VARNA V3 (Darty et al., 2009).

Evolutionary conservation of BoHV-1 miRNA. To examine the evolutionary conservation of BoHV1-encoded miRNAs in other herpesviruses within the genus Varicellovirus, the sequences of the BoHV-1 pre-miRNA hairpin structures were used to search the following herpesvirus genome sequences: BoHV-5 (GenBank accession no. NC_001491), equid herpesvirus 4 (GenBank accession no. NC_001844), suid herpesvirus 1 (GenBank accession no. BK001744) and HHV-3 (GenBank accession no. NC_001348). The search was performed using BLAT with the following parameters: -noHead -minMatch=1 -oneOff=1 -minIdentity=80 -tileSize=8. Sequence alignments covering at least 80% of the length of BoHV-1 pre-miRNA were considered as potential orthologues and used in further RNA secondary structure analyses (Kent, 2002).

BoHV-1 miRNAs. All sequences identified as new miRNA precursors and the most frequently sequenced tags for each mature miRNA were submitted to the miRNA database miRBase (http://www.mirbase.org).

Northern blot analysis. For the detection of pre-miRNA and mature miRNA species by Northern blot analysis, 5–10 μg small RNA fractions were separated on a denaturing 17% polyacrylamide gel containing 7 M urea. The gels were pre-run for 15 min prior to sample loading followed by 8 h electrophoresis at 380 V. After electrophoresis, the RNA was transferred onto positively charged nylon membrane (Boehringer Mannheim) for 1 h at constant voltage (22 V, limited to 1.1 mA) using an electroblot Bio-Rad semi-dry apparatus. The membrane was cross-linked with UV light at 125 mJ on a Bio-Rad GS Gene Linker. Nylon membranes were pre-hybridized with ULTRAhyb-oligo solution (Ambion) at 40 °C for 1 h. The membranes were hybridized overnight with radioactively end-labelled antisense oligonucleotides at 40 °C and then washed twice for 15 min with 2 x SSC/1% SDS at room temperature. The blots were subjected to autoradiography using the film Agfa Curix Ortho HT-G. Antisense oligonucleotide probes complementary to the mature miRNA sequences (Supplementary Table S1, available in JGV Online) were end-labelled using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions. A probe specific for the U6 snRNA was used as a loading control (Supplementary Table S1).

qPCR. qPCR assays were designed and performed as described by Varkonyi-Gasic et al. (2007). Minor modifications to this method included the use of Taqman Mastermix (Applied Biosystems) and the cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min. Cycling and analysis were conducted on a Corbett RotorGene 3000. Oligonucleotides used for these amplifications are shown in Supplementary Table S1. The relative expression of each BoHV-1 miRNA was calculated using the bovine miRNA, bta-miR-25 as the host reference gene and normalized to the uninfected cell extracts using the 2–△△Ct method (Livak & Schmittgen, 2001). The expression of bta-miR-25 was determined using the commercially available assay for human miRNA hsa-miR-25 (Applied Biosystems) as these miRNAs share 100% identity.

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