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Research article

Bovine herpesvirus 4 immediate early 2 (Rta) gene is an essential gene and is duplicated in bovine herpesvirus 4 isolate U

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ABSTRACT

The ORF50/*Rta* gene has been shown to be an essential gene for many gammaherpesviruses. Although the BoHV-4 ORF50/*Rta* homolog, immediate early gene 2 (IE2), has been shown to activate several BoHV-4 early and late promoters in cotransfection assays, there is no direct proof of its indispensability for progression of the virus to the lytic replication cycle in the context of the viral genome. In the present communication, replication defective BoHV-4-V.test IE2 mutants were efficiently rescued, with respect to production of infectious virus and DNA replication, upon the expression of BoHV-4 ORF50/*Rta* in *trans*. Surprisingly, in the course of our studies, we discovered that the IE2 gene is duplicated in the genome of BoHV-4-U.

1. Introduction

Herpesvirus immediate early (IE) genes do not require prior viral protein synthesis for their expression because their expression is activated by host cell transcription factors, already present at the moment of infection, and are thus expressed "immediately" during cell infection (Staudt and Dittmer, 2007). BoHV-4 IE2 protein (homologous to Epstein-Barr virus replication and transcription activator, Rta, and thus designated BoHV-4 Rta) encoded by open reading frame 50 (ORF 50) is well conserved among gammaherpesviruses (Zimmermann et al., 2001). BoHV-4 IE2 RNA is a spliced, 1.8 kb RNA, which is transcribed from left to right on the restriction map of the BoHV-4 genome from DNA contained in the 8.3 kb *Hin*dIII fragment F, and is the less abundant of two IE RNAs encoded by BoHV-4 (van Santen, 1991, 1993). The predicted amino acid sequence of the protein encoded by IE2 RNA reveals that it could encode a 61-kDa protein with amino acid sequence homology to the Epstein-Barr virus (EBV) transactivator R and its homologs in other gammaherpesviruses, including herpesvirus saimiri (HVS), equine herpesvirus 2 (EHV-2), murine herpesvirus 68 (MHV-68), rhesus rhadinovirus (RRV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Studies of MHV68, KSHV and RRV demonstrated that the expression of ORF50/*Rta* is essential for viral replication. Recombinant viruses in which ORF50 was deleted or inactivated by a premature stop codon within its open reading frame were replication-defective, but were efficiently rescued, with respect to production of infectious virus and DNA replication, upon the expression of ORF50/*Rta* in *trans* (Pavlova et al., 2003; Xu et al., 2005; Zhou et al., 2010). Transactivation studies have shown that BoHV-4 Rta specifically transactivates expression of reporter genes linked to the promoter regulatory regions of all BoHV-4 early (E) and late (L) genes examined, including those encoding thymidine kinase and the BoHV-4 homologue of the herpes simplex virus type 1 (HSV-1) major DNA

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binding protein (Bermudez-Cruz et al., 1997, 1998; van Santen, 1993; Zhang and van Santen, 1995). BoHV-4 Rta expression plays a primary role in initiating viral lytic replication, not only during reactivation of latently infected non-permissive cells but also during *de novo* infection of permissive cells (Donofrio et al., 2004; van Santen, 1993). These results indicate that ORF50/*Rta* is essential for lytic viral reactivation and transactivation of viral genes contributing to DNA replication. Although the BoHV-4 IE2 gene has been well characterized in terms of gene structure, transcription and RNA post transcriptional processing (van Santen, 1993), it has not been demonstrated that the expression of ORF50/*Rta* is required for progression to the lytic replication cycle in the context of the viral genome. One study focused on the effects of exogenous expression of BoHV-4 ORF50/*Rta*, and demonstrated the activation of BoHV-4 lytic replication in a non-permissive cell line by over-expression of ORF50/*Rta* (Donofrio et al., 2004), beginning to demonstrate the direct role of BoHV-4 ORF50/*Rta* for the progression of the virus into the lytic cycle. The cloning of different isolates of BoHV-4 genome as bacterial artificial chromosomes (BAC) (Donofrio et al., 2008, 2009; Gillet et al., 2005) allowed, in the present work, the generation of viral mutants targeting specific parts of the IE2 locus within the BoHV-4 genome, which provided further evidence that BoHV-4 ORF50/*Rta* has a direct role in initiating and allowing progression of BoHV-4 lytic replication.

2. Materials and methods

2.1. Cells and stable cell lines generation

Bovine Embryo Kidney [(BEK) from M. Ferrari, Istituto Zooprofilattico Sperimentale, Brescia, Italy], and BEK/*cre* cell lines (Donofrio et al., 2008) were cultured in Eagle's Minimal Essential Medium (EMEM) (Lonza) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin (SIGMA) and 100 μg/ml streptomycin (SIGMA). BEK/IE2 were cultured in Eagle's minimal essential medium (EMEM) (Lonza) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 IU/ml penicillin (SIGMA). For establishment of stable IE2-expressing cell lines, confluent BEK in a 75 cm^2 flask were detached with trypsin, washed with Dulbecco's modified essential medium/high (D-MEM/high), and transfected with p2xCMVeIE2*neo*, pCMV-ORF50-IRES-*neo*, or pCMV-ORF50-IRES-*hygro*. DNA plasmids (15 μg) in 500 μl D-MEM/high without serum were electroporated (Equibio apparatus, 270 V, 960 μF, 4-mm gap cuvettes) into BEK cells; electroporated cells were immediately returned to a 25 cm^2 flask in complete EMEM with FBS. A control BEK 25 cm^2 flask was also seeded with untransfected cells for every transfection. Cells were incubated at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Six hours after electroporation cells were subjected to selection by specific antibiotics: BEK transfected with p2xCMVeIE2*neo* were selected in 700 μg/ml of G418, BEK transfected with pCMV-ORF50-IRES-*neo* with 900 μg/ml of G418, and BEK transfected with pCMV-ORF50-IRES-*hygro* with 200 μg/ml of Hygromycin B; control untransfected BEK were subjected to the same selection as the transfected ones. These concentrations of selective antibiotics resulted in death of all the control cells. Cells were sub-cultured to a fresh culture vessel when growth reached 70–90% confluence (i.e., after 15 days) and seeded in a new flask; single colonies were recovered and screened by RT-PCR for the presence of IE2 gene. Stable cell lines containing p2xCMVeIE2*neo* were named BEK/IE2, and those containing pCMV-ORF50-IRES-*hygro* or *neo* were named BEK/ORF50 (*hygro* or *neo*). They were maintained in complete EMEM complemented with the same concentrations of antibiotics used for selection.

2.2. Viruses

BoHV-4-U, BoHV-4-U/IE2pr, BoHV-4-UΔORF50, BoHV-4-V.test, BAC-BoHV-4-V.test/IE2pr, and BAC-BoHV-4-LVRΔORF50 were propagated by infecting confluent monolayers of BEK, BEK/IE2 or MDBK cells at a multiplicity of infection (m.o.i.) of 0.5 50% tissue culture infectious doses ($TCID_{50}$) per cell and maintained in Eagle's minimal essential medium (EMEM) (Lonza) with 2% FBS for 2 h. The medium was then removed and replaced by fresh EMEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited CPE (approximately 72 h post-infection), the virus was prepared by freezing and thawing cells three times and pelleting the virions through 30% sucrose, as described previously (Donofrio et al., 2006). Virus pellets were resuspended in cold MEM without FBS. $TCID_{50}$ were determined with BEK, BEK/IE2, and BEK/ORF50 cells by limiting dilution.

2.3. Plasmid constructs

pCMV-ORF50-IRES-*neo* or *hygro* were obtained by subcloning a 1669 bp *Nhe*I/*Bam*HI fragment, amplified by PCR from BoHV-4 DNA using a forward primer containing an *atg* translational initiation codon and matching the beginning of the ORF 50 second exon (Full-IE2-sense-*Nhe*I, Table 1), and a reverse primer matching the end of the second exon including the *stop* codon (Full-IE2-anti-*Bam*HI, Table 1), into the Multiple Cloning Site (MCS) of bicistronic vectors pIREShyg2 or pIRESneo2 (Clontech).

pIE2*neo* was obtained by subcloning the 3442 bp *Eco*RI/*Pst*I fragment of BoHV-4 IE2 (van Santen, 1993) in pEGFP-C1, used as a vector backbone containing a *neo* resistant cassette for G418 drug selection, cut with *Eco*RI and *Pst*I.

p2xCMVeIE2*neo* was obtained by subcloning first the 231 bp *Nde*I/*Bgl*II fragment of hCMV enhancer promoter generated from pEGFP-C1 by PCR, using the primer pair *Nde*I-hCMV-sense/*Bgl*II-hCMV-anti (Table 1), then the 438 bp *Bgl*II/*Hin*dIII fragment of hCMV enhancer promoter generated from pEGFP-C1 by PCR, using the primer pair *Bgl*II-hCMV-sense/*Hin*dIII-hCMV-anti, in pIE2*neo* (Table 1).

pIE2prKanaGalKIE2pr was obtained by subcloning first a 593 bp *Eco*RI/*Pst*I fragment of BoHV-4 IE2 (TargI-sense/Anti I amplicon, Table 1, Accession number: NC002665) in pTZΔlinker KanaGalk vector, cut with *Eco*RI and *Pst*I, and then a 932 bp *Kpn*I/*Hin*dIII fragment (Sense II amplicon/Anti II amplicon, Table 1), into the vector, cut with *Kpn*I and *Hin*dIII.