High-level expression of biologically active bovine alpha interferon by *Bovine herpesvirus 1* interferes only marginally with recombinant virus replication *in vitro*

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An artificial open reading frame (ORF) for bovine alpha interferon (boIFN-α) with the codon preference of *Bovine herpesvirus 1* (BHV-1) glycoprotein B was constructed to assess the effect of expression of boIFN-α by BHV-1 from an expression cassette. Transient expression of the ORF revealed that transfected cells secreted substantial amounts of biologically active boIFN-α, which moderately inhibited replication of BHV-1 after stimulation of bovine cells with 10^6 U ml^-1. The boIFN-α-encoding expression cassette was recombined into the glycoprotein E locus of the glycoprotein E-negative BHV-1 vaccine strain GKD. Cells infected with the resulting recombinant BHV-1/boIFN-α secreted up to 10^7 U boIFN-α per ml cell culture supernatant, which is about 40- to more than 100-fold the activity reached with other virus expression systems. Bioassays demonstrated that the BHV-1-expressed interferon induced a rapid and sustained antiviral state in stimulated bovine cells. Analysis of the *in vitro* growth properties of the recombinant revealed, depending on the cell line used, no or only slight inhibition in direct spreading from cell to cell and a modest delay in virus egress from infected cells. Final titres, however, were comparable to those reached by the parent strain. Penetration into cells was not affected. The results from this study demonstrate that BHV-1/boIFN-α expresses high levels of boIFN-α, grows to high titres in cell culture and thus represents a potential alternative means to deliver endogenously produced boIFN-α *in situ* for a period of time.

INTRODUCTION

The first host cell responses to virus infections include expression and secretion of interferons (IFNs), which lead to an antiviral state in cells and contribute to induction and regulation of the subsequent antiviral immune response. Based on the cellular receptors used for signal transduction and on sequence homology, IFNs are assigned to type I IFN or type II IFN; gamma IFN (IFN-γ) is the sole member of the latter type. Type I IFNs bind to a common receptor and include beta IFN (IFN-β), omega IFN, kappa IFN, tau IFN and, depending on the animal species, up to 24 isotypes of alpha IFN (IFN-α). Binding of type I IFNs to their receptor results in activation of the JAK/STAT pathway, which finally ends in the activation of IFN-α/β-responsive genes (for reviews see Caraglia et al., 2005; Malmgaard, 2004; Stark et al., 1998). Since IFN-α/β rapidly induces an antiviral state within cells, sensitive viruses must have developed mechanisms to prevent the innate antiviral response mediated by IFN-α/β (for reviews see Biron & Sen, 2001; Goodbourn et al., 2000). Different strategies have evolved to achieve this goal. For example, *Vaccinia virus*, the prototypic member of the poxvirus family, expresses an IFN-α/β receptor, the B18R gene-encoded protein, that neutralizes the antiviral effects of extracellular IFN (Alcamì et al., 2000; Colamonici et al., 1995; Symons et al., 1995), whereas paramyxoviruses like *Human* and *Bovine respiratory syncytial virus*, *Simian virus 5* or *Newcastle disease virus* block IFN production and/or IFN-α/β-induced intracellular signalling by the expression of accessory proteins (Didcock et al., 1999a, b; Huang et al., 2003; Schlender et al., 2000; Spann et al., 2004; Valarcher et al., 2003). *Foot-and-mouth disease virus* (FMDV), a picornavirus highly sensitive to IFN-α/β (Sellers, 1963; Chinsangaram et al., 2001), expresses proteases L and 3C that cleave host translation initiation factor eIF-4G (Strong & Belsham, 2004) and thus suppresses translation of IFN-α/β mRNAs (Chinsangaram et al., 1999).

Herpesviruses, although in general less sensitive to IFN-α/β in cell culture than RNA viruses, have also evolved mechanisms to counteract IFN production and IFN-induced host
responses. It has been shown that herpes simplex virus 1 (HSV-1) expresses several proteins that interfere with the innate immune response in vitro (summarized in Barreca & O'Hare, 2004; Chee & Roizman, 2004; Melroe et al., 2004) and in vivo in a mouse model system (Leib et al., 1999).

Because the anti-IFN mechanisms are largely inefficient once cells are primed to the antiviral state by activation of IFN-α/β-stimulated genes, use of IFNs to control or ameliorate the outcome of virus diseases of both humans and animals has attracted considerable interest since the discovery of IFN by Isaacs & Lindemann (1957). In most studies, recombinant purified IFN-α/β was used for local or systemic application. However, their field of application appears limited because of unwanted side-effects and the need for repeated inoculations of high doses due to their short serum half-lives (Bielefeldt Ohmann et al., 1987; Iqbal Ahmed & Johnson, 2003; Jonasch & Haluska, 2001; Samuel, 2001). Although recombinant DNA technology facilitated production of large quantities of pure IFNs, their therapeutic use in animal husbandry would still be too costly. Recently, it was shown that inoculation of replication-defective human adenovirus 5-expressing porcine IFN-α (Ad5-pIFNα) protected pigs from foot-and-mouth disease (Chinsangaram et al., 2003) and delayed and reduced disease signs in cattle after challenge with FMDV (Wu et al., 2003), suggesting that infection with recombinant viruses might be an alternative approach for the delivery of type I IFNs to rapidly induce resistance against virus infections (Chinsangaram et al., 2003). However, to our knowledge only a few additional IFN-α/β-expressing viruses have been reported to date, e.g. replication-competent and replication-incompetent HSV vectors, which lead to secretion of only relatively low IFN activities after infection of cultured cells (Mester et al., 1995; Weir & Elkins, 1993), and recombinant Bovine herpesvirus 1 (BHV-1). The latter, named BHV-1/ gB2FullIFN-α, used glycoprotein B (gB) to express biologically active bovine IFN-α (boIFN-α) as furin-excisable polypeptide (Keil et al., 2005) and IFN-α-activity secreted from BHV-1/ gB2FullIFN-α-infected MDBK cells (about 10^5 U ml^-1) was comparable to the activity reported for MDBK cells infected with Ad5-pIFN-α (Chinsangaram et al., 2003).

BHV-1, a member of the subfamily Alphaherpesvirinae of the family Herpesviridae, is an economically important pathogen of cattle and causes infectious bovine rhinotracheitis and infectious pustular vulvovaginitis. BHV-1 has proven to be a suitable vector for the efficient in vitro and in vivo expression of bovine cytokines and both wild-type virus genomes and vaccine virus genomes have been used for the integration of expression cassettes encoding bovine interleukins-1β, -2, -4, -6 and -12 and bovine IFN-γ (König et al., 2003; Kühnle et al., 1996; Raggio et al., 1996, 2000). To elucidate whether high-level expression of boIFN-α from an expression cassette by recombinant BHV-1 is compatible with efficient virus propagation in cell culture, a prerequisite for in vivo applications, an expression cassette encoding boIFN-α was integrated into the genome of a glycoprotein E (gE)-negative BHV-1 marker vaccine strain. It has been shown here that, although BHV-1 is sensitive to boIFN-α, secretion of up to 10^7 U boIFN-α per ml culture medium of cells infected with the recombinant BHV-1/boIFNα has only marginal effects on virus replication in vitro.

**METHODS**

**Cells and viruses.** Madin-Darby bovine kidney (MDBK) cells were kindly provided by A. Metzler (Zürich, Switzerland) and grown in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin ml^-1 and 100 μg streptomycin ml^-1. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO2. Bovine pharyngeal cell line 244 (KOP/R) and Crandle feline kidney (CRFK) cells (kindly provided by R. Riebe, The Collection of Cell Lines in Veterinary Medicine, Insel Riems, Germany) were maintained under the same conditions. The gE-negative BHV-1 strain GKD, a commercially available vaccine virus (Bovilis IBR marker; Intervet International), has been described in detail previously (König et al., 2003). Vescular stomatitis virus (VSV) strain Indiana was kindly provided by H. Schirmeirer (Insel Riems, Germany).

**Construction and cloning of the synthetic ORF encoding boIFN-α.** The amino acid sequence of boIFN-α subtype C (Velan et al., 1985) was back-translated using the codon preference of gB of BHV-1 and the resulting artificial ORF was assembled from synthetic oligonucleotides by a combination of ligase chain reaction (LCR) and PCR essentially as described previously (Au et al., 1998). First, DNA fragments A and B were generated by combining 30 pmol of each of the oligonucleotides A1–A13 and B1–13 (Table 1), respectively, in 50 μl reaction mixes containing 8 U Pfu DNA ligase (Stratagene) and the supplied reaction buffer. All oligonucleotides except A1, A7, B1 and B7 were 5’-phosphorylated. Oligonucleotides A1–A6 and B1–B6 represent the sense strands; oligonucleotides A7–A13 and B7–B13 represent the respective complementary strands. To create a 25 bp overlap between fragments A and B, the 25 nt at the 3’-end of A6 and the 5’-end of B1 were identical and complementary to A7 and B13, which have the same sequence (Table 1). After 30 cycles of 95° C for 60 s and 55° C and 70° C for 90 s each, LCR products were combined, purified by extraction with phenol and precipitated with ethanol, and used for a fusion PCR using Pfu polymerase (Invitrogen), as recommended by the supplier, in 50 μl reaction volume and 33 cycles of 94° C for 30 s, 53° C for 45 s and 72° C for 90 s. Finally, 5 μl fusion PCR products were used as template for a PCR using Pfu polymerase and primers IFN-α^+ and IFN-x^-, which provided NcoI and NotI cleavage sites for subsequent cloning. The product of the last PCR was purified after 2% agarose gel electrophoresis, cleaved with NcoI and NotI, and integrated into the expression vector pieces (Keil et al., 2005) cleaved with the same enzymes. The resulting plasmid was named pieIFN-α. All cloning procedures were carried out according to standard methods (Sambrook et al., 1989). The correct sequence of the artificial boIFN-α ORF was verified by automated nucleotide sequencing of both strands.

**Electroporation.** Confluent CRFK cells were trypsinized, washed twice with PBS and resuspended in PBS at approximately 1-5 x 10^6 cells ml^-1. Aliquots (200 μl) were mixed with 2-5 μg plasmid DNA and electroporated using an EasyjetT electroporation system (Equibio) and default program G at 300 V. Six aliquots were combined after electroporation and seeded into 50 cm² cell culture dishes. After attachment of the cells, monolayers were washed twice.
Table 1. Sequences of oligonucleotides used in this study

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<th>Oligonucleotides for LCR</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>A2</td>
<td>CCGGATCTGACGCTTGCCACTGCGGCCAACACGACGCTGGAG</td>
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<td>A3</td>
<td>ACGCCCGGCTGATGTCGCTGAGGCGCTGCGGCGCTGAGGCGAGC</td>
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<td>A5</td>
<td>CGGCCAGCGACGCGCTTGACGAGGCGCTGCGGCGCTGAGGCGAGC</td>
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<tr>
<td>A6</td>
<td>CGAGCAGCAGGTACGGCTGACGAGGCGCTGCGGCGCTGAGGCGAGC</td>
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<td>A7/B13</td>
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<tr>
<td>B12</td>
<td>CTCGAGACGCTGTTGGAGCTTGGTGTCACCTCTGGACGACGCTGAGTGGC</td>
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PCR primer*  
IFN-α+      | TAACTGGGCGCTGCGGCGGGAAGC |
IFN-α-      | ATGGCGCCCGGCGTTGGAGCTTCGGCTGCTGGTCGCTGAGTTGCA |

* Cleavage sites for NcoI (IFN-α+) and Ncol (IFN-α-) used for cloning are typed in bold face; artificial ORF sequences are underlined.

with culture medium without FBS and incubated in medium without FBS for 48 h. Culture supernatants were then clarified by low-speed centrifugation and stored at −20 °C until use.

Construction of BHV-1/boIFN-α. To yield recombination plasmid pBIV-α, the artificial ORF encoding boIFN-α was isolated from pieIFN-α by cleavage with NcoI and Ncol. After generation of blunt ends with Klenow polymerase, the purified fragments were ligated into the KpnI-cleaved and blunt-ended GKD transfer vector p6vec (König et al., 2003). This vector was designed to integrate heterologous ORFs into the gE locus of BHV-1/GKD and contains the murine cytomegalovirus (MCMV) e1 promoter (Bühler et al., 1990) followed by a short polylinker sequence with cleavage sites for Ncol (IFN-α+) and Ncol (IFN-α-) used for cloning are typed in bold face; artificial ORF sequences are underlined.

Second dimension gel electrophoresis according to Bühler et al., 1990) followed by a short polylinker sequence with cleavage sites for Ncol (IFN-α+) and Ncol (IFN-α-) used for cloning are typed in bold face; artificial ORF sequences are underlined.
Laemmli (1970) was done using linear gradient gels from 7.5 to 15% acrylamide in Mini Protean slab gel chambers (Bio-Rad). Proteins were visualized by colloidal Coomassie staining (Neuhoff et al., 1988). Protein spots were excised and digested with trypsin using the Montage In-Gel Digest-ZP kit (Millipore) and processed for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Peptide mass fingerprint spectra were obtained on an Ultraflex instrument (Bruker) and processed by flexAnalysis and BioTools software (Bruker). Database queries were performed with MASCOT Server 2.0.0 software (Matrix Science; Perkins et al., 1999) using the MSDB database.

**Immunoprecipitation.** Cells were infected and proteins were metabolically labelled with [35S]methionine and [35S]cysteine. Immunoprecipitation of proteins from cell lysates was performed as described by Fehler et al. (1992) using a monospecific rabbit serum directed against a synthetic peptide representing amino acids Leu910 to Asn928 of the carboxy terminus of BHV-1 gB (anti-gb serum). Labelled proteins were visualized after SDS-PAGE by using a Fuji FLA3000 fluorescence scanner and Aida 2D gel evaluation software.

**Determination of boIFN-α activity.** Secretion of biologically active boIFN-α into the cell culture medium was analysed by a VSV plaque reduction assay. Supernatants from transfected cells were serially diluted in normal cell culture medium and added to KOP/R cells or MDBK cells in six-well plates. Cultures were incubated for 24 h at 37 °C and then infected with approximately 100 p.f.u. VSV. Supernatants were removed 1 h post-infection (p.i.) and semi-solid methylcellulose-containing medium was added. Plaques were counted after 24 h incubation at 37 °C. The dilution of supernatant resulting in a 50% plaque reduction was defined as 1 U boIFN-α activity. Media from infected cells were sterilized by UV-light prior to dilution. Comparison of boIFN-α activity with or without UV-light treatment revealed no differences between the samples (not shown).

**Western blotting.** Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-Mx mAb M143 (kindly provided by O. Haller, Freiburg, Germany) using the SuperSignal West Pico chemiluminescence kit (Pierce) as recommended by the supplier.

**Analyses of cell culture characteristics.** For single-step growth curves, MDBK cultures were infected with 10 p.f.u. per cell. At 2 h p.i., cells were incubated for 2 min with low pH citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3-0) to inactivate extracellular virions (Fehler et al., 1992). Cells were washed twice with cell culture medium and incubated until the times indicated in Fig. 7 when supernatants and cells were harvested and stored at −70 °C. Cells were incubated for 2 min with low pH citrate buffer before harvest. Serial dilutions were titrated on MDBK cells and cultures were incubated under semi-solid medium containing methylcellulose. Plaques were counted after 2 days.

**Determination of plaque diameters.** MDBK cells or KOP/R cells were infected with diluted virus stocks and incubated under semi-solid medium containing methylcellulose for 2 days. Diameters of 100 randomly selected plaques were determined under a microscope using a graduated ocular.

**Penetration kinetics.** MDBK cells were pre-cooled at 4 °C for 30 min and further incubated at 4 °C for 2 h after addition of about 200 p.f.u. of the respective viruses to allow adsorption. Cultures were then shifted to 37 °C and extracellular virions were inactivated at the indicated times by incubation of the monolayers with low pH citrate buffer for 2 min. Cells were washed twice with cell culture medium and incubated under semi-solid medium containing methylcellulose. Plaques were counted after 2 days. Plaque count of untreated cultures was set at 100% penetration.

**RESULTS AND DISCUSSION**

For expression of boIFN-α by BHV-1, an artificial ORF with the codon usage of the BHV-1 gB ORF was assembled from synthetic oligonucleotides. This approach was chosen to create an ORF that is composed of the codons preferred by a highly expressed BHV-1 gene and with a G+C content of 69 mol%, which is close to the G+C content (72 mol%) of the BHV-1 genome (BHV-1 complete genome sequence: GenBank accession no. AJ004801). For transient expression of boIFN-α, the synthetic ORF was integrated into the expression vector pieces (Keil et al., 2005) under the control of the MCMV major immediate early 1 promoter (Dorsch-Häsler et al., 1985). Polyadenylation of transcripts is directed by the polyadenylation signal of the BHV-1 glycoprotein D gene (Kühne et al., 1996). To test for the expression of boIFN-α, the resulting plasmid pieIFN-α or the vector plasmid pieces was transfected by electroporation into CRFK cells. CRFK cells were used because they showed the highest transfection rate (approx. 90% of the surviving cells) among a number of cell lines tested (data not shown). Culture media were harvested 48 h later and clarified by low-speed centrifugation. To identify the boIFN-α molecule, 100 μl transfected cell supernatants were concentrated and analysed by 2D gel electrophoresis. This revealed the presence of an additional protein spot in the pieIFN-α-transfected cell supernatant at pH 8.4 with an apparent molecular mass of 16 kDa (Fig. 1), which corresponded to predictions for boIFN-α subtype C and was not detectable among proteins secreted from pieces-transfected cells (Fig. 1). Peptide mass fingerprint analysis and a subsequent database search identified the protein in the spot circled in Fig. 1 as boIFN-α subtype C with high significance (MOWSE score of 98, 10 positive peptides covering over 50% of the sequence).

Due to the lack of antibodies, the presence of biologically active boIFN-α could not be demonstrated directly. The capability of the pieIFN-α-transfected-cell supernatant to induce Mx protein expression, which is regarded as indicative of the presence of IFN-α/β (Haller et al., 1998), was therefore tested. The supernatant was serially diluted in normal cell culture medium and added to MDBK cells for 24 h. Cells were lysed and proteins were analysed for the induction of Mx protein expression by Western blotting using Mx-specific mAb M143, which recognizes the 78 kDa apparent molecular mass Mx1 protein expressed by MDBK cells (Müller-Doblies et al., 2002). Fig. 2(a) shows that a 106 dilution, which contained the equivalent of about 2 U boIFN-α (see Fig. 2b), was sufficient to induce synthesis of detectable amounts of Mx1 protein, which increases in abundance in parallel with the increase in boIFN-α added. This result agrees well with those reported for rIFN-α-mediated induction of Mx1 protein expression in MDBK cells (Müller-Doblies et al., 2002). Mx1 protein expression was not detected after incubation of MDBK cells with culture medium from pieces-transfected cells (not shown). Additional signals in Fig. 2(a), originating from lower molecular mass proteins and generated by non-specific
Fig. 1. Identification of boIFN-α in culture medium from cells transfected with pieIFN-α. Concentrated culture media from CRFK cells transfected by electroporation with pieIFN-α (right) or the vector plasmid piecas (left) were analysed by 2D gel electrophoresis. Proteins were visualized by Coomassie staining. For clarity, parts of the gels containing an additional protein of the predicted size of boIFN-α (seen only on the right) are enlarged. The protein contained in the circled spot was subjected to peptide mass fingerprint analysis.

Fig. 2. (a) Induction of Mx protein expression. MDBK cells were incubated with normal culture medium (M) or with serially diluted pieIFN-α-transfected CRFK culture medium for 24 h as indicated. Cells were harvested and proteins were analysed by Western blotting using mAb M143 for detection of bovine Mx1 proteins (boMx1). Note that the image of the Western blot was electronically compressed. (b) Secretion of antiviral activity from cells transfected with pieIFN-α. Culture media from CRFK cells transfected by electroporation with pieIFN-α were clarified by low-speed centrifugation and the supernatants (triangles and circles) or rIFN-α as a control (squares and stars) were serially diluted and added to confluent MDBK cells (circles and squares) or KOP/R cells (triangles and stars). Approximately 100 p.f.u. VSV was added to each culture 2 h later. At 1 h p.i., the inoculum was replaced by semi-solid methylcellulose-containing medium and plaques were counted at 24 h p.i. Plaque counts of untreated cells were set as 0 % plaque reduction. (c) Sensitivity of BHV-1 to boIFN-α. MDBK cells (filled bars) and KOP/R cells (open bars) were incubated with or without 10^4 U boIFN-α per ml culture medium, infected with dilutions of BHV-1–GFP 24 h later and incubated under methylcellulose-containing medium. At 2 days p.i., diameters of 100 autofluorescent plaques were measured using a graduated ocular and a fluorescence microscope. Mean diameters are shown in arbitrary units.
BHV-1 is substantially inhibited in boIFN-α-treated cells. Additional experiments revealed that the plating efficiency was 90% reduced by boIFN-α pre-treatment, which also resulted in about a 100-fold decrease in virus yield 24 h after infection at an m.o.i. of 0·1 (data not shown). These results showed that BHV-1 replication in MDBK and KOP/R cells is sensitive to boIFN-α, which is in good agreement with earlier reports on the susceptibility of BHV-1 to bacterially expressed bovine IFN-α (Bielefeldt Ohmann et al., 1984; Babiuk et al., 1985). In contrast to replication of VSV, which is severely inhibited, replication of BHV-1 is less sensitive to the antiviral effect of boIFN-α. In comparison to the alphaherpesvirus HSV-1 (Lipp & Brandner, 1985; Mossman et al., 2000; Nicholl & Preston, 1996), however, IFN-α sensitivity of BHV-1 appears to be more pronounced. It should be considered, however, that these differences in sensitivity might be due to the specific cells and IFN preparations used in the respective studies.

Recently, Abril et al. (2004) reported that efficient replication of wild-type BHV-1 in mice was only achieved in animals that lacked a functional IFN-α/β system, which contrasts with the situation in cattle and indicates that BHV-1 encodes species-specific functions that enable virus replication following an IFN response in the natural host but are not functional in mice. Indirect evidence led Geiser et al. (2005) to suggest that bICP0 is the major viral protein involved in inhibition. Whether bICP0 alone and/or other BHV-1 gene products contribute to blocking of the IFN response needs to be clarified.

To elucidate whether expression of boIFN-α by BHV-1 influences replication of BHV-1 in vitro, plasmid pf6recIFN-α was co-transfected with purified genomic DNA of BHV-1/GKD. BHV-1/GKD was chosen as the progenitor strain to provide the same genetic background for animal experiments used with the previously tested bovine cytokine-progeny virions by dot-blot hybridization and plaque-purified to homogeneity as described previously (König et al., 2003). The recombinant, BHV-1/boIFN-α, was identified among the progeny virions by dot-blot hybridization and plaque-purified to homogeneity as described previously (König et al., 2003). To test whether antiviral activity was released from BHV-1/boIFN-α-infected cells, KOP/R cells in 24-well culture dishes were infected at an m.o.i. of 10 with purified BHV-1/GKD or BHV-1/boIFN-α. Culture media were collected at 2, 4, 6, 8, 24 and 48 h p.i., sterilized by UV irradiation after ultracentrifugation and tested for antiviral activity by a VSV plaque reduction assay. The results are shown in Fig. 3. Antiviral activity was observed already at 2 h p.i., increased until 24 h p.i., and remained constant until 48 h (for clarity, the curve for 48 h p.i. was not included in Fig. 3). No antiviral activity was detected when supernatants were collected immediately after infection with BHV-1/boIFN-α and in the supernatants of parallel cultures infected with BHV-1/GKD (data not shown), demonstrating that the antiviral activities observed at early times p.i. were not due to residual boIFN-α within the BHV-1/boIFN-α virus stock or to induction of IFN by BHV-1.

To verify that boIFN-α was secreted from cells infected with BHV-1/boIFN-α, 100 μl culture medium from cells infected with BHV-1/boIFN-α or BHV-1/GKD for 24 h was concentrated and analysed by 2D gel electrophoresis. Three proteins with apparent molecular masses of about 16 kDa and isoelectric points around pH 8·4 were detected only in the medium of BHV-1/boIFN-α-infected cells (Fig. 4) and analysed by peptide mass fingerprint and subsequent database search. No significant matches were found for the two less abundant proteins (marked by arrowheads in Fig. 4), whereas the protein in the spot marked by an arrow was identified as boIFN-α subtype C, proving that BHV-1/boIFN-α-encoded boIFN-α is secreted from infected cells. Kinetics of secretion of boIFN-α from BHV-1/boIFN-α-infected cells was monitored in comparison to intracellular transport of BHV-1 gB by pulse–chase experiments (Fig. 5). KOP/R cells were infected with BHV-1/GKD or BHV-1/boIFN-α, incubated for 30 min with [35S]methionine/[35S]cysteine and then chased with normal cell culture medium for the times indicated in Fig. 5 (0–90 min) when supernatants and cells were harvested. Secreted proteins were precipitated with acetone prior to PAGE and labelled cellular proteins were immunoprecipitated from cell lysates with a polyclonal rabbit anti-gB serum raised against the carboxy terminus of gB (Keil et al., 2005). Intracellular transport and processing of the 117 kDa gB precursor molecules to the 130 kDa form, which is then cleaved into the respective NH2- and COOH subunits, were comparable for gB of BHV-1/GKD and BHV-1/boIFN-α, indicating that expression of boIFN-α does not interfere with intracellular transport processes (Fig. 5b). In the supernatants of
BHV-1/boIFN-α-infected cells, two proteins with slightly different apparent molecular masses of about 16 kDa became visible after 15 min chase and both increased in abundance until 90 min chase (Fig. 5a). It is currently not clear whether these proteins represent isoforms of mature boIFN-α, generated for example by differential intracellular processing, since the fate of the boIFN-α precursor during intracellular transport could not be followed. It is assumed that the slightly slower migrating molecules are the same as have been detected after 2D gel electrophoresis at a more neutral isoelectric point (Fig. 4, closed arrowhead) and could not be identified by mass spectrometry. The differences in the relative abundances of these two proteins at early and late times after infection could indicate that the slower migrating protein is less stable or that its relative expression level increased later in the infection. The appearance of boIFN-α in the culture medium after 15 min chase is significantly faster than formation of the secreted form of BHV-1 glycoprotein G, which became detectable only after 60 min chase (Keil et al., 1996). Thus, the results of the pulse–chase experiments demonstrate that BHV-1-expressed boIFN-α is efficiently transported and secreted from infected cells without apparently affecting transport and maturation of BHV-1 gB (Fig. 5b) or BHV-1 gD (data not shown), which are both essential for BHV-1 penetration and cell-to-cell spread.

To analyse the efficacy of BHV-1/boIFN-α-expressed boIFN-α, KOP/R cells were incubated with the serially diluted supernatant from BHV-1/boIFN-α-infected cells, which was also used for 2D gel electrophoresis. Cultures were washed twice with normal culture medium 1, 4 or 24 h later and infected with approximately 100 p.f.u. VSV. Plaques were counted 24 h p.i. (Fig. 6a). Induction of the antiviral state of the KOP/R cultures had already started after 1 h incubation and was nearly complete after 4 h. The

![Fig. 4. Identification of boIFN-α in supernatants from cells infected with BHV-1/boIFN-α. Concentrated culture media from KOP/R cells infected with BHV-1/boIFN-α (left) or the parent strain BHV-1/GKD (right) were analysed by 2D gel electrophoresis. For clarity, parts of the gels containing additional proteins of the predicted size of boIFN-α (left) are enlarged. Proteins subjected to peptide mass fingerprint analysis are marked: arrowheads indicate proteins that could not be identified; the arrow denotes the protein spot containing boIFN-α subtype C.](http://vir.sgmjournals.org)

![Fig. 5. Rapid secretion of boIFN-α into the culture medium of BHV-1/boIFN-α-infected cells. KOP/R cells were infected with BHV-1/GKD (left) or BHV-1/boIFN-α (right) at 10 p.f.u. per cell and proteins were labelled with [35S]methionine/[35S]cysteine at 6 h p.i. for 30 min, washed and further incubated with normal cell culture medium as indicated. (a) Proteins from the respective supernatants were precipitated with 3 vols acetone for 30 min at −70°C after the addition of 2.5 μg BSA. Precipitated proteins were pelleted by centrifugation, washed in 80% ethanol, resuspended in sample buffer and separated by 12.5% SDS-PAGE. (b) Cell lysates were incubated with polyclonal anti-gB serum and immunoprecipitated proteins were separated by 7.5% SDS-PAGE. Positions of the gB precursor (pgB), uncleaved gB (gB), and the NH2- and COOH-subunits of cleaved gB are shown on the left. Apparent molecular masses of the proteins are indicated in kDa on the right.](http://vir.sgmjournals.org)
antiviral activity of this preparation, determined after 24 h incubation, corresponded to about 10^7 U boIFN-α ml^{-1}. Maintenance of the antiviral state of the cultures was determined by incubation of KOP/R cells with the same dilutions as above for 24 h. Cells were washed twice with normal culture medium and incubated further for 8, 28 and 72 h. Approximately 100 p.f.u. VSV was then added to the cultures and plaques were counted 24 h later (Fig. 6b). The remarkable resistance to VSV plaque formation at 72 h after removal of boIFN-α from the cells indicates that reversal from the antiviral state is a slow process in bovine cells, which is in good agreement with the sustained up-regulation of the Mx1 gene in MDBK cells after stimulation with rIFN-α (Müller-Doblies et al., 2002). It should be noted that nearly identical results were obtained using boIFN-α from transfected CRFK cells (data not shown), indicating that the biological activity of boIFN-α is not significantly affected by expression of BHV-1. A surprising result was the high expression level of boIFN-α, which greatly exceeds the IFN-α/β activities induced by other virus vectors (Chinsangaram et al., 2003; Mester et al., 1995; Weir & Elkins, 1993). Although not addressed experimentally, it is hypothesized that this may at least be partly due to the use of the codon-adapted ORF, which makes the boIFN-α mRNAs similar to authentic BHV-1 transcripts. Experience with the efficient expression of other codon-adapted ORFs by BHV-1 (König et al., 2002; Kühnle et al., 1998; Schmitt et al., 1999) supports this assumption.

