Caractérisation des gènes UL12, UL25 et UL28 du virus de l'herpès bovin 1 et fonction du gène UL28 dans la réplication virale.

Par

Nathalie Desloges

Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph. D.) en virologie et immunologie

Jury d'évaluation

Président du jury et examinateur interne	Peter Tijssen INRS-Institut Armand-Frappier
Examinateur externe	Sylvia Vidal Université d'Ottawa, Ottawa
Examinateur externe	Louis Flamand Centre Hospitalier Universitaire Laval (C.H.U.L.), Québec
Directrice de recherche	Claire Simard INRS-Institut Armand-Frappier

INRS-Institut Armand-Frappier
Université du Québec
Mai 2002

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABRÉVIATIONS

ADCC	lyse des cellules dépendante des anticorps "antibody-dependent cell-mediated cytotoxicity"
ADN	acide désoxyribonucléique
ARN	acide ribonucléique
ARNm	ARN messager
ATP	adénosine 5'-triphosphate
β-gal	β-galactosidase
BHV1	virus de l'herpès bovin 1
BRSV	virus respiratoire syncytial bovin
BSA	albumine sérique bovine
Btif	facteur de trans-induction des gènes alpha "bovine alpha gene trans-inducing factor"
BVDV	virus de la diarrhée virale bovine
Cdk2	kinase 2 dépendante des cyclines "cyclin-dependent kinase 2"
CMH	complexe majeur d'histocompatibilité
DAB	3,3'-diaminobenzidine tétrahydrochloride
dCTP	désoxycytosine 5'-triphosphate
DIG	Digoxigenin
DMEM	milieu Eagle modifié par Dulbecco "Dulbecco's modified Eagle medium"
DMSO	diméthyl sulfoxide
E. coli	*Escherichia coli*
EHV1	virus de l'herpès équin 1
ELISA	"enzyme-linked immunosorbent assay"
EtBr	bromure d'éthidium
FBS	sérum bovin fœtal
HRP	péroxydase de raifort "horseradish peroxidase"
HSV1	virus de l'herpès simplex 1
HSV2	virus de l'herpès simplex 2
IBR	rhinotrachéite infectieuse bovine

ICTV	Comité International sur la Taxonomie des Virus
IFN	interféron
IL	interleukine
IPTG	"isopropyl beta-D-thiogalactopyranoside"
IPV	exanthème coïtal "infectious pustular vulvovaginitis"
IR	région répétée interne
kb	kilobases
kDa	kiloDalton
kpb (kbp)	kilopaires de base
LiDS	dodécylsulfate de lithium
MAb	anticorps monoclonal
MDBK	cellules de rein de bœuf "Madin Darby bovine kidney cells"
m.o.i.	multiplicité d'infection
MW	poids moléculaire
NK	cellules tueuses naturelles
ORF	cadre de lecture ouvert "open reading frame"
PAA	acide phosphonoacétique
pac	signal d'encapsidation
pb (bp)	paires de bases
PBS	solution saline tamponnée au phosphate
PCR	réaction de polymérisation en chaîne "Polymerase Chain Reaction"
PEG	polyéthylène-glycol
p.i.	post-infection
PI-3V	virus parainfluenza-3
PRV	virus de la pseudorage porcine
PVDF	polyfluorure de vinylidène
RK13	cellules de rein de lapin "Rabbit kidney cells"
SDS	dodécylsulfate de sodium
SDS-PAGE	gel d'électrophorèse en gel de polyacrylamide-SDS
SIV	virus de l'immunodéficience simienne
TBS	solution saline tamponnée au Tris

TR	région répétée terminale
tTA	transactivateur contrôlé par la tétracycline "tetracycline-controlled transactivator"
UL	segment unique long
US	segment unique court
Vhs	arrêt de synthèse des protéines de l'hôte causé par le virion "virion host shut-off"
VZV	virus de la varicelle-zona
WT	type sauvage "wild-type"
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

SOMMAIRE

Le virus de l'herpès bovin 1 (BHV1) est responsable de maladies respiratoires et génitales sévères du bétail, ce qui occasionne des pertes économiques considérables pour les éleveurs. Le génome viral est à présent complètement caractérisé, ce qui a permis la localisation des différents cadres de lecture ouverts (ORF; "open reading frame") par analogie avec les autres virus alphaherpès. Mon projet de recherche avait comme objectif d'étudier la fonction des ORFs UL12, UL25 et UL28 dans la réplication du BHV1 sachant que leurs homologues chez le virus de l'herpès simplex 1 (HSV1) sont impliqués dans le processus de clivage et d'encapsidation de l'ADN viral (Goldstein et Weller, 1998a; McNab *et al.*, 1998; Tengelsen *et al.*, 1993). Tout d'abord, dans le but de démontrer hors de tout doute que les ORFs ciblés étaient bel et bien fonctionnels chez le BHV1, j'en ai identifié les transcrits, les sites d'initiation de la transcription et les produits de traduction. L'identification des protéines virales potentielles requérant le développement d'antisérums spécifiques, j'ai procédé à l'expression d'une partie des séquences codantes chez *E. coli* pour ensuite immuniser des souris avec les protéines recombinantes purifiées. Les résultats obtenus démontrent clairement que les ORFs ciblés représentent bel et bien des gènes.

