"But I being poor, have only my dreams,

I have spread my dreams under your feet,

tread softly because you tread on my dreams".

W.Yeats

UNIVERSITÀ DEGLI STUDI DI PARMA

Department of Biochemistry and Molecular Biology

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PhD course in

Biochemistry and Molecular biology

BOVINE HERPESVIRUS 4 IMMEDIATE EARLY 2 GENE

IS ESSENTIAL AND CAN BE DUPLICATED

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- ✓ *Bovine herpesvirus 4 immediate early 2 (Rta) gene is an essential gene and is duplicated in bovine herpesvirus 4 isolate U*. **Franceschi V**, Capocefalo A, Ravanetti L, Vanderplasschen A, Gillet L, Cavirani S, van Santen VL, Donofrio G. Vet. Microbiol. 2011 Mar 24;148(2-4):219-31.

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Abstract

Bovine herpesvirus 4 (BoHV-4) is a virus with a worldwide distribution in cattle population that has been isolated from a lot of different tissues and samples from animal with various clinical manifestations, ranging from conjunctivitis, ocular discharge and genital diseases as post-partum metritis or abortion, but even in apparently healthy animal.

Even if a clear correlation between BoHV-4 and any pathologies has never been demonstrated; BoHV-4 is most consistently associated with metritis, a very common postpartum disease of the uterus, in which the role of BoHV-4 as a secondary pathogen is well documented.

BoHV-4 has a well known tropism for endometrial stromal and epithelial cells, in which causes non-apoptotic cell death and *de novo* virus production associated with an increased prostaglandin-endoperoxide synthase 2 protein, cyclo-ossigenase 2 (COX2) and prostaglandin E_2 production and secretion from stromal cell. BoHV-4 activation and replication in the bovine endometrium is associated with the early transactivation of the BoHV-4 IE2 gene promoter from endometrial cells. BoHV-4 IE2 gene promoter transactivation and viral replication were associated also with extracellular stimuli belonging to the intrauterine microenvironment such as *E. coli* LPS and PGE.

A model that fits well endometrial BoHV-4 disease is described in literature, involving a vicious circle comprising of bacterial endometritis leading to secretion of PGE, then PGE and LPS stimulating virus replication, which causes further endometrial tissue damage and inflammation. The existence of a virus patho-biotype causing uterine disease appears possible; virus strain adaptation to an organ, tissue or cell type is infact a very important issue for the study of pathology, even because the function of most of the viral genes remains still unknown.
In this study a BoHV-4 strain was isolated from the uterus of a persistently infected cow affected with non-responsive post-partum metritis and designated BoHV-4-U. After the characterization this uterine strain was cloned as a bacterial artificial chromosome (BAC) to easily manipulate and study the viral genome.

The feasibility of using BoHV-4-U for mutagenesis was demonstrated using the BAC recombineering system, that allows to study single or multiple gene disruptions, opening the way to the clarification of the interactions between BoHV-4 infection and the host endometrial cells.

After the generation of the BAC-BoHV-4-U the first gene we decided to investigate was the ORF50/*Rta* gene, that has been shown to be an essential gene for DNA replication and latency reactivation in 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 co-transfection assays, there is no direct

proof of its real indispensability for progression of the virus to the lytic replication cycle in the context of the viral genome.

Through different strategies the ORF50 gene was interrupted generating before some mutants, replication defective BoHV-4-V.test /IE2 mutants, as a control viruses, and then some BoHV-4-U/IE2 mutants.

The BoHV-4-V.test/IE2 mutants was efficiently rescued, with respect to the production of infectious virus and DNA replication, upon the expression of the BoHV-4 ORF50/*Rta* protein in *trans* by different complementing cell lines; the BoHV-4-U/IE2 mutants surprisingly were all able to growth, even in non-complementing cell lines. The generation of a suitable probe led to discover that the IE2 gene is duplicated in the genome of BoHV-4-U.

These data demonstrated that in BoHV-4, too, ORF50/Rta is the master replication switch gene and, that can be duplicated in some strains, as in our uterine strain.

The deletion of the second gene copy of BoHV-4-U Rta unable the virus the capacity to replicate, and this inability was completely complemented by the *in trans* expression of ORF50/Rta.

The generation of this completely replication un-competent virus maybe will provide a new powerful tool for recombinant vaccine production, even in respect to the rescue of the virus through the expression, under a strictly inducible promoter, of the Rta protein.

Riassunto

Bovine herpesvirus 4 (BoHV-4) appartiene alla famiglia dei gammaherpesvirus ed è ampiamente distribuito nella popolazione bovina. Questo virus è stato isolato da moltissimi tessuti e campioni provenienti sia da animali apparentemente sani, che da animali che mostravano una varia sintomatologia clinica, che spaziava da problemi oculari, come la congiuntivite, fino a problemi genitali, il più comune dei quali risulta essere la metrite post-parto.

Non è stata tutt'ora dimostrata l'evidenza di una chiara correlazione di causa-effetto tra l'infezione da BoHV-4 e alcuna patologia, anche se, l'associazione tra BoHV-4 e la metrite post-parto, una patologia molto comune a carico dell'utero bovino in fase post-parto, è ben documentata e ha suggerito come BoHV-4 possa essere considerato un patogeno secondario nell'insorgenza di questa patologia.

BoHV-4 mostra uno spiccato tropismo per le cellule endometriali stromali e epiteliali uterine, nelle quali causa morte cellulare per via non-apoptotica, insieme ad una produzione di virus strettamente associata con un'aumentata produzione di COX2 (ciclo-ossigenasi 2) e prostaglandina endoperossido sintasi 2, oltre che di un'aumentata secrezione di prostaglandina E2 da parte delle cellule stromali.

La rapida attivazione di BoHV-4 e consequente replicazione nell'endometrio bovino risulta essere strettamente associata alla precoce transattivazione del promotore del gene Immediate early II (IE2) di BoHV-4 da parte delle cellule endometriali; che è inoltre associata anche con stmoli extracellulari come il Lipopolisaccaride (LPS) e PGE, rilasciati nell'micro-ambiente uterino, ad esempio in corso di infezione baterica.

Sulla base dei risultati ottenuti in precedenza, è stato ipotizzato un modello che sembra ben descrivere le connessioni presenti tra BoHV-4 e l'endometrio; questo modello svela l'esistenza di un sistema che si autoalimenta, sostenuto dalla secrezione di PGE da parte di patogeni batterici, PGE e LPS a loro volta stimolano la replicazione del virus, che causa così ulteriori danni tissutali e infiammazione. Appare quindi possibile che esista un biotipo virale adattato all'utero e in grado di dare patologie uterine; la possibilità che i ceppi virali si adattino ad un organo, come anche ad un tessuto o a un tipo cellulare risulta essere molto importante nella comprensione della patologia, anche nell'ottica della prevenzione e della terapia.

In questa tesi è stato dapprima isolato un ceppo di BoHV-4 residente nell'utero di una vacca affetta da una metrite post-parto non responsiva ai farmaci, che è stato designato BoHV-4-U; dopo la sua caratterizzazione questo ceppo è stato clonato come Cromosoma Artificiale Batterico (BAC), per poterlo studiare e manipolare agevolmente. Tramite un sistema di Ricombinazione Omologa, è stata testata la validità di questo BAC-BoHV-4-U, al fine di permettere lo studio di delezioni di geni singoli o

multipli, che saranno importanti per comprendere le interazioni che si instaurano tra BoHV-4 e le cellule ospiti uterine.

Il primo gene sul quale si è concentrata la nostra attenzione, dopo la generazione del BAC-BoHV-4-U è il gene codificato dall'ORF50 del virus, la proteina Rta, che è stata dimostrata essere essenziale per la replicazione del DNA e la riattivazione dalla latenza per molti altri gammaherpesviruses.

Per quanto riguarda BoHV-4, studi precedenti hanno dimostrato come il gene Orf50/Rta sia in grado di trans-attivare alcuni promotori di geni precoci e tardivi di BoHV-4, in saggi di co-transfezione; tuttavia non è ancora disponibile alcuna prova diretta che questo gene sia responsabile della progressione del virus verso un ciclo litico nel contesto del genoma virale.

Abbiamo quindi proceduto ad inattivare il gene tramite differenti strategie, generando dapprima un mutante di controllo, difettivo nel gene IE2, appartenente al ceppo di referenza V.test, per poi procedere con la delezione del gene nel nostro isolato uterino.

Nel ceppo di referenza IE2 si è dimostrato essere indispensabile per la replicazione del virus, in effetti era possibile ricostituire il virus solo fornendo *in trans* la proteina Rta, tramite delle linee cellulari complementate; nel ceppo uterino abbiamo avuto risultati discordanti, in quanto la delezione del gene IE2 non sembrava interferire con la replicazione virale, rendendo il virus capace di crescere anche in linee cellulari non complementate con Rta. Tramite la generazione di un'opportuna sonda, che ha permesso di effettuare analisi approfondite, è emerso come nel nostro ceppo uterino, BoHV-4-U, il gene IE2 fosse duplicato. La delezione anche della seconda copia genica di IE2 ha evidenziato come anche in questo ceppo IE2 sia un gene essenziale per la replicazione del virus; anche in questo caso la capacità replicativa del virus veniva ristabilita fornendo *in trans* la proteina Rta.

La generazione di un virus replicazione-incompetente, costituirà un nuovo e interessante mezzo per la produzione di vaccini ricombinanti, anche nell'ottica di poter ottenere il rescue del virus, attraverso l'espressione della proteina Rta, posta sotto il controllo di un promotore strettamente cellula o organo inducibile.

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Chapter 1

Introduction

1.1. *Herpesviridae* family

The *Herpesviridae* family comprises more than 120 viruses with linear double-stranded DNA genomes and common characteristics. According to their biological properties, host range, multiplication rate, cytopathology and latency site this family has been divided in three subfamilies, named *Alpha*-, *Beta*- and *Gammaherpesvirinae*.

All members of this viral family share a common morphology based on an icosaedral capsid symmetry. Usually the spherical virion comprises core, capsid, tegument and envelope. The core consists of the viral genome packaged as a single, linear, double-stranded DNA molecule into preformed capsid. Capsid assembles by co- condensation around a protein scaffold to form a pro-capsid in which the subunities are weakly connected. Proteolitic cleavage of the scaffolding protein triggers the loss of the scaffold and the reorganization of the shell into the characteristic capsid form. The structure of the tegument is poorly defined, with evidence of symmetry only in the region immediately surrounding the capsid; many proteins are contained in the tegument, not all required for virions formation. Enveloped tegument structure lacking capsids can be assembled and are released from cell along with virions. The envelope has a lipid bilayer structure and is strictly associated with the outer surface of the tegument; contains different integral viral glycoproteins.

Herpesviruses are widely distributed in nature and have a wide range of vertebrate hosts, currently one herpesvirus, l'Ostreid Herpesvirus 1 (OsHV-1) can infect invertebrates (Roizman, 2001). As a general rule, the natural host range of individual viruses is highly restricted, and also co-evolution between herpesviruses and single host species occurs, through occasional transfer to other species in nature. Even if considering experimental animal and *in vitro* systems the host range varies remarkably, the same general rule holds true: members of the subfamily *Alpha*- often infect a wide variety of animal species and cell line from different species, whereas members of the subfamily *Beta*- and *Gammaherpesvirinae* exhibit a very narrowed experimental host range.

These viruses produce lesions in man and animals ranging from localized vesicular eruptions of surface epithelia to diffuse wide spread damage in the mucosa of the respiratory, digestive and genitals tracts; from localized giant cell proliferation in glandular epithelium to necrosis of liver, lymphoid and other tissues; and from specific neuronal damage to diffuse meningoencephalitis. Infection of the fetus may lead to fetal death, abortion or in newborn diseases. Since the tissue tropism of various herpesviruses does not differ greatly, they tend to produce similar diseases in different animal species. Animal herpesviruses can thus cause disease such as bovine, feline, canine and equine rhinotracheitis.

A variety of immune evasion mechanisms have been identified in different viruses, including the evasion of complement, antibody, MHC class I presentation and Natural Killer cells killing; however the ability of these

viruses to survive to immune system is strictly connected to their ability to establish life-long latent infection; Alpha- in neuronal cells, Beta- in monocytes and Gammaherpesvirus in lymphocytes (Fauquet, 2005).

Alphaherpesvirinae

Short replication cycle, wide host range, latency in sensorial ganglions and lysis of the infected cells are the hallmarks of this subfamily. Examples are Human Herpesvirus-I (HHV-1, also known as Herpes Simplex virus 1), Human Herpesvirus-3 (HHV-3, also known as Varicella–zoster virus, VZV), Bovine Herpesvirus-1 (BoHV-1) and Caprine herpesvirus-1 (CaHV-1).

Betaherpesvirinae

This subfamily presents a slow replication cycle, narrow host range, and latency in monocytes, secretory glands, kidney and other tissues. The prototype virus of this subfamily is Human Herpesvirus-5 (HHV-5, or Human Cytomegalovirus, HCMV): has a high prevalence and cause severe and even fatal diseases in immunologically immature or compromised patients. HHV-5 has the largest genome among mammalian DNA viruses, with a 235 kb double-stranded DNA molecule; due to its genome size and its slow replicative cycle is extremely difficult to obtain HHV-5 mutants.

Gammaherpesvirinae

In this subfamily are grouped viruses that usually have a host range limited to the family or the order of the natural host, latency in lymphocytes or in lymphoid tissues and oncogenic properties; moreover many of them do not easily replicate in cultured cells, some of them seemingly not at all (Ackermann, 2006).

This subfamily is divided in two genera: lymphocryptovirus (or gamma-1-herpesvirus) and rhadinovirus (or gamma-2 herpesvirus). The etiological agent of infective mononucleosis, Human Herpesvirus-4 (or Epstein Bar Virus, EBV) groups in the lymphocryptovirus genus and is associated with oncogenic potential in lymphoid tissues.

Members of the Rhadinovirus genus have been isolated in many mammalian species, like Saimiriine Herpesvirus-2 (SaHV-2 or Herpesvirus Saimiri, HVS) in monkeys, Equid Herpesvirus-2 (EHV-2) in equines, Murid Herpesvirus-4 (MuHV-4 or Murine gammaherpesvirus-68, MuHV-68) in mice, Bovine herpesvirus-4 (BoHV-4) in cattle and Human Herpesvirus-8 (HHV-8 or Kaposi's sarcoma associated herpesvirus, KSHV) in man.

Extensive studies have made apparent that, in the course of their evolution, herpesviruses have undergone considerable diversification with respect to their antigenic and biological properties and to composition, size and arrangement of DNA sequences. However the morphology of the virions has been preserved.

The genomes are composed of linear double-stranded DNA ranging from 125 to 240 Kb in size and from 32-75% in G+C content. All herpesviruses share long regions with repeated sequences in their genome: the number, position and the sequence of these regions vary according to subfamily, genus and species which the virus belongs to; there is also a minor variability even among different strains of the same species. Roizman and Batterson (Roizman, 2001), presented a schematic arrangement of herpesvirus genomes; genomic organization is moreover used to classified herpesviruses, infact only five general genomic structures have been identified (Fig. 1).

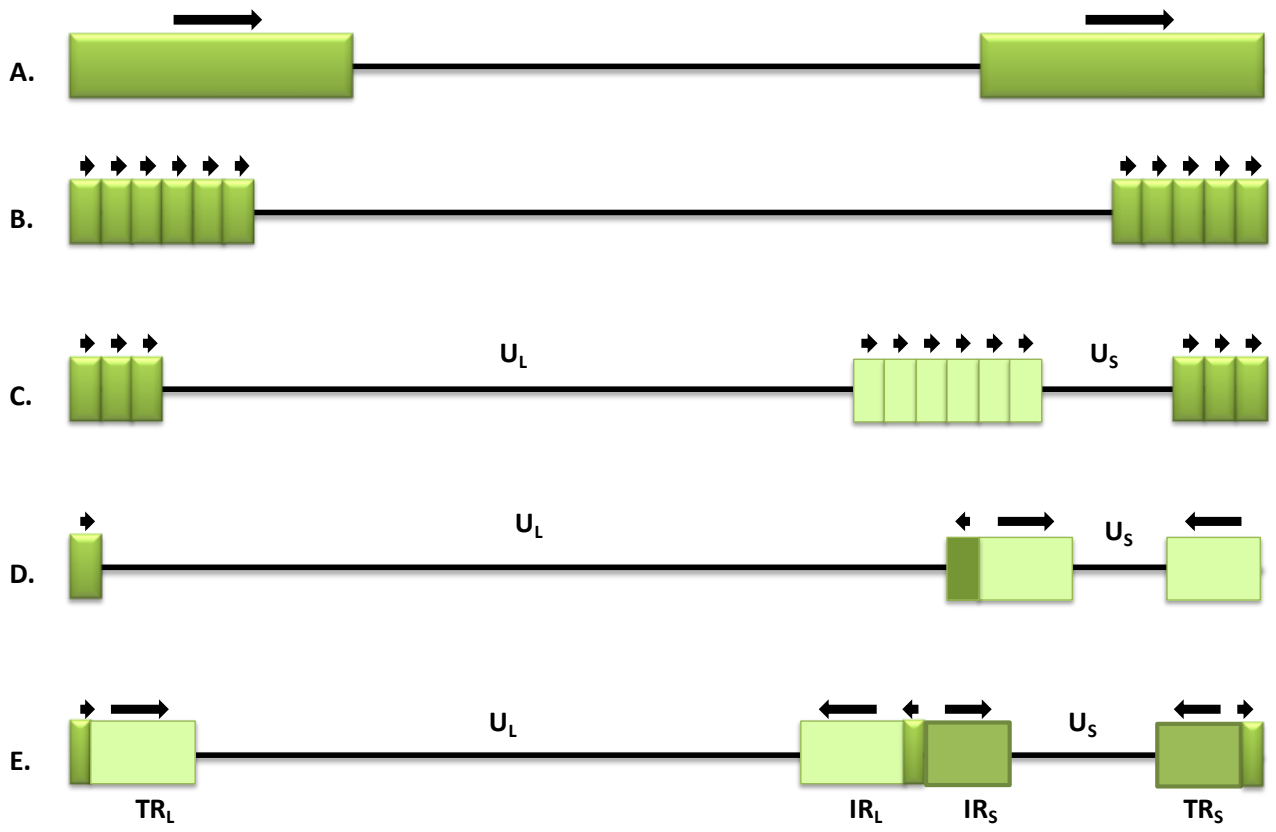

Fig. 1. Herpesvirus genomic organization (adapted from (Fauquet, 2005)

Structure A shows a unique sequence flanked by a direct repeat that may be larger than 10kb (HHV-6) or as short as 30bp (MuHV-1); rhadinoviruses in general present a type B structure, with one unique long region flanked by direct terminal repeats variable in numbers; HHV-4 has a type C structure, with two unique region divided by a set of direct repeat and flanked by direct terminal repeats. HHV-3 exemplifies type D structure, that presents different elements at each terminus, which are present internally in inverted orientation and separate the two long unique regions; while viruses with a type E structure are HHV-1 and HHV-5, in which the terminal reiterations are also repeated in inverted orientations internally.

As a matter of fact closely related herpesvirus tend to share the same genomic structure, even if exceptions can be found; HHV-1 and HHV-5 share the same genomic organization even if they are distantly related, reflecting probably that they have evolved independently into the same organization (Fauquet, 2005).

1.2. Bovine Herpesvirus 4 (BoHV-4)

The interest in bovine herpesviruses stems from their considerable economic importance rather than from their contribution to the biological understanding of Herpesviridae. All bovine herpesviruses conform to the other members of the family on the basis of morphology, uniqueness in using the nucleus for replication and maturation, and the ability to become latent in infected hosts (Roizman and Morse, 1978). Both domestic livestock and exotic ruminants are susceptible to herpesvirus infection, with a wide range of clinical manifestations (see Table 1).

The term "Bovid" was introduced to accommodate viruses isolated from all members of Bovidae; cattle, sheep and goats. Bovid herpesviruses (BHV) were characterized on the basis of the serology and restriction-enzyme analysis of DNA.

Virus	Natural host	Disease Conditions
BHV-1	Cattle	Infectious pustular vulvovaginitis, infectious bovine rhinotracheitis, abortion, respiratory disease, conjunctivitis, meningoencephalitis
BHV-2	Cattle	Bovine herpesmammillitis, pseudo-lumpy skin disease
BHV-3	Wildebeest	Malignant catarrhal fever
BHV-4	Cattle	Unapparent infection, abortion, metritis, mammary pustular dermatitis, respiratory and enteric infection
BHV-5	Sheep	Pulmonary adenomatotis or Jaagsiekte
BHV-6	Goat	Latent infections, enteritis

Table.1. Classification of bovine herpesviruses, adapted from (Ludwig, 1982)

The BHV-4 group includes numerous herpesviruses isolates obtained from both diseased and apparently healthy cattle and buffaloes.

This virus was isolated the first time in 1963 by Bartha et al. (prototype strain Movar 33-63) (Bartha et al., 1966), in calves showing respiratory and conjunctival signs. It has been classified in the Herpesviridae family after the microscope observation of the viral particle. In the first times various isolates were observed with

worldwide distribution, and were called with different names, such as “orphan herpesvirus”, “Movar-type herpesvirus”, “Bovid cytomegalovirus” and “Bovid Herpesvirus-4”, generating confusion about the nomenclature, partly because of the specific acronyms used by various investigators in designating viral isolates. Bartha reviewed the nomenclature of all these isolates recommending for all “Movar-Type” the term “Bovine herpesvirus-4” (BHV-4) (Bartha et al., 1987). The international committee of viral Taxonomy decided then to adopt the official name of BoHV-4 in 2000 (Fauquet, 2005).

BoHV-4 was firstly proposed to be a bovine cytomegalovirus because of its morphogenesis in infected cells (presence of inclusion body in cytoplasm and nucleus of infected cells and of giant cells) as well as its slow growth in cell culture (Storz et al., 1984).

Studies on the genome organization and determination of first gene sequence revealed that BoHV-4 is a member of the *Gammaherpesvirinae:* (i) the virus has a B-type genome structure (Roizman, 2001) with a long unique genome region(LUR) flanked by poly-repetitive DNA (prDNA) elements, (ii) the genome contains a thymidine kinase gene (Kit et al., 1986), (iii) gene sequenced so far are strictly homologous to those of HHV-8 and SaHV-2, and (iv) the gene arrangement is collinear with those of other gammaherpesviruses (Bublot et al., 1992; Goltz et al., 1994).

On the similarity of its biological properties and genome structure to those of SaHV-2, it has been proposed, lately in the Eighty’s, that BoHV-4 was included in the gammaherpesvirus, specifically in the gamma-2 genus, also called Rhadinovirus (Kit et al., 1986). In ruminants other four rhadinovirus have been identified: Alcelaphine herpesvirus 1(AlHV-1), Ovine herpesvirus 2 (OvHV-2), Bovine lymphotrophe herpesvirus (BLHV) and Caprine Herpesvirus 2 (CpHV-2); BoHV-4 seems not to be phylogenetically related to neither of them (McGeoch et al., 2005), so far its evolutive origin is still unclear (Dewals et al., 2006).

Besides cattle, BoH-4 has been detected in a variety of ruminants. In particular BoHV-4seems to be highly prevalent among wild African Buffalo (*Syncerus Caffer*), which could be considered as the natural reservoir of the virus. Overall, more than 40 BoHV-4 strains have been isolated across the world; these can be classified in three groups: the European strain (or Movar 33-63 like strain), the American strain (or DN599-like strain) and the African Buffalo strains (Dewals et al., 2006).

1.2.1. BoHV-4 genome

BoHV-4 genome has been completely sequenced: in 2001 the sequence of BoHV-4 North American strain 66-p-347 was completed (Broll et al., 1999; Zimmermann et al., 2001) and more recently, in 2011 that of BoHV-4 European strain V-test (Palmeira et al., 2011).

BoHV-4 as a typical herpesvirus B genome organization; its linear, double-stranded DNA of 144±6 kb consists of a long central unique region (LUR~110 Kb) with a low G+C content which is flanked by two stretches of tandem repeats with high G+C content (Fig. 2). These stretches have been designated polyrepetitive DNA (prDNA); it has been demonstrated that the frequency of prDNA units at both genomic termini is different between individual molecules of different strains; but the overall numbers is relatively constant (Ehlers et al., 1985).

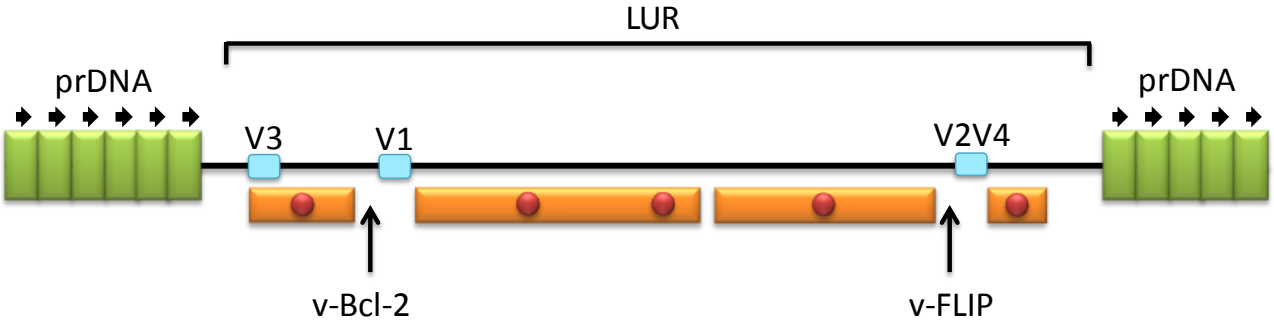

Fig. 2. BoHV-4 genomic organization (the orange bars represent the 5 conserved blocks within the LUR; V1-V4 the variable regions between strains).

BoHV-4 prDNA size varies from 1450 to 2850 bp according to the strains; each unit is formed by a repeated sequence of approximately 200bp at the 3' of each unit (repeated 3 times completely and 1 partially), and the other of 25 bp at the 3'of the unit (repeated 2 times); in strain 66-p-343 there are 15 unit of prDNA (Broll et al., 1999). prDNA does not contain any typical coding sequences (Chang and Van Santen, 1992), but are important during viral DNA replication; the cleavage/packaging site necessary to cleave the viral concatamers formed during rolling circle replication and involved also in viral incapsidation are infact located at the 5' and 3' termini of each repeated unit of prDNA. These site are called *pac 1* and *2* and have been individuated in all herpesvirus; *pac-1* consists in a T-A rich region located near the left end of the region and *pac-2* in a T motif near the right end; a 443 bp region in the prDNA is sufficient for cleavage and packaging of BoHV-4 genome (Broll et al., 1999; Palmeira et al., 2011).

prDNA acts as a genome-size buffer, ensuring the packaging of a complete copy of the LUR flanked by prDNA (Broll et al., 1999; Ehlers et al., 1985); in other gammaherpesvirus it has been demonstrated that prDNA can have also other functions: during latency should be an anchorage point for viral episomes to

host chromosomes (Fejer et al., 2003); moreover could contain an *oriP* latency-associated origin of replication, like in SaHV-2 (Collins et al., 2002).

The LUR of BoHV-4 strain 66-p-347 consists in 108.873 bp, with a 41.4% G+C content, as determined trough shotgun analysis by Zimmermann et al, in 2001. Only 1% of CpG dinucleotide frequency is described in this LUR, confirming data recorded in other *Gammaherpesvirinae* (Zimmermann et al., 2001). V-test strain sequencing revealed a 108.241 bp LUR with a average G+C content of 41.21%; the overall nucleotide identity between the two strain is high to 99.1%, but shows a large variability at the genome level (Palmeira et al., 2011).

The conserved gene of gammaherpesviruses are arranged in a common block organization, but there are individual differences in number, position and orientation of subgroup-specific or individual ORFs (Zimmermann et al., 2001). These common blocks are arranged in 5 blocks (1 to 5 in 5'-3' direction), within each of them ORFs tend to have the same organization and orientation (Bublot et al., 1992; Bublot et al., 1990). Blocks 1, 2 and 4 group genes "core", conserved in all *Herpesviridae*, involved in viral capsid formation, in viral replication and in DNA encapsidation, but also in capsid release from the nucleus. Probably these genes have been inherited from an ancestor, common to all herpesvirus; otherwise ORFs in blocks 3 and 5 group gene proper of gammaherpesvirus. There are some region called "inter-block" (from A to F in 5'-3' direction), coding gene unique to one or few phylogenetically related species; often they code for gene of cellular origin (Lomonte et al., 1995; Raftery et al., 2000). Two regions of multiple direct repeats have been identified within the LUR, called R1 and R2. R1 consists of several stretches of several complete and incomplete direct repeats of 23, 25 and 65 bp in length; its length and sequence differ between the strains; R2 consists of two different repeat stretches, R2a and R2b. R2a consists of 28 perfect and 3 imperfect direct repeats of 22-23 bp and R2b contains several different repeats from 8 to 68 bp in length as well as one inverted repeat with an hairpin-loop predicted structure. Interestingly R2 is connected with a notably change in G+C content: whereas R2a has a high G+C content of 71%, a 750-bp sequence stretch directly upstream of R2 has only a 30% G+C content. Analysis of positional homology and the fine mapping of BoHV-4 sequence have led to the identification of a putative *Ori Lyt,* mapping downstream of the G+C-rich R2a stretch, in the R2b region with the predicted hairpin-loop structure, partially overlapping with the gene Bo11 and Bo12. The sequencing of these regions in the V-test strain showed a high divergence, up to more than 40% as well as large gaps. The palindromic region forming a perfect hairpin-loop structure is conserved in V-test genome; even if the R2 region shows a high divergence the palindromic motif essential for viral replication and present in putative *Ori Lyt* is conserved; therefore this structure can represent a common secondary one used by all herpesvirus and need to be tested as a candidate for an essential *Ori Lyt* motif (Palmeira et al., 2011).

The analysis of BoHV-4 sequence revealed the presence of at least 79 ORFs, 62 homologous to that of other rhadinovirus, 17 unique to BoHV-4 (Fig.3). ORFs homologous to other herpesviruses have been named from ORF1 to ORF 79; so ORFs homologous in SaHV-2 and in BoHV-4 have the same name; otherwise those peculiar of BoHV-4 have been named from Bo1 to Bo17, according to their position in the genome from 5' to 3' end. Only Bo5 has a corresponding gene in SaHV-2. The central part of the LUR contains a stretch of 54 genes with the same orientation (ORFs 16 to 69), within it there are only two genes without genetic but with positional and orientational homology to SaHV-2 (Bo9 and Bo10). In all other herpesviruses sequenced till now. Differences in the presence and/or position of ORF 16 (v-Bd-2) and in the number of individual or subgroup-specific genes between ORF50 and ORF69 are observed.

BoHV-4 genome in respect to those of SaHV-2 seems to lack a lot of genes involved in modulation of the immune response, in cellular growth and survival and in nucleotide metabolism, the majority of them in SaHV-2 are located in genomic region outside the conserved gammaherpesvirus blocks (Lomonte et al., 1995). BoHV-4 have no genes coding for cytokines or cytokines receptor (while SaHV-2, HHV-8 and EHV-2 have), for interleukine receptors (while SaHV-2, HHV-8, MuHV-68 and EHV-2 have), for chemokines or viral macrophage inflammatory protein (while HHV-8 have) (Lomonte et al., 1995). In this respect BoHV-4 appears most comparable to AlHV-1, more than HHV-8 or SaHV-2 (Markine-Goriaynoff et al., 2003a and b).

BoHV-4 moreover has not a gene coding for cycline D or for a complement regulatory protein and is also absent the gene for dehydrofolate reductase and for thymidilate synthetase, while are present both in SaHV-2 and in HHV-8.

To date BoHV-4 has never been associated with lympho-proliferative disorders or with transforming capability classically associated to other gammaherpesviruses like HHV-8 or MuHV-68. BoHV-4 genome codes for two ORFs with a potential involvement in cell survival: a v-Bcl-2 (ORF 16) and a v-FLIP (ORF17) have been identified, they should have a role in prevent apoptosis, one of the early stage of cellular transformation; but it has been demonstrated that the virus can induce apoptosis at a late stage of infection in permissive cells (Sciortino et al., 2000).

BoHV-4 contains 17 unique ORFs, without positional and sequence homologous in others herpesviruses, except for Bo5, as described above. Among these ORFs are seven that have never been described in literature: Bo1, -3, -6, -7, -12, -13 and -15.

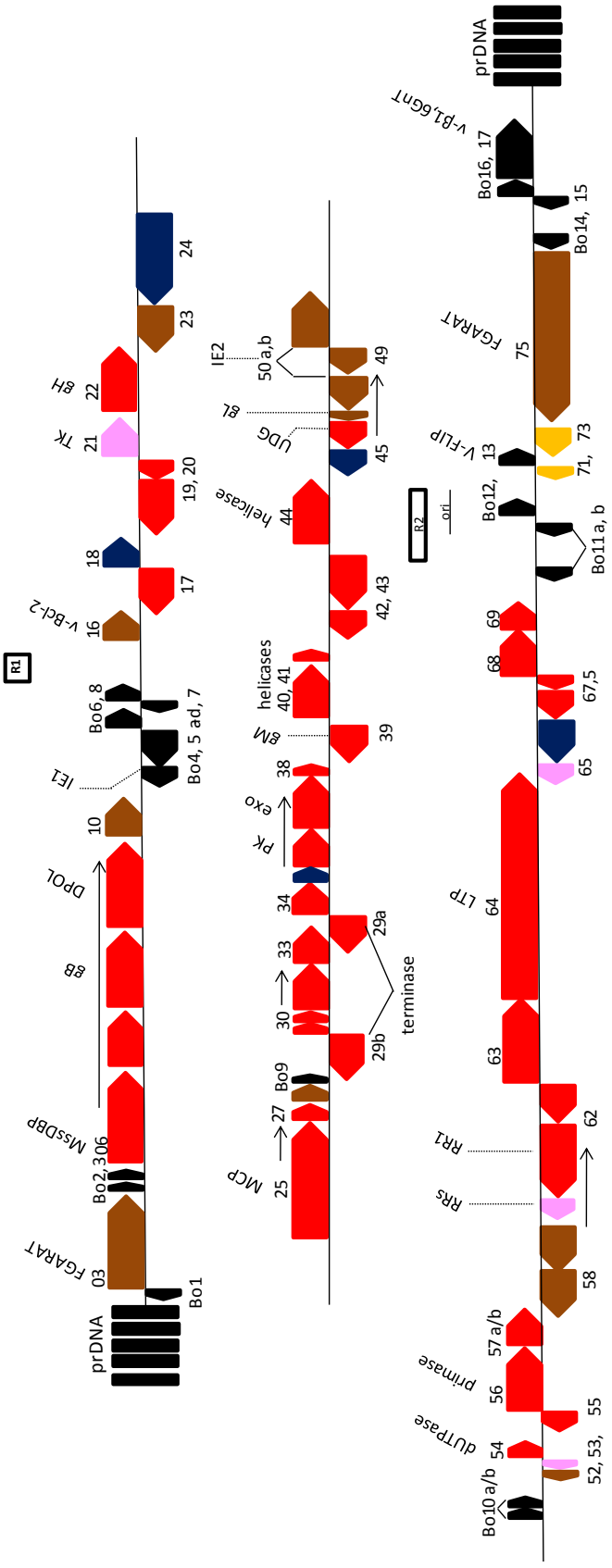

Fig. 3. BoHV-4 genomic organization, not to scale, adapted from (Zimmermann et al., 2001). In Red are represented the ORFs common to all herpesviruses, in Blue those to β and γ; in Pink those to α and γ; in Brown those peculiar to γ herpesviruses. In yellow are the ORFs of γ2 herpesviruses; in black are the gene unique for BoHV-4, designed from Bo1-17. (R1/2 are the repeated sequences R1 and R2,ori is the Ori of replication; DPOL:Dna polymerase; dUTPase:dUTP nucleotide hydrolase; exo: exonucleotide alkaline; FGARAT: α-N-formylglycineamide ribonucleotide aminotransferase; gB/H/L/M, glycoproteins B, H, L and

M; LTP:long Tegument protein;MCP:major capsid protein; MssDBP:major single strand DNA binding protein; PK:protein kinase; RRs and RR1: ribonucleotide reductase subunity; TK: timidina kinase; UDG: uracil DNA glicosidase; v-Bcl-2: viral B cell lymphoma gene; v-FLIP: viral Flice inhibitory protein.

ORF Bo4 and the spliced Bo5 are part of the Major Immediate Early I transcript (IE1), as described by Van santen; they exhibit partial amminoacid homology and may be involved in differential splicing (van Santen, 1991). ORF Bo8 overlaps partially with the 1.7kb late gene, described by Bermudez-cruz et al (Bermudez-Cruz et al., 1997).

Bo10 encodes a non essential viral envelope protein, gp180, that regulates viral tropism; recently it was demonstrated that gp180 interacts with glycosaminoglycans (GAGs), hiding a critical cell-binding epitope on virions until displaced by GAGs (Machiels et al., 2011a). Machiels et al., developed two Bo10 mutant viruses that showed that gp180 is dispensable for establishment and maintenance of latency in vivo, but drastically reduced the susceptibility of BoHV-4 to neutralization by immune serum on various cell line; gp180 seemed to hide partially several epitopes on gB, gH and gL, providing a O-glycan shield able to hide these epitopes (Machiels et al., 2011b).

Bo11 is encoded by a previously described IE2-transactivated, spliced, 1.1kb RNA (Bermudez-Cruz et al., 1998); Bo17 encodes a viral β-1,6-N-acetylglucosaminyltransferase (β-1,6GnT), that is 81,1% homologous with the human one, involved in cellular differentiation and in immunitary processes. Interestingly, BoHV-4 is the only virus in which this gene has been identified (Vanderplasschen et al., 2000).

RNAs of six genes were experimentally demonstrated to be spliced; besides Bo5, Bo10 and Bo11, these are ORF29, 50 and 57. ORF29 encodes a terminase, and is present and spliced in all herpesviruses sequenced till now. The ORF50 product is a putative R transactivator, coded by the spliced IE2; the intron of the IE2 transcript contains the complete ORF49, coding for a product of 299aa, conserved in other herpesviruses (van Santen, 1991, 1993). ORF57 codes for a putative post-transcriptional transactivator; in HVS is responsible for redistribution of the spliceosome complex, modulating mRNA processing in infected cells (Cooper et al., 1999).

BoHV-4, as all other herpesviruses, possesses a Thymidine Kinase gene (TK), involved in pyrimidine metabolism, more precisely in the salvage pathway of pyrimidine biosynthesis (Lomonte et al., 1992). TK is encoded by ORF21 and is transactivated by IE2 gene product (Zhang and van Santen, 1995); its disruption is not incompatible with virus replication (Kit et al., 1986), and TK gene has been chosen as a target site for foreign DNA insertion in vectorializing strategies (Donofrio et al., 2008c).

1.2.2. BoHV-4 glycoproteins

BoHV-4 particle has an icosaedric capsid, which is composed by 150 hexamers and 12 pentamers; the nucleocapsid has a diameter of 90-100 nm, and is surrounded by an n envelope, that usually contains the viral glycoproteins involved in attachment, penetration, budding and diffusion of the viral particle.

In herpesviruses there are 5 glycoproteins extremely conserved in all herpesviruses: gB, gH, gL, gM and gN. In BoHV-4 29 polypeptides were identified, all of them present in the envelope are glycosilated (Dubuisson et al., 1989).

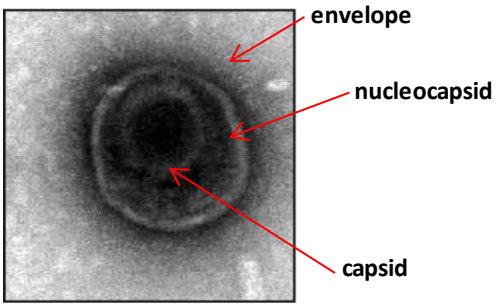

Fig. 4. BoHV-4 particle as appear at electronic microscope adapted from (Machiels et al., 2011b)

Four major glycoprotein have been studied: the first is the complex formed by gp6/gp10/gp17 (150kDa/120kDa/51kDa); gp10-gp17 are bounded by disulfides bonds and gp6 by non covalent bonds. The heterodimer gp10-gp17 derives from the proteolitical cleavage of BoHV-4 gB gene product, conserved in all herpesviruses and essential for host cell penetration (Dubuisson et al., 1989; Goltz et al., 1994). The disruption of this gene is not compatible with virus life and replication, and usually the complementation with gB from even others gammaherpesviruses is not able to rescue the virus (personal data). Another complex studied is that formed by gp11/VP24 (120kDa/16,5kDa), in which gp11, that is glycosilated, is bound to VP24, not glycosilated, through a non covalent bond, at least six neutralizing epitopes were identified in this complex (Dubuisson et al., 1989). The third major glycoprotein of BoHV-4 envelope is gp8 (135kDa), involved in heparin-like molecules contacting at the cell surface; this protein is poorly glycosilated and is present at both the viral envelope and in the culture medium (Egyed, 2000; Vanderplasschen et al., 1993). Gp8 is a late protein and its secreted form can interferes with the immune humoral response of the host (Dubuisson et al., 1992); a lot of studies have demonstrated that the protein identified as gp8 in reality is another form of gp6/gp10/gp17 protein. The last major protein characterized is gp1 (>300kDa), a strictly late protein as described by Dubuisson et al. (Dubuisson et al., 1991).

Other BoHV-4 glycoproteins have moreover been described: gp21 (26-27kDa), described in the sera of infected animals; gp110, corresponding to the gH glycoprotein of the other gammaherpesviruses and gp31-35 or gp45-65, corresponding to gL protein. gH is important for viral envelope fusion with host cell membrane and propagation between cells; gL has not still a so clarified role, but is clear that can associate with gH. A recent paper demonstrated that a gL deficient virus displayed a growth deficit associated with a default in virus entry; it impaired endocytosis and endosome migration; moreover a deficit in gL altered also gH glycosilation, clearing that there is a strict correlation between gH and gL (Lete et al., 2011).

gM and gN are not so studied in gammaherpesviruses, and their function has never been determined; they can complex and maybe act in budding of virions *in vivo*.

Bo10, as described before, codes for a gp180 glycoprotein , that is expressed as a late gene, incorporated in the virion and function as a non-essential structural protein for BoHV-4 replication *in vitro* (Machiels et al., 2011 a, b).

BoHV-4 does not present gD or gE homologous; glycoprotein D is the major immunogen of BoHV-1 and gE is used as a marker in vaccine against BoHV-1. As a matter of fact there are any antigenic relationships between BoHV-1 and BoHV-4, even because they belong to different subfamilies; BoHV-1 to *alpha*- and BoHV-4 to *gammaherpesviruses*.

BoHV-4 shares instead some epitopes with AlHV-1, a gammaherpesvirus with whom it can also be observed a cross reaction in serology, maybe protective (Osorio et al., 1985).

1.2.3. BoHV-4 *in vitro* infection

1.2.3.1. *In vitro* host range

BoHV-4 replicates in primary and immortalized cultures of bovine cells: in kidney, tests, lungs, skin, mammary gland, nasal turbinate, endothelial cells, B-T lymphocytes, macrophage-derived cells, histiocytes, thyroid, embryo tracheal primary cells, fetal bovine bone marrow, in lymphosarcoma calf thymus; also in different kidney's continuous lines (MDBK for Madin Darby Bovine Kidney, GBK for Georgia Bovine kidney and BEK for Bovine Embryonic cells) or lung's derived (EBL for embryonic bovine lung). BoHV-4 can establish persistent infection in bovine macrophage cells (BoMac) (Donofrio and van Santen, 2001).

BoHV-4 can also infect cells from different origins: buffalo, sheep, goat, swine, cat, dog, rabbit, mink, turkey, ferret, horse, chicken rat, mouse and hamster (Bartha et al., 1966; Egyed, 2000; Markine-Goriaynoff, 2003b).

Recent studies have demonstrated that BoHV-4 can infect human cells and that some human cells lines are sensitive or even permissive to BoHV-4 (Donofrio et al., 2000b; Egyed and Bartha, 1998; Gillet et al., 2004). The replication of the virus in a primary cell line of glioblastoma and in human cell lines MRC-5 and WI-38, from embryonic lung was evidentiated. A recombinant BoHV-4 expressing a resistance gene for neomycin has established a persistent infection in RD4 (Rhabdomyosarcoma cells 4 line) cells, under the selective pressure of neomycin (Donofrio et al., 2000b).

These observations led to the hypothesis that cross-species transmission of BoHV-4 could lead to human infection: BoHV-4 has infact a high prevalence in cattle population and no eradication scheme is directed against this virus; is frequently isolated from bovine serum as well as in the milk of cows affected by mastitis or apparently healthy, making possible the transmission of virus to human trough a lot of transmission route. To date there are no reported cases of human infection with BoHV-4. Scientists demonstrated that human serum neutralizes efficiently BoHV-4 through activation of complement by natural antibodies raised against host cell derived epitopes expressed on the virion surface, such as the Galα1-3Gal epitope (Machiels et al., 2007).

The ability of BoHV-4 to infect human cells has also investigated to address the potential of BoHV-4 recombinant strains for viro-oncolytic treatment of human cancer. BoHV-4 can induce apoptosis *in vitro* in some human carcinoma cell lines, A59 and OVCAR, through a pathway involving caspase 10 activity (Gillet et al., 2005b). Recently a work was published in which, a BoHV-4 vector armed with HSV-1 TK gene, was able to reduce glioma in mice and rats (Redaelli et al., 2011; Redaelli et al., 2010).

The fact that BoHV-4 can infect such a broad host range, in contrast to other gammaherpesviruses, is probably related to the interaction of virus with host cell heparan-like structures as a first contact (Vanderplasschen et al., 1993). Heparan-like proteins are infact present on the surface of the majority of vertebrate's cells, and this explains why BoHV-4 has a so wide host range.

1.2.3.2. Viral replication *in vitro*

Kinetics of virus production extra- and intracellular show that BoHV-4 is a cells-associated virus with a slow growth (Storz et al., 1984). Its replication and the synthesis of late protein is restricted by DNA Synthesis Dependence on the S phase of the cell cycle; moreover the slow replicative cycle could be due to its slow rate of thymidine kinase induction in infected cells (Kit et al., 1986); BoHV-4 does not induce a host cell shut-off, allowing cell to go through the S phase (Vanderplasschen et al., 1995).

Cythopatic effect (CPE) appears usually in infected cells 48 to 72 hours after infection and is characterized by rounded cells, dispersed in the monolayers, without formation of classical plaques.

The expression of herpesvirus proteins is temporally regulated. The proteins are classified into three kinetic classes depending on the order of their synthesis during *in vitro* infection. They are chronologically expressed as immediate early (IE or alpha), early (E or beta) and late (L or gamma) proteins. Immediate early proteins are expressed directly after release of the viral genome from the capsid into the nucleus. While E protein expression occurs after synthesis of the IE protein, L protein expression depends on the expression of both IE ad E protein and viral DNA synthesis.

Herpesvirus can establish two different types of infection: productive or latent infection. Typically cells infected by either alpha- r beta- herpesviruses support lytic replication, while only specific subsets of cells normally harbor latent virus. Gammaherpesviruses seem to initially favor the establishment of latency, while only a subset of cells supports lytic replication (Ackermann, 2006).

- ✓ Productive infection: BoHV-4 binds to the host cellular surface through the weak interaction between gB and heparan sulfate-like structures; it is not clarified if are involved other interactions for a stable attachment to the host cell, and if there is a specific receptor for BoHV-4.

  Immediately after the attachment, the envelope fuses with the plasma membrane, and the nucleocapsid and the envelope protein are released in the cytoplasm and transported to the nucleus through the interaction with microtubules. Viral DNA is released into the nucleus passing through a nuclear pore; then circularized without synthesis of new DNA. Three classes of genes have been individuated, differentially expressed during the time: α or IE genes; β or E genes and γ or L genes (Chang and Van Santen, 1992). After the circularization of DNA in the nucleus some proteins of the envelope interact with the host cell transcriptional machinery to stimulate the transcription of IE genes by the host RNA polymerase II. IE1 and IE2 proteins are the two major immediately early proteins of BoHV-4, are produced in the cytoplasm and migrate to the nucleus where they block the expression of IE gene and activate the one of E and L genes. E genes are expressed at the major level from 4 to 8 hours after the infection; they work in the metabolism of nucleotides and in DNA replication. Between gene γ, we distinguish γ1, or partial late and γ2, real late. γ1 expression is amplified by viral DNA synthesis, and γ2 is totally dependent by viral DNA synthesis. γ proteins are produced in the cytoplasm and represent the majority of structural protein, which can migrate to the nucleus where they assemble in the new forming capsids.

  Viral DNA circularized in the nucleus and replication starts from the *Ori* of replication and proceeds with the rolling circle mechanism that produces a lot of viral concatamers, complex structure composed by genomic units disposed head to tail. These replication intermediates are cleaved and packaged in genomic unit in the new capsid. The new-formed capsids are then transported to the periphery of the nucleus, passed through the lumen of endoplasmic reticulum where they probably acquire some protein of the envelope but it is still not so clear how the capsid exit from the nucleus

and acquire the envelope. Two models have been proposed to clarify this process: the first is the luminal model, that hypotheses that full capsids embedded with viral DNA could acquire the envelope through passing the nuclear membrane and the ER; in this case the proteins composing the envelope will be the precursors of the final proteins. Enveloped capsids then arrive to the Golgi passing through the connection intra ER and Golgi or transport vesicles. The precursor proteins will then modified at the surface of the virion and the virions released by exocytose. The second model is based on the fusion of the virion envelope with the nuclear membrane and then to the ER membranes to release free capsids in the cytoplasm; then passing through the Golgi's vesicles acquire the final envelope.

Recently it was demonstrated that BoHV-1 capsids arrive to the cytoplasm through some nuclear pores, then reach the cellular surface passing in the Golgi's vesicles (Wild et al., 2005).

The lytic cycle in gammaherpesviruses is initiated only on rare occasion; the least contribution to pathogenicity has to be expected from this stage. In BoHV-4 are known three transactivating proteins, transcribed from immediate early genes that initiate the lytic cycle. Two of them are encoded by conserved open reading frames: ORF50 (Rta/IE2 protein) and ORF57 (Mta/IE1), and the third by a non conserved gene: Bo4/5. Promoter activation by these protein has also a strong effect on DNA synthesis from the *Ori* of lytic replication; as a result, virions are generated and released from the productively infected cells (Ackermann, 2006). The typical inclusion bodies appear in the cytoplasm 48 to 72 hours after the infection and are composed by viral particles at different stages of maturity, with capsids empty or full, enveloped or not; in the eighty's that was considered a proof that BoHV-4 was a bovine cytomegalovirus, because of its similarity with HCMV (Storz et al., 1984).

- ✓ Latent infection: all the herpesviruses can give raise to latency state; apparently the default pathway used by the gammaherpesviruses will most often proceed with the establishment of latency instead of the lytic state (Ackermann, 2006). There is no common pattern of gene expression required for establishment, maintenance, and reactivation of latency. Some herpesviruses synthesize a lot of protein during latency, while others do not synthesize any. In rhadinoviruses the latency is characterized by the combination of viral sequences required for genome maintenance and genes that mediate cell survival by affecting signaling pathways and manipulation of the immune response (Field et al., 2003). Generally latency consists in the absence of virus replication or very low levels of it. The beginning of a latent infection is in common with lytic one; when the viral DNA arrive to the nucleus it does not start the classical amplification of signal through the transcription of alpha and beta gene; however it seems that a weak level or transcription of alpha and beta can occur, but it is not enough to start a lytic cycle. During latency

the viral genome persists in the nucleus as an episome, a circularized form of viral DNA. In rhadinoviruses during cell division episomes are distributed to the new formed cells thanks to the protein LANA (latency associated nuclear antigen, coded by ORF73) or its orthologues. LANA binds the cellular chromatin with its N-terminal and interacts with different sequences in prDNA with its C-terminal. In this way it starts the viral replication and is also permitted the anchorage of episome to cellular chromosomes. In BoHV-4 ORF73 deletion does not affect the virus replication *in vitro*, but affects virus persistence *in vitro* in cells persistently infected, and prevented latent infection *in vivo* in a rabbit model (Thirion et al., 2010).

Recently Donofrio et al. have demonstrated that in RD4 and BoMac cell persistently infected with a recombinant BoHV-4 carrying the neomycin-resistance gene and drug selected with geneticin, the viral DNA can exist as an episome or can be integrated in the cellular DNA, at random sites. Further studies are required to corroborate if the integration occur also in vivo and to verify if it is random or in specific chromosome loci (Donofrio et al., 2010a).

- ✓ Susceptibility and permissiveness: generally the host range of a virus is determined by the susceptibility and permissiveness of the cells targeted; classically gammaherpesviruses are considered to have a very narrow host range. BoHV-4 is convenient to study because has the ability to replicate *in vitro* in a wide range of cultured cells of different origins. A cell is defined susceptible when possesses on its surface the receptor required for virus attachment and entry; a susceptible cell became a permissive one if support a complete cycle of productive infection. Two types of non-productive infections have been described: the latent infection that can be reactivated from different exogenous stimuli in a productive infection; and the abortive one, in which the viral replication is irreversible blocked, that can evolve to cellular death or to a totally unapparent infection. Non permissive infection of human cells by BoHV-4 was indeed observed (Gillet et al., 2004); and the overexpression of Immediate Early protein 2 (IE2) was able to induce virus replication in non-permissive cell lines (Donofrio et al., 2004a).

1.2.4. BoHV-4 *in vivo* infection

1.2.4.1. *In vivo* host range

In contrast to other gammaherpesviruses, that have a restricted or preferential host, BoHV-4 has been isolated in several animals.

The widespread distribution of BoHV-4 in cattle (*Bos Taurus and Bos indicus*) populations justified the fact that the bovines species is considered the natural hosts; hence the nomenclature of the virus. The virus has been isolated in a variety of ruminants, other than the cattle: in African buffalo (*Syncerus caffer*) has a high

prevalence (Rossiter et al., 1989), higher than that observed in cattle, suggesting that maybe the African buffalo can be the original host species for BoHV-4, and that other species, including domestic cattle, may have acquired the virus more recently (Dewals et al., 2006).

BoHV-4 has also been isolated in the American bison (*Bison bison*) and in zebus (*Bos indicus*)(Moreno-Lopez et al., 1989); sheep and goat are sensitive to BoHV-4 infection, but there are not a lot of data about wild ruminant.

The virus has been isolated in felines too: in lions (Egyed et al., 1997), and in a cat suffering from urolithiasis (Fabricant et al., 1971); felines are very far from phylogenetical point of view from cattle, but they feel to be sensitive to the virus. Experimental infection of cats with BoHV-4 gave different results depending on the strain used, maybe because of the adaptation that occurs between virus and host (Thiry et al., 1990). The same virus isolated from the lion was inoculated in new born cats and caused severe pathologies, varying from conjunctivitis to pneumonia , often with a lethal prognosis (Egyed et al., 1997). It is not clear how the virus can have infected for the first time felines: in reality there could be a strain circulating and adapted to felines or a contamination with infected biological materials. BoHV-4 has infact been isolated in fetal bovine serum, used as a supplement for culture medium and also in formulation of a lot of substances, opening the problem that contamination can be the leading trail for BoHV-4 infection in species not naturally susceptible, as felines or maybe humans.

Simian herpesvirus aotus 2, isolated from the kidney of an apparently healthy owl monkey (*Aotus trivirgatus*), was proven to be a BoHV-4 strain (Bublot et al., 1991). In monkeys this virus is neither pathogen not oncogenic, and a high prevalence has been demonstrated, such as 42%, but it is not clear if this virus can give experimental infection and if these data are real or coming from a contamination or the circulation of a primate herpesvirus that is not yet identified. This monkey isolate replicated in different monkey's cell lines from kidney, intestines and lung, as in rabbit cells and goat cells, where CPE and inclusion bodies were observed. No CPE was seen in a primary culture of whole embryo cells (Barahona et al., 1973).

An herpesvirus related to BoHV-4 could circulate between exotic animals such as Asian elephants (*Elephas maximus*), that presented antibodies against BoHV-4 (Metzler et al., 1990) and in black rhinoceros (*Diceros bicornis*), in which a DNA polymerase strictly related to that of BoHV-4 was identified.

Other species are susceptible to BoHV-4 infection, even if the virus has not been isolated in field conditions; these species are considered experimental animals to study this virus. A model studied was the lapin, considered a good model to study BoHV-4 biology (Egyed, 2000; Egyed and Baska, 2003; Egyed et al., 1997; Lin et al., 2000).

Experimental inoculations of BoHV-4 in hamster, rats and chicken have produced no clinical signs (Gibbs, 1977). As a model to study BoHV-4 pathogenesis was chosen the rabbit, that successfully support the infection with BoHV-4; lymphoid organs were the site for viral replication and/or persistence; the virus was infact isolated from conjunctival swabs and spleen, but during latency also in bone marrow, lung, kidney, salivary glands and liver with a low titer (Osorio et al., 1982).

Recently we reported the use of transgenic mice, like IFNAR$^{-/-}$ mice (knockout for IFN alpha and beta receptor) that showed no clinical signs of infection and support virus replication in selected organ, as spleen and lymph nodes (Franceschi et al., 2011).

1.2.4.2. Clinical signs and pathologies

BoHV-4 has been isolated from cattle suffering from a lot of different diseases and from healthy cattle. Although a possible role in respiratory and genital tracts infections has been reported and the virus has been isolated from several clinical conditions of cattle, experimental inoculation of most of the isolates of BoHV-4 in cattle and in laboratory animals do not produce clinical disease. The pathogenic role of this virus, therefore, is still not defined.

Various conditions from which BoHV-4 has been isolated are:

a) Ocular and respiratory diseases: the virus was firstly isolated in an animal suffering from keratoconjunctivitis (strain Movar 33/63) and from respiratory illness, characterized by nasal discharge, cough, dyspnoea and pulmonary lesions (Strain DN599) (Bartha et al., 1966; Mohanty et al., 1971). Intranasal and intratracheal inoculation of BoHV-4 reproduced the majority of symptoms and led to death some calves (Mohanty et al., 1972), the role of virus in this study remains unclear because even *Pastorella multocida* was isolated in some cases of this experiment. In other cases the inoculums of BoHV-4 resulted only in mild respiratory symptoms.

b) Genital diseases (orchids, vaginitis, aborts, and metritis): since from the Eighty's several BoHV-4 strains were isolated in Africa in animals showing symptoms of the "epivag" syndrome : the bullets showed epididymitis and the calves vaginitis, post partum metritis and abortion. Inoculation of virus isolated from a metritis in fetuses caused the death of two fetuses at 3 and 4 month of gestation, showing lymphoreticular activation in the lung and in the lymph nodes (ParK and Kendrick, 1973).

   BoHV-4 has been also isolated in case of abortion, in association with Bovine Viral Diarrhea Virus (BVDV) (Reed et al., 1979). Association of BoHV-4 with other pathogens in cases of abortion has

been consistently reported in the past although the role of this association is not yet fully understood. BoHV-4 has been associated with metritis, especially post-partum metritis, with or without peritonitis.

c) Mastitis: the virus has been isolated in case of mastitis and was detected in the cellular fraction of milk (Donofrio et al., 2000a). The experimental intranasal and intra-mammary inoculation has reproduced in several cases a subclinical mastitis (Wellenberg et al., 2002), but it is common though that BoHV-4 is not the causative agent of mastitis, but only a secondary pathogens.

d) Skin lesions (lumpy skin disease, mammary pustular dermatitis): BoHV-4 has been isolated from skin lesions, ulcerative mammilitis, or mammary pustular dermatitis. When experimental infection was attempted with a BoHV-4 isolate recovered from a case of mammary pustular dermatitis, a febrile response was observed; in another study intradermal inoculation in the udder produced vesicular lesions but no definite role of BoHV-4 could be assessed (Osorio and Reed, 1983).

e) Enteric diseases: DN599 strain was isolated from the feces of an adult cow with diarrhea, no other bacteria or parasites were found; however experimental inoculation failed to reproduce enteric symptoms in cattle (Eugster, 1978/1979). Another strain has been detected in samples from a tumors of the urinary bladder and rumen (Kaminjolo et al., 1972).

f) Other pathologies: several strains of BoHV-4 have been isolated in animals with different pathologies and clinical signs: neoplastic diseases as lymphosarcoma, ocular carcinoma, T cell lymphoma and some others (Anson et al., 1982; Kaminjolo et al., 1972; Toho, 1985).

1.2.4.3. BoHV-4 Pathogenesis

The natural infection probably occurs by direct contact through the genital or the respiratory route and the respiratory and alimentary tracts, by inspired air or by the swallowing of virus aerosol or virus-infected cells. The virus replicates in epithelial cells of the intestines, larynx, trachea and bronchioles, causing slight catarrhal symptoms and fever. From the apparently healthy animals the virus is continuously detected from the blood leukocytes, spleen and lymph nodes for months (Osorio et al., 1982). BoHV-4 is normally isolated from upper respiratory tracts, and from genital tracts; if nasal transmission has been proven experimentally (Dubuisson et al., 1989)**,** genital transmission is not yet totally understood. Recently a work by Egyed et al. demonstrated the intra-uterine transmission of virus between cows and fetus; viral genomes were recovered from semen cells and from spleen of stillborn calves (Egyed et al., 2011).

In general, the diseases that result from infection with BoHV-4 appear to be consequent of cell destruction resulting from virus replication in mononuclear blood cells. In immunologically weakened host BoHV-4 can cause an organ specific, lytic infection; it is possible that the virus enters the body via the oronasal route, multiplies in the mononuclear cells and disseminates in all the body via these cells.

Primary multiplication of the virus usually occurs at the entry site, then virus multiplication occurs in peripheral blood leukocytes, in the monocity/macrophage lineage, and the virus is disseminated in the host by these cells. The presence of viral DNA in blood of infected animal is detectable 10 to 30 days after the infection (Egyed et al., 1999), viremia is not always detectable and can re-appear at different times. Also endothelial cells are sensitive of BoHV-4 infection, probably the virus spread from the mononuclear blood cells to the endothelial cells, opening the route to infect tissues and organs.

The virus preferentially replicates in conjunctiva, upper respiratory tract mucosa, and genital mucosa, resulting in ocular, nasal and vaginal excretions; these tissues can be considered the primary and secondary multiplication sites for BoHV-4.

In experimentally infected rabbits BoHV-4 actively replicates in the spleen and also in the macrophages; splenic macrophages can be the site of both acute and latent infections; identification of BoHV-4 in non-T, non-B cells located in the marginal zone of the spleen of persistently infected cattle and rabbits have implied that cells of the monocytes/macrophage lineage are a site of persistent BoHV-4 infection (Osorio et al., 1982; Osorio et al., 1985).

After the first infection BoHV-4 establishes a latent infection, as all the other herpesviruses. Different tissues have been proposed to be the site of latency: nervous system, lymph nodes, amygdale, upper respiratory tracts, lungs, bone marrow, conjunctives, bilious vesicles (Boerner et al., 1999). Splenic mononuclear cells (macrophages) could be the main site for viral persistence (Lopez et al., 1996); nervous tissues were also considered as sites of viral latency, although the role of the nervous system in acute and latent BoHV-4 infections is still questionable. Several studies have tried to verify if nervous system is a possible site of latency, but no clear data were acquired. Asano et al. recovered the viral genome in animals euthanized more than one year after the experimental infection; specifically in lymph nodes and medulla, spinal cord and trigeminal ganglion, supporting that even the nervous system may be a latency site (Asano et al., 2003).

Lymphoid organs and mononuclear blood cells have been suggested as the sites of viral latency in cattle because the virus was recovered from co–cultures and organ explants of spleen from latently infected cattle (Castrucci et al., 1987a). The association of BoHV-4 with the lymphoid organs and a prolonged viremia associated with the mononuclear cells, with consequent permanent transport through lymphatic

and haematogenous routes for long periods of times, may explain the reason for the recovery of virus from diverse tissues and syndromes (Osorio and Reed, 1983).

Donofrio et *al.* demonstrated that BoHV-4 can establish a persistent infection in vitro in a bovine macrophage cell line (BoMac) (Donofrio and van Santen, 2001).

External stimuli, natural as the parturition, or experimental as dexamethasone treatment, can reactivate BoHV-4 from latency. Then the virus can be re-isolated from nasal secretion, mononuclear blood cells, and bone marrow cultures (Dubuisson et al., 1989).

1.2.5. BoHV-4 correlates with post-partum metritis

Half of all dairy cattle after parturition suffer from uterine microbial diseases often cause of infertility by disrupting uterine and ovarian function; metritis is defined how the uterine disease that appears usally within one week and 21 days after parturition, but is most common within 10 days, and is present in the 40% of cows (Sheldon et al., 2009). Metritis is characterizedb by an enlarged uterus with a red-brown, watery and fetid fluid to viscous off-white purulent uterine discharge; grade 1 metritis show no systemic symptoms, while grade 2 experienced decrease in milk yeald, dullness anf fever; and grade 3 signs of toxemia such as inappetance, cold extremities, depression and/or collapse, with a poor prognisis.

The most prevalent bacteria isolated from the uteus lumen of cattle suffering from uterine diseases, are *Escherichia coli* and *Arcanobacterium pyogenes*, followed by some anaerobic bacteria such as *Prevotella* species, *Fusobacterium necrophorum* and nucleatum.

BoHV-4 was isolated for the first time in a metritis case in 1973 (ParK and Kendrick, 1973) and to date is the only virus usually associated with uterine diseases in the post-partum period in cattle (Ackermann, 2006; Thiry et al., 1992); Monge et al. reported that there is a clear association between BoHV-4 infections and acute post-partum metritis in dairy cows, and that BoHV-4 associated post-partum metritis appeared to be an emerging syndrome, even because often be refractory to antibiotic treatment (Monge et al., 2006).

Wellemans et al. demonstrated the strict correlation between BoHV.4 and post-partum metritis: they inoculated several cattle with the virus and all cows developed ulcerative post-partum metritis (Wellemans et al., 1986); a recent paper reported that 15/22 cows affected by metritis were BoHV-4 positives, but that they recovered clinically and the course of metritis was not severe after a early and prolonged treatment with antibacterial and $PGF_{2\alpha}$ (Nak et al., 2011).

BoHV-4 is highly tropic for endometrial stromal and epithelial cells, led to non-apoptotic cell death and *de novo* viral production associated with increased cyclo-ossigenasi 2 synthesis (COX-2) and stromal cell prostaglandin-endoperoxide synthase 2 (PTGS2) protein and stimulated prostaglandin E_2 (PGE) secretion (Donofrio et al., 2007a; Donofrio et al., 2008b). Post-entry events are responsible for BoHV-4 successfull

replication in bovine endometrial cells, with quick viral reconstitution following the electroporation of nude viral DNA into endometrial stromal and epithelial cells (Donofrio et al., 2007a; Donofrio et al., 2008b).

The high efficiency of BoHV-4 replication in endometrial cells was associated with strong transactivation ot the IE2 gene; on IE2 promoter was also identified a putative endometrial specific TATA box; furthermore, BoHV-4 IE2 gene promoter transactivation and viral replication were associated with extracellular stimuli belonging to the intrauterine microenvironment such as *E. coli*, LPS and PGE (Donofrio et al., 2005). BoHV-4 replication was also reactivated in latently infected macrophages by stromal cells in co-cultivation (Donofrio et al., 2007a; Donofrio et al., 2008b). A model for endometrial BoHV-4 disease was proposed, where viral particles infecting the endometrium, could stimulate PGE production and establish a positive feedback loop between PGE production and viral replication, which causes further endometrial tissue damage and inflammation (Donofrio et al., 2005; Donofrio et al., 2007a; Donofrio et al., 2008b).

Bacterially induced metritis in cattle persistently infected with BoHV-4 could be exacerbated or become chronic after the recruitment of macrophages, that are the site for BoHV-4 latency, from the blood stream to the site of inflammation; because opportunistic infection can increase PGE production, it is supposed to have a correlation between opportunistic infection of the uterus, PGE2 production and BoHV-4 replication, with the generation of a loop system involved in generating and exacerbating uterine e diseases.

The chemokine Interleukin 8 (IL-8) is involved in the recruitment of granulocytes, such as macrophages and neutrophils, in the site of Infection; it is stated that endometritis is associate with an increased IL8 mRNA expression in the endometrium, and that the intrauterine infusion of the chemokine, can reproduce the disease (Zerbe et al., 2003). A recent study confirmed that IL-8 is constitutively expressed and secreted in bovine endometrial stromal cells (BESC) and that the induction of IL-8 in BoHV-4 infected cells is mediated by Orf50/Rta at the transcriptional level, leading to the identification of a Rta responsive element on IL-8 promoter (Donofrio et al., 2010b). These observations may represent an endometrial defence mechanism against viral infection or a virulence mechanism by which viral replication stimulates a chemokine to attract more susceptible host cells to the endometrium; targeting chemokines and/or their receptor might provide a successful therapeutic approach.

Based on this *in vitro* knowledge, it appears evident that the existence of a patho-biotype of BoHV-4 causing uterine disease, can not be ignored.

1.2.6. Immune response to BoHV-4

After BoHV-4 infection in cattle a weak or null production of serum neutralizing antibodies can be observed; high titre of anti-BoHV-4 can be identified through indirect immuno-fluorescence test (IFI) (Castrucci et al., 1987a; Castrucci et al., 1987b; Storz et al., 1984). When a response is detectable, serum neutralizing antibodies (SNA) appeared from 22 to 34 days after the first infection; addiction of complement increases the titre and enables SNA to be detected earlier and with higher titles after infection. It is not clear if the weak humoral immune response is because of the strong cell association of BoHV-4 which may result in minimum exposure of the virus to the immune system.

The role of BoHV-4 in cell-mediated immunity has not yet been investigated; Mohanty et al. reported the absence of antibodies in BoHV-4 infected calves but, when challenged with the virus 8 weeks after the primary infection, the surviving calves were found to be immune to the virus (Mohanty et al., 1972).

Serum-neutralization test is not used to detect the virus in infected animal because has been demonstrated that the domains responsible for virus attachment and penetration are not so accessible to monoclonal antibodies. Instead other techniques as IFI or ELISA are able to detect a seroconversion from 14 to 20 days after the primary infection (Dubuisson, 1987; Osorio and Reed, 1983).

Since the immune system plays a key role in protection against infection, viral damage to its components can exacerbate the severity of disease or predispose the animal to super-infection with other viral or non-viral agents. Viral replication may lead to temporarily suppressed humoral and/or cell-mediated immune response. Some viruses have as preferential target the immune cells, as Marek's disease and BVDV. Similarly, BoHV-4 is associated with mononuclear blood cells and lymphoid tissues both in acute and latent infections which may have implications for the regulation of humoral and cellular immune responses in cattle (Osorio et al., 1985). Similar information about BoHV-4 pathogenesis is not yet available.

1.2.7. BoHV-4 Diagnosis and prophylaxis

Not many tests are available for the detection of BoHV-4 or its antigen in fluids and tissues of infected animals. Several tests have been standardized to determine the level of BoHV-4 specific antibody in serum samples. Isolation of BoHV-4 has been successfully determined from different clinical specimen by using conventional cell culture inoculation and/or co-cultivation of infected cells with susceptible cells and organ explantation, and then analyzed by Immunofluorescence.

Complement fixation, dot-immunoblotting assay, virus neutralization, Immunofluorescence (IFAT) and ELISA tests have been used for serological studies (Naeem and Goyal, 1990); IFAT and are used extensively and a good correlation between this two tests has been demonstrated; the seroconversion indicates that a

primary infection, a re-infection or a reactivation is in course. Later during the infection also the complement-dependent neutralization can be successfully used, even if the virus elicits a poor neutralizing antibody response. A recent study has demonstrated how a serum neutralization test can be very effective, in animal immunized with a recombinant BoHV-4, deleted in Bo10 gene, responsible for hiding the serum neutralizing epitopes of gB and gH/gL (Machiels et al., 2011b).

The viral genome can be detected by specific PCR, with primers designed over BoHV-4 selective sequences as the gB (Boerner et al., 1999), the thymidine kinase (Egyed et al., 1996; Egyed and Bartha, 1998; Wellenberg et al., 2001); even the in situ ibridization, combined with PCR data was used to assess the presence of BoHV-4 genome (Egyed et al., 1997).

Restriction analysis can also be utilized to characterize different BoHV-4 isolates for diagnostic or epidemiological purposes, is infact described a distinct difference between the patterns of different herpesviruses (Ludwig, 1982).

Since the aetiopthologic role of BoHV-4 has not yet fully understood, no attempt has been made to develop a vaccine. Castrucci et al. observed a variation in the pathogenic potential of several isolates (Castrucci et al., 1987a; Castrucci et al., 1987b), indicating that it might be possible to develop a vaccine by using one of the non-pathogenic isolates as a vaccine. In America have been developed some vaccines against BoHV-4, but it is clear that control of BoHV-4 infection will be provided only by hygienic measures; trough the identification of latently infected animals and the physical separation with serum-negative animals. The serum-positive cows that are in the post-partum period have to be controlled in a particular way, because they can excrete a lot of virus, for long period in the uterine exudates (Thiry, 2000).

1.2.8. BoHV-4 as a vector

Several characteristics made BoHV-4 attractive as a vector: (i) its ability to establish a persistent infection, both in the natural host, the cattle, and in the experimental host, the rabbit, (ii) to maintain the viral genome as an episome in the cell host , (iii) the lack of oncogenicity, (iv) the possibility to delete even the 30% of the viral genome without consequence on the viral replication and so, (v) the huge capacity to transport exogenous DNA, (vi) the free access to the complete genome sequence of two different isolates types and (vii) the possibility to easily manipulate its genome as a Bacterial artificial chromosome (BAC). Moreover BoHV-4 has to date no clear disease correlation (an exception has to be done for the correlation as a secondary pathogen in uterine diseases) and can be isolated from a variety of samples and tissues from different species of animal; it is also able to replicate *in vitro* and cause CPE in a number of primary cell cultures and immortalized cell lines.

The possibility to manipulate BoHV-4 as a BAC has led the way to generate recombinant viruses able to target exogenous antigenic protein for vaccinal purposes or therapeutical agents for cancer treatment.

For example the potential of BoHV-4 for the treatment of glioma has been investigated. The suitability of BoHV-4 as a vector was first evaluated *in vitro*, using the rat glioma F98 cell line and then in vivo, injecting the virus into rat brains. In F98 cells the virus was able to induce cell death by non-apoptotic way but, *in vivo*, showed a non permissive behavior, because the virus did not spread through the cerebral parenchyma and not cause clinical signs. The ability to establish permissive infection in cultures of glioma cells, and the absence of pathogenicity in the rat brain, make the virus an ideal candidate as a gene delivery or oncolityc vector for gliomas (Redaelli et al., 2008). BoHV-4 showed also the capability to infect selectively gliomas induced in the rat brain *in vivo;* a recombinant virus, expressing an enhanced green fluorescent protein (EGFP) was able to infect the glioma cells and was also confined to the tumor cells, being safe for the nervous system (Redaelli et al., 2010).

Taking into account these data a new recombinant virus was generated, that expressed the thymidine kinase gene of HSV-1 (TK-HSV-1) and was used to target glioma cells growing in the brain of mice and rats. After the injection of the virus in the brain, the animal were treated with ganciclovir, that selectively targets the glioma cells because can be converted in a toxic agent by the thymidine kinase gene product. BoHV-4 used as an oncolityc virus has led to a highly reduction of the gliomas in the mice, but especially in the rats, with no consequence on nervous system cells, implying that BoHV-4 is a good candidate to be used as a vector for cancer treatment in the nervous system (Redaelli et al., 2011).

BoHV-4 has also been tested for vaccinal purposes: Donofrio et al., infact have explored the potential to target the antigenic protein of different viruses as BVDV (Bovine Viral Diarrhea Virus) or BoHV-1 (Bovine Herpesvirus 1), respectively gE2 and gD, to generate serum-neutralizing antibodies. A fusion peptide, obtained by the fusion of the ectodomain of the gE2 protein and the ectodomain of the gD protein, deprived of the signal peptide, but with the trans-membrane domain, was generated and tested in several different animal species, varying from rabbits, swine, goats and sheep; in every cases good title of serum-neutralizing antibodies were obtained, against the two protein both. These data have to be supported and validated through challenge experiments that have to be planned (Donofrio et al., 2009b).

Another study focused about the generation of a vector for Bluetongue vaccination: the VP2 protein of the serotype 8 of Bluetongue Virus (BTV-8) was used to create a recombinant BoHV-4, that was experimented to vaccinate transgenic mice $IFNAR^{-/-}$, then exposed to a challenge with a lethal dose of BTV-8. Only the 17% of the animals survived the challenge, showing that a partial protection has been achieved, all the animals showed moreover a delay in the clinical signs and in the blood viremia; probably this vector have to be

hampered combining the expression of more than one antigenic protein, such as VP7 and NS1 (Franceschi et al., 2011).

The feasibility of BoHV-4 as a vector for vaccination has also been tested in chicken; although the virus was able to replicate, leading to CPE effect in a chicken fibroblast cell line (DF-1) and can infect chorion allantoic membrane of embryonated eggs, it did not replicate and was not pathogenic, even at very high dose, in chickens, even if a good humoral immune response was achieved against the antigen carried on by the virus, suggesting that can be a good candidate for chicken vaccination (Donofrio et al., 2008a).

1.3. BAC as a tool for herpesvirus genetics

To date the complete sequence of the genome of many herpesviruses has been determined, but the precise function for many of the viral gene, both *in vitro*, and in particular *in vivo*, has not fully elucidated; this knowledge is important not only to understand their role in pathogenesis, but also to study them as potential therapeutic target, and even for the rational design of new vaccines.

The disruption of herpesvirus genome is usually achieved by the integration of a marker gene into the viral genome through homologous recombination in permissive cells; this technique can not be applied to all type of viruses, especially viruses like HCMV that shows slow replication kinetics and is strictly cell associated, rendering the isolation of viral mutants very difficult. Conventional mutagenesis is usually followed by the phenotype analyses, both *in vitro* and *in vivo*; and is achieved through chemical mutagenesis, site-directed mutagenesis by homologous recombination in eukaryotic cells and manipulating virus genome using overlapping cosmid clones. All these methods are very useful to generate mutants, but often these procedure are inefficient, laborious and time consuming; they require to select the mutants from the parental virus, and this selection can be assessed only at the very end of the experimental procedure (Adler et al., 2003).

Cosmids are basically plasmids with *cos* sequences (cohesive end site) derived from λ phage; the presence of this sites lead to an increase of insert size till to 35 kb, these sites can be also used to pack the plasmid in phage particles *in* vitro, that can transform with a high efficiency bacteria.

Even the cloning of infectious viral genomes as a yeast artificial chromosome (YAC) has been described, for example for adenovirus (Ketner et al., 1994), but remains unclear if large herpesvirus genome can be cloned as YACs and whether these genomes are stable in yeast. YACs can accommodate up to 2Mb of DNA fragment, and include all the sequence necessary to maintain the vector as a chromosome: an origin of replication, a centromere, two telomeres (YAC are amplified as linear DNA molecules) and several selectable markers. The transformation in yeast is a process not so efficient as in bacteria, and YAC are very

difficult to isolate intact; they are also highly susceptible to shearing, since they are cloned as linear molecules; and genomic rearrangements and contamination with parental yeast DNA is however frequent.

BACs are single copy cloning vectors derived from *E.coli* F plasmid; circular DNA molecules of about 100kb, able to persist as an episome or to integrate in bacterial chromosome; the high capacity vector derived from this plasmid contain the F replication and partitioning system that ensure low copy number and faithful segregation.

BACs have been widely used thanks to their great capacity to accommodate large inserts (>300kb) and to be genetically stable in bacteria; viral BACs containing *E.coli* can infact be stored at -80°C, and new viruses can be quickly produced from them; the low and the controlled copy number of such vector has made them superior to cosmid and YAC (Shizuya et al., 1992). BACs are also suitable to clone viral genome, as first demonstrated by Luckow *et al.* (Luckow et al., 1993) with baculovirus. Exploiting bacterial genetics allowed the introduction of every type of mutation (deletion, insertion or point mutation) into the cloned viral genomes; manipulated genomes can moreover be characterized prior to the virus reconstitution.

Ever since the creation of the first herpesvirus bacterial artificial chromosome (BAC) mutant, for murine cytomegalovirus, over a decade ago, mutagenesis using BAC technology has been proven to be an important tool to study herpesviruses pathogenesis. The availability of viral mutants would significantly contribute to the understanding of viral gene functions and to the evaluation of their role in pathogenesis.

Since the cloning of the murine cytomegalovirus genome (MCMV) as the first herpesviral infectious BAC, a lot of DNA viruses have been cloned as BACs, including HCMV, HSV-1, EBV, HHV-8, Pseudorabies virus, Marek's disease virus, MuHV-68, RRV, VZV, SaHV-2 and BoHV-4 (Adler et al., 2003; Borst et al., 1999; Gillet et al., 2005a; Messerle et al., 1997; Zhou et al., 2010).

A typical BAC cassette is about 10 kb and must have an origin of replication, the genes necessary for BAC replication (such as *redF* and *repE*), and genes to control the rate of replication in order to limit the copy number per cell (such as *parA* and *parB*). Even an antibiotic resistance marker must also be contained in the BAC cassette; and must be flanked by 500-1000bp that are homologous to the target sequence where the BAC vector will be inserted. The insertion of a selectable marker, such as betagalactosidase or GFP, is necessary to isolate BAC-containing recombinant virus.

It is desirable to reconstitute the complete wild type genome of the virus and so, to excise the BAC cassette from the virus, because the extra genetic materials might overcame the tolerable size limit of the viral genome, causing genomic instability and inefficient packaging. The excision of the BAC cassette can be achieved providing the BAC cassette of two *loxP* sites, exploiting the Cre-loxP system; or inserting a fragment of identical viral sequences on the opposite of BAC cassette; or inserting the cassette in the

tandem repeated area of the viral genome, that spontaneously tend to excise the cassette or exploiting the system of mini F-replicon (Zhou et al., 2010).

The BAC cassette, flanked by viral sequences, is usually transfected in infected cells, resulting in the insertion of the BAC cassette in viral genomes by homologous recombination (Fig.5). During replication herpesviruses form circular intermediates that can be extracted from cells and used to transform *E.coli;* resulting in a shuttle vector containing the viral genome cloned as a BAC, and subsequently can be manipulated in bacteria cells, amplifying it. The so purified BAC clone can be transfected in eukaryotic permissive cells to reconstitute the recombinant virus; if the BAC cassette was provided of two *loxP* sites, the infection of permissive cell line expressing the recombinase *cre*, will lead to the reconstitution of a viable virus.

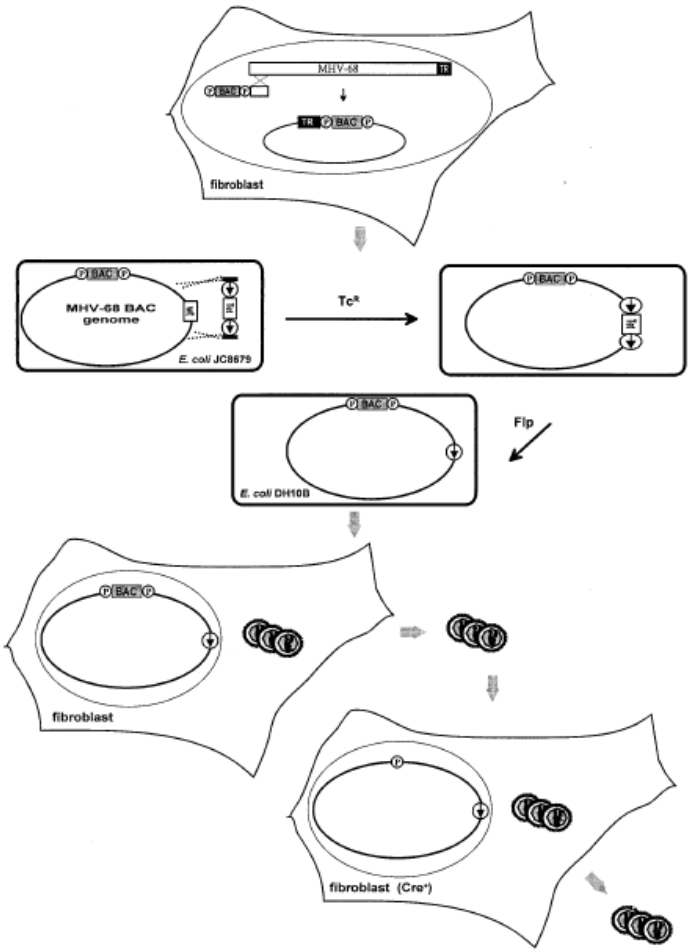

Fig.5. Strategy for the construction of an infectious herpesvirus BAC clone , adapted from (Adler et al., 2003)

BoHV-4 was cloned as a BAC for the first time in 2005 by Gillet *et al.* (Gillet et al., 2005a), using an approach similar to those adopted by Messerle *et al.* to clone MCMV (Messerle et al., 1997). The BoHV-4 *EcoRI* fragment containing a *XhoI* site from the right end of the viral DNA was isolated and cloned in a plasmid to generate pGEM-T-EcoRI G. *XhoI* was then used to clone the BAC cassette in the plasmid; co-transfection with the linearized plasmid and purified viral DNA resulted in homologous recombination in MDBK cells (Madin Darby Bovine Kidney cells). The integration took place in a non-coding region at the junction of right prDNA and the unique central region ofBoHV-4 (Fig.6).The BAC cassette was flanked by two loxP sites to excise the BAC cassette, resulting in the removal of the BAC cassette after transfection in a permissive cell line expressing the *cre* recombinase (BEKfincre, Bovine embryo kidney cells expressing the *cre* recombinase). In the BAC vector is inserted moreover an expression cassette for the EGFP protein (enhanced green fluorescent protein), to easily visualize the cells infected by the recombinant BAC through fluorescence microscopy analysis.

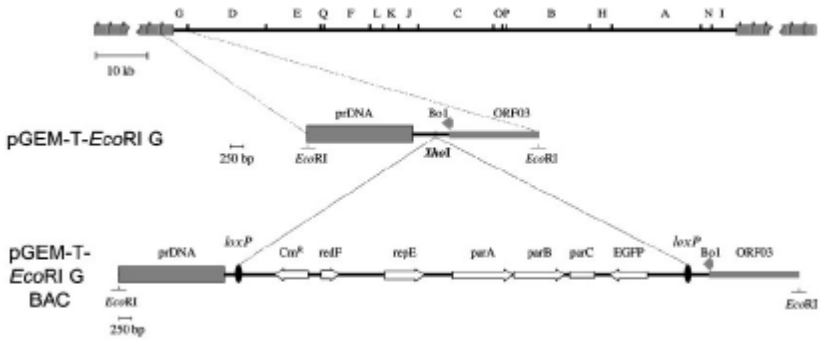

Fig.6.Diagram showing the insertion of a BAC cassette at the left end of the BoHV-4 genome, adapted from (Gillet et al., 2005a)

There are two commonly used methods to mutate BAC DNA: random transposition and site direct mutagenesis. Site-direct mutagenesis exploits homologous recombination to create specific mutation in viral genes; transposition creates a diversity of BAC mutants, random mutated, and sequencing or PCR is required to determine the mutation site (Warden et al, 2011.).

Homologous recombination is the process of exchanging DNA between two molecules through regions of identical sequences; the primary role of homologous recombination in the cell is almost certainly the repair of the DNA damage; most of the time occurring at the replication fork. In *E.coli* most recombination depends on RecA protein that binds to single stranded DNA, forming DNA-protein filaments that have the ability to look for other DNA molecules, showing sequence homology, leading the pairing between the strands. Then the RecBCD enzyme initiates the recombination at dsDNA generating 3' single strand overhangs, helping RecA to promote strand exchange and recombination. The intermediates formed are

resolved by the process of branch migration catalyzed by RuvAB or RecG, and the endonucleolytic cleavage catalyzed by RuvC.

Phage-mediated recombination systems can be provided in *E.coli* by the λ phage Red functions, *Exo* and *Beta*, that can also generate recombinants in the complete absence of RecA, through a process called single strand annealing. If two homologous DNAs both receive a double strand break, *Exo* can degrade the 5'ended strands exposing 3' overhangs, that can be bound by *Beta*, that anneals the two strands and generate recombinants. The third gene involved in recombination is *Gam* that encodes a RecBCD exonuclease inhibitor and protect linear DNA-targeting cassette from degradation by RecBCD. These three genes can be expressed from a stably integrated defective λ prophage, where exo, bet and gam are controlled by the strong phage promoter *pL*, under stringent control of the temperature-sensitive repressor,*cI857*. In this system exo, bet and gam are not expressed when the bacteria are kept at 32°C, the induction at 42°Cper 15 minutes, rapidly induce at high level these genes and homologous recombination is efficiently performed. Genetic engineering with phage-encoded recombination functions that utilizes short homologies sequences, till to 50 bp, has been named "Recombineering", a conventional term to describe homology-dependant, recombination-mediated, genetic engineering (Court et al., 2002; Warming et al., 2005).

Warming et al., described the development of a novel Galk-based selection system for BACs manipulation in *E.coli* SW102. The E.coli galactose operon consists of 4 genes, GalE, GalT, Galk and GalM, necessary for the utilization of galactose as the unique carbon source. The Galk gene, codes for galactokinase, an enzyme able to convert the galactose in galactose-1-phosphate; is also able to catalyze the phosphorylation of a galactose analog, 2-deoxy-galactose (DOG) in a toxic compound. The *E.coli* SW102 strain is $Galk^-$ and is unable to use galactose as carbon source; leading to positive clone selection in galactose enriched minimal medium and the negative one in medium enriched with DOG (Warming et al., 2005). Donofrio et al., added another selection step, flanking the Galk cassette with the gene that confers resistance to Kanamicine; the addiction of a negative selection n kanamicine reduces the number of background colonies growing during the selection step (Donofrio et al., 2009b).

1.4. Gammaherpesviruses Immediate early 2 gene (IE2)

Herpesviruses have large complex DNA genomes and transcription is strictly regulated during *in vitro* infection. Three phases occurs during infection and are sequentially regulated; genes are so divided into three classes: immediate early (IE), early (E) and late (L), on the basis of their time of expression. The temporal regulation of gene transcription is accomplished through transactivation of viral transcriptional promoters by viral genes specific products. IE proteins are usually considered as transactivator proteins; their expression does not require a prior viral DNA synthesis, and are also the only genes transcribed when cells are treated with cycloheximide, a protein synthesis inhibitor. Proteins encoded by the IE genes are necessary for the expression of E and L genes; L genes synthesis is restricted by viral DNA replication and infact are not expressed when DNA polymerase inhibitors are used (Hay and Hay, 1980).

Herpesviruses lytic gene expression follows this temporal and sequential cascade, ultimately resulting in the production of progeny virions and destruction of and egress from the infected host cells.

Immediate early genes define mRNAs that are transcribed in the presence of protein synthesis inhibitors, this applies both to herpesviruses genes after *in vitro* infection of permissive cells and to cellular genes after serum stimulation (Roizman, 1996). Herpesviruses immediate-early genes transcription is necessary and sufficient to initiate viral replication(McKnight et al., 1987).

Rta/Orf50 is an immediate-early protein that is essential to initiate lytic replication of all studied gammaherpesviruses: Epstein Barr virus (EBV), Kaposi's sarcoma associated herpesvirus (HHV-8), rhesus rhadinovirus (RRV), herpesvirus saimiri (SaHV-2) and murid herpesvirus 68 (MuHV-68) (Staudt and Dittmer, 2006). Ectopic expression of Rta/Orf50 will reactivate virus from latency; its deletion or inhibition by a dominant-negative mutant will prevent lytic and reactivation replication. There are other IE genes that are transcribed in presence of cycloheximide, but their presence is not enough to reactivate virus from latency.

All gammaherpesviruses encode an Rta/Orf50 homologue, and each has been proven to play a pivotal role in the initiation of viral lytic gene expression and lytic reactivation from latency.

Rta (replication and transcription activator) is the gene product of ORF50 gene and is the only demonstrated essential latent/lytic switch protein for the gamma-2-herpesviruses (rhadinoviruses); instead of gamma-1- or lymphocryptovirus, in which are present two IE protein, Zta and Rta, that independently can reactivate EBV from latency. The two groups of gammaherpesviruses can so have evolved different molecular mechanisms of virus-mediated lytic reactivation.

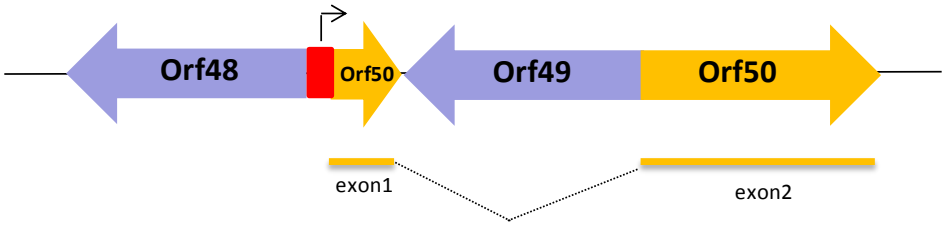

Fig. 7. Classical architecture of Gammaherpesviruses Orf50 gene.

The Orf50 transcripts from all gamma-2-herpesviruses share similarities in the genomic location, amino acid sequence and splicing patterns, and a typical architecture, essentially comprised of two exons, separated from an intron, that contains ORF49 gene, located in opposite orientation; its promoter is probably located within the Rta ORF. Splicing of the two exons and excision of the intron results in a single, major Rta transcripts, but differential splicing can also occur, for example in HHV-8 (Damania, 2004; Damania et al., 2004).

Amino acid sequence alignments of the Rta homologues revealed that the most conserved regions are at the N-terminus. This conserved portion of Rta in EBV is required for dimerization and DNA binding; another well conserved region is at the C-terminus and is rich in acidic residues, probably indicating a transcriptional putative activation domain. The Rta homologues of gammaherpesviruses share similar amino acid sequences and may also have similar functions in activating viral and possibly cellular promoters. It is not yet fully understood whether the Rta protein are capable of substituting for each other to transactivate virus-specific promoters; it has been shown that EBV Rta could not transactivate the viral promoters activated by SaHV-2 Rta or HHV-8 and vice versa (Damania, 2004; Damania et al., 2004).

The exact mechanism whereby Rta/Orf50 activates expression are still not clear, but two possibilities can be supported by the existing data: (ì) Rta can transactivate by either binding directly to target gene promoters through specific RRE (Rta Responsive Element) target sequences to stimulate transcription, or (ìì) by interacting with cellular factor and or other viral factors to enhance transcription indirectly; for example HHV-8 Rta can interact with the RBP-Jk cellular protein, that can bind to promoter DNA in the RRE of Orf50 (Chang et al., 2005).

1.4.1. EBV Rta/Orf50

The major viral lytic reactivation protein is considered to be Zta (or Zebra, BZLF-1 or Z protein), that is a bZIP-type (basic zipper) transcriptional transactivators (Cox et al., 1990). The EBV Orf50 homologue, the BRLF1 gene product Rta, is also a sequence specific DNA-binding protein, with transactivator's functions. Independently Zta or Rta can both initiate lytic gene expression; Rta can act on E gene promoters in cell-specific manner. The common thinking is that Zta and Rta act synergistically to induce viral reactivation from latently infected cells. Rta alone can activate a number of lytic promoters without Zta actions; and Zta alone can activate other that does not require Rta; Zta can moreover suppress the transactivation function of Rta. A deletion mutant of either Zta or Rta is defective for viral replication (Feederle et al., 2000).

A recent study revealed that the expression of Zta and Rta substantially interfered with the cell cycle regulatory machinery in Raji (EBV-positive human Burkitt's lymphoma –derived cells) cells, strongly inhibiting the expression of Rb and p53 and inducing that of E2F1; down regulation of Rb was further demonstrated to be mediated by proteosomal degradation, and p543 and op21 affected at transcription levels (Guo et al., 2010).

A recombinant baculovirus was engineered to express a EBV Rta expression cassette and OriP and was specifically able to inhibits the growth of EBV-positive NPC (nasopharyngeal carcinoma) cells *in vitro* and *in vivo*, suggesting that may have potential application in NPC gene therapy (Wang et al., 2008).

1.4.2. HHV-8 Rta/Orf50

The HHV-8 ORF50 gene encodes for a 691 amino acid (aa) protein that is highly phosphorylated and localizes within the nucleus of mammalian cells, its expression can be detected as early as 1 hour after induction of viral replication and in the presence of the protein synthesis inhibitor (Lukac et al., 2001). Deletions of 160 aa in the C-terminal activation domain results in a stable but truncated form of Rta, that forms multimers with the wild type protein and functions as a dominant-negative inhibitors of Rta transactivation (Lukac et al., 1998). Expression of this truncated form leads to the suppression of both spontaneous and chemically induced viral reactivation. Viral reactivation can also be blocked by expression of human RNaseP (Wang et al., 2004). These data, taken together with a lot of other studies demonstrated that Rta is the switch protein involved in HHV-8 lytic reactivation.

Also the BAC strategy was explorated; a mutant in which the ORF50 was deleted, was generated and tested in human cells; even these types of experiments demonstrated the role of Rta protein, because this virus was unable to reactivate on chemical studies; *in trans* expression of Rta was able to abolish both defects (Xu et al., 2005).

A recombinant BACmid with a deletion in bZIP ORF, a protein able to repress Rta transactivational activities for some promoters, was also generated and displayed an enhanced growth phenotype with respect to virus production and accumulation of virus encoded mRNA; bZIP can influence LANA localization and be involved in persistency state; this study also demonstrated that bZIP may facilitate the interaction of Rta with *OryLyt* (Kato-Noah et al., 2007; Lefort and Flamand, 2009).

Extensive studies have demonstrated that HHV-8 Rta but not EBV Rta was able to induce MuHV-68 lytic viral proteins and DNA replication and processing and produce viable virions from infected cells; through the induction of MuHV-68 proper Rta; the species specificity of MHV-68 Rta respect to those of EBV or HHV-8 resides in the N-terminal DNA binding domain (Rickabaugh et al., 2005).

HHV-8 Rta supports a lot of post-translational modifications, as phosphorylation in first attempt, that can regulate the function, and also the stability of the protein; Rta can infact modulate host gene transcription, as interleukine-6, or STAT3 transactivators activities; can also induce CD21 and CD23a and inhibits p53 tumor suppressor protein (Gwack et al., 2003).

HHV-8 Rta is a strong transactivators of many viral promoters, as demonstrated in transient transfection assays; 19 direct sites for Rta binding have been identified on different genes, including *OryLyt* (Chen J., 2009) and Mta (ORF57), Kaposin, v-MIP-1, thymidine kinase, v-IL-6, LANA and a lot of other genes, as reported by Staut et al. (Staudt and Dittmer, 2006). Rta also acts on its own promoters through an Oct-1, Sp-1 mediated mechanism (Wang et al., 2003).

Two RNAs antisense to HHV-8 Rta have been identified, seemingly they do not regulate Rta transcription but are mRNA encoding small peptides: these RNAs accumulate during lytic infection, were 5' co-terminal, unspliced, and differentially poly-adenilated; they appear in polysomes in infected cells and internal AUG codons can be assessed by translating ribosomes, leading to the synthesis of small peptides of 17 to 48 amino acids that can have a putative effect *in vivo*, maybe contributing to T cell epitope repertoire, generated in both infected and non infected cells (Xu and Ganem, 2010).

Even two miRNAs (small single stranded non coding RNAs that post-transcriptionally regulate the expression of specific mRNAs by pairing to complementary target sequences within related mRNAs) were identified to directly target and inhibit HHV-8 Rta 3' un-translated region, in a sequence-dependent manner, promoting the maintenance of the latent state by translationally regulating the Rta expression. These miRNAs can act suppressing viral lytic replication via activation of NF-kB, that is a strong negative Rta regulator through forming complex with RBP-Jk, one of the major co-activator of Rta (Lin et al., 2011).

1.4.3. MuHV-68 Rta/Orf50

Wu et al. demonstrated that even the Rta protein encoded by MuHV-68 Orf50 is sufficient to induce lytic reactivation in latently infected cells (Wu et al., 2000), through the ectopic expression of the protein they verified that lytic protein were activated from Rta. They constructed also two mutants of MuHV-68 Rta, with deletions at the C-terminus and demonstrated that the mutants were no more able to transactivate some promoters as the Orf57 ones, and also that these mutants can function as dominant negative mutants, inhibiting trans-activation of wild type Rta, and the progression of a productive infection (Wu et al., 2001).

A lot of studies have been published that clarified how Rta is necessary for viral reactivation, even studying the disruption of the ORF with BAC technologies (Pavlova et al., 2005; Pavlova et al., 2003) or by RNA interference knockout (Jia et al., 2004); definitively proving that Rta is essential for virus replication.

A mutant called M50 was generated to express constitutively the protein, by insertion of a new promoter element in the 5'-UTR of the Orf50 promoter; this mutant was unable to establish the latency and, when used in immunization protocol in mice, conferred partial protection against wild type infection (Boname et al., 2004).

A Rta/Orf50 null mutant established long-term latency in mice lung B cells, but failed to vaccinate against a wild type virus challenge, therefore implicating the necessity of lytic replication to generate a protective immune response (Moser et al., 2006).

Assay studies on Rta transactivational activity showed that, if Rta is over-expressed, can up-regulate nearly every MuHV-68 gene, supporting the data that even in MuHV-68 Rta is the most important IE transactivators proteins (Martinez-Guzman et al., 2003). A recombinant virus over-expressing MuHV-68 Rta greatly accelerated the expression of specific lytic cycle ORFs and down-regulated transcription of the major latency gene, ORF73; also cellular genes were up-regulated, as FgF10, VEGFs and VCAM-1, important in cell growth, differentiation and cell adherence (Hair et al., 2007).

Recently a recombinant virus deleted in Orf50 promoter was generated; it retained the ability to replicate *in vitro*, even with a slow growth rate, and revealed the presence of a distal upstream Orf50 promoter, it was able to establish latency in the spleen of infected mice, but unable to reactivate from latently infected splenocytes. This distal promoter is partially methylated in vivo and heavily in latently-infected cells, suggesting that DNA methylation may serve to silence the activity of this distal promoter during latency (Gray et al., 2009). Another study clarified better that MuHV-68 Rta promoter is hypomethylated during lytic infection in mice lung and that methylation level increases with virus latent infection in the spleen; histone acetylation, but not DNA demethylation, is sufficient to reactivate the virus from latency in cells (Yang et al., 2009).

Lee et al., clarified the role of Orf49, that resides adjacent to and in opposite direction from Rta; demonstrating that a mutant harboring a transposon at the Orf49 locus evidenced a highly attenuated *in vitro* growth; they generate also a FLAG-tagged protein and were able to state that this protein enhanced the Rta ability to transactivate downstream promoter, and also increased he level of auto-transcription of Rta; cooperating with Rta protein in regulating virus replication (Lee et al., 2007).

To date it was demonstrated that MHV-68 Rta interacts with ORF57 promoters, ORF72 (v-Cyclin) (Pavlova et al., 2005) with MK3 promoter and ORF18 (trans-factor for late gene expression) (Hong et al., 2011).

1.4.4. SaHV-2 Rta/Orf50

The SaHV-2 R-transactivator protein is homologous to BRLF1 gene product (Rta) that is translated from a bicistronic mRNA which also encodes another IE product, BZLF1. The SaHV-2 R gene is proposed to be spliced with more than one exon; therefore it was clarified that two transcripts of ORF50 are expressed individually from a promoter 5' to the exon 1 and an internal promoter within exon 2 and were called Orf50a and Orf50b, respectively. These transcripts are expressed at different phases during the lytic cycle; Orf50a as an IE gene and Orf50b as a E gene, supporting the idea that Orf50a is the real switch protein from latent to lytic replication, and Orf50b maybe a spliced form or a protein able to auto-regulate itself (Wellemans et al., 1986).

Two isoforms of SaHV-2 Rta/Orf50 were isolated: Orf50a and Orf50b that show a high divergence in their sequence of about 30% in different strains. Orf50a protein efficiently activates early genes, and is able to reactivate lytic replication in persistently infected cells, whereas Orf50b shows contrasting activities in the different strains; maybe Orf50 may be involved in cell transformation and acts as an oncogene (Ensser et al., 2003). There is no evidence that Rta can transform cells in culture or can cooperate with other oncogene to do so; rather it seems that long term stable expression of Rta/Orf50 was incompatible with continued cell growth.

The SaHV-2 Rta transactivates its own promoter, as well as the early ORF6, ORF57 and ORF9 viral promoters (Thurau et al., 2000). The Rta/Orf50 has an AT-hook DNA binding domain required to interact with ORF6 and ORF7 promoters. Rta/Orf50 was able to up-regulate its own promoter by use of a 36 bp RRE, that has no significant homology to previously reported RREs of any of others gammaherpesviruses; this sequence conferred a responsiveness to an enhancer-less than SV40 minimal promoter (Walters et al., 2004). SaHV-2 Rta was shown to bind TATA-binding protein *in vitro*, and this provides another way to act of Rta (Hall et al., 1999).

1.4.5. RRV Rta/Orf50

RRV has also been shown to transactivate a promoter-reporter constructs in transient transfection assays. The R8, ORF57 and gB promoters of RRV were highly activated and the vIRF promoter was slightly activated by Rta (DeWire et al., 2002). In contrast to HHV-8, RRV Rta does not auto-activate its own promoter.

RRV ORF50 can be dramatically inhibited by the homologous to HHV-8 LANA protein, called R-LANA, through recruiting the histone deacetilase complexes; the repressive action of R-LANA on ORF50 may down-regulate the transcription of the early genes at late times during the lytic cycle and also help to maintain viral latency by preventing virus reactivation (DeWire and Damania, 2005).

1.4.6. BoHV-4 Rta/Orf50

Studies of RNAs transcribed from the BoHV-4 genome during lytic infection in MDBK (Madin Darby bovine kidney) cells led to the identification of the two major IE RNA and protein, by van Santen in 1991 (van Santen, 1991). The BoHV-4 major IE RNA is a spliced, 1.7kb RNA transcribed from right to left on BoHV-4 restriction map. The nucleotide sequence of the DNA encoding the major IE early RNA, approximately 1.1kb 5' flanking sequences, and 0.15 kb 3'flanking sequences, contains 60% A+T and 40 G+C nucleotides. This 5'flanking sequence does not contain multiple repeat motifs characteristic of the major IE promoter region of human, murine and simian cytomegalovirus (Thomsen et al., 1984). The nucleotide sequence of the DNA encoding the major IE RNA predicts that it encodes a protein of 284 amino acid and 33KDa, called E1.

IE1 is a putative DNA binding protein and is infact similar to IE110 protein of HSV-1 and could be involved in zinc finger structures formation near its N-teminal.

The other IE RNA, transcribed from left to right on BoHV-4 genome, is about 1.8-kDa, and encodes a 61 kDa protein that shows amino acid homology sequence with EBV transactivating protein R, Rta (van Santen, 1991; van Santen and Chang, 1992) and also with SaHV-2 Rta. IE2 RNA has a short, mostly un-translated, 5' exon, similar to the structure of the 5'portion of EBV R RNA and of those of SaHV-2. The initiation codon of BoHV-4 IE2 is contained in the first exon, while the remaining codons are in the second exon. BoHV-4 IE2 does not transactivate promoters transactivated by SaHV-2 Rta and EBV Rta, and EBV Rta does not transactivate BoHV-4 or SaHV-2 targets; BoHV-4 IE2 moreover does not transactivate the homologous SaHV-2, that are not transactivate by the SaHV-2 homolog to BoHV-4 IE2 (Nicholas et al., 1991).

Transactivation studies showed that the BoHV-4 IE2 gene product specifically transactivates the promoter-regulatory region of the BoHV-4 early gene homologous to the major DNA-binding protein of HSV-1 (van Santen, 1993). Also the thymidine kinase gene was proven to be transactivated by IE2 protein in its promoter regulatory region within a 72 bp fragment 5' to the start of transcription (Zhang and van Santen,

1995). Bermudez and Cruz identified another RNA that is transactivated from BoHV-4 IE2: the promoter of the 1.1kb poly-adenilated RNA, coded by BORFE1, is infact transactivated by IE2; even in this case a responsive element was identified, clarifying that IE2 functions as a promoter factor, rather than an enhancer factor (Bermudez-Cruz et al., 1998).

Donofrio et al., were able to activate the lytic replication of the virus in a non-permissive cell line, RD4 (human rhabdomyosarcoma cells) by overexpression of BoHV-4 IE2 gene, provided *in trans* by a plasmid vector; suggesting that in BoHV-4 too, Rta was the master switch protein involved in virus replication, not only during reactivation of latently infected non-permissive cells, but also in *de novo* infection of permissive cells (Donofrio et al., 2004a).

Chapter 2

Aim of work

BoHV-4 is a virus widely distributed in cattle population. It shows a lot of attractive features as a vector for vaccinal and/or gene therapy purposes. A paper was recently published in which the generation of a mutant in ORF73, unable to establish latency in an animal model, is described; opening the way to generate replication or latency un-competent viruses. The feasibility to manipulate BoHV-4 as a BAC, described for the first time in 2005 by Gillet et al. (Gillet et al., 2005a), has led to the development of new strategies to generate viral mutants.

BoHV-4 has been isolated, across the years, from a lot of different tissues and samples from animal with various clinical manifestations, ranging from conjunctivitis, ocular discharge and genital diseases as post-partum metritis or abortion, but even in apparently healthy animal. The real correlation between BoHV-4 as a causative agent of any pathologies, has never been clearly demonstrated, even if in case of post-partum metritis, the role of BoHV-4 as a secondary pathogen is strictly defined (Donofrio et al., 2005; Donofrio et al., 2008b). BoHV-4 has a well known tropism for endometrial stromal and epithelial cells, in these cell types leads to non-apoptotic cell death and *de novo* viral production associated with an increased secretion of inflammatory proteins. The rapid activation of BoHV-4 replication in the bovine endometrium is associated with the transactivation of the BoHV-4 IE2 gene promoter from endometrial cells (Donofrio et al., 2008b); and BoHV-4 IE2 gene is supposed to be the master gene involved in the switch of viral replication, as for other herpesviruses (van Santen, 1991). BoHV-4 IE2 gene promoter transactivation and viral replication were associated also with extracellular stimuli belonging to the intrauterine microenvironment such as *E. coli* LPS and PGE (Donofrio et al., 2008b).

Sheldon et al. proposed a model that describes well endometrial BoHV-4 disease, involving a vicious circle comprising of bacterial endometritis leading to secretion of PGE, then PGE and LPS stimulating viral replication, which causes further endometrial tissue damage and inflammation (Sheldon et al., 2009).
Even if BoHV-4 has been isolated from different lesions and from healthy animals, the existence of a patho-biotype of BoHV-4 causing uterine disease, can not, consequentially, be ignored.

The aim of this study was to generate a BAC from a uterine BoHV-4 isolate to characterize and study the interactions between the virus and the endometrium. The first part of this thesis describes how such a BAC was generated and characterized; the second lead to affirm that in BoHV-4 too, as for the other gammaherpesviruses, IE2 is the master gene switch and is necessary for viral replication. Surprisingly we found that in our uterine isolate IE2 gene is duplicated, providing information about the preferential tropism for endometrial cells, and we were able to disrupt all the two gene copies, generating a totally replication un-competent virus, complemented by *in trans* expression of Rta protein. This replication un-competent virus would probably have some applications as oncolityc vector for gene therapy.

Chapter 3

Experimental Section

3.1

Isolation and characterization of bovine herpesvirus 4 (BoHV-4) from a cow affected by post partum metritis and cloning of the genome as a Bacterial Artificial Chromosome: a versatile molecular tool to study interactions between BoHV-4 and the endometrium.

Abstract

Bovine herpesvirus 4 (BoHV-4) is worldwide distributed in cattle and is the virus most consistently associated with metritis, a very common postpartum disease of the uterus. Virus strain adaptation to an organ, tissue or cell type is a very important issue for the study of pathology and the function of most of the viral genes remains unknown.

A strain of the virus was isolated from the uterus of a persistently infected cow affected with non-responsive post-partum metritis and designated BoHV-4-U. The isolate was studied by RFLP-PCR and sequencing using the TK locus as a genetic marker region for the BoHV-4 genome.

To easily manipulate and study the viral genome the BoHV-4-U DNA was cloned as a bacterial artificial chromosome (BAC) and the stability of the pBAC-BoHV-4-U clone was confirmed over twenty passages, and viral growth was not affected compared with the wild type virus.

The feasibility of using BoHV-4-U for mutagenesis was demonstrated using the BAC recombineering system, that allows to study single or multiple gene disruptions, clarifying the interactions between BoHV-4 infection and the host endometrial cells.

Introduction

Uterine infections are important because they disrupt not only the function of the uterus, but also the ovary and the overarching higher control centres in the hypothalamus and pituitary (Sheldon et al., 2009). The inflammatory and immune response to uterine infection compromises animal welfare as well as causing sub-fertility and infertility. Indeed, uterine disease causes infertility at the time the infection is present and sub-fertility even after successful resolution of the disease. Understanding the mechanisms underlying the effect of microbial infection and the associated immune response on bovine reproduction is important to develop new treatments and disease prevention strategies (Dobson et al., 2003).

Abortion may follow infection with a variety of alpha-, beta- and gammaherpesvirus, but viral causes of uterine disease are seldom investigated in cattle. Although postpartum metritis affects up to 40% of cattle, it has been assumed that the majority of diseases are of bacterial origin and virus isolation or serology is rarely considered (Sheldon et al., 2009). However, bovine herpesvirus 4 (BoHV-4) has often been implicated in cases of bovine metritis. The first reported isolation of BoHV-4 from a case of bovine metritis was in 1973 (ParK and Kendrick, 1973). Later several other isolates were obtained from cows with reproductive disorders from different countries, including Italy (Castrucci et al., 1986) and India (Mehrotra et al., 1986). In Belgium, BoHV-4 seroprevalence was associated with postpartum metritis, and chronic infertility of cattle (Czaplicki and Thiry, 1998). Postpartum metritis has also been associated with BoHV-4 in the USA (Frazier et al., 2001; Frazier et al., 2002), Spain (Monge et al., 2006) and Serbia (Nikolin et al., 2007).

BoHV-4 is tropic for endometrial stromal and epithelial cells, leading to non-apoptotic cell death and *de novo* viral production associated with increased stromal cell prostaglandin-endoperoxide synthase 2 (PTGS2) protein and stimulated prostaglandin E_2 (PGE) secretion (Donofrio et al., 2007a; Donofrio et al., 2008b). The successfull replication of BoHV-4 in bovine endometrial cells was shown to be attribuited to post-entry events, with rapid viral reconstitution following the electroporation of nude viral DNA into endometrial stromal and epithelial cells (Donofrio et al., 2007a; Donofrio et al., 2008b). A plausible mechanism underling this rapid activation of BoHV-4 replication in the bovine endometrium was associated with the capability of endometrial cells to transactivate the BoHV-4 immediate early 2 (IE2) gene promoter (Donofrio et al., 2008b), which is the molecular master swich gene for BoHV-4 replication (van Santen, 1991). Furthermore, BoHV-4 IE2 gene promoter transactivation and viral replication were associated with extracellular stimuli belonging to the intrauterine microenvironment such as *E. coli* LPS and PGE (Donofrio et al., 2008b). BoHV-4 replication was also reactivated in latently infected macrophages by stromal cells in co-cultivation (Donofrio et al., 2007a; Donofrio et al., 2008b). Based on this knowledge, a model for endometrial BoHV-4 disease was proposed (Sheldon et al., 2009), involving a vicious circle comprising of

bacterial endometritis leading to secretion of PGE, then PGE and LPS stimulating viral replication, which causes further endometrial tissue damage and inflammation.

Although BoHV-4 has been isolated from different lesions and from healthy animals, the existence of a patho-biotype of BoHV-4 causing uterine disease can not be ignored. Thus, in the present study, BoHV-4 was isolated from a cow affected with non-responsive post-partum metritis, characterized and the genome cloned as bacterial artificial chromosome (BAC). This new uterine BAC-BoHV-4 clone represents a versatile molecular tool for functional genomic study of BoHV-4 genes adapted to the endometrium, which will lead to new insights into the relationship between BoHV-4 and postpartum metritis.

Materials and Methods

Herd screening and isolation of BoHV-4

A dairy herd comprising of 73 cows that had a high incidence of postpartum metritis, abortion, and infertility was screened by indirect fluorescent antibody test (IFAT) for BoHV-4 antibodies. Briefly, IFAT was performed as follow: BoHV-4 infected and uninfected BEK cells were seeded onto 18-wells glass slides fixed in cold acetone and stored a -20 °C until used. Sera were serially diluted in phosphate buffered saline (PBS) and 30 µl added to the infected and uninfected cells. For each slide a negative and a positive serum was introduced, as well as a control made by treating a slide well containing BoHV-4 infected and a one containing uninfected cells only with the secondary antibody. After 1 h incubation at 37 °C, the slides were washed three times in PBS and incubated 1 h at 37 °C with fluorescein isothiocyanate conjugated anti-bovine immunoglobulin G. At the same time, cells were counterstained with Evans Blue Dye (EBD; Sigma, St. Louis, USA). Fluorescence was detected using a Zeiss Axiovert epifluorescent microscope and images acquired with the Axiocam Zeiss system. Sera were considered positive for BoHV-4 antibody when green fluorescence was detected at a dilution ≥ 1:40, with no fluorescence for uninfected cells at 1: 20.

Endometrial cells collection by uterine trypsin flushing

A cow identified by IFAT as persistently infected with BoHV-4 and with a history of metritis, was treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Estrumate, SCHERING PLOUGH) and 4 days later endometrial cells were collected by flushing the uterus. Briefly, a Foley catheter was introduced through the cervix into the uterine lumen guided by rectal palpation and 50 ml of a trypsin EDTA solution (0,25% trypsin, 1 mM EDTA in PBS) infused and the uterus gently massaged for 2 min before aspiration into a 50 ml tube. After collection, 1 ml FBS was added and endometrial cells collected by centrifugation at 2,000 RPM for 5 min. The cells were washed twice with 10 ml medium comprising of Dulbecco's modified essential medium (D-MEM; Sigma), 10% FBS (Sigma), 50 IU/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma) and 2.5 µg/ml Amphotericin B (Sigma), re-suspended in 2 ml of medium and stored at -80°C.

BoHV-4-U isolation

The following BoHV-4-sensitive cell lines were obtained: bovine arterial endothelial (BAE-7372) from Prof. S. Grolli (Veterinary Biochemistry Institute, Parma University, Italy); Madin Derby Bovine Kidney (MDBK CCL-22) from ATCC (ATCC American Type Culture Collection, Manassas, USA); and bovine embryo kidney

(BEK) and bovine embryo lung (BEL) cells from M. Ferrari (Istituto Zooprofilattico Sperimentale, Brescia, Italy). All cells were cultured in D-MEM (Sigma) containing 10% FBS, 2 mM of l-glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 2.5 µg/ml of Amphotericin B. The uterine endometrial cells were co-cultured with BAE-7372, MDBK, BEL and BEK cells in culture flasks until plaques characteristic of a cytopathic effect (CPE) developed. Single plaques were purified using 0.2% agarose gel three times. Flasks containing plaques were frozen in liquid nitrogen and thawed, the medium containing cell plaques centrifuged at low speed and the supernatant filtered through a 0.2 µm syringe filter (Minisart, Sartorius) and stored at -80°C.

BoHV-4-U plaque immuno-peroxidase staining

The cell plaques were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at 37°C, washed twice with PBS, 0.1% BSA, and incubated 5 min at 20°C with the same solution. Cells were then incubated 5 min at 20°C with PBS, 0.3% Triton X-100, washed three times with PBS, and incubated 10 min at 37°C with 0.15% H_2O_2 in PBS. Rabbit anti-BoHV-4 hyper-immune serum, diluted 1:500 in PBS, was incubated with the cells for 2 h at 37°C. After three washes with PBS, cells were incubated with peroxidase-conjugated secondary antibody, diluted 1:500 in PBS, for 1 h at 37°C, and washed three times with PBS. Secondary antibody was detected by development in 250 µg/ml DAB (Sigma), 0.015% H_2O_2, 50 mM Tris pH 7.4, for 10 min at 20°C.

BoHV-4-U characterization

Viral infected cells were lysed overnight in TEL (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) buffer containing 0.5% sodium dodecyl sulphate (SDS) and 100 µg of proteinase K per ml at 37 °C. Nucleic acids were extracted by treatment with phenol–chloroform and precipitated with ethanol. Treatment with 100 µg/ml of RNAse A (Sigma) was performed for 1 h, after which the DNA was extracted with phenol and precipitated with ethanol again. The samples were kept at −20 °C. One microgram sample DNA was amplified over 30 cycles, each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and chain elongation with 1 U of Taq polymerase (Roche) at 72 °C for 2 min. PCR amplification was carried out in a final volume of 50 µl of 10 mM Tris–hydrochloride pH 8.3 containing 0.2 mM deoxynucleotide triphosphates, 3 mM $MgCl_2$, 50 mM KCl and 0.25 µM of each primer. In the first cycle, the samples were denatured at 94 °C for 5 min, and in the last cycle the extension step was increased to 7 min. The primers used for amplification were selected from the published sequence of BoHV-4 genome (Zimmermann et al., 2001) (sense primer:5′-cgaattctagtctaaagtcatcctc-3′; antisense primer: 5′-cgaattccattggcttcatcccaca-3′. Accession number: NC002665). The PCR products were electrophoresed in 1% agarose gel and visualized

after ethidium bromide staining. The amplified 2538-bp TK fragment was extracted from the agarose gel, digested with HindIII restriction endonuclease, and analyzed on 1.5% agarose gel in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA) containing ethidium bromide for DNA staining. The specificity of the PCR product was determined by sequencing.

RNA extraction and RT-PCR

Total RNA from infected and uninfected cells was extracted with TriPure reagent (Roche) and 2 µg of total RNA were reverse transcribed using Ready-To-Go, T-Primed First-Strand Kit (Amersham Biosciences). Three microliters of reverse transcribed RNA were amplified over 30 cycles, each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and chain elongation with 1 U of Taq polymerase (Boehringer-Diagnostics) at 72 °C for 2 min. PCR amplification was carried out in a final volume of 50 µl of 10 mM Tris–hydrochloride, pH 8.3, containing 0.2 mM deoxynucleotide triphosphates, 3 mM $MgCl_2$, 50 mM KCl and 0.25 µM of each primer (sense primer: 5′-acaaacacacagaccagtca-3′; antisense primer: 5′-gtttcacaacagattgagca -3′. Accession Number NC002665). Primers were selected from the published sequence of BoHV-4 genome (Zimmermann et al., 2001).

BAC-BoHV-4-U generation

For BAC cloning BoHV-4-U genome, a strategy employed to BAC clone BoHV-4-A genome was used (Donofrio et al., 2008c). The intergenic region between the ORF2 and ORF3, previously shown to be suitable for the introduction of the BAC cassette was chosen. pSP72Bo2-*lox*GFP-BAC*lox*-Bo3 was excised out from pSP72Bo2-*lox*GFP-BAC*lox*-Bo3 (Donofrio et al., 2008c) with PvuII/ClaI restriction digestion and the gel-purified fragment was co-electroporated with 1 µg of purified BoHV-4-U DNA, obtained by sucrose purified viral particles, in BEK cells. GFP positive plaques were under agar isolated and transferred onto newly prepared BEK cells. After several rounds of isolation, circular viral DNAs intermediates were isolated from infected cells by the Hirt isolation method (Hirt, 1967) and transferred into *Escherichia coli* DH10B. The transformants were plated on agar containing 17 µg/ml chloramphenicol. DNA was extracted from each positive clone and was restriction enzyme digested and analyzed by electrophoresis on 1% agarose gel. DNA fragments were visualized with a UV trans-illuminator.

Cell culture electroporation and recombinant virus reconstitution

BEK or BEK*cre* cells were maintained as a monolayer with complete medium containing 90% D-MEM, 10% FBS, 2 mM l-glutamine, 100 IU/ml penicillin and 10 µg/ml streptomycin. Cells were sub-cultured to a fresh culture vessel when growth reached 70–90% confluence (i.e., every 3–5 days) and were incubated at 37 °C

in a humidified atmosphere of 95% air–5% CO_2. BAC-BoHV-4 plasmid DNA (5 µg) in 500 µl D-MEM without serum was electroporated (Equibio apparatus, 270 V, 960 µF, 4-mm gap cuvettes) into BEK or BEK*cre* cells from a confluent 25-cm^2 flask. Electroporated cells were returned to the flask, fed the next day, and split 1:2 when they reached confluence at 2 days post-electroporation. Cells were left to grow until CPE appeared. Recombinant viruses were propagated by infecting confluent monolayers of BEK or MDBK cells at a m.o.i. of 0.5 $TCID_{50}$ per cell and maintaining them in MEM with 10% FBS for 2 h. The medium was then removed and replaced by fresh MEM 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 virions through 30% sucrose. Virus pellets were resuspended in ice cold D-MEM without FBS. $TCID_{50}$ were determined on MDBK cells by limiting dilution.

BAC-BoHV-4-U genome modification by recombineering

Recombineering was performed as described by Warming et al., (Warming et al., 2005) with some modifications: Five hundreds µl of a 32°C overnight culture of SW102 containing BAC-BoHV-4-U, were diluted in 25 ml Luria-Bertani (LB) medium with or without chloramphenicol (SIGMA) selection (12.5 µg/ml) in a 50 ml baffled conical flask and grown at 32°C in a shaking water bath to an OD_{600} of 0.6. Then, 10 ml were transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for exactly 15 min in a shaking water bath. The remaining culture was left at 32°C as the un-induced control. After 15 min the two samples, induced and un-induced, were briefly cooled in ice/water bath slurry and then transferred to two 15 ml Falcon tubes and pelleted using 5000 r.p.m. (eppendorf centrifuge) at 0°C for 5 min. The supernatant was poured off and the pellet was resuspended in 1 ml ice-cold ddH_2O by gently swirling the tubes in ice/water bath slurry. Subsequently, 9 ml ice-cold ddH_2O were added and the samples pelleted again. This step was repeated once more, the supernatant was removed and the pellet (50 µl each) was kept on ice until electroporated with gel-purified ~4.3 kb fragment (TK-KanaGalK-TK) obtained by cutting pTK-KanaGalK-TK with XhoI/EcoRI (ROCHE). An aliquot of 25 µl was used for each electroporation in a 0.1 cm cuvette at 25 µF, 2.5 kV and 201 Ω. After electroporation, the bacteria were recovered in 1 ml LB (15 ml Falcon tube) for 1 h in a 32°C shaking water bath. For the counter selection step (see below), the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32°C shaking water bath.

After the recovery period, the bacteria were washed twice in sterile 1× M9 salts (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl and 0.5 g/l NaCl,) (SIGMA) as follows: 1 ml culture was pelleted in an eppendorf tube at 13,200 r.p.m. for 15" and the supernatant was removed with a pipette. The pellet was resuspended in 1 ml of 1× M9 salts, and pelleted again. This washing step was repeated once more. After the second wash, the

supernatant was removed and the pellet was resuspended in 1 ml of 1× M9 salts before plating serial dilutions (100 µl each of 1:10, 1:100 and 1:1000 dilutions) on M63 minimal medium plates (agar 15 g/l; (DIFCO, BD Biosciences), 0.2% D-galactose (SIGMA), 1 mg/l D-biotin (SIGMA), 45 mg/l L-leucine (SIGMA) and 50 mg/l kanamycin (SIGMA)). Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal medium plates. Plates were incubated 3–5 days at 32°C. Several selected colonies were picked up, streaked on McConkey agar indicator plates (DIFCO, BD Biosciences) containing 50 µg/ml of kanamycin and incubated at 32°C for 3 days until red colonies appeared. Red colonies were grown over night in 5 ml of LB containing 50 µg/ml of kanamycin and BAC-BoHV-4-U was purified and analyzed through HindIII restriction enzyme digestion for TK-KanaGalK-TK fragment targeted integration into the BoHV-4 TK locus.

Results

BoHV-4 isolation from the uterus of an infected animal

A cow was identified from a herd with a high incidence of metritis; the animal had with a history of 2 years of reproductive failure, was affected by post partum metritis and was BoHV-4 IFAT positive twice at 6-month interval (Fig. 8).

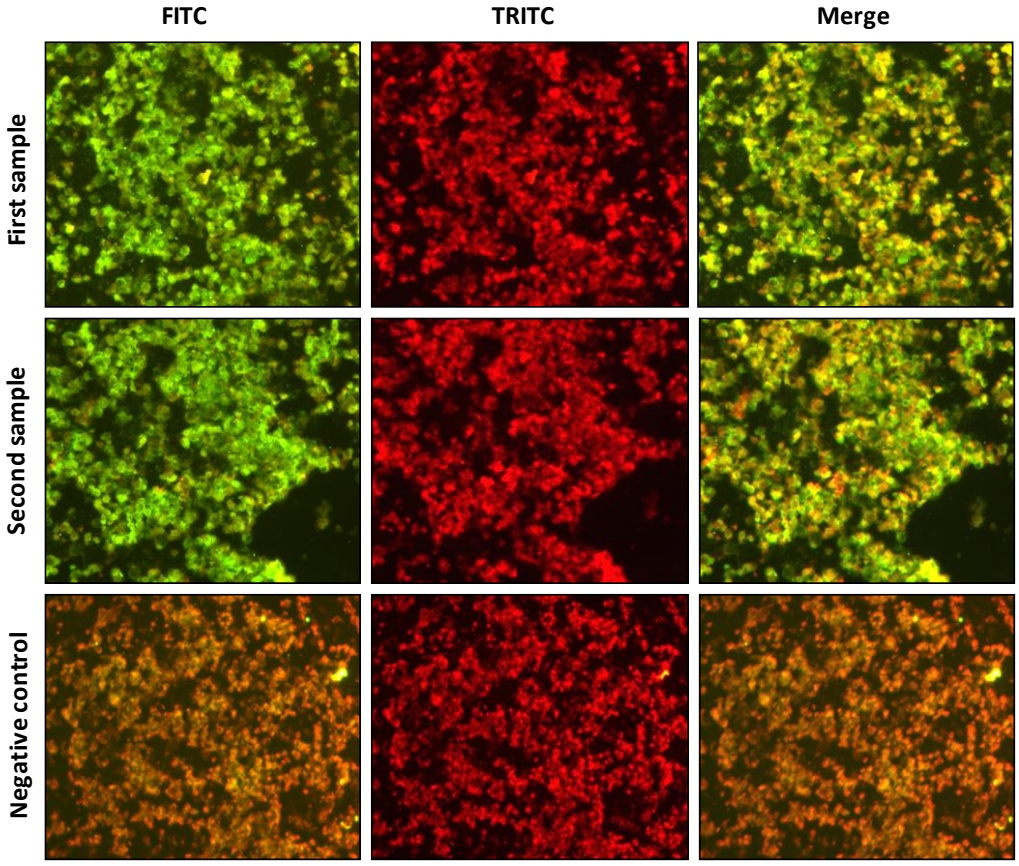

Fig. 8. Representative microscopic (10X) IFAT images of sera from the cow which was used to isolate BoHV-4-U. The presence of anti-BoHV-4 antibodies in the first and second sample (6 months following the first) are detectable by green cells when observed with a FITC filter and the negative controls appears brown due to the Evans blue dye. Counterstaining with Evans blue dye observed with a TRITC/filter (red pictures) was used to monitor the integrity of the cell substrate. The merged pictures (FITC/TRITC) allow co-localization of the anti-BoHV-4 antibody staining with the cellular substrate.

Uterine cells were collected from this animal and co-cultivated with BoHV-4 permissive cell lines. Ten days after co-culture and one cell passage, a CPE typical of BoHV-4 appeared in the BEK cells (Fig. 9A). The presence of BoHV-4 was confirmed by immuno-peroxidase staining of the plaques with anti-BoHV-4 antibodies (Fig. 9B).

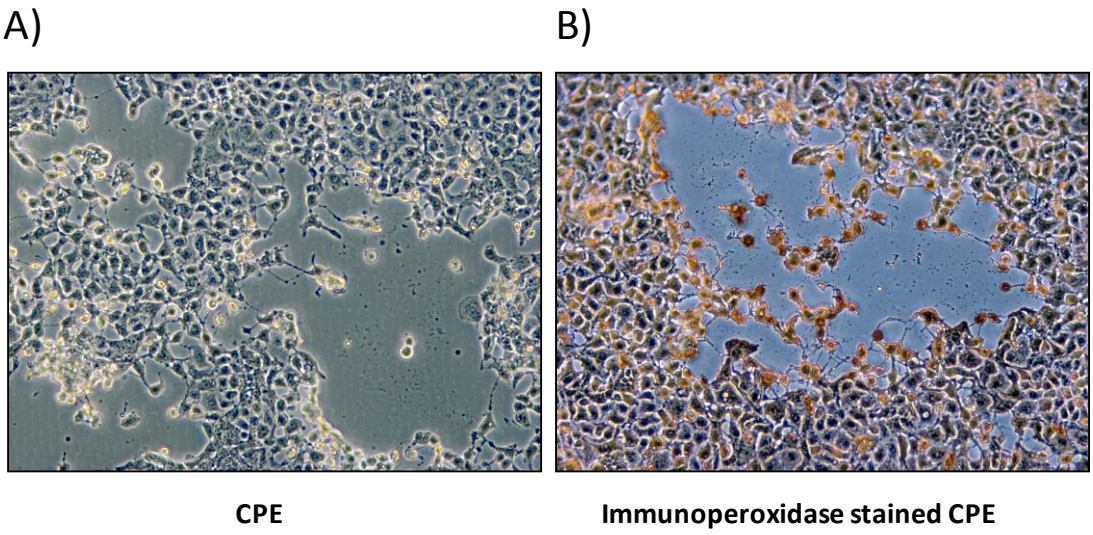

Fig 9. A) Representative phase contrast microscopic images (10X) of BoHV-4-U cytopathic effect generating plaques immuno-peroxidase stained with anti-BoHV-4 antibodies (B).

BoHV-4 isolate characterization

To confirm the specificity of the BoHV-4 isolate, single-plaque purified virus was analyzed by RFLP-PCR of the TK locus (Fig. 10A) and RT-PCR for the spliced form of the Immediate Early 2 (IE2) gene (Fig. 10B), which is a specific marker for BoHV-4 immediate early gene expression (van Santen, 1991). Both methods confirmed the specificity of the uterine isolate, which was called BoHV-4-U. A restriction profile comparing the BoHV-4-U viral genome with reference strains (LVR, DN 599 and Movar), classified the isolate as Movar-like (data not shown), which was in agreement with the geographical distribution of the Movar strain in the north of Italy (Donofrio et al., 2000a).

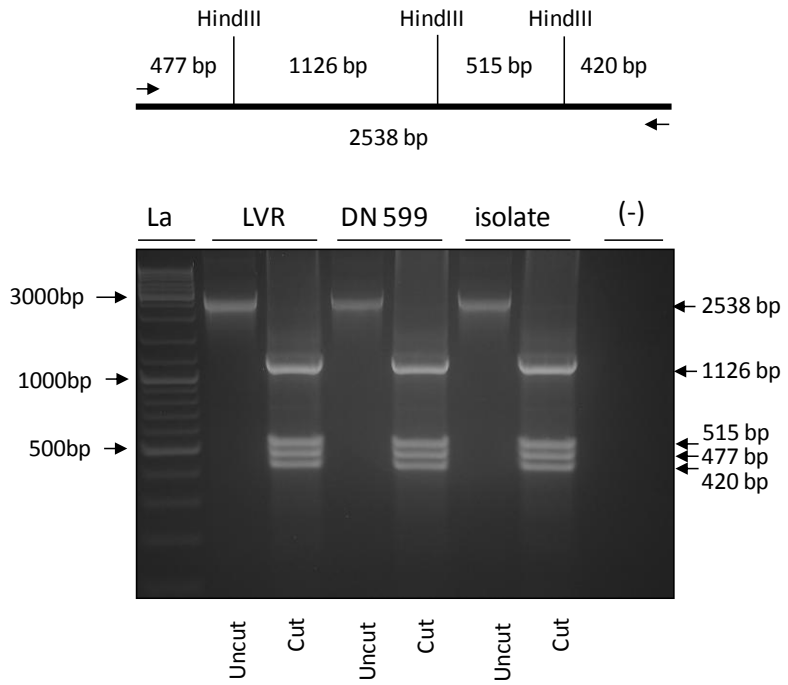

Fig.10A) Predicted location of HindIII restriction sites and respective expected restriction fragment sizes (not shown to scale). Ethidium bromide stained agarose gel electrophoresis of specific amplification of BoHV-4 DNA fragment from LVR, DN599 reference strains and the BoHV-4-U isolate. PCR amplified 2,538-bp fragment (Uncut) and digested with HindIII restriction enzyme (Cut). Lanes (-) corresponds to negative controls and La to 1 kb ladder molecular size marker.

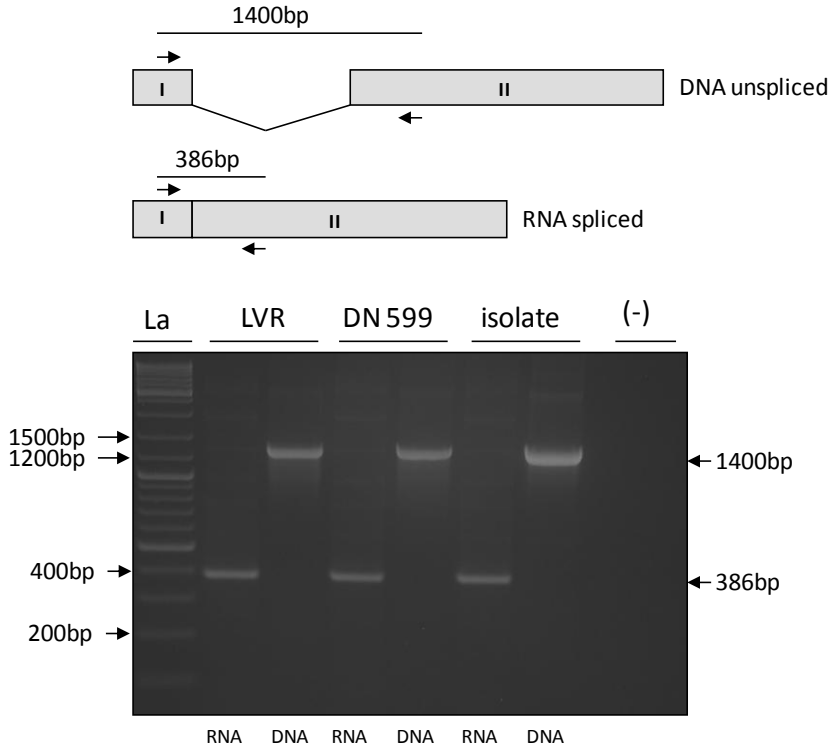

Fig. 10B) Diagram showing the genomic region containing the BoHV-4 IE2 gene (not to scale), where the exon II contains most of the IE2 ORF, except the translation initiation codon contained into the exon I. During splicing, an intron is removed and the two exon (I and II) are joined together to generate the IE2 ORF. The positions of the primers used to amplify the region containing IE2 are shown by arrows. The selected primers generate an amplicon of 1400 bp from the viral IE2 genomic region, while the amplicon is only 386 bp in length from the spliced product of IE2 transcript, thus allowing distinction between viral genomic DNA and the reverse transcribed IE2 RNA. Ethidium bromide stained agarose gel electrophoresis of RT-PCR or PCR of RNA and DNA from LVR, DN599 reference strains and the new isolate BoHV-4-U infected cells.

Cloning of BoHV-4-U as a bacterial artificial chromosome

To facilitate further characterization and manipulation of BoHV-4-U, the genome was cloned as a bacterial artificial chromosome. The intergenic region located between Bo2 and Bo3 ORFs (situated to the left end of the viral genome, Fig. 11A) (Zimmermann et al., 2001) was selected as a target site to introduce the BAC cassette. The plasmid pBo2-EGFP-BAC-Bo3, containing homologous regions flanking the floxed BAC cassette for *cre* excision and an EGFP expression cassette, was linearized and co-transfected with BoHV-4-U purified genome into BEK cells. Recombinant virus was isolated from single plaques expressing EGFP and the structure of the recombinant virus was assessed by HindIII restriction profiling, PCR and sequencing. Circular intermediates of the recombinant BoHV-4-U were isolated from newly infected BEK cells and electroporated into *E. coli* DH 10B to generate pBAC-BoHV-4-U (Fig. 11B). HindIII restriction profile (Fig. 11C), Southern blotting, PCR and partial sequencing (data not shown) confirmed the integrity of the clone.

Clone stability and viral reconstitution

The stability of the pBAC-BoHV-4-U clone in bacteria was confirmed over twenty passages (Fig. 12A). Electroporation of the pBAC-BoHV-4-U plasmid into BEK cells, allowed infectious virus reconstitution as observed by plaques expressing green fluorescent protein formation and CPE spreading through the cell monolayer over time (Fig. 12B). The BAC cassette was successfully excised from the BAC-BoHV-4-U, as monitored by the lost of GFP expression, when BAC-BoHV-4-U virus was growth on BEK cells expressing *cre* or pBAC-BoHV-4-U plasmid was electroporated into BEK cells expressing *cre* (Fig. 12C). No difference in growth characteristics between BoHV-4-U, BAC-BoHV-4-U and BoHV-4-UΔBAC was observed (Fig. 13).

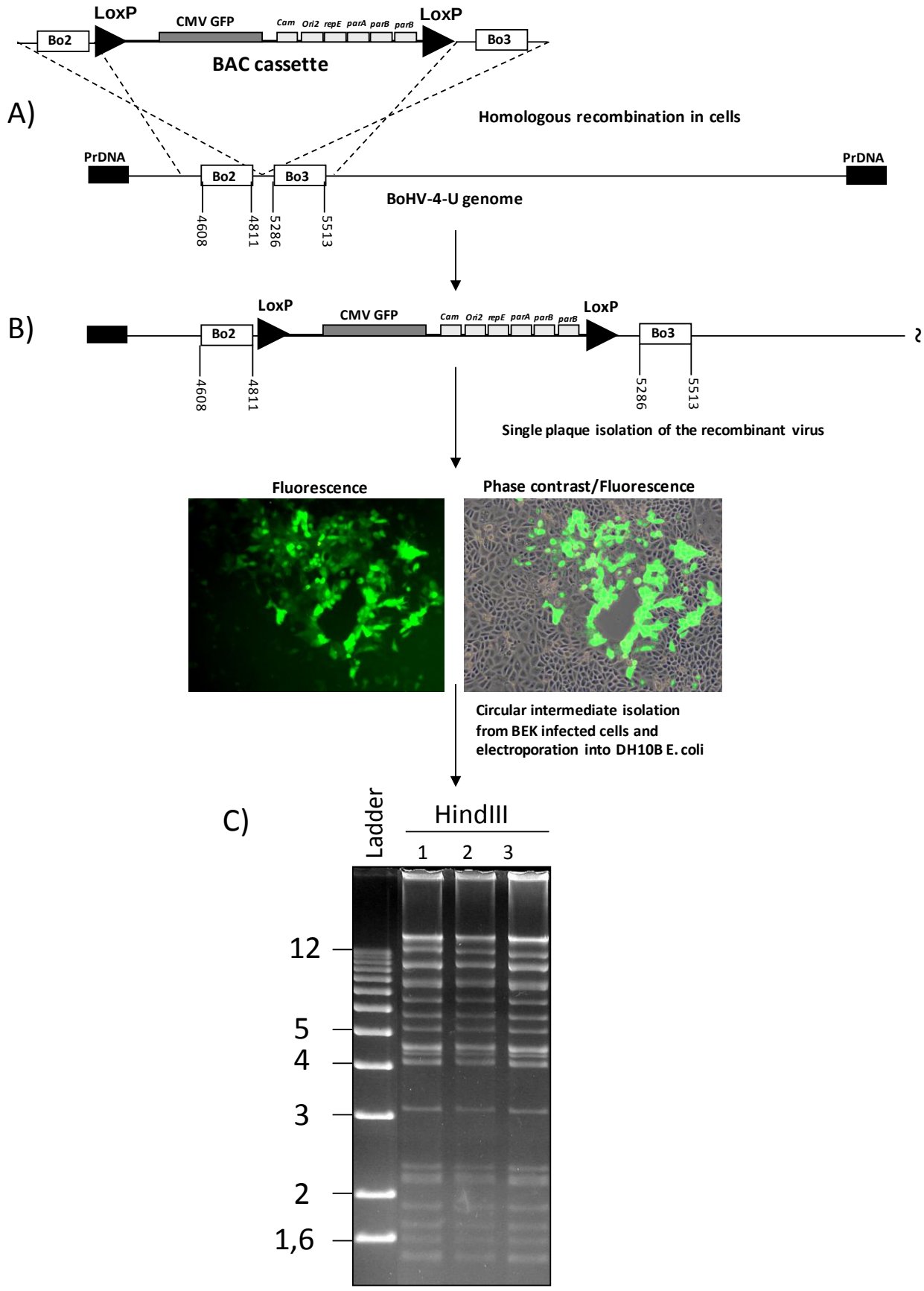

Fig. 11) Representative flow chart of the strategy used to generate pBAC-BoHV-4-U. A) Through classical homologous recombination in eukaryotic cells obtained by co-transfection, the floxed (*loxP* sites indicated by triangles) CMV-GFP BAC cassette containing F1 plasmids elements (chloramphenicol resistant gene, Cam; F1 origin of replication, Ori2; partitioning protein genes, repE, parA, parB and parC), flanked by two homologous BoHV-4-U regions, Bo2 and Bo3, was introduced into the

intergenic region of the BoHV-4-U genome [Numbers indicates sequence location in BoHV-4 genome based on the Zimmermann's sequence (Zimmermann et al., 2001)]. B) Diagram of the recombinant BoHV-4-U and images of a single plaque expressing GFP (Fluorescence and phase contrast merged on fluorescence. 10X). C) Ethidium bromide stained agarose gel of three (1, 2, 3) representative HindIII digested pBAC-BoHV-4-U clones.

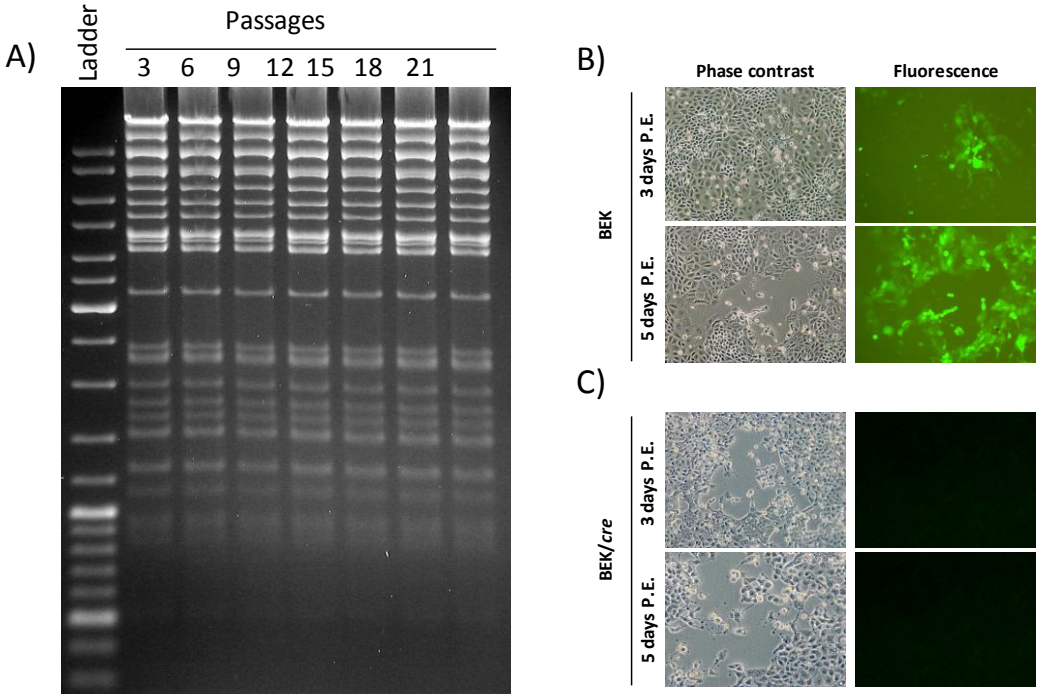

Fig. 12) A) Stability of the pBAC-BoHV-4-U plasmid in *E. coli* DH10B. DH10B containing the pBAC-BoHV-4-U were passaged for 21 consecutive days, BAC DNA from the culture was prepared on the indicated days (3, 6, 9, 12, 15, 18 and 21), HindIII digested and analyzed on ethidium bromide stained agarose gel. B) BEK or C) BEK expressing *cre* cells (BEK/*cre*) at 3 and 5 days post electroporation with pBAC-BoHV-4-U, where BAC-BoHV-4-U virus is reconstituted causing CPE and spreading through the cellular monolayer as shown by both phase contrast and fluorescence images (in B). Whereas, due to the expression of *cre* and the BAC cassette containing GFP is removed from the BAC-BoHV-4-U reconstituted, CPE is appreciable in phase contrast images but not in fluorescence (in C).

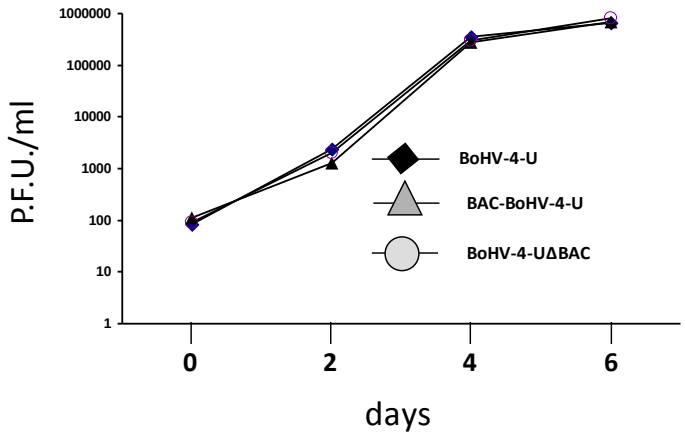

Fig.13. Replication kinetics of BoHV-4-A-U reconstituted virus in BEK/cre cells (the BAC cassette has been removed by cre), compared with those of the parental BAC-BoHV-4-U (virus reconstituted on un-expressing cre BEK cells) still containing the BAC cassette and the BoHV-4-U isolate. The data presented are the means ± standard errors of triplicate measurements, P>0,05 for all time points as measured by Student's *t* test.

Insertional mutagenesis into BoHV-4-U genome cloned as Bacterial Artificial Chromosome.

To test the feasibility of BoHV-4-U for mutagenesis, the BAC recombineering system approach (Warming et al., 2005), modified by the introduction of a kanamycin selection step, was used for this purpose. Accordingly, the galactokinase cassette (*Gal*K) along with a kanamycin resistance cassette, both under the control of constitutively active prokaryotic promoters, were subcloned in pINT2 (Donofrio et al., 2002) to be flanked by the BoHV-4 TK gene and adjacent sequences. The generated targeting vector (TK-KanaGalK-TK) was excised out from the plasmid backbone and used for heat-inducible homologous recombination in SW102 *E.coli* containing the BAC BoHV-4 genome (Fig. 14).

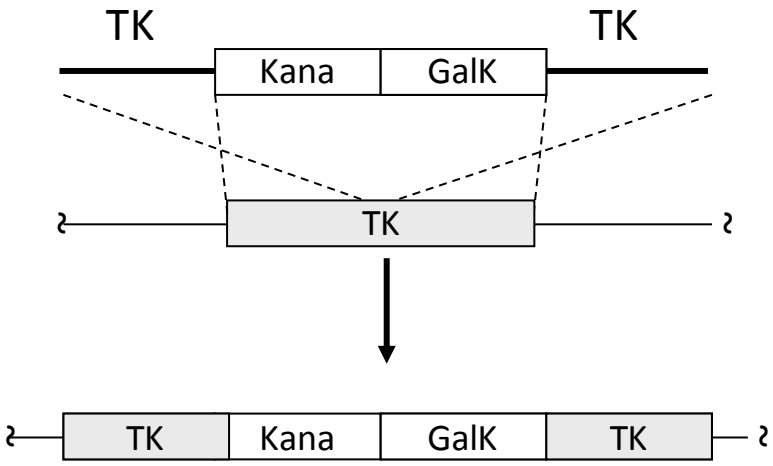

Fig. 14. Kanamycin resistant cassette (Kana) adjacent to the galactokinase cassette (GalK) and flanked by BoHV-4 thymidine Kinase gene and adjacent sequences (TK), are introduced into the TK gene of BoHV-4-U genome cloned as bacterial artificial chromosome (BAC-BoHV-4-U) via heat inducible homologous recombination in SW 102 *E.coli*.

Following a positive selection in minimal medium containing galactose (on solid agar plate) and a second positive selection with medium containing kanamycin (liquid medium), all the clones analyzed displayed the correct targeting (Fig.15).

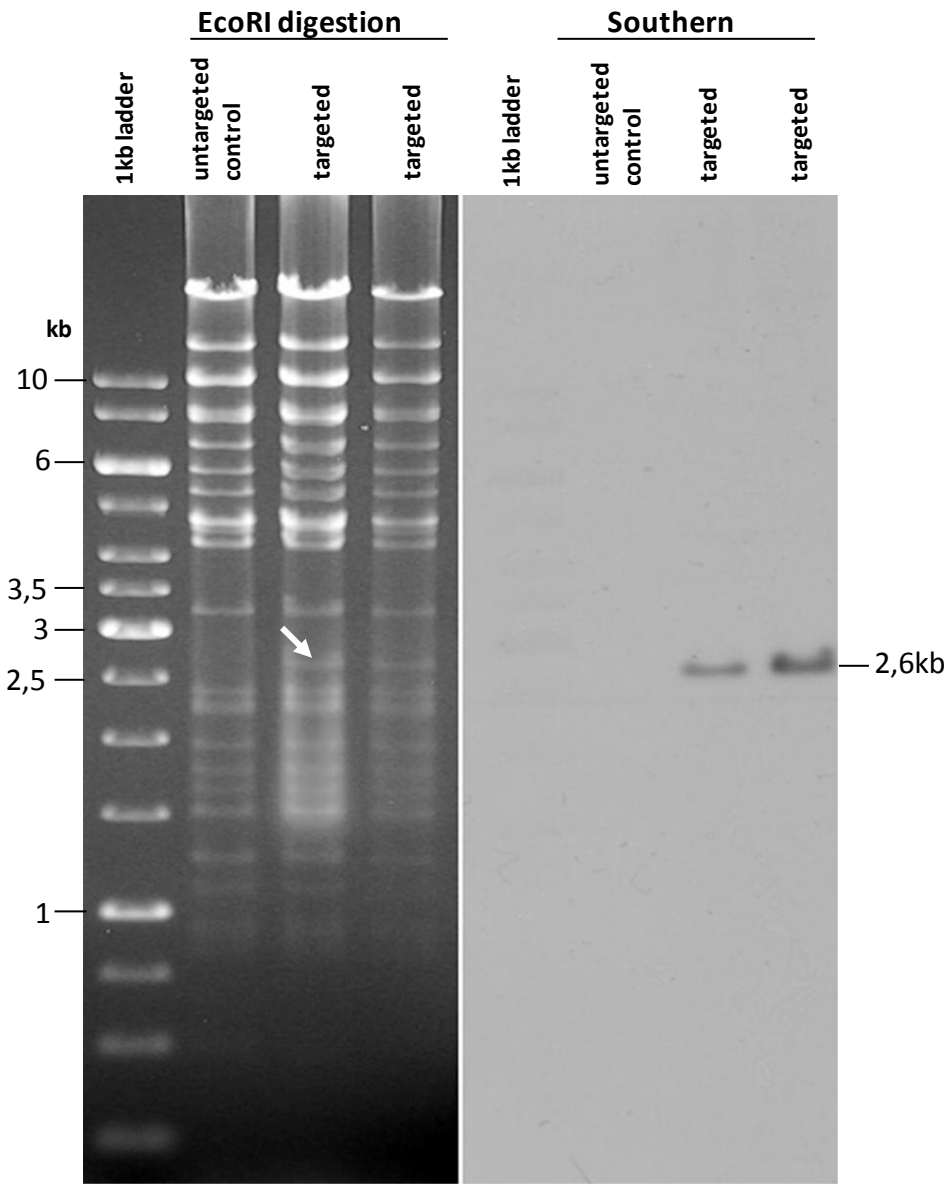

Fig.15. HindIII restriction enzyme analysis of 2 clones out of hundreds, displaying the right targeting, where a new 2.6 kb band (indicated by arrow) corresponding to the insertion of the Kana/GalK cassettes is present but missing in the untargeted control. Southern hybridization with a Kanamycin specific probe confirms the specific targeting.

Discussion

BoHV-4 is a herpesvirus belonging to the gammaherpesvirus family, genus *Rhadinovirus* (Zimmermann et al., 2001). Although BoHV-4 has been isolated from animals with various clinical symptoms and also from apparently healthy animals, BoHV-4 isolation from animals with disease of the female genital tract is the best documented and one of the few cases where disease could be reproduced (Wellemans et al., 1986). Wellemans et al, (1986) reproduced metritis by infecting cows at various times before parturition; no clinical signs were seen after primary infection but metritis was observed after parturition. It is likely that BoHV-4 causes post-partum metritis along with other microbes such as the bacteria *E. coli* and *A. pyogenes* (Donofrio et al., 2008b).

In the present study a BoHV-4 strain (BoHV-4-U) was isolated from the uterus of a persistently infected cow, affected by postpartum metritis. Restriction pattern analyses by RFLP-PCR and sequencing using a large 2,538 bp conserved region (TK locus) as a genetic marker region for the BoHV-4 genome, (Lomonte et al., 1996), including the 3' end of ORF1 (homologous to the EBV BVRF1 gene), ORF2 (homologous to the EBV BXRF1 gene), ORF3 (TK gene) and ORF4 (gH gene) 5' end, confirmed that the isolate was a BoHV-4 strain

Even the spliced form of the Immediate Early 2 (IE2) gene, a specific marker for BoHV-4 immediate early expression (van Santen, 1993) was analyzed by RT-PCR and sequencing.

The BoHV-4-U genome was cloned as a bacterial artificial chromosome (BAC) and the stability of the pBAC-BoHV-4-U clone was confirmed over twenty passages, and viral growth was not affected compared with the wild type virus. The feasibility of using BoHV-4-U for mutagenesis was demonstrated using the BAC recombineering system.

Virus strain adaptation to an organ, tissue or cell type is a very important issue for pathology. Differences observed in the disease potential of BoHV-4, as is the case of post-partum metritis, may constitute an adaptation of the virus to different selection pressures that may increase the frequency of the most efficient genetic variants. These selection pressures might include the different endometrial cells and their endocrine functions, physical and chemical conditions. To understand the effects of BoHV-4 on uterine function it is therefore important to use a specific BoHV-4 isolate from a case of post-partum metritis. Nuget et al. (2006) (Nugent et al., 2006), for example, found that non-neuro-pathogenic EHV-1 strains encode A in the position 2,254 (amino acid N752) of the genome corresponding the viral DNA polymerase (*pol*) ORF. Whereas, neuro-pathogenic EHV-1 strains encode G in that position (amino acid D752). This suggested that the ancestral EHV-1 virus probably encoded D752 and the variants expressing N 752 arose

subsequently, possibly due to selective advantage. The relationship between polymorphism in EHV-1 *pol* and neuro-pathogenicity was verified by targeted mutagenesis of a single nucleotide in the DNA genome of the virus cloned as bacterial artificial chromosome (Goodman et al., 2007), confirming the functionality of such single nucleotide polymorphism. Starting from the assumption that the uterine isolate of BoHV-4 genome could be cloned as bacterial artificial chromosome, the viral genome could then be modified by homologous recombination and the virus reconstituted in eukaryotic cells following transfection.

The primary method for investigating the function of individual herpesvirus genes is mutagenesis. Mutated viruses are usually constructed by homologous recombination following the co-transfection of viral genomic DNA and a mutated allele on a separate DNA fragment (Roizman, 1996). Recombinant viruses are either screened or selected during several sequential rounds of plaque purification. However, if the mutation results in growth defects relative to wild-type parental virus, the mutant virus may be difficult or impossible to purify. Lethal mutations are often detected only indirectly by the unsuccessful isolation of the desired mutant virus. Such mutants can be isolated and confirmed as lethal only by the construction of a complementary cell line expressing the wild-type allele. To overcome these limitations, many herpesviruses have been cloned as an infectious bacterial artificial chromosome (BAC).

Although the genome of BoHV-4 has been sequenced completely (Zimmermann et al., 2001) the function of most of the gene remains unknown. Fewer than 75 ORFs have been characterized in BoHV-4, based on amino acids homology and genome co-location with the other gammaherpesviruses. While most of the genes have no apparent homology to known viral or cellular genes, some are known to be important for virus–host interaction. For example, IE2 gene, is the molecular master switch for BoHV-4 replication, and the capability of endometrial cells to transactivate the IE2 promoter was previously investigated by transient transfection assay using a reporter construct containing the IE2 promoter electroporated into endometrial stromal and epithelial cells (Donofrio et al., 2008b). Therefore the mutation of the IE2 gene in BoHV-4-U would be of great interest, to understand its function in the context of the interaction between a specific uterine strain (BoHV-4-U) and uterine cells.

The functional analysis of BoHV-4-U genes in endometrial cells is a fundamental prerequisite for regulatory region discovery. Gene trap technology (Zambrowicz et al., 1997) in combination with transposition technology, site direct mutagenesis and homologous recombination on BAC-BoHV-4-U genome, will provide an important tool to carry out a large-scale search and analysis of BoHV-4-U regulatory regions, in the context of which, factors belonging to the uterus microenvironment, like hormones, cytokines, prostaglandins and others, can be tested. Targeting genes and regions of gene expression regulation and the phenotypic analysis of the BoHV-4-U mutants represents a powerful tool to study the direct and indirect interactions of genes and genomic elements in a biological context as the uterus in this particular case. The classification of equivalent phenotypes carrying different types of mutations will provide insight

into functional networks and their constituting elements. A classification of mutants into functional clusters will contribute to identifying uncharacterized genes responsible for BoHV-4 strain adaptation to the uterine microenvironment. The established BoHV-4-U mutant's library together with different endometrial cell types will cover a substantial amount of biological information of intrinsic value concerning the functional relationship of the targeted genes and the host background. The results obtained in the large-scale gene mutation analysis will be investigated as a whole for their functional relationship.

The uterine adapted BoHV-4-U genome cloned as bacterial artificial chromosome, represents an important tool to understand the complex interactions between BoHV-4-U and uterine host cells and the effect of viral infection on endometrial metabolic and endocrine function, and antigen-processing pathways.

3.2

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

Abstract

The ORF50/*Rta* gene has been shown to be an essential gene for DNA replication and latency reactivation in 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 co-transfection assays, there is no direct proof of its real indispensability for progression of the virus to the lytic replication cycle in the context of the viral genome.

In this study, a replication defective BoHV-4-V.test IE2 mutant was efficiently rescued, with respect to the production of infectious virus and DNA replication, upon the expression of BoHV-4 ORF50/*Rta* in *trans*.

This mutant was able to normally growth and replicate only if Rta was provided *in trans*, demonstrating that even in BoHV-4, ORF50/Rta is the master replication switch gene.

Surprisingly, in the course of our studies, we discovered that the IE2 gene is duplicated in the genome of BoHV-4-U.

Introduction

Herpesvirus immediate early (IE) genes are expressed "immediately" during cell infection without requiring any prior viral protein synthesis for their expression; they are infact activated by host cell transcription factors, already present at the moment of 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 *HindIII* 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 homolog's in other gammaherpesviruses, including herpesvirus saimiri (SaHV-2), equine herpesvirus 2 (EHV-2), murine herpesvirus 68 (MuHV-68), rhesus rhadinovirus (RRV) and Kaposi's sarcoma-associated herpesvirus (HHV-8). Studies of HHV-8, MuHV68 and RRV demonstrated that the expression of ORF50/*Rta* is essential for viral replication; several recombinant viruses with deletion or inactivation by a premature stop codon insertion within its open reading frame were generated and were all replication-defective; but were efficiently rescued, with respect to production of viable 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 binding protein (Bermudez-Cruz et al., 1997; Bermudez-Cruz et al., 1998; van Santen, 1993; Zhang and van Santen, 1995). BoHV-4 Rta expression plays a pivotal 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., 2004a; Donofrio et al., 2004b; Donofrio et al., 2004c; 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.

We have demonstrated previously the activation of BoHV-4 lytic replication in a non-permissive cell line by over-expression of ORF50/*Rta* (Donofrio et al., 2004a), 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., 2009a; Donofrio et al., 2008c; Gillet et al., 2005a) 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* is necessary to initiate and allow progression of BoHV-4 lytic replication.

Materials and Methods

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., 2008c) 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 cells 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 un-transfected 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 Hygromicin B; control un-transfected 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.

Viruses

BoHV-4-U, BoHV-4-U/IE2pr, BoHV-4-UΔORF50, BoHV-4-LVR, BAC-BoHV-4-LVR/IE2pr, and BAC-BoHV-4-LVRΔORF50 and BAC BoHV-4 U ΔORF50 $IgKE_2VP_2^{2800}gD^{106}$ and BAC BoHV-4 U ΔORF50-double mutants 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., 2009b) . Virus pellets were resuspended in cold MEM without FBS. $TCID_{50}$ were determined with BEK, BEK/IE2, and BEK/ORF50 cells by limiting dilution.

Plasmid Constructs

pCMV-ORF50-IRES-neo or hygro were obtained by subcloning a 1669 bp NheI/BamHI 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-NheI, Table 2), and a reverse primer matching the end of the second exon including the stop codon (Full-IE2-anti-BamHI, Table 2), into the Multiple Cloning Site (MCS) of bicistronic vectors pIREShyg2 or pIRESneo2 (Clontech).

pIE2neo was obtained by subcloning the 3442 bp EcoRI/PstI 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 EcoRI and PstI.

p2xCMVeIE2neo was obtained by subcloning first the 231 bp NdeI/BglII fragment of hCMV enhancer promoter generated from pEGFP-C1 by PCR, using the primer pair NdeI-hCMV-sense/ BglII-hCMV-anti (Table 2), then the 438 bp BglII/HindIII fragment of hCMV enhancer promoter generated from pEGFP-C1 by PCR, using the primer pair BglII-hCMV-sense/ HindIII-hCMV-anti, in pIE2neo (Table 2).

pIE2prKanaGalKIE2pr was obtained by subcloning first a 593 bp EcoRI/PstI fragment of BoHV-4 IE2 (TargI-sense/Anti I amplicon, Table2, Accession number: NC002665) in pTZΔlinker KanaGalk vector, cut with EcoRI and PstI, and then a 932 bp KpnI/HindIII fragment (Sense II amplicon/Anti II amplicon, Table 2), into the vector, cut with KpnI and HindIII.

pTZKanaGalK was obtained by subcloning the 2231 bp galactokinase prokaryotic expression cassette (GalK), along with the kanamycin resistant expression cassette (KanaGalK) by PCR from pINT2KanaGalK (Donofrio et al., 2007b), into pTZ57R cut by KpnI and PstI. pTZKanaGalK was cut with EcoRI/KpnI to insert the first IE2 1001 bp amplicon (Delta ExonII A/Delta Exon II B, Table 2), amplified by PCR and cut with the same enzymes; in this intermediate construct, cut with PstI/HindIII, was sub-cloned the second IE2 1038 bp amplicon, amplified by PCR (Delta Exon II C/Delta Exon II D, Table 2) and cut with PstI/HindIII to generate pORF50KanaGalK.

pTK-KanaGalK-TK, was generated by sub-cloning the galactokinase prokaryotic expression cassette (GalK), along with the kanamycin resistant expression cassette into the pINT2 shuttle vector (Donofrio et al., 2007b).

The AseI/MluI fragment from pIgK$E_2VP_2^{2800}$gD^{106} (Capocefalo et al, 2010.) was sub-cloned in pORF50KanaGalK after a KpnI/PstI double digestion and by a blunt-end reaction, to obtain pORF50LEFT/IgK$E_2VP_2^{2070-2083}$gD^{106}/ORF50RIGHT.

pORF50KanaGalK was further modified through the insertion firstly of the 273bp X-amplicon (EcoRI-X-sense, KpnI-X anti), restricted with EcoRI/KpnI, in the vector backbone, restricted with the same enzyme; and then of the 261bp Y-amplicon (PstI-Y-sense, HindIII-Y-anti), cut with PstI/HindIII and inserted in the vector cut with the same enzyme, to generate pORF50-X-KanaGalK-ORF50-Y. X and Y have been designed to amplify the IE2 region of the II exon, deleted during the first step of targeting.

Transient Transfection

Confluent BEK cells in twenty four-well plates were co-transfected with 0.5 μg of pIE2prom-Luc (containing 137, 337, 537 or 1122 nt of the IE2 promoter region) or 0.5 μg pGL3 empty vector, as a negative control and 0.05 μg of pRK-Renilla to normalize the efficiency of transfection, using LTX transfection reagent (Invitrogen, UK) as suggested by the manufacturer. Transfection mixture was prepared in DMEM/high without serum and antibiotics and left on the cells for 6 h at 37°C, 5% CO_2 in air, in a humidified incubator. After 6 h, the transfection mixture was replaced with complete medium (EMEM, 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml Amphotericin B) and left to recover for 18 h at 37°C, 5% CO_2 in air, in a humidified incubator. 24 h post transfection, cells were treated with different reagents to perform luciferase assay.

Luciferase reporter assay

Luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega) with minor modifications. Following treatments, cells were washed with PBS, lysed with 100 μl of lysis passive buffer by freeze-thawing at -80 °C. 10 μl of the cell lysate was added to 50 μl of Luciferase Assay Reagent (LAR) and Luciferase activity was determined with a PerkinElmer Victor^3 Multilabel Counter, according to the manufacturer's specifications. Individual assays were normalized for *Renilla* luciferase activity with a second reading, adding 50 μl of Stop & Glo substrate.

Experiments were performed with 4 replicates and each experiment repeated three times and statistical differences were tested by Student's *t* test

RNA extraction and RT-PCR

Total RNA from BEK/IE2 and BEK/ORF50 cells was extracted with TriPure reagent (Roche) and 2 µg of total RNA were reverse transcribed using Ready-To-Go, T-Primed First-Strand Kit (Amersham Biosciences). Two microliters of resulting cDNA were amplified in a final volume of 50 µl of 10 mM Tris–hydrochloride, pH 8.3, containing 0.2 mM deoxynudeotide triphosphates, 3 mM $MgCl_2$, 50 mM KCl, 1 U of Taq polymerase (Invitrogen) and 0.25 µM of each primer (IE2-A-sense/IE2-B-antisense, Accession Number NC002665, Table 2). Primers were selected from the published sequence of BoHV-4 genome (Zimmermann et al., 2001). The amplification program was 30 cycles, each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and chain elongation with at 72 °C for 2 min.

BAC BoHV-4-A recombineering and selection

Recombineering was performed as previously described (Donofrio et al., 2009a; Donofrio et al., 2008c) with some modifications. SW102 bacteria containing BAC-BoHV-4-U and BAC-BoHV-4-LVR were grown, heat induced and electroporated with a gel-purified 3146 bp fragment (IE2prKanaGalKIE2pr) obtained by cutting pIE2prKanaGalKIE2pr (pTZΔlinker I-II amplicon IE2) with *Eco*RI (Fermentas). After recovery of 1 h in a 32°C shaking water bath, bacterial serial dilutions were plated on M63 minimal medium plates (agar 15 g/l; (DIFCO, BD Biosciences), 0.2% D-galactose (SIGMA), 1 mg/l D-biotin (SIGMA), 45 mg/l L-leucine (SIGMA) and 50 mg/l kanamycin (SIGMA). Plates were incubated 3–5 days at 32 °C. Several selected colonies were picked, streaked on McConkey agar indicator plates (DIFCO, BD Biosciences) containing 12.5 µg/ml of chloramphenicol and incubated at 32 °C for 3 days until white colonies appeared. White colonies were grown in duplicate for 5–8 h in 0.5 ml of LB containing 50 µg/ml of kanamycin or LB containing 12.5 µg/ml of chloramphenicol. Only those colonies growing on chloramphenicol and on kanamycin were kept and grown overnight in 5 ml of LB containing 12.5 µg/ml of chloramphenicol. BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-LVR/IE2pr were purified and analyzed through *Eco*RI restriction enzyme digestion for pIE2prKanaGalKIE2pr fragment targeted integration.

To delete IE2 Exon II, SW102 bacteria containing BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-LVR/IE2pr were grown, heat induced and electroporated with a ~4.2 kb gel purified fragment (pORF50KanaGalkORF50) obtained by cutting pORF50KanaGalK with *Hin*dIII/*Eco*RI (FERMENTAS). Selection step, plating of dilutions, and selection of clones were performed as above. BAC-BoHV-4-UΔORF50 and BAC-BoHV-4-LVRΔORF50 were purified and analyzed through *Pst*I or BamHI restriction enzyme digestion for pORF50KanaGalKORF50

fragment targeted integration. Retargeting in BAC-BoHV-4-UΔORF50 was done electroporating the ≈4428bp PvuII fragment excised out from pORF50LEFT/ IgKE$_2$VP$_2^{2070\text{-}2883}$gD^{106}/ORF50RIGHT in SW102 bacteria containing BAC-BoHV-4-UΔORF50 and heat induced. After a recovery step of 4 hours bacteria serial dilution were plated on M63 minimal medium plates (agar 15 g/l; (DIFCO, BD Biosciences), 0.2% Deoxy-2-galactose (DOG)(SIGMA), 1 mg/l D-biotin (SIGMA), 45 mg/l L-leucine (SIGMA) and 12.5 mg/l chloramphenicol (SIGMA) and incubated at 32 °C for 5 days until white colonies appeared. White colonies were grown in duplicate for 5–8 h in 0.5 ml of LB containing 50 µg/ml of kanamycin or LB containing 12.5 µg/ml of chloramphenicol. Only those colonies growing on chloramphenicol but not on kanamycin were kept and grown overnight in 5 ml of LB containing 12.5 µg/ml of chloramphenicol. BAC-BoHV-4 U ΔORF50-IgKE$_2$VP$_2^{2070\text{-}2883}$gD^{106}were purified and analyzed through *BamHI* restriction enzyme digestion for -IgKE$_2$VP$_2^{2070\text{-}2883}$gD^{106} fragment targeted integration.

To delete the second copy of IE2 Exon II, SW102 bacteria containing BAC-BoHV-4 U ΔORF50-IgKE$_2$VP$_2^{2070\text{-}2883}$gD^{106} were grown, heat induced and electroporated with a ~2.74 kb gel purified fragment (pORF50X-KanaGalkORF50-Y) obtained by cutting pORF50-X-KanaGalK-y with *Hin*dIII/*Eco*RI (FERMENTAS). Selection step, plating of dilutions, and selection of clones were performed as above, for the description of BAC-BoHV-4-UΔORF50 generation.

Original detailed protocols for recombineering can also be found at the recombineering website (http://recombineering.ncifcrf.gov).

Restriction enzyme analysis and non-isotopic Southern hybridization

BAC-BoHV-4-U, BAC-BoHV-4-U/IE2pr, BAC-BoHV-4-UΔORF50, BAC-BoHV-4-LVR, BAC-BoHV-4-LVR/IE2pr, BAC-BoHV-4-LVRΔORF50, BAC-BoHV-4 U ΔORF50-IgKE$_2$VP$_2^{2070\text{-}2883}$gD^{106} and BAC-BoHV-4-UΔORF50-double mutant recombinants were characterized as previously described (Donofrio et al., 2009b). Fifteen microliters of DNA prepared from bacteria containing pBAC-BoHV-4-U and pBAC-BoHV-4-LVR and derivatives, were restriction enzyme digested, separated by electrophoresis overnight in a 0.8% agarose gel, stained with ethidium bromide, capillary transferred to a positively charged nylon membrane (Roche), and cross-linked by UV irradiation by standard procedures. The membrane was pre-hybridized in 50 ml of hybridization solution (7% SDS, 0.5 M phosphate, pH 7.2, 1 mM EDTA) for 2 h at 65 °C in a rotating hybridization oven (Techna instruments). Probe preparation and digoxigenin non-isotopic labelling was performed by PCR (Donofrio et al., 2009a), using ORF50-del-sense and ORF50-del-antisense primers or Kana primers (SmaI-AseI-kana sense: 5'-aac ccc cgg gat taa tcc gga att gcc agc tgg gg-3'; SmaI-MluI-Kana anti: 5'-cca acc cgg gac gcg tga aat tgt aag cgt taa taa t-3'). Five microliters of the probe were added to 100 µl of ddH_2O, denatured in boiling water for 5 min, and cooled on ice for another 2 min. Denatured

probe was added to hybridization solution and membrane hybridized overnight at 65 °C in a rotating hybridization oven (Techna Instruments). Following hybridization, the membrane was washed and blocked in 50 ml of blocking solution. Anti-digoxigenin Fab fragment (150 U/200 µl [Roche]), diluted 1:15,000 in 50 ml of blocking solution, was applied to the membrane and detection was performed following equilibration of the membrane in detection buffer for 2 min at room temperature. Chemiluminescent substrate (CSPD, Roche) was added by scattering the drops over the surface of the membrane after placement of the membrane between two plastic sheets, and any bubbles present under the sheet were eliminated with a damp lab tissue to create a liquid seal around the membrane. Signal detection was obtained by exposing the membrane to X-ray film. The exposure time was adjusted with the intensity of the signal.

Cell culture electroporation and recombinant virus reconstitution

BEK and BEK/*cre* cells were cultured and transfected as previously described (Donofrio et al., 2009b). BEK or BEK/*cre* cells were maintained as a monolayer with complete medium containing 90% EMEM, 10% FBS, 2 mM l-glutamine, 100 IU/ml penicillin and 10 µg/ml streptomycin. Cells were sub-cultured to a fresh culture vessel when growth reached 70–90% confluence (i.e., every 3–5 days) and were incubated at 37 °C in a humidified atmosphere of 95% air–5% CO_2.

BAC-BoHV-4-U, BAC-BoHV-4-LVR, BAC-BoHV-4-U/IE2pr, BAC-BoHV-4-LVR/IE2pr, BAC-BoHV-4-UΔORF50, BAC-BoHV-4-LVRΔORF50, BAC-BoHV-4 U ΔORF50-IgK$E_2VP_2^{2070\text{-}2883}gD^{106}$,BAC-BoHV-4-UΔORF50-double mutant, BAC-BoHV-4-UΔTK and BAC-BoHV-4-LVRΔTK DNA plasmid (5 µg) in 500 µl D-MEM/high without serum were electroporated (Equibio apparatus, 270 V, 960 µF, 4-mm gap cuvettes) into BEK or BEK*cre* cells from a confluent 25-cm^2 flask. Electroporated cells were returned to the flask, fed the next day, and split 1:2 when they reached confluence at 2 days post-electroporation. Cells were left to grow until CPE appeared. Recombinant viruses were propagated by infecting confluent monolayers of BEK cells at a m.o.i. of 0.5 $TCID_{50}$ per cell and maintaining them in EMEM with 10% 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 virions through 30% sucrose. Virus pellets were resuspended in ice cold EMEM without FBS. $TCID_{50}$ were determined on BEK or MDBK cells by limiting dilution.

5 µg of BAC-BoHV-4-UΔORF50, BAC-BoHV-4-LVRΔORF50, BAC-BoHV-4 U ΔORF50-IgK$E_2VP_2^{2070\text{-}2883}gD^{106}$ and BAC-BoHV-4-UΔORF50-double mutant in 500 µl DMEM/high without serum were also electroporated (Equibio apparatus, 270 V, 960 µF, 4-mm gap cuvettes) into BEK/IE2 or BEK/ORF50 cells from a confluent 25-cm^2 flask. Following electroporation, cells were recovered in a new 75-cm^2 flask with 25 ml of EMEM and 10% fetal bovine serum to allow cells to attach. Cells were left to grow until CPE appeared. Recombinant

viruses were propagated by infecting confluent monolayers of BEK/IE2 or BEK/ORF50 cells at a m.o.i. of 0.5 $TCID_{50}$ per cell. When complete CPE appeared, the virus was prepared as described before. $TCID_{50}$ were determined on BEK/IE2 or BEK/ORF50 cells by limiting dilution.

Viral growth curves

BEK/IE2 and BEK/ORF50 cells were infected with BAC-BoHV-4-LVR and BAC-BoHV-4-LVRΔORF50 at a MOI of 1 $TCID_{50}$/cell and incubated at 37 °C for 4 hours. Infected cells were washed with serum free EMEM and then overlaid with EMEM containing 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 IU/ml penicillin (SIGMA), 100 µg/ml streptomycin (SIGMA) and 2.5 µg/ml Amphotericin B. The supernatants of infected cultures were harvested after 12 hours and then every 24 hours, and the amount of infectious virus was determined by limiting dilution on BEK/IE2 or BEK/ORF50 cells.

Results

Firstly, based on the hypothesis that the IE2 gene is essential for initiation and progression of BoHV-4 lytic replication, IE2 expressing cell lines were generated to rescue IE2-defective BoHV-4 in *trans*.

A cell line, BEK/IE2, was created, which contains a 3442-bp *Eco*RI/*Pst*I BoHV-4 genome fragment including the full BoHV-4 IE2 gene and its promoter-regulatory region (van Santen, 1993) (Fig. 16A).

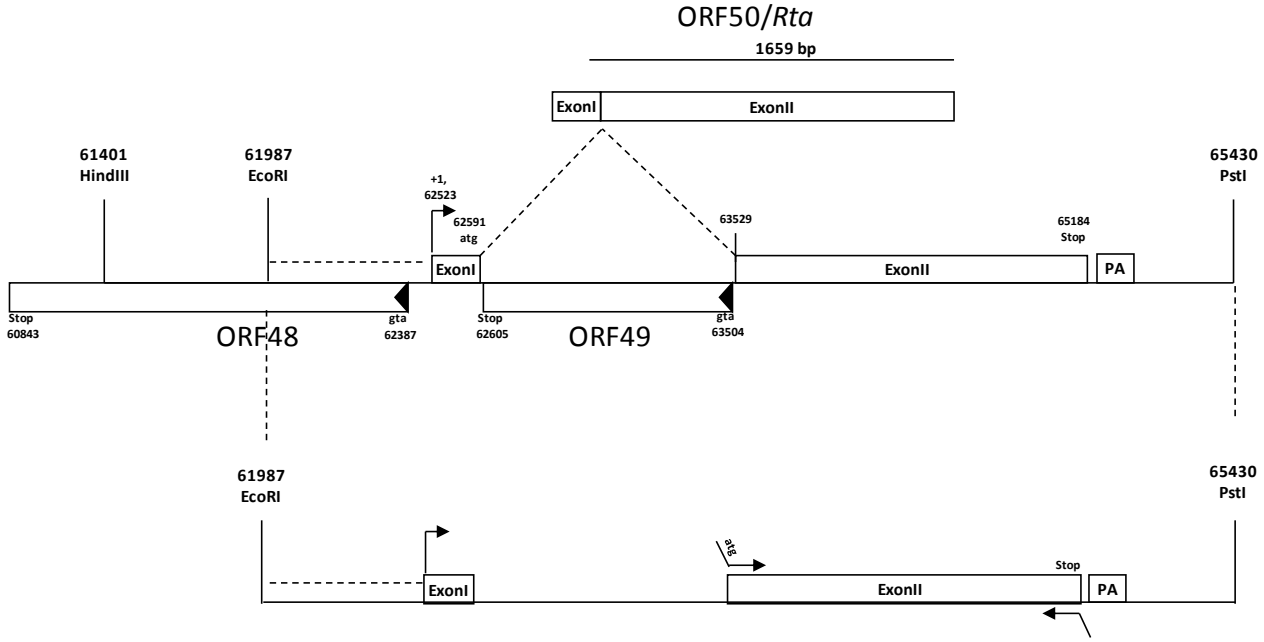

Fig. 16. A) BoHV-4 IE2 (ORF 50) genomic locus schematic diagram (not to scale) based on the BoHV-4-66p347 complete genome published sequence (GenBank accession number AF318573). ORF 50 is formed by splicing of Exon I, beginning at the transcriptional start site (+1, 62523) indicated by an arrow and containing the atg (62591), to Exon II. ORFs 48 and 49 are located on the opposite strand as ORF50; ORF 49 is contained within the ORF 50 intron, below, the *EcoRI/PstI* fragment containing the minimal promoter (dashed line) and employed to generate pIE2*neo* construct (in C).

The transcriptional activity of the IE2 promoter region was evaluated by Luciferase assay, by subdoning different region of the promoter: 137 bp, 337 bp, 537 bp and 1122 bp containing the TATA box, the transcriptional start site and the first 15 non-coding nudeotides (UTR) of the first exon were obtained by PCR and sub-cloned in front of the Luciferase ORF (Luc) in the pGL3 basic vector. Constructs were transiently co-transfected with pTK-Renilla for data normalization, into Human Embryo Kidney cells (ATCC, CRL-1573, USA) and Luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit.

537 bp 5' to the IE2 transcription start site in the 3442-bp *Eco*RI/*Pst*I fragment were shown to comprise the minimal IE2 promoter-regulatory region giving the maximal signal in a Luciferase reporter assay (Fig. 16B).

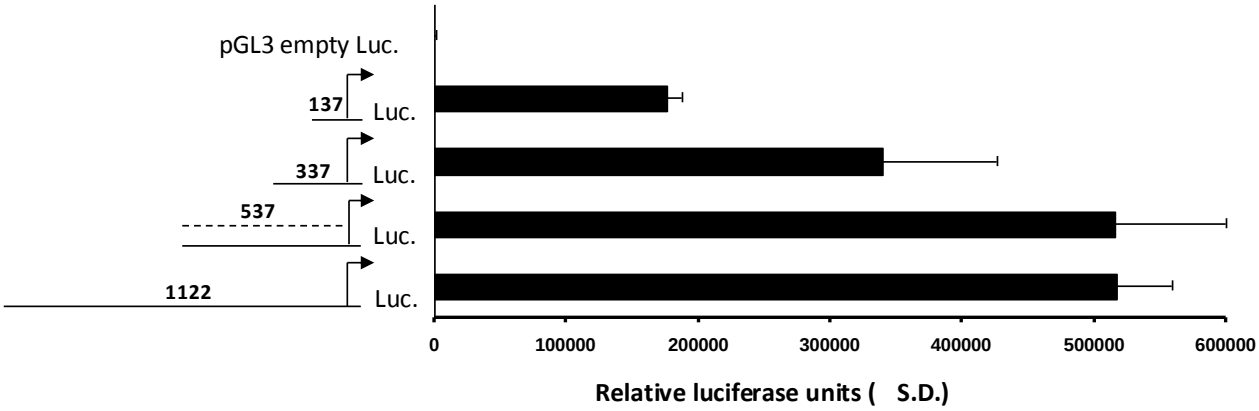

Fig.16.B) Transcriptional activity of different IE2 promoter region sub-cloned in front of the Luciferase ORF (Luc.) in the pGL3 basic vector. Each reaction was done in quadruplicate, and each point represents the average ± standard deviation (±S.D.) from three experiments (* *P<0.001*).

To increase IE2 expression, two copies of a 427-bp sequence containing the hCMV enhancer but lacking the basal promoter (TATA box and transcriptional start site) were inserted in front of the 3442-bp *Eco*RI/*Pst*I BoHV-4 genome fragment and subcloned into a vector backbone containing a *neo* resistant cassette for G418 drug selection (p2xCMVeIE2*neo*) (Fig. 16C and D). IE2 mRNA was expressed at higher levels from p2xCMVeIE2*neo* than from the un-enhanced expression construct, pIE2*neo* (Fig. 16C), containing only the 3442-bp EcoRI/PstI BoHV-4 genome fragment, when tested by transient transfection assay and RT-PCR (Fig. 16E). p2xCMVeIE2*neo* was used to generate BEK/IE2, stably expressing IE2.

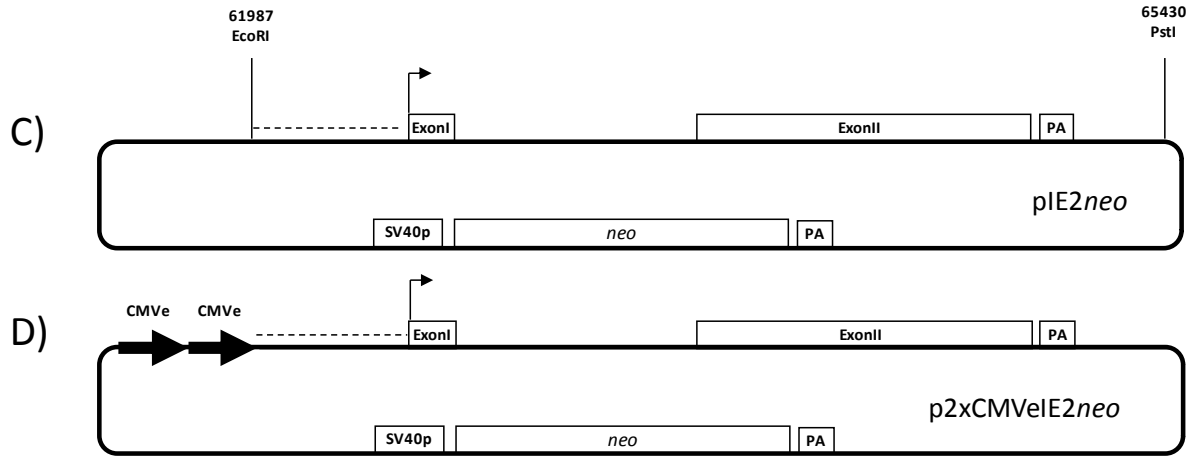

Fig.16.C) pIE2*neo* vector diagram (not to scale) showing the *Eco*RI/*Pst*I fragment (in A, bottom), sub-cloned into a vector containing a *neo* expression cassette for G418 selection. D) p2xCMVeIE2 vector diagram (not to scale) where the human cytomegalovirus enhancer (CMVe), obtained from pEGFP-C1 vector (Clontech), has been inserted in duplicate upstream of the *Eco*RI/*Pst*I fragment into pIE2*neo*.

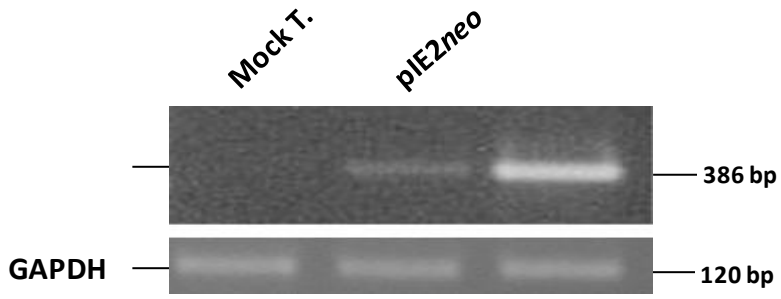

Fig.16. E) Semi-quantitative differential expression between pIE2neo and p2xCMVeIE2*neo* tested by RT-PCR. The primers employed generate an amplicon of 386-bp in length from the spliced product of IE2 transcripts. The experiment was repeated three times giving similar results. RT-PCR detecting RNA transcribed from GAPDH house-keeping gene is shown as a control.

BoHV-4 IE2 gene contains even the ORF49 within its intron, which is transcribed in the opposite direction of ORF50 (Fig. 16A) so, to exclude the possibility that a defect in the expression of ORF 49 expression could be responsible for or contribute to a replication defective phenotype; a cell line (BEK/ORF50) expressing only the ORF50 was generated. The complete ORF 50 that encodes only the complete Rta protein was generated by PCR using a forward primer containing an *atg* translational initiation codon and the rest of the primer sequence matching the beginning of the ORF 50 second exon, and a reverse primer matching the end of the second exon including the *stop* codon. The amplicon was cloned into a bi-cistronic vector containing an internal ribosomal entry site (IRES) followed by a selectable marker (*neo* or Hygromycin) (pCMV-ORF50-IRES-*neo* and pCMV-ORF50-IRES-*Hygromicin*; Fig. 16F) and used to generate a stable cell line expressing only ORF50/Rta (BEK/ORF50). Both BEK/IE2 and BEK/ORF50 stably expressed ORF50 through at least six passages when tested by RT-PCR (Fig. 16G). Furthermore, a morphological change characterized by increased cell sprouting and shrinking, although completely compatible with cell survival and replication, was observed for BEK/IE2 and BEK/ORF50 cell lines (data not shown).

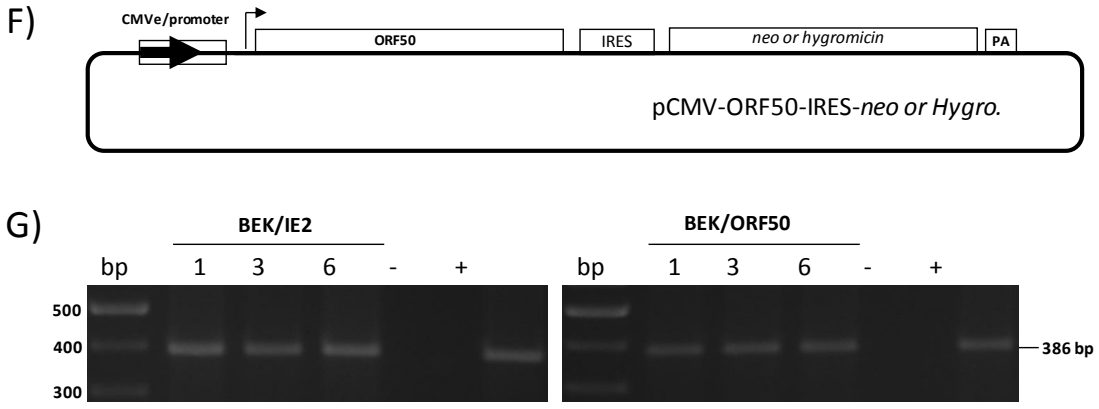

Fig.16. F) Diagram (not to scale) of the bi-cistronic expression vector, pCMV-ORF50-IRES-*neo* or *Hygro,* made by sub-cloning the ORF50 coding region obtained by PCR, into pIRES-*neo* or pIRES-*Hygro* (Clontech, Mountain View, USA). G) RT-PCR analysis of ORF 50 mRNA expression in BEK cells stably transfected with p2xCMVeIE2 *neo* (BEK/IE2) and pCMV-ORF50-IRES-neo (BEK/ORF50) at passages 1, 3 and 6.

To investigate the role of ORF50/*Rta* expression in initiating the BoHV-4 transcriptional/replication cycle, the BoHV-4 IE2 gene was interrupted in its 5' untranslated region by site specific insertional mutagenesis mediated by heat inducible homologous recombination into the genome of two strains of BoHV-4 cloned as bacterial artificial chromosome (BAC): the BAC-BoHV-4-U genome, cloned from a BoHV-4 uterine isolate (Donofrio et al., 2009a) and the BAC-BoHV-4-V.test genome, cloned from the BoHV-4-V.test reference strain (Gillet et al., 2005a), respectively. A targeting construct, pIE2prKanaGalKIE2pr, containing the 2004-bp KanaGalK DNA stuffer double selecting cassette (Donofrio et al., 2007b) flanked by two BoHV-4 IE2 gene homologous regions, was inserted between the putative IE2 transcriptional start site [based on the IE2 transcriptional start site identified in the BoHV-4 DN-599 isolate (van Santen, 1993)] and the IE2 translational start site (ATG) positioned in the IE2 gene first exon (Fig. 17A).

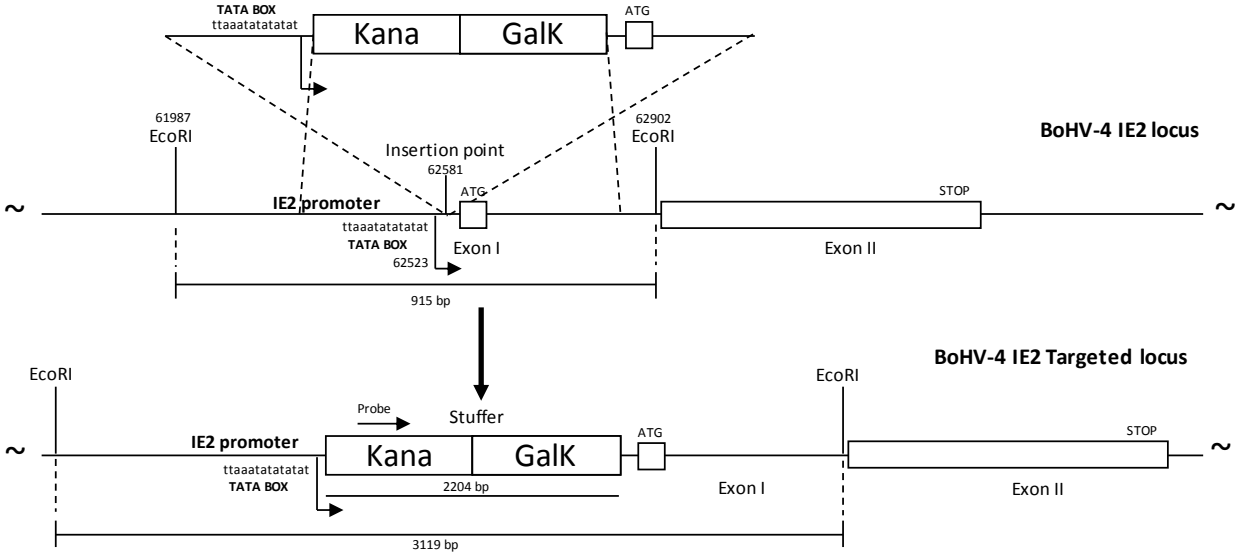

Fig.17. A) The 2204-bp Kana/GalK selectable DNA stuffer, flanked by IE2 homologous regions, was introduced at position 62582, between the IE2 gene transcriptional start site and the first atg of the BoHV-4-U and BoHV-4-V.test strains cloned as BAC. The expected IE2 targeted locus (A, bottom) has an increased size of the *Eco*RI fragment (3119 bp instead of 915 bp), generated by *Eco*RI restriction enzyme digestion (diagrams are not to scale).

Then, the targeting cassette was electroporated into SW102 *E. coli* containing BAC-BoHV-4-U and BAC-BoHV-4-V.test genomes, to produce BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-V.test/IE2pr. Selected targeted clones were analyzed by PCR and sequencing, *Eco*RI restriction enzyme digestion, and Southern hybridization. All the clones analyzed displayed the expected targeting (Fig. 15B).

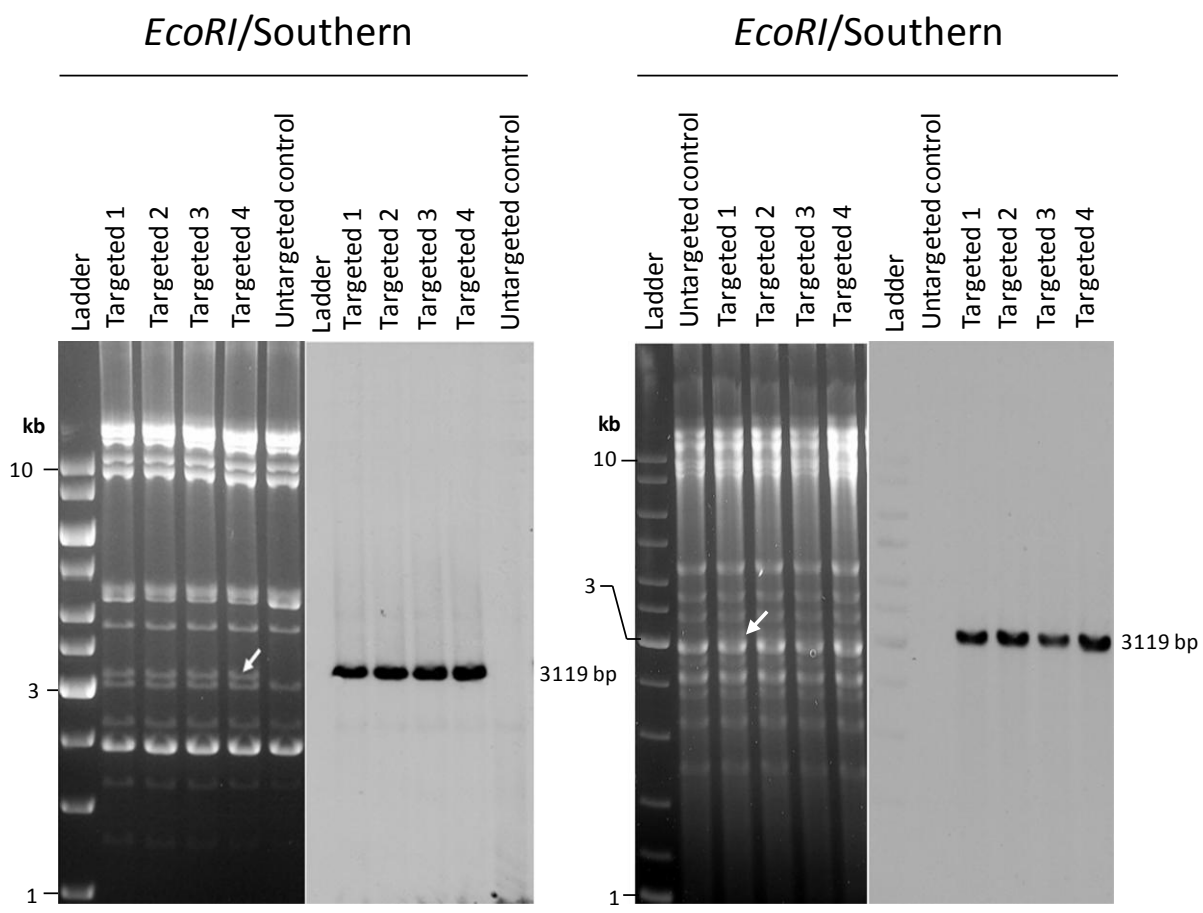

Fig.17. B) *Eco*RI restriction profile and Southern blotting of 4 representative targeted clones compared to the untargeted control. The expected targeting, shows a new 3119 bp band (indicated by arrow) that misses in the untargeted control, Southern blotting with a probe (indicated in Fig.15.A) matching the Kana/GalK sequence, confirmed the data.

Two control mutant viruses, constructed by the insertion of the same DNA stuffer into the BAC-BoHV-4-U (BAC-BoHV-4-UΔTK) (Donofrio et al., 2009a) and BAC-BoHV-4-V.test (BAC-BoHV-4-V.testΔTK) (Gillet et al., 2005a)TK ORF were employed as controls in a viral reconstitution assay to test the competence of BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-V.test/IE2pr DNA to reconstitute infectious replicating viral particles (Infectious Replicating Viral Partides, IRVPs). BEK cells or BEK expressing *cre*, to excise the floxed BAC cassette (Donofrio et al., 2008c), were electroporated with BAC-BoHV-4-U, BAC-BoHV-4-V.test, BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK, BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-V.test/IE2pr DNA. As expected, the BAC-BoHV-4-V.test/IE2pr genome could not reconstitute IRVPs when electroporated into either BEK or BEK/*Cre* cells.

Although a phenotype identical to BAC-BoHV-4-V.test/IE2pr was expected for BAC-BoHV-4-U/IE2pr, to the contrary, BAC-BoHV-4-U/IE2pr could reconstitute IRVPs efficiently as well as control BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK, BAC-BoHV-4-U and BAC-BoHV-4-V.test DNA, as shown by forming plaques on the cell monolayer and the spreading of the cytopathic effect (CPE) over time (Fig. 18.A).

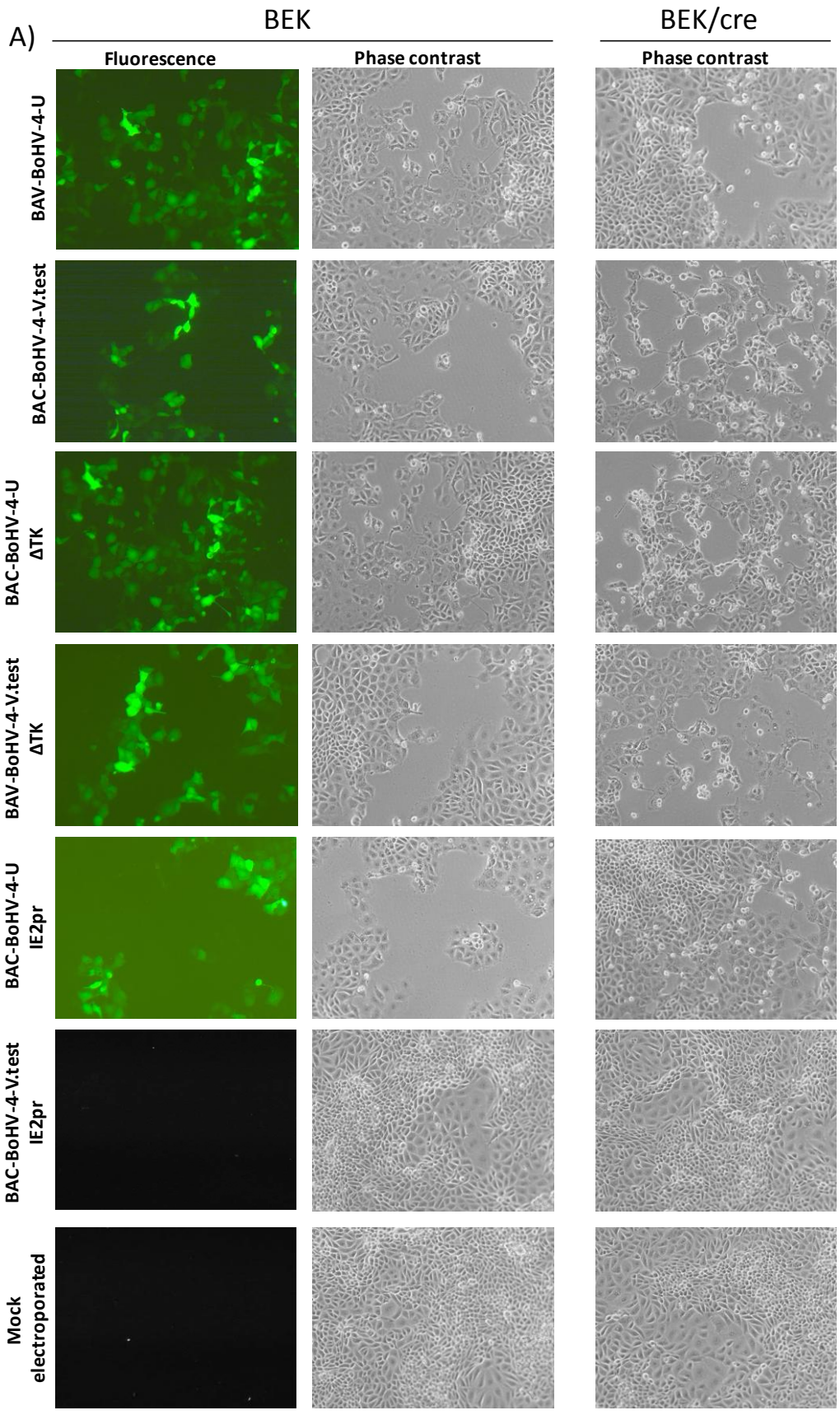

Fig. 18. A) Representative images (10X) of BEK and BEK expressing cre (BEK/cre) electroporated with BAC-BoHV-4-U, BAC-BoHV-4-V.test, BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK, BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-V.test/IE2pr DNA. CPE induced by reconstitution of IRVPs is recognizable only for BAC-BoHV-4-U, BAC-BoHV-4-V.test, BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK, BAC-BoHV-4-U/IE2pr and not for BAC-BoHV-4-V.test/IE2pr The test was repeated three times always giving identical results.

BoHV-4-U/IE2pr reconstituted progeny viruses could still transcribe the IE2 gene, while BAC-BoHV-4-V.test/IE2pr could not, as expected (data not shown). When BAC-BoHV-4-V.test/IE2pr DNA was electroporated into BEK/IE2 **(Fig. 18.B)** and BEK/ORF50 (not shown) complemented cells, IRVPs were reconstituted efficiently, thus linking BoHV-4-V.test viral reconstitution and replication with IE2 expression.

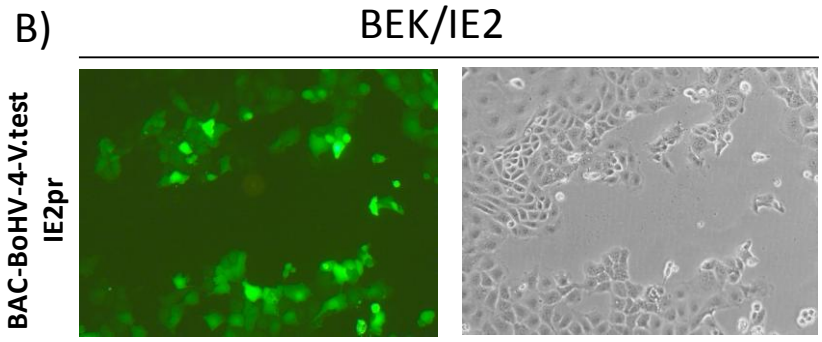

Fig.18 B) Representative images (10X) of CPE induced by reconstitution of IRVPs, rescued in cells expressing ORF50 (BEK/IE2 cells) are shown. Similar results were obtained in BEK/ORF50 cells).

An alternative approach to disrupt the IE2 gene was then employed. BAC-BoHV-4-U and BAC-BoHV-4-V.test genomes were generated that contained a 488-bp deletion in the second exon of IE2; through the insertion of the 2204-bp Kana/GalK selectable DNA stuffer between positions 63905 and 64393 of the BoHV-4-U and BoHV-4-V.test strains cloned as BAC (Fig. 19A). Selected targeted clones were carefully analyzed by restriction enzyme digestion, Southern hybridization (Fig. 19B), PCR and sequencing (data not shown).

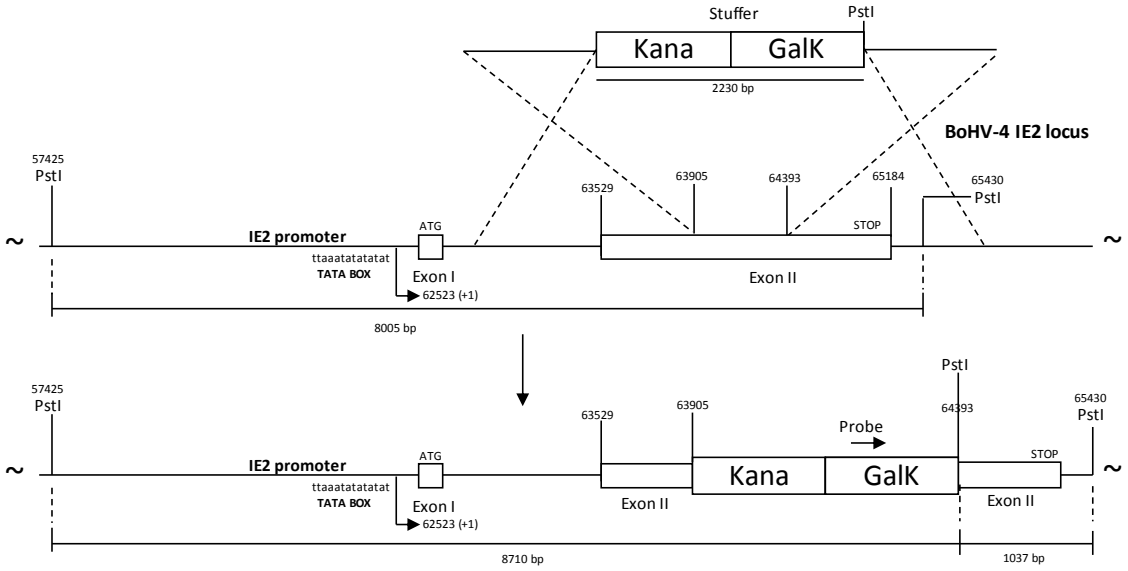

Fig. 19. A) Overall strategy to delete a 488-bp sequence from the IE2 gene Exon II, via heat inducible homologous recombination. The expected IE2 targeted locus (A, bottom) has an increased size of the *Pst*I fragment (8710 bp instead of 8005 bp), generated by *Pst*I restriction enzyme digestion (diagrams are not to scale).

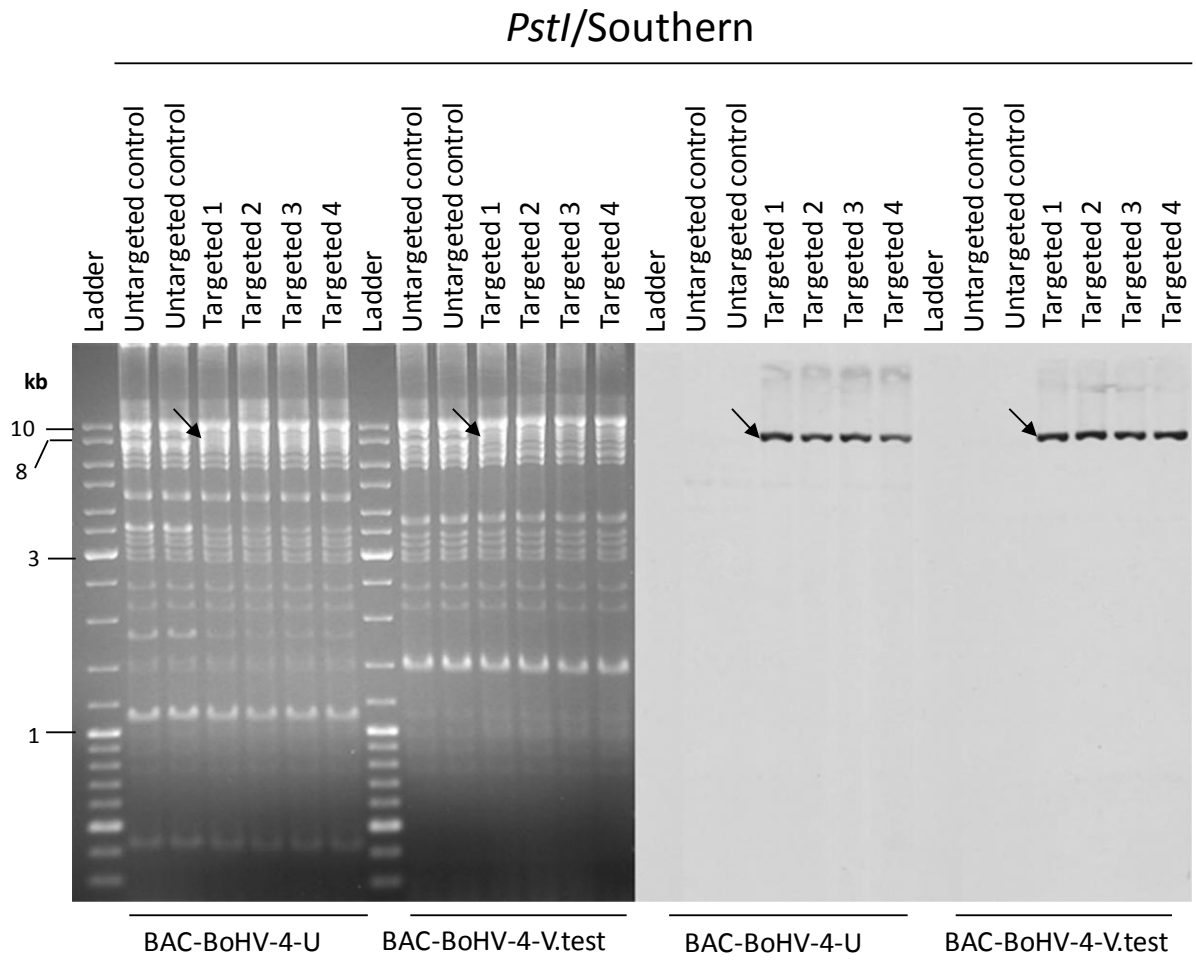

Fig.19.B) *Pst*I restriction profile and corresponding Southern blotting of representative targeted clones compared to the untargeted controls. The targeted clones display the expected targeting, where a new 8710-bp band (indicated by arrow) is present but missing in the untargeted control and confirmed by Southern blotting with a probe (indicated in Fig.17.A) matching the Kana/GalK sequence.

Representative targeted clones were finally electroporated into BEK cells expressing or not expressing *cre*. As before, BAC-BoHV-4-UΔORF50 but not BAC-BoHV-4-V.testΔORF50, maintained the capability to reconstitute IRVPs (Fig. 20A). Again, when BAC-BoHV-4-V.testΔORF50 DNA was electroporated into BEK/IE2 and BEK/ORF50 complementing cells, IRVPs were reconstituted efficiently (Fig. 20B), as well as control BAC-BoHV-4-U, BAC-BoHV-4-V.test, BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK DNA (data not shown).

BAC-BoHV-4-V.testΔORF50 replication characteristics were identical to BAC-BoHV-4-V.test ones, when the replication curve was performed in BEK/IE2 cells (Fig. 20C), whereas, an accelerated replication phenotype of the BAC-BoHV-4-V.test was observed when replicated in BEK/IE2 cells compared to replication in BEK cells.

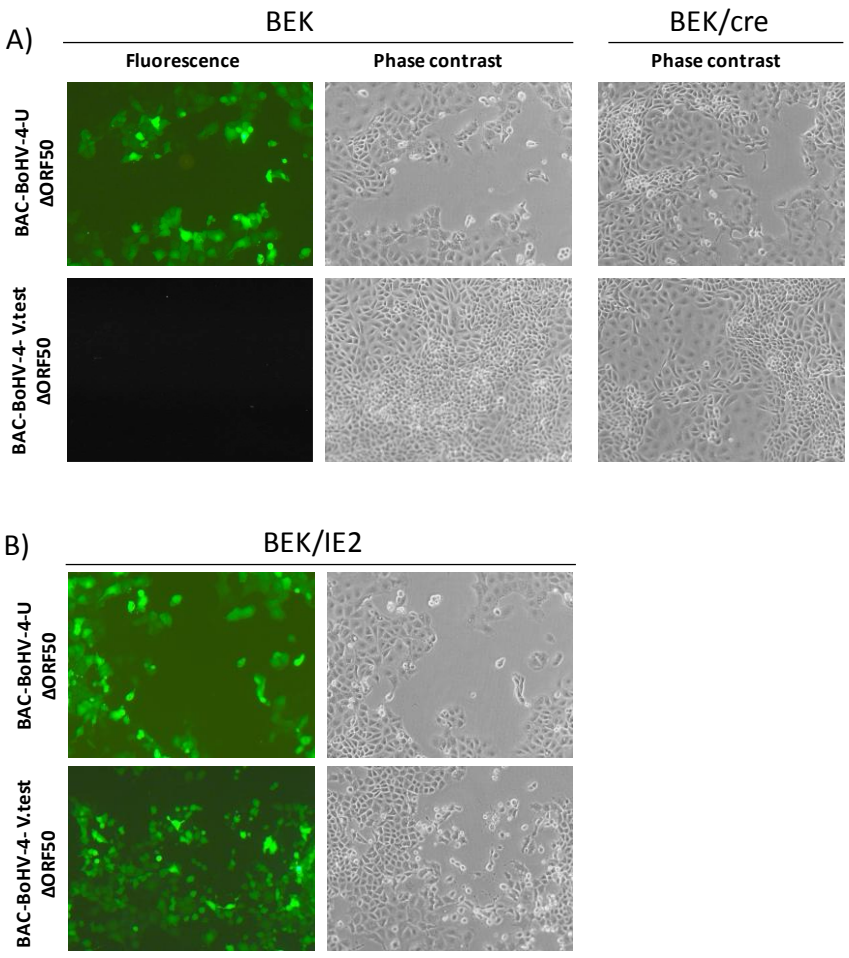

Fig. 20. A) Representative images (10X) of BEK and BEK expressing cre (BEK/cre),electroporated with BAC-BoHV-4-UΔORF50 and BAC-BoHV-4-V.testΔORF50 DNA. CPE induced by reconstitution of IRVPs is recognizable only for BAC-BoHV-4-UΔORF50 and not for BAC-BoHV-4-V.testΔORF50. The test was repeated three times, giving always identical results; and B) Representative images (10X) of CPE induced by reconstitution of IRVPs, rescued in cells expressing ORF50 (BEK/IE2 cells are shown. Similar results were obtained in BEK/ORF50 cells).

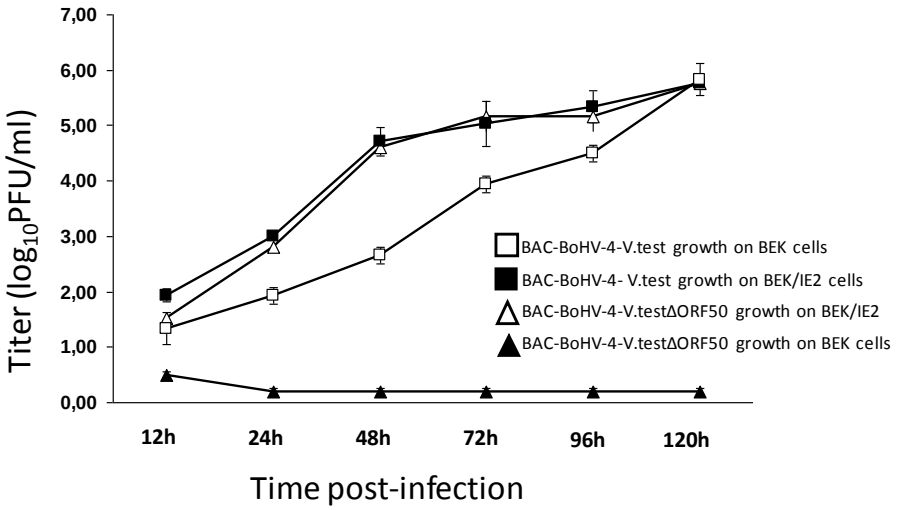

Fig.20. C) Replication kinetics of BAC-BoHV-4-V.test in BEK cells (white squares), BAC-BoHV-4-V.test in BEK/IE2 cells (black squares), BAC-BoHV-4-V.testΔORF50 in BEK cells (black triangles) and BAC-BoHV-4-V.testΔORF50 in BEK/IE2 (white triangles).

RT-PCR revealed that mRNA containing the full-length 1659 bp ORF50 was still expressed in BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-UΔORF50 but not in BAC-BoHV-4-V.test/IE2pr and BAC-BoHV-4-V.testΔORF50 (results not shown). Therefore, a possible duplication of IE2 gene in the BoHV-4-U genome was hypothesized. To determine whether the BoHV-4-U genome contains duplicate IE2 genes, a probe was generated that hybridizes to Exon II and thus to the fragment containing the BoHV-4 IE2 locus when cut with *Pst*I and *Bam*HI restriction enzymes (Fig. 21A).

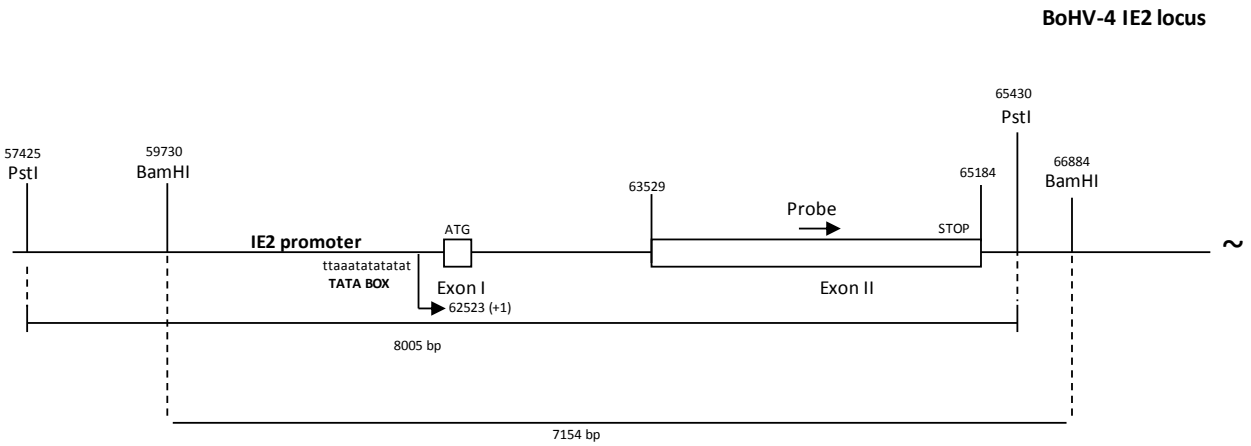

Fig. 21. A) BoHV-4 IE2 genomic locus schematic diagram (not to scale) with *Pst*I and *Bam*HI restriction site positions. The predicted sizes of the restriction fragments (8005 bp for PstI and 7154 bp for BamHI) are based on the BoHV-4-66p347 complete genome published sequence (accession number AF318573).

When analyzed by *Pst*I and *Bam*HI restriction digestion and Southern blotting both the BoHV-4-U isolate and the BAC-BoHV-4-U genome showed the presence of two bands **(Fig. 21B)**; such duplication was absent in the BoHV-4-V.test genome, where just a single band appeared.

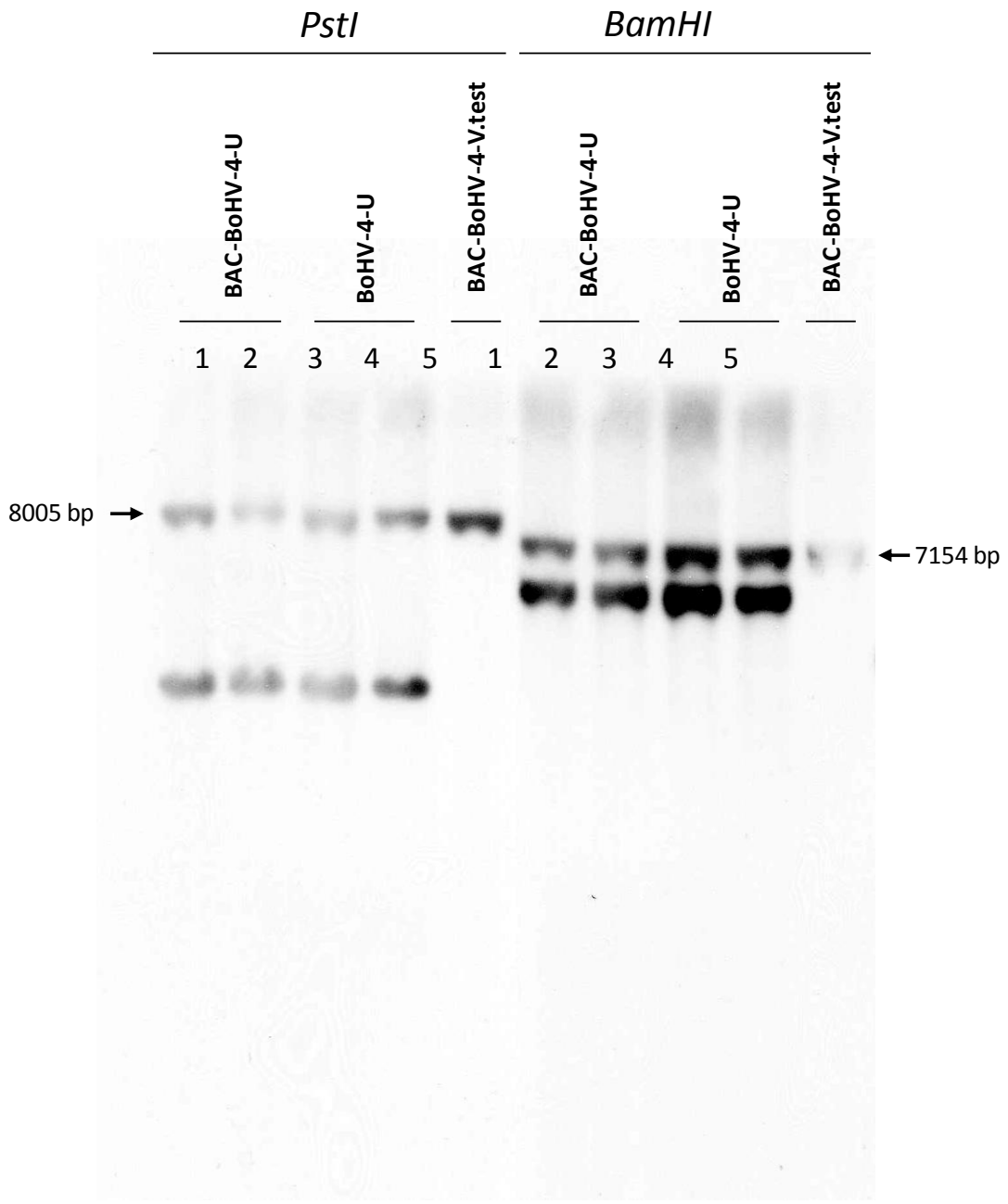

Fig.21.B) Southern blotting of BAC-BoHV-4U (Lanes 1 and 2), BoHV-4-U isolate (Lanes 3 and 4) and control BAC-BoHV-4-V.test (Lane 5) DNA, cut with *Bam*HI and *Pst*I and hybridized with the IE2 exon II-specific probe indicated in Fig.19.A).

To delete even the second gene copy of IE2 gene, a re-targeting construct, pORF50LEFT/IgK$E_2VP_2^{2070\text{-}2083}gD^{106}$/ORF50RIGHT, in which IgK$E_2VP_2^{2070\text{-}2883}gD^{106}$ expression cassette (Capocefalo et al., 2010) is flanked by two BoHV-4 IE2 gene homologous region sequences, was generated.

The 2204-bp Kana/GalK DNA stuffer between positions 63905 and 64393 of the BAC-BoHV-4-UΔORF50 (Fig. 22) was replaced by the $IgKE_2VP_2^{2070\text{-}2883}gD^{106}$ expression cassette. Selected targeted clones were carefully analyzed by restriction enzyme digestion, Southern hybridization (Fig. 22B), PCR and sequencing (data not shown).

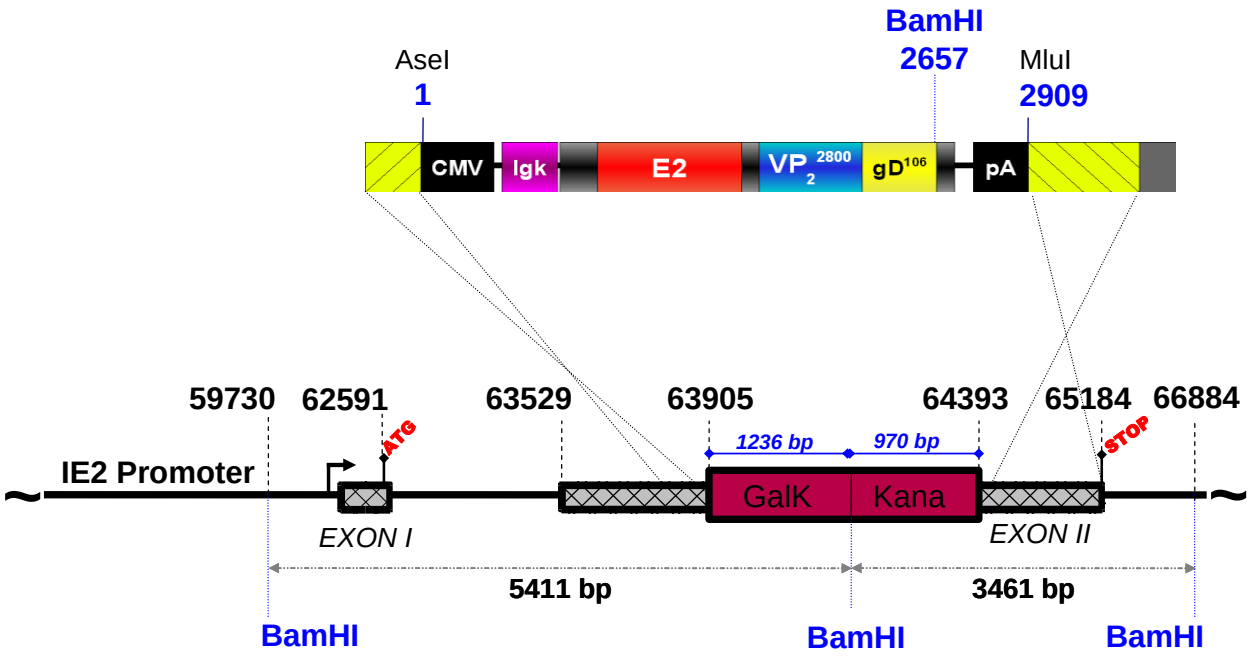

Fig. 22. A) Overall strategy to replace the KanaGalk stuffer inserted in the IE2 gene Exon II, via heat inducible homologous recombination (diagrams are not to scale).

When pBACBoHV-4 U ΔORF50-$IgKE_2VP_2^{2070\text{-}2883}gD^{106}$ was electroporated into BEK or BEKcre cells viable virus was obtained in both case and the depletion of the floxed BAC cassette in the progeny virus was assessed by loss of the green fluorescence in the viral plaques in consequence of the excision of the CMV-GFP expression cassette contained in the BAC plasmid backbone (data not shown).

The duplicated IE2 gene was deleted via homologous recombination trough the insertion of a 2206 bp KanaGalk cassette stuffer between position 63.905 and 64.393, that is the region already deleted in the IE2 first copy gene (Fig.23A).

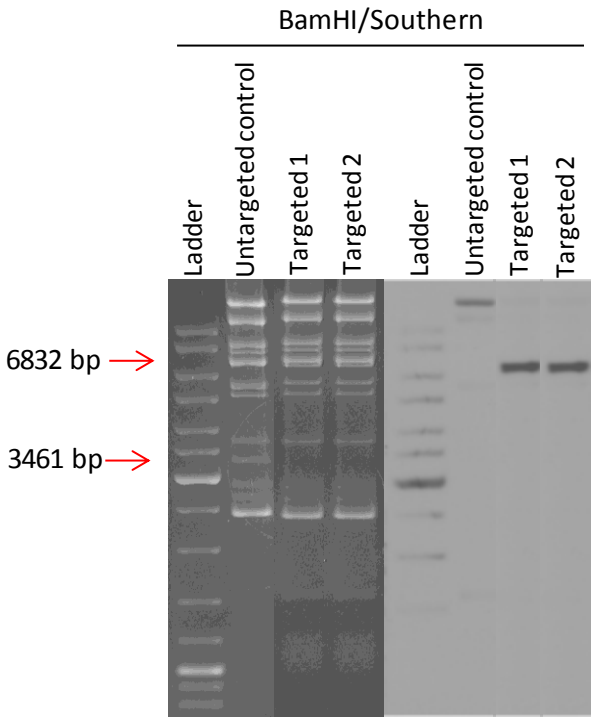

Fig.22.B) *BamHI* restriction profile and corresponding Southern blotting of representative targeted clones compared to the untargeted controls. The targeted clones display the expected targeting, where a new 6832-bp band (indicated by arrow) is present but missing in the untargeted control and confirmed by Southern blotting with a probe (indicated in Fig.20.A) matching the GalK sequence.

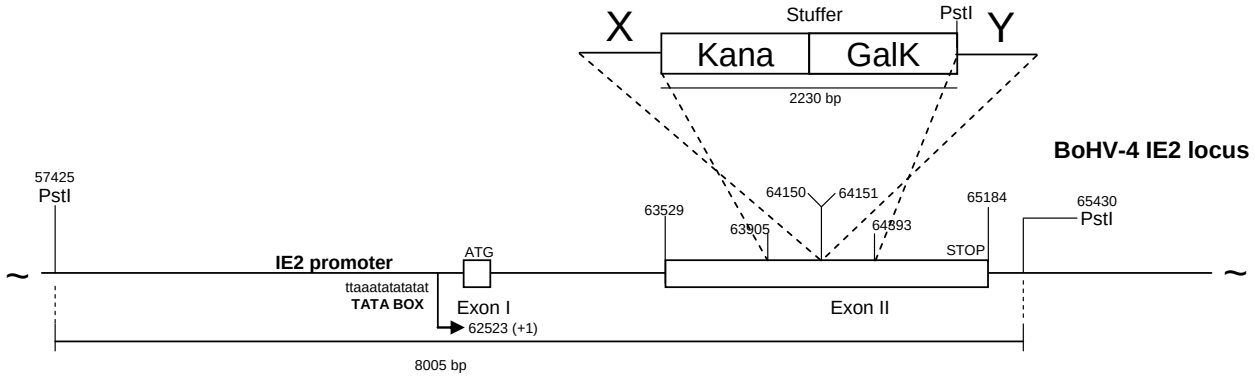

Fig. 23.A) Overall strategy to insert the kanaGalk stuffer in the second gene copy of IE2 gene Exon II, via heat inducible homologous recombination (diagrams are not to scale).

Selected targeted clones were carefully analyzed by restriction enzyme digestion, Southern hybridization **(Fig. 23B)**, PCR and sequencing (data not shown).

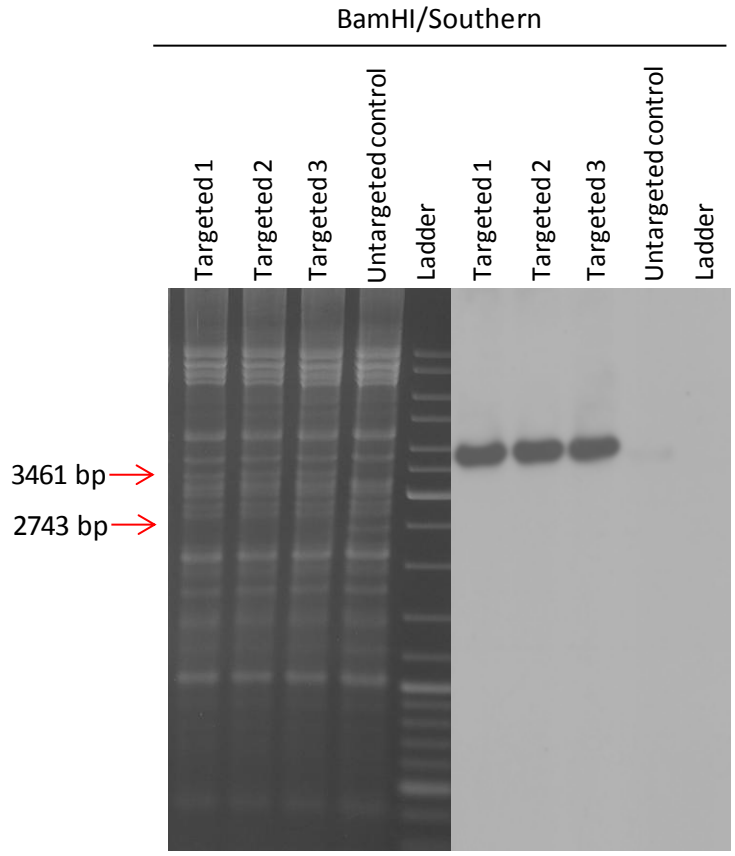

Fig.23.B) ***EcoRI*** **restriction profile and corresponding Southern blotting of representative targeted clones compared to the untargeted controls. The targeted clones display the expected targeting, where a new 3461bp band (indicated by arrow) is present but missing in the untargeted control and confirmed by Southern blotting with a probe matching the GalK sequence.**

Only the electroporation of pBACBoHV-4 U ΔORF50-double mutant in BEK/IE2 and BEK/ORF50 cells was able to rescue a viable virus from the BAC clones; as supposed it was not able to growth neither in BEK cells nor in BEK fincre cells (data not shown).

Discussion

BoHV-4 IE2 protein (*Rta*) is encoded by ORF 50 and is well conserved among γ-herpesviruses (Zimmermann et al., 2001); studies from closely related viruses, as MuHV68, HHV-8 and RRV demonstrated that the expression of ORF50/*Rta* was essential for viral replication and lytic reactivation. Transactivation studies have shown that BoHV-4 Rta specifically transactivates expression of a reporter gene linked to the promoter regulatory region of the BoHV-4 early (E) gene encoding the BoHV-4 homologue of herpes simplex virus type 1 (HSV-1) major DNA binding protein. 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 *denovo* infection of permissive cells (Donofrio et al., 2004b; van Santen, 1993). Although BoHV-4 IE2 gene was well characterized in terms of gene structure, transcription and RNA post transcriptional processing (van Santen, 1993), it has not been yet demonstrated that the expression of ORF50/*Rta* is required for progression to the lytic replication cycle in the context of the viral genome.

A prior study focused on the results of exogenous expression of BoHV-4 ORF50/*Rta*, that lead the activation of BoHV-4 lytic replication in a non-permissive cell line by over-expression of ORF50/*Rta* (Donofrio et al., 2004a), this study was the first attempt to demonstrate the direct role of BoHV-4 ORF50/*Rta* for the progression of the virus into the lytic cycle.

BoHV-4 IE2 promoter was previously shown to be transcriptionally up-regulated in bovine endometrial stromal cells (Donofrio et al., 2007a; Donofrio et al., 2008b). To better understand the importance of such promoter in the context of viral replication on the endometrial cellular substrate we generate pBAC-BoHV-4-U, starting from a uterine virus isolated from a cow affected by post-partum metritis (Donofrio et al., 2009a).

In the present work, we generated several viral mutants targeting specific parts of the IE2 locus within the BoHV-4 genome, demonstrating that ORF50/*Rta* has a direct role in initiating and progressing BoHV-4 lytic replication.

Based on the hypothesis that IE2 gene is essential for initiation and progression of the BoHV-4 lytic replication, before to attempt the generation of BoHV-4 mutants un-expressing or lacking IE2 gene products, IE2 complemented cell lines were generated to rescue in *trans* the defect in IE2 gene. A cell line, BEK/IE2, was created with a 3442 bp EcoRI/PstI BoHV-4 genome fragment containing the full BoHV-4 IE2 gene (van Santen, 1993) and expressing its ORF50 splicing product; to force IE2 expression a 427 bp sequence containing the hCMV enhancer promoter but lacking the basic transcriptional apparatus (TATAbox and transcriptional start site) was dimerized in front of the BoHV-4 537 bp IE2 promoter, which

was shown to be the minimal promoter size giving the maximal signal in a Dual Luciferase reporter assay, and present at the 5' end of the 3442 bp EcoRI/PstI BoHV-4 genome fragment and subcloned into a vector backbone containing a *neo* resistant cassette for G418 drug selection (p2xCMVeIE2*neo*).

p2xCMVeIE2*neo* was much better expressed then the un-enhanced version, pIE2*neo* containing only the 3442 bp EcoRI/PstI BoHV-4 genome fragment when tested by transient transfection assay and RT-PCR.

Because the BoHV-4 IE2 gene contains the ORF49 within its intron, which is transcribed on the opposite direction of the ORF50, it is theoretically possible that BEK/IE2 cells express both ORF49 *and* ORF50 products. Therefore, to exclude the possibility that defect in the expression of other genes present into the IE2 gene locus, specifically the ORF 49, which could be responsible or contribute for a replicating defective phenotype, as shown for MHV-68 (Lee et al., 2007), a cell line (BEK/ORF50) expressing only the ORF50 was generated. The ORF 50 spliced transcript that encodes the only and complete Rta protein was amplified by PCR and subcloned into a bi-cistronic vector generating pCMV-ORF50-IRES-neo that was used to generate a stable cell line expressing only the ORF50/Rta. Both these two cell lines, BEK/IE2 and BEK/ORF50/Rta stably expressed ORF50 through the passages when tested by RT-PCR and moreover**,** a morphological change, characterized by an increased cell sprouting and shrinking, but completely compatible with cell survival and replication, was observed for BEK/IE2 and BEK/ORF50 cell lines; such morphological change was probably due to IE2 over-expression, as previously observed for MHV-68 ORF50 (Staudt and Dittmer, 2007), but it requires further investigated to be fully stated.

As a first step toward understanding the pivotal role of ORF50/*Rrta* expression in initiating BoHV-4 transcriptional/replication cycle, BoHV-4 IE2 gene promoter was interrupted by site specific insertional mutagenesis mediated by heat inducible homologous recombination into the genome of two strains of BoHV-4 cloned as bacterial artificial chromosome (BAC) (Warming et al., 2005).The first targeting construct, pIE2prKanaGalKIE2pr, containing the KanaGalK 2004 bp DNA staffer double selecting cassette (Donofrio et al., 2007b) flanked by two BoHV-4 IE2 gene homologous regions, was inserted between the putative IE2 transcriptional start site and the IE2 translational start site (ATG) positioned into the IE2 gene first exon (van Santen, 1993)

The functionality of the targeting construct, in terms of transcriptional blocking was initially tested by transient transfection assay and RT-PCR and no ORF50/*RTA* spliced transcript could be detected (data not shown). BAC recombineering of the targeting cassette in the BAC-BoHV-4-U and BAC-BoHV-4-LVR genome, get BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-LVR/IE2pr. Because the introduction of a 2004 bp staffer into the viral genome could impairs the viral replication due to the enlargement of the viral genome size, thus confusing the phenotypic effect induced by the inactivation of the IE2 promoter, two control parental mutant viruses, built by the insertion of the same 2004 bp staffer into the BAC-BoHV-4-U (BAC-BoHV-4-

UΔTK) (Donofrio et al., 2009a) and BAC-BoHV-4-LVR (BAC-BoHV-4-LVRΔTK) (Donofrio et al., 2007b) TK ORF, which was already shown to be a BoHV-4 not dispensable gene (Donofrio et al., 2002), were employed as a control in a viral reconstitution assay to test the competence of the recombinant viruses. After electroporation in BEK cells or BEK expressing *cre*, BAC-BoHV-4-LVR/IE2pr genome could not reconstitute IRVPs, as expected. Although an identical phenotype to BAC-BoHV-4-LVR/IE2pr was expected for BAC-BoHV-4-U/IE2pr, to the contrary, BAC-BoHV-4-U/IE2pr cold well reconstitute IRVPs, as well as control BAC-BoHV-4-UΔTK, BAC-BoHV-4-LVRΔTK, BAC-BoHV-4-U and BAC-BoHV-4-LVR DNA. Furthermore still unexpectedly, BoHV-4-U/IE2pr reconstituted progeny virus could still transcribe IE2 gene, rather BAC-BoHV-4-LVR/IE2pr could not and when BAC-BoHV-4-LVR/IE2pr DNA was electroporated into BEK/IE2 and BEK/ORF50 complemented cells, IRVPs were well reconstituted, linking BoHV-4-LVR viral reconstitution and replication with IE2 expression.

For some reasons, the IE2 promoter abrogation strategy gave a non replicating competent phenotype just for BAC-BoHV-4-LVR but not for BAC-BoHV-4-U. Such reasons could be differently explained: ì) merely for a technical problem or badly designed strategy and experiments, but those did not seem to be the case because the targeting construct as well as the targeted viral genomes were finely checked by PCR, sequencing, restriction enzyme analysis and southern hybridization and not mistakes were found. ìì) for strain variations in terms of IE2 product requirement, where IE2 expression is dispensable for BAC-BoHV-4-LVR strain but not for BAC-BoHV-4-U.

Taking into account the later hypothesis, a forward approach to the problem was employed. The IE2 second exon containing most of the IE2 ORF was 488 bp deleted both in BAC-BoHV-4-U and BAC-BoHV-4-LVR genome. As before, the so generated BAC-BoHV-4-UΔORF50 but not BAC-BoHV-4-LVRΔORF50, maintained the capability to reconstitute IRVPs; and also when BAC-BoHV-4-LVRΔORF50 DNA was electroporated into BEK/IE2 and BEK/ORF50 complemented cells, IRVPs were well reconstituted, as well as control BAC-BoHV-4-U, BAC-BoHV-4-LVR, BAC-BoHV-4-UΔTK, BAC-BoHV-4-LVRΔTK DNA. Even the, growth characteristics were identical to the LVR ones when growth curve was performed on BEK/IE2 cells. These growth kinetics data were completely in agreement with those of a MHV-68 mutant transcribing ORF50 at high level and showing more rapid lytic spread in permissive fibroblast cultures (May et al., 2004).

Although with a different strategy, the necessity of IE2 gene integrity was shown only for BoHV-4-LVR but not for BoHV-4-U. Because the full length 1659 bp ORF50 mRNA was still expressed in BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-UΔORF50 but not in BAC-BoHV-4-LVR/IE2pr and BAC-BoHV-4-LVRΔORF50; therefore, a possible duplication of IE2 gene in BoHV-4-U genome was hypothesized.

This hypothesis was confirmed by generating a probe spanning the deleted region within the IE2 exon 2, and, as expected, was absent into BoHV-4-LVR genome.

The deletion of the other gene copy unable the replication competence of the virus; infact the recombinant virus was able to growth only in the lines complemented in *trans* with Rta protein.

Although the full BoHV-4-U genome sequence would be necessary to finely allocate and define such duplication in the contest of the entire genome, this work represent the final proof on the essentiality of IE2 gene for BoHV-4, in agreement with what was found for other gamma herpesviruses like MuHV-68 (Pavlova et al., 2003), HHV-8 (Xu et al., 2005) and RRV (Zhou et al., 2010).

Appendix 1:

Primer name	Sequence
Full-IE2-sense-NheI	5’-gct agc tag cca cca tga agg gga tta ttt atc cc-3’
Full-IE2-anti-BamHI	5’-gcg gga tcc tta cac aaa caa atc gag ggg gtt-3’
NdeI-hCMV-sense	5’-ggt gga gta ttt acg gta aac tgc cca-3’
BglII-hCMV-anti	5’-gga aga tct gca gtt acc ctc aaa caa aac-3’
BglII-hCMV-sense	5’-gga aga tct cat agc cca tat atg gag ttc-3’
HindIII-hCMV-anti	5’-gca gtt acc ctc aaa caa aac aag ctt ggg-3’
TargI-sense	5’-atg tgc ctg aca aac ttt ttt gtg gga-3’
Anti I amplicon	5’-aat ttg ctg cag tgt gtt tgt ttc ttg tgg ctg-3’
Sense II amplicon	5’-ttg tat ggt acc cag acc agt cat ggt aag aat-3’
Antisense II amplicon	5’-acc cag aag ctt gat acc aat atg aat aca ggt-3’
Delta ExonII A	5’-gtt gtc cag aaa att gct tga ttg aaa aac-3’
Delta ExonII B	5’-gcc ggt acc ttt ctg tga gat gtt tca caa cag-3’
Delta ExonII C	5‘-caa act gca gtc tga acc aca gac cat tct caa ac-3’
Delta ExonII D	5’-ggc caa gct ttt agt cag ttg aaa ggt gca gag g-3’
IE2-A-sense	5’-aca aac aca cag acc agt ca-3’
IE2-B-antisense	5’-gtt tca caa cag att gag ca-3’
ORF50-del-sense	5’-tgt att gca ctg aca agt ttt tta taa act-3’
ORF50-del-antisense	5’-cag ttt ctc cag atc tgg tcc ttt gtt tgc-3’
EcoRI-X-sense	5’-ccc gaa ttc gaa tgg tgt att gca ctg aca agt ttt ta-3’
KpnI-X-anti	5’-ccc ggt acc ctt tat aaa tgc ctt aca ttt gtc atc aat-3’
PstI-Y-sense	5’-ccc ctg cag ctc acc ttt cct tac ctt aat ctt gag act-3’
HindIII-Y-anti	5’-gcc caa gct tta aca gtt tct cca gat ctg gtc ctt tgt t-3’

Table 2. List of primers used in this work.

Appendix 2:

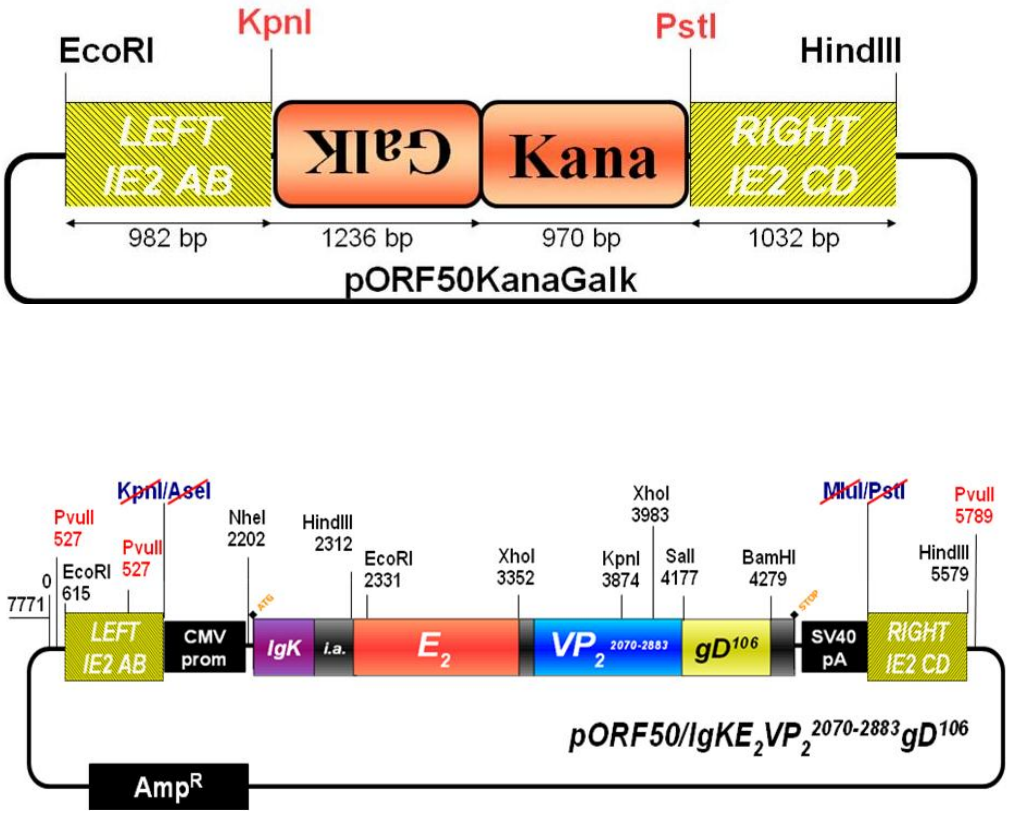

Fig.24. Schematic representation (not to scale) of two constructs used in this work.

Chapter 4

General Discussion and Conclusions

The present doctoral thesis covers researches lead to underline the relationship between BoHV-4 and the uterine microenvironment, involved in the generation of pathologies, such as post-partum metritis and others uterine diseases.

In the first study we characterized a uterine BoHV-4 isolate, thanks to RFLP and PCR analysis and we cloned this strain as a Bacterial Artificial Chromosome (BAC), to easily manipulate the viral genome; in the second study we demonstrated that this BAC, called BAC-U, can be further studied through the knock-out of viral specific genes. Infact we successfully interrupted the Immediate Early II gene (IE2) of BAC-U, demonstrating that this gene is the real master switch of the virus replication; moreover in this uterine strain the IE2 gene was surprisingly duplicated, suggesting that gene duplication may provide a selective gain for the virus to persist in the uterine microenvironment.

BoHV-4 is an herpesvirus belonging to the gammaherpesvirus family, genus *Rhadinovirus* (Zimmermann et al., 2001), as HHV-8, MuHV-68 and SaHV-2, that are extensively studied even because of their oncogenic potential; BoHV-4 as the same genome structure and gene co-linearity and homology with these viruses have been described (Zimmermann et al., 2001a), but its absence of oncogenicity and of a clear correlation of pathologies, has prompted the attention on BoHV-4, as a model for human viruses and for vaccinal purposes (Donofrio et al., 2006; Donofrio et al., 2009b).

Although BoHV-4 has been isolated from animals with various clinical symptoms and also from apparently healthy animals, BoHV-4 isolation from animals with disease of the female genital tract is the best documented and one of the few cases where disease could be reproduced (Wellemans et al., 1986); the pathogenic role of BoHV-4 remains otherwise still undear, and the correlation of the virus with pathologies is at the time unresolved, even by experimental infections.

A study by Wellemans *et al.*, (Wellemans et al., 1986) were able to reproduce metritis by infecting cows at various times before parturition; no clinical signs were seen after primary infection but metritis appeared after parturition. BoHV-4 has often been implicated in cases of bovine metritis; the first reported isolation of this virus from a case of bovine metritis was in 1973 (ParK and Kendrick, 1973). Later several other isolates were obtained from cows with reproductive disorders from different countries, including Italy (Castrucci et al., 1986), India (Mehrotra et al., 1986), in the USA (Frazier et al., 2001; Frazier et al., 2002), in Spain (Monge et al., 2006) and Serbia (Nikolin et al., 2007). It is the common thinking that BoHV-4 was not the primary cause of metritis, but can have a secondary pathogenic role, causing post-partum metritis along with other microbes such as the bacteria *E. coli* and *A. pyogenes* (Donofrio et al., 2008b), that are the most prevalent bacteria isolated from the uterus of cattle suffering from uterine diseases, followed by a range of anaerobic bacteria, such as *Prevotella* species, *Fusobacterium necrophorum* and *nucleatum* (Sheldon 2009).

The infections of the uterine microenvironment alter not only the function of the uterus, but also the ovary and the overarching higher control centres in the hypothalamus and pituitary glands (Sheldon et al., 2009). Sub-fertility and infertility follow often the inflammatory and immune response to uterine infection and compromise even animal welfare. Infertility is caused at the time during the course of the infection; and sub-fertility often after successful resolution of the disease.

Uterine diseases, such as metritis, can cause infertility that is strictly associated to financial losses; failure to conceive, reduced milk production and the costs for treatments increased the amount of losses (Sheldon 2009). The clarification of the mechanisms involving the effect of microbial infection and the associated immune response on bovine reproduction is necessary to develop new treatments and prevention strategies (Dobson et al., 2003).

Abortion may follow infection with a variety of alpha-, beta- and gammaherpesvirus, but viral causes of uterine disease are seldom investigated in cattle. Although postpartum metritis affects up to 40% of cattle, it has been assumed that the majority of diseases are of bacterial origin and virus isolation or serology is rarely considered (Sheldon et al., 2009).

BoHV-4 has a strictly tropism for endometrial cells, infact can infect purified population of bovine endometrial stromal and epithelial cells, leading to non-apoptotic cell death and *de novo* viral production that is also associated with an increased production of COX-2 and prostaglandin-endoperoxide synthase 2 (PTGS2) protein from stromal cells and with prostaglandin E_2 (PGE) secretion (Donofrio et al., 2007a; Donofrio et al., 2008b).

Post-entry events are responsible of the succesfull replication of BoHV-4 in bovine endometrial cells as demonstrated by the rapid viral reconstitution following the electroporation of nude viral DNA into endometrial stromal and epithelial cells (Donofrio et al., 2007a; Donofrio et al., 2008b); the tropism should not infact be determined only by the receptor has never been identified.

In a precedent study a construct containing the Immediate Early II gene (IE2) of BoHV-4, labelled with an EGFP, was used to transfect endometrial stromal and epithelial cells; in 24 hours, the EGFP robustly accumulated in the cytoplasm of both cells type (Donofrio et al., 2007a), suggesting that a mechanism underling the rapid activation of BoHV-4 replication in the bovine endometrium was associated with the capability of endometrial cells to transactivate the BoHV-4 IE2 gene promoter (Donofrio et al., 2008b), which is supposed to be the molecular master swich gene for BoHV-4 replication (van Santen, 1991).

BoHV-4 IE2 gene promoter transactivation and viral replication are moreover associated with extracellular stimuli belonging to the intrauterine microenvironment such as *E. coli* LPS and PGE (Donofrio et al., 2008b) and BoHV-4 replication is also reactivated in latently infected macrophages by a co-cultivation with stromal cells (Donofrio et al., 2007a; Donofrio et al., 2008b); but is also stimulated by the addiction of exogenous PGE (Donofrio et al., 2005).

All these data led to the description of a model that fits well BoHV-4 replication and uterine diseases, involving the secretion of PGE from bacteria causing endometritis, then PGE and LPS can stimulate viral replication or re-activation from the latency site, the macrophages, in the site of infection; with further endometrial tissue damage and inflammation (Sheldon et al., 2009).
Starting from this model, the evidence of the existence of a patho-biotype of BoHV-4 causing uterine disease can not be ignored, although BoHV-4 has been isolated from different lesions and from healthy animals.

Thus, in the first study here presented, BoHV-4 was isolated from a cow affected with non-responsive post-partum metritis, and after the characterization, its genome was cloned as bacterial artificial chromosome (BAC). The so generated BAC-BoHV-4-U clone could be a useful molecular tool to study the functional genomic of BoHV-4 genes adapted to the endometrium, which will be used to clarify the relationship between BoHV-4 and postpartum metritis.

In the first study a BoHV-4 strain (BoHV-4-U) was isolated from the uterus of a BoHV-4 persistently infected cow, with a history of postpartum metritis. This isolate was confirmed to be a BoHV-4 strain after restriction pattern analyses by RFLP-PCR and sequencing using a large 2,538 bp conserved region (TK locus) including the 3' end of ORF1 (homologous to the EBV BVRF1 gene), ORF2 (homologous to the EBV BXRF1 gene), ORF3 (TK gene) and ORF4 (gH gene) 5' end (Lomonte et al., 1996).

Also the IE2 gene was analyzed by RT-PCR and sequencing, specifically for its spliced form, that is a specific marker for BoHV-4 immediate early expression (van Santen, 1993), confirming that this uterine isolates belongs really to BoHV-4.

After the so described characterization, the BoHV-4-U genome was cloned as a bacterial artificial chromosome (BAC) and the stability of the pBAC-BoHV-4-U clone was assessed over twenty passages, and also the viral growth was not affected compared with the wild type virus.

The use of a specifical BoHV-4 uterine isolate is necessary to well understand and underline the effects of the virus on the uterine environment and functions. In pathology is otherwise a very important issue the virus strain adaptation to an organ, tissue or cell type for the knowledge and description of the disease. The fact that BoHV-4 has not clearly fully correlated with a pathology, even in case of post-partum metritis, suggests that maybe the virus can be adapted to uterine stimuli and micro-environmental selective pressures given by endometrial cells and also from external or chemical, physical conditions; that may increase the frequency of the most efficient genetic variants.

An important tool to study viral genome, even in attempt to characterize gene mutation or polymorphism, is the possibility to clone the genome of herpesvirus as bacterial artificial chromosome, and then to modify

the viral genome by homologous recombination , being than possible to reconstitute a viable virus in eukaryotic cells following transfection (Messerle et al., 1997).

The disruption of herpesvirus genome is usually achieved by mutagenesis through the integration of a marker gene into the viral genome through homologous recombination of co-transfected viral genomic DNA and a mutated allele on a separate DNA fragment in permissive cells; this technique could be applied to all type of viruses, especially viruses showing a slow replication kinetics and that are strictly cell associated, rendering the isolation of viral mutants very difficult. Recombinant viruses are either screened or selected with sequential rounds of plaque purification but, if the case that mutation results in growth defects relative to wild-type parental virus, the mutant virus may be difficult or impossible to purify. Lethal mutations are often detected only indirectly by the unsuccessful isolation of the desired mutant virus; these mutants can be successfully isolated and confirmed to be lethal only after the construction of a complementary cell line expressing the wild-type allele.

Conventional mutagenesis is usually followed by the phenotype analyses, both *in vitro* and *in vivo*; that is achieved through chemical mutagenesis, site-directed mutagenesis by homologous recombination in eukaryotic cells and manipulating virus genome using overlapping cosmid clones. All these methods are very useful to generate mutants, but often these procedure are inefficient, laborious and time consuming; even because they require to select the mutants from the parental virus, and this selection can be assessed only at the very end of the experimental procedure (Adler et al., 2003).

To overcome these limitations, many herpesviruses have been cloned as an infectious bacterial artificial chromosome (BAC) (Adler et al., 2003; Borst et al., 1999; Gillet et al., 2005a; Messerle et al., 1997; Zhou et al., 2010).

The genome of BoHV-4 has been sequenced completely (Palmeira et al., 2011; Zimmermann et al., 2001) but the function of most of the gene remains still unknown; the generation of a BAC even for BoHV-4, was firstly described by Gillet *et al.*, in 2005, targeting the homologous recombination of an engineered cassette expressing EGFP with the non-coding region at the junction of right prDNA and the unique central region ofBoHV-4 (Gillet et al., 2005a).

To date the analysis of BoHV-4 sequence has revealed the presence of at least 79 ORFs, 62 homologous to that of other rhadinovirus, 17 unique to BoHV-4, based on amino acids homology and genome co-location with the other gammaherpesviruses. Homology data with others rhadinoviruses and precedent work has led to the hypothesis that IE2 gene could be the molecular master switch protein for BoHV-4 replication. Endometrial cells can rapidly transactivate the IE2 promoter as previously stated by transient transfection assay using a reporter construct containing the IE2 promoter (Donofrio et al., 2008b). Therefore the mutational analysis of the IE2 gene in BoHV-4-U would be of great interest, to understand its functions in

the context of the interaction between uterine cells and a virus specifically adapted to the uterus, as a BoHV-4-U.

The analysis of the functionality of BoHV-4-U genes in endometrial cells would be of great interest to discover regulatory regions; transposition technology, site direct mutagenesis and homologous recombination on BAC-BoHV-4-U genome will provide an important tool to carry out a large-scale research and analysis of BoHV-4-U regulatory regions, analysing even the effect of factors belonging to the uterus microenvironment, like hormones, cytokines, prostaglandins and others.

The classification of mutants into functional clusters will contribute to identify uncharacterized genes possibly responsible for BoHV-4 strain adaptation to the uterine microenvironment. The so established BoHV-4-U mutant's library together with different endometrial cell types will cover a substantial amount of biological information of intrinsic value concerning the functional relationship of the targeted genes and the host background. The results obtained in the large-scale gene mutation analysis will be investigated as a whole for their functional relationship.

The uterine adapted BoHV-4-U genome cloned as bacterial artificial chromosome, represents indeed a very useful tool to understand the complex interactions existing between BoHV-4-U and uterine host cells and microenvironment, and to study the effect of viral infection on endometrial metabolic and endocrine function, and also on antigen-processing pathways.

After the cloning of BoHV-4-U as a Bacterial as a Bacterial artificial chromosome and the assessment of its potential to study BoHV-4 genome and the relationships between the uterine microenvironment and an adapted virus strain, we started to analyse single genes, in order to demonstrate their role in the emerging of uterine diseases.

The first gene we decided to submit to an extensive study was the Immediate Early II gene; previously we infact demonstrated the activation of BoHV-4 lytic replication in a non-permissive cell line by over-expression of ORF50/*Rta* (Donofrio et al., 2004a), beginning to demonstrate the direct role of BoHV-4 ORF50/*Rta* for the progression of the virus into the lytic cycle.

In the second study several viral mutants were generated targeting specific parts of the IE2 locus within the BoHV-4 genome, which provided further evidence that BoHV-4 ORF50/*Rta* is necessary to initiate and allow progression of BoHV-4 lytic replication.

Herpesvirus immediate early (IE) genes are expressed "immediately" during cell infection without the requirement of prior viral protein synthesis for their expression; host cell transcription factors, already present at the moment of infection, are able to activate them (Staudt and Dittmer, 2007).

The Open Reading Frame 50 (Orf50) gene product, also known as Rta, Replication and Transcription Activator, is an Immediate Early gene that is extremely conserved in functions but not always in sequence,

in all Rhadinoviruses (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 homolog in other gammaherpesviruses, including herpesvirus saimiri (SaHV-2), equine herpesvirus 2 (EHV-2), murine herpesvirus 68 (MuHV-68), rhesus rhadinovirus (RRV) and Kaposi's sarcoma-associated herpesvirus (HHV-8).

Studies on several Rhadinoviruses, closely related to BoHV-4, have demonstrated that the expression of ORF50/*Rta* is essential for viral replication; recombinant viruses with deletion or inactivation by a premature stop codon insertion within its open reading frame were generated and were all replication-defective; but were efficiently rescued, with respect to production of viable virus and DNA replication, upon the expression of ORF50/*Rta* in *trans* (Pavlova et al., 2003; Xu et al., 2005; Zhou et al., 2010).

Precedent 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 binding protein (Bermudez-Cruz et al., 1997; Bermudez-Cruz et al., 1998; van Santen, 1993; Zhang and van Santen, 1995) supporting the idea that BoHV-4 Rta expression could play a pivotal 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., 2004a; Donofrio et al., 2004b; van Santen, 1993).

A prior study focused on the results of exogenous expression of BoHV-4 ORF50/*Rta*, that lead the activation of BoHV-4 lytic replication in a non-permissive cell line by over-expression of ORF50/*Rta* (Donofrio et al., 2004a), this study was the first attempt to demonstrate the direct role of BoHV-4 ORF50/*Rta* for the progression of the virus into the lytic cycle.

BoHV-4 IE2 gene was already well characterized in terms of gene structure, transcription and RNA post transcriptional processing (van Santen, 1993), but it has not been clearly demonstrated that the expression of ORF50/*Rta* is required for progression to the lytic replication cycle in the context of the viral genome.

BoHV-4 IE2 promoter was also previously shown, as described above, to be transcriptionally up-regulated in bovine endometrial stromal cells (Donofrio et al., 2007a; Donofrio et al., 2008b). To better understand the importance of such promoter in the context of viral replication on the endometrial cellular substrate we generated pBAC-BoHV-4-U, starting from a uterine virus isolated from a cow affected by post-partum metritis (Donofrio 2009).

In the second work here presented, we generated several viral mutants targeting specific parts of the IE2 locus within the BoHV-4 genome, providing the proof of principle that ORF50/*Rta* has a direct role in initiating and progressing BoHV-4 lytic replication.

Firstly we generated IE2 complemented cell lines, to be able to rescue *in trans* the defect in IE2 genes in the mutants and reconstitute the virus, hypothizing that even in BoHV-4 the IE2 gene should be essential for initiation and progression of the viral lytic replication.

The cell line BEK/IE2, was created expressing the spliced form of the Orf50 gene product, Rta, contained in the 3442 bp EcoRI/PstI BoHV-4 genome fragment (van Santen, 1993); an expression cassette was engineered to force IE2 expression, inserting a 427 bp sequence containing the hCMV enhancer promoter but missing the basic transcriptional apparatus (TATAbox and transcriptional start site), that was dimerized in front of the BoHV-4 537 bp IE2 promoter, which was shown to be the minimal promoter size giving the maximal signal in a Dual Luciferase reporter assay, and was present at the 5' end of the fragment; after the subcloning into a vector backbone containing a *neo* resistant cassette for G418 drug selection p2xCMVeIE2*neo* vector was generated. A transient transfection assay and RT-PCR demonstrated that p2xCMVeIE2*neo* was much better expressed then the un-enhanced version, pIE2*neo* containing only the 3442 bp EcoRI/PstI BoHV-4 genome fragment.

BoHV-4 IE2 gene contains also the ORF49 within its intron, that is transcribed in the opposite direction respect to ORF50 gene, and was described to cooperate with Rta protein in MuHV-68, facilitating the auto-activation of Rta and cooperating with Rta in regulating virus replication by downstream actions (Lee et al., 2007).

BEK/IE2 cells express the full length Orf50 gene, and it's in theory possible that both ORF49 *and* ORF50 are expressed. Thus, to exclude the possibility that defect in the expression of ORF 49, present into the IE2 gene locus, could be responsible or contribute to a replicating defective phenotype, as shown for MHV-68 (Lee et al., 2007), a new cell line (BEK/ORF50) expressing only the ORF50 ORF was generated.

The ORF50 spliced transcript that encodes the complete Rta protein was amplified by PCR and subcloned into a bi-cistronic vector generating pCMV-ORF50-IRES-neo, used then to generate a stable cell line expressing only the ORF50/Rta protein. Both these two cell lines, BEK/IE2 and BEK/ORF50/Rta stably expressed ORF50 when tested by RT-PCR; moreover a morphological change, characterized by an increased cell sprouting and shrinking, but completely compatible with cell survival and replication, appeared in BEK/IE2 and BEK/ORF50 cell lines; such morphological change was probably due to IE2 over-expression, as previously observed for MHV-68 ORF50 (Staudt and Dittmer, 2007), but it requires to be further investigated to be understood.

As a first step in understanding the role of ORF50/*Rta* expression in promoting BoHV-4 transcriptional/replication cyde, BoHV-4 IE2 gene promoter was interrupted by site specific insertional mutagenesis mediated by homologous recombination into the genome of V.test BoHV-4 and BoHV-4-U cloned as bacterial artificial chromosome (BAC) (Warming et al., 2005).

The first targeting construct, pIE2prKanaGalKIE2pr, contained the KanaGalK 2004 bp DNA staffer double selecting cassette (Donofrio et al., 2007b) flanked by two BoHV-4 IE2 gene homologous regions, and was inserted between the IE2 putative transcriptional start site and the ATG, the translational start site positioned into the IE2 gene first exon (van Santen, 1993)

This targeting construct was tested by transient transfection assay and RT-PCR and no ORF50/*RTA* spliced transcript could be detected (data not shown).

BAC recombineering of the targeting cassette in the BAC-BoHV-4-U and BAC-BoHV-4-V.test genome, get BAC-BoHV-4-U/IE2pr and BAC-BoHV-4-V.test/IE2pr.

Also two control parental mutant viruses were generated through the insertion of the same DNA staffer into the Timidine kinase ORF of BAC-BoHV-4-U (BAC-BoHV-4-UΔTK) (Donofrio et al., 2009a) and BAC-BoHV-4-V.test (BAC-BoHV-4-V.testΔTK) (Donofrio et al., 2007b), to verify if the introduction of the DNA staffer could be detrimental for virus replication, due to the enlargement of the viral genome size.

After electroporation in BEK cells or BEK expressing *cre* of BAC-BoHV-4-V.test/IE2pr DNA, the virus could not be reconstituted, as expected.

To the contrary, BAC-BoHV-4-U/IE2pr could well reconstitute a virus progeny, as well as the control BAC-BoHV-4-UΔTK, BAC-BoHV-4-V.testΔTK, BAC-BoHV-4-U and BAC-BoHV-4-V.test.

Surprisingly, even the BoHV-4-U/IE2pr reconstituted progeny virus could still transcribe IE2 gene, rather BAC-BoHV-4-V.test/IE2pr could not; moreover when BAC-BoHV-4-V.test/IE2pr DNA, that was unable to reconstitute viable virus, was electroporated into BEK/IE2 and BEK/ORF50 complemented cells, viable viruses were well reconstituted, connecting BoHV-4-V.test viral reconstitution and replication with IE2 expression. This is the first proof of principle that the IE2 gene is the master gene switch, involved in activating virus replication, in gammaherpesviruses and in BoHV-4, too.

We had then to explain why the IE2 promoter inactivation strategy gave a non replicating competent phenotype for BAC-BoHV-4-V.test but not for BAC-BoHV-4-U.

We hypothesed several reason to explain this unexpected result: firstly we thought about a technical problem or a badly designed strategy and experiments, but all the targeting construct as well as the targeted viral genomes were attentively checked by PCR, sequencing, restriction enzyme analysis and

southern hybridization and no mistakes were found; secondly we supposed that strain variations in terms of IE2 product requirement, could be possible, rendering Rta protein expression indispensable for BAC-BoHV-4-V.test strain but not for BAC-BoHV-4-U.

Taking into account the later hypothesis, another approach to solve the problem was employed, deciding to interrupt only the IE2 second exon that contains most of the IE2 ORF, indeed not touching regulatory region and the first exon, as well the intron region, coding for Orf49.

488 bp were then deleted both in BAC-BoHV-4-U and in BAC-BoHV-4-V.test genomes; after transfection in permissive cells, the so generated BAC-BoHV-4-UΔORF50 but not BAC-BoHV-4-V.testΔORF50, still was able to reconstitute viable viruses; and also the electroporation of BAC-BoHV-4-V.testΔORF50 DNA into BEK/IE2 and BEK/ORF50 complemented cells rescued well the viral phenotype, as well as the control BAC-BoHV-4-U, BAC-BoHV-4-V.test, BAC-BoHV-4-UΔTK and BAC-BoHV-4-V.testΔTK DNA.

Comparing the growth characteristics we found that identical growth kinetics were defined for V.tests ones when growth curve was performed on BEK/IE2 cells These growth kinetics data resembled those of a MuHV-68 mutant transcribing ORF50 at high level, that showed more quick lytic spread in permissive fibroblast cultures (May et al., 2004). Even in this case, the necessity of IE2 gene integrity was shown only for BoHV-4-V.test but not for BoHV-4-U.

We also demonstrated that the full length 1659 bp Orf50 mRNA was still expressed in BAC-BoHV-4-U mutants but not in V.tests ones (through RT-PCR analysis); these data begin to support that IE2 gene could be duplicated in BoHV-4-U genome but not in BoHV-4-V.test.

This hypothesis was confirmed through the generation of a probe spanning the 488bp deleted region within the IE2 exon 2, and, as expected, the probe revealed a signal only in the BAC-BoHV-4-U mutants, but was absent into BoHV-4-V.test genome.

We disrupted also the additional gene copy of IE2 gene, present in BAC-BoHV-4-U, through the generation of another targeting cassette to exchange the DNA staffer present in the first IE2 locus, with a new generated cassette, leading the same homologies region; and then we targeted the second gene copy with a different targeting cassette, that lead as homology regions, the 488bp deleted during the first targeting.

This BoHV-4-U-IE2 double mutant was totally unable to growth, an efficient rescue of the virus was achieved only after the transfection of the mutant into the cell lines complemented in *trans* with Rta protein.

Although the full BoHV-4-U genome sequence would be necessary to finely allocate and define such duplication in the contest of the whole genome, this thesis presented the final proof on the essentiality of

IE2 gene for BoHV-4 replication, in agreement with what was found for other gamma herpesviruses like MuHV-68 (Pavlova et al., 2003), HHV-8 (Xu et al., 2005) and RRV (Zhou et al., 2010).

Moreover, the generation of a viral vector, totally unable to growth that requires the ectopic expression of Rta protein to be rescued, opened the way to the generation of replication conditional viruses that could be important tool to be used as viral vaccine or as oncolityc viruses.

Such a vector could combine the potential of BoHV-4 as a vector, in terms of absence of oncogenicity, no clear correlation with any pathologies, and the possibility to delete up to the 30% of the genome, and to manipulate it as a BAC; with the ability to be activated and to replicate only in a defined area, organ or tissues, perhaps in a cell-specific manner, through the ectopic expression of Rta protein, guided by an inducible promoter or a cell-specific one.

Chapter 5

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Appendix: List of papers published during the 3 years of the PhD course in "Biochemistry and Molecular Biology" (2009-2011)

1. p21(Waf1/Cip1) as a molecular sensor for BoHV-4 replication. Capocefalo A, Franceschi V, Whitelaw CB, Vasey DB, Lillico SG, Cavirani S, Donofrio G. J Virol Methods. 2009 Nov;161(2):308-11.
2. Isolation and characterization of bovine herpesvirus 4 (BoHV-4) from a cow affected by post partum metritis and cloning of the genome as a bacterial artificial chromosome. Donofrio G, Franceschi V, Capocefalo A, Cavirani S, Sheldon IM. Reprod Biol Endocrinol. 2009 Aug 19;7:83.
3. Cellular targeting of engineered heterologous antigens is a determinant factor for bovine herpesvirus 4-based vaccine vector development. Donofrio G, Franceschi V, Capocefalo A, Taddei S, Sartori C, Bonomini S, Cavirani S, Cabassi CS, Flammini CF. Clin Vaccine Immunol. 2009 Nov;16(11):1675-86.
4. Expression and secretion of Bluetongue virus serotype 8 (BTV-8) VP2 outer capsid protein by mammalian cells. Capocefalo A, Franceschi V, Mertens PP, Castillo-Olivares J, Cavirani S, Di Lonardo E, Leni Z, Donofrio G. J Virol Methods. 2010 Nov;169(2):420-4.
5. The chemokine IL8 is up-regulated in bovine endometrial stromal cells by the BoHV-4 IE2 gene product, ORF50/Rta: a step ahead toward a mechanism for BoHV-4 induced endometritis. Donofrio G, Capocefalo A, Franceschi V, Price S, Cavirani S, Sheldon IM. Biol Reprod. 2010 Dec;83(6):919-28..
6. Integration of bovine herpesvirus 4 genome into cultured persistently infected host cell genome. Donofrio G, Capocefalo A, Franceschi V, De Lorenzi L, van Santen V, Parma P. Virol J. 2010 Sep 21;7:246.
7. Virally and physically transgenized equine adipose-derived stromal cells as a cargo for paracrine secreted factors. Donofrio G, Capocefalo A, Franceschi V, Morini G, Del Bue M, Conti V, Cavirani S, Grolli S. BMC Cell Biol. 2010 Sep 23;11:73.
8. Bovine herpesvirus 4 immediate early 2 (Rta) gene is an essential gene and is duplicated in bovine herpesvirus 4 isolate U. Franceschi V, Capocefalo A, Ravanetti L, Vanderplasschen A, Gillet L, Cavirani S, van Santen VL, Donofrio G. Vet Microbiol. 2011 Mar 24;148(2-4):219-31.
9. Swine adipose stromal cells loaded with recombinant bovine herpesvirus 4 virions expressing a foreign antigen induce potent humoral immune responses in pigs. Donofrio G, Taddei S, Franceschi V, Capocefalo A, Cavirani S, Martinelli N, Ottonello S, Ferrari M. Vaccine. 2011 Jan 29;29(5):867-72.
10. Cytokine expression, glucocorticoid and growth hormone changes after porcine reproductive and respiratory syndrome virus (PRRSV-1) infection in vaccinated and unvaccinated naturally exposed pigs. Borghetti P, Saleri R, Ferrari L, Morganti M, De Angelis E, Franceschi V, Bottarelli E, Martelli P. Comp Immunol Microbiol Infect Dis. 2011 Mar;34(2):143-55.
11. HOXB7 expression by myeloma cells regulates their pro-angiogenic properties in multiple myeloma patients. Storti P, Donofrio G, Colla S, Airoldi I, Bolzoni M, Agnelli L, Abeltino M, Todoerti K, Lazzaretti M, Mancini C, Ribatti D, Bonomini S, Franceschi V, Pistoia V, Lisignoli G, Pedrazzini A, Cavicchi O, Neri A, Rizzoli V, Giuliani N. Leukemia. 2011 Mar;25(3):527-37.

12. Immunization of knock-out α/β interferon receptor mice against lethal bluetongue infection with a BoHV-4-based vector expressing BTV-8 VP2 antigen. Franceschi V, Capocefalo A, Calvo-Pinilla E, Redaelli M, Mucignat-Caretta C, Mertens P, Ortego J, Donofrio G. Vaccine. 2011 Apr 5;29(16):3074-82.
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And thanks to you, my love

Thank you so much

To myself I wish to

Stay hungry, stay foolish