To test for the influence of boIFN-α-expression on the cell culture characteristics of the BHV-1 recombinant, single-step growth and plaque formation were analysed. BHV-1/GKD and BHV-1/boIFN-α penetrated comparably into MDBK cells (data not shown), suggesting that the formation of infectious virions was not impaired. Single-step growth was analysed on MDBK cells after infection of cultures with BHV-1/GKD or BHV-1/boIFN-α at an m.o.i. of 10. Non-penetrated virions were inactivated by low pH treatment at 2 h p.i. and infectivity in cells and supernatants was determined at the times given in Fig. 7(a). In contrast to cell-associated infectivity, which showed comparable kinetics for both virions, release of BHV-1/boIFN-α appeared delayed since it took about 24 h until extracellular infectivity surpassed the titre of intracellular virions, whereas BHV-1/GKD reached this point approximately 8 h earlier. Nevertheless, final titres in the culture supernatants at 32 h p.i. were comparable. This result may indicate interference of boIFN-α-activity with the egress of BHV-1. However, similar delayed release of infectious

![Fig. 6. Rapid and sustained induction of the antiviral state of KOP/R cells treated with BHV-1/boIFN-α]-expressed boIFN-α. The culture medium from KOP/R cells infected with BHV-1/boIFN-α was clarified by ultracentrifugation and sterilized by UV-irradiation, serially diluted and added to confluent KOP/R cells. (a) Cultures were washed 1 h (triangles), 4 h (squares) or 24 h (dots) later and approximately 100 p.f.u. VSV was added to each culture. At 1 h p.i., the inoculum was replaced by semi-solid methylcellulose-containing medium and plaques were counted at 24 h p.i. (b) Cultures were infected for 24 h, washed and further incubated with normal cell culture medium for 8 h (diamonds), 28 h (asterisks) or 72 h (stars) prior to the addition of approximately 100 p.f.u. VSV to each culture. The inoculum was replaced by semi-solid methylcellulose-containing medium at 1 h p.i. and plaques were counted at 24 h p.i. Plaque counts of untreated cells were set as 0% plaque reduction.

![Fig. 7. Cell culture characteristics of BHV-1/boIFN-α. (a) Growth curves. MDBK cells were infected with BHV-1/GKD (squares) and BHV-1/boIFN-α (circles) with 10 p.f.u. per cell. Cells were treated with low pH buffer to inactivate non-penetrated virions at 2 h p.i. Cells (closed symbols) and supernatants (open symbols) were harvested at the times indicated and titrated on MDBK cells. Cultures were overlaid with semi-solid methylcellulose-containing medium and plaques were counted 2 days later. Arrows indicate the time point when extracellular BHV-1/GKD (closed arrow) or BHV-1/boIFN-α (open arrow) infectivity surpassed the respective intracellular infectivity. Results shown represent typical experiments. (b) Plaque size determination. KOP/R cells and MDBK cells were infected with dilutions of BHV-1/GKD (closed bars) or BHV-1/boIFN-α (open bars) and incubated under semi-solid methylcellulose-containing medium. At 2 days p.i., diameters of 100 plaques for each virus were measured using a graduated ocular. Mean diameters are shown in arbitrary units. Plaque sizes of the parent strain BHV-1/GKD were set as 100%.
virions from recombinant BHV-1-infected cells was also observed with other BHV-1 recombinants expressing heterologous viral glycoproteins or cytokines (Keil et al., 2005; Kühnle et al., 1998; Schmitt et al., 1999; G. M. Keil, unpublished results). The delay might therefore be due to the association of newly synthesized (glyco)proteins with cellular compartments involved in BHV-1 glycoprotein processing and viral envelope formation.

Determination of the sizes of plaques formed under a methylcellulose-containing semi-solid medium revealed that on KOP/R cells BHV-1/boIFN-α induced smaller plaques that reached approximately 70% of the BHV-1/GKD-induced plaque diameters (Fig. 7b). No significant differences between the plaque sizes were observed on MDBK cells, which correlates with the lower responsiveness of MDBK cells to boIFN-α in comparison to KOP/R cells (Fig. 2c).

In conclusion, it has been shown that BHV-1 is a suitable vector for the expression of high levels of boIFN-α. Secretion of up to 10^7 U ml^-1 into recombinant virus-infected culture medium did only marginally affect in vitro growth of the recombinant, although BHV-1 appears moderately sensitive to the antiviral effect induced by boIFN-α in target cells prior to infection. In a pilot experiment aimed to test for the safety in cattle, it was observed that antiviral activity in sera increased temporarily to about 1000 U ml^-1 after infection with BHV-1/boIFN-α, which apparently did not interfere with virus replication since the titres in nasal swabs were comparable to those obtained in earlier studies using BHV-1 mutants with the same genetic background (unpublished results; König et al., 2003). Thus, BHV-1/boIFN-α constitutes a good candidate for the delivery of boIFN-α in vivo in particular situations where a rapid and sustained induction of an antiviral state is needed to support disease control.

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REFERENCES


C. Höhle and others


Strong, R. & Belsham, G. J. (2004). Sequential modification of translation initiation factor eIF4G1 by two different foot-and-mouth...