Par la suite, les études fonctionnelles des gènes UL25 et UL28 ont été entreprises. Étant donné qu'une demande de brevet concernant les résultats obtenus pour le gène UL25 est en préparation et afin de ne pas nuire à la prise de ce brevet, seuls les résultats concernant le gène UL28 sont présentés dans cette thèse.

Afin d'étudier son rôle, le gène UL28 du BHV1 a été éliminé du génome viral permettant ainsi la création d'un mutant de délétion spécifique. Une lignée cellulaire permissive au BHV1 sauvage (lignée RK13; "rabbit kidney cells") a été génétiquement modifiée pour lui faire exprimer le produit du gène UL28 du virus (lignée RK13/UL28^{+}). Le développement de cette lignée de complémentation avait pour but d'obtenir une lignée cellulaire qui soit capable de supporter la croissance du mutant de délétion viral désiré (BHV1ΔUL28) car, s'il s'avérait que ce gène soit absolument requis à la réplication du virus dans les cellules infectées, sa délétion du génome viral aurait été létale. Suite à l'obtention de la lignée, j'ai

pu entreprendre la construction proprement dite du mutant de délétion désiré en transfectant la lignée avec l'ADN viral sauvage purifié et un plasmide recombinant dont le gène UL28, bordé des séquences proximales intactes, avait été préalablement remplacé par une cassette d'expression de la β-galactosidase (β-gal) de *E. coli*. Les virus régénérés suite à une recombinaison homologue entre les deux molécules d'ADN, présentaient un phénotype β-gal$^+$ ce qui a facilité leur isolation. Le mutant de délétion UL28 est incapable de se reproduire sur la lignée cellulaire RK13 qui ne complémente pas, signifiant que ce gène est essentiel à la réplication du BHV1. J'ai voulu déterminer à quelle étape le cycle réplicatif avorte pour ce mutant. La détection spécifique de la protéine tardive Btif encodée par le gène UL48 a démontré qu'une mutation du gène UL28 ne compromet pas l'expression des gènes viraux de la classe tardive et que par conséquent, l'expression des gènes des classes précédentes ne serait pas compromise non plus. Des analyses de type Southern ont démontré que la délétion de ce gène ne compromet pas la synthèse d'ADN viral. Une déficience du gène UL28 n'empêcherait également pas une encapsidation précoce d'ADN viral. Cet ADN serait possiblement synthétisé au début de la réplication par un mécanisme non-classique du cercle roulant où des génomes linéaires seraient détachés dès la fin de leur synthèse et encapsidés. Cependant, lors de la réplication par le mécanisme du cercle roulant classique générant de longs concatémères d'ADN, la présence d'UL28 serait requise pour le clivage de ces molécules d'ADN.

En plus d'accroître les connaissances sur les processus de réplication, de clivage et d'encapsidation de l'ADN du BHV1, ce projet a le potentiel extrêmement prometteur de permettre le développement d'une toute nouvelle catégorie de vaccins qui auraient l'avantage de combiner la sécurité (intransmissibilité, innocuité) associée aux vaccins de type inactivés à l'efficacité des vaccins de type atténués.

INTRODUCTION

Le virus de l'herpès bovin 1 (BHV1) est un membre de la sous-famille des *alphaherpesvirinae* de la famille des *Herpesviridae*. Le BHV1 est un pathogène important des bovins associé à la rhinotrachéite infectieuse bovine (IBR) et la vulvovaginite pustulaire infectieuse (IPV, balanoposthite chez les taureaux). Le BHV1 peut également causer des conjonctivites, des avortements et possiblement des encéphalites. Le BHV1 serait également le pathogène viral majeur impliqué dans la maladie respiratoire bovine, connue aussi sous le nom de fièvre des transports, qui occasionne des pertes économiques importantes aux éleveurs de bovins.

Une grande variété de vaccins atténués et inactivés ont été développés afin de lutter contre le BHV1. Ces vaccins préviennent les signes cliniques sévères associés à l'infection et ainsi, diminuent les pertes économiques. Cependant, malgré la disponibilité de ces vaccins conventionnels, la maladie est toujours en progression dans les cheptels. La recherche pour le développement de vaccins anti-BHV1 de nouvelle génération est donc pertinente. L'une des avenues les plus prometteuses consisterait à inactiver des gènes viraux dont les produits sont essentiels à la réplication du BHV1. De tels mutants rendus inaptes à se répliquer présenteraient l'avantage d'être sécuritaires et efficaces à induire une réponse immunitaire chez les animaux.

Pour créer un mutant inapte à se répliquer, un gène absolument requis à la réplication du virus doit être supprimé du génome viral afin de provoquer l'avortement prématuré du cycle réplicatif. Il est donc essentiel de connaître au préalable la fonction des gènes viraux dans le processus de réplication virale. Mon projet de recherche visait à étudier la fonction des gènes UL12, UL25 et UL28 dans la réplication du BHV1 et cela, en les supprimant individuellement du génome viral afin de développer des mutants de délétion spécifiques. Puisque les différents ORFs du BHV1 ont été localisés sur le génome par comparaison de séquences avec les autres virus herpès, il fallait, pour commencer, démontrer que les ORFs ciblés représentaient bel et bien des gènes. Pour ce faire, j'ai identifié les transcrits, les sites d'initiation de la transcription et les produits de traduction démontrant ainsi hors de tout doute que les gènes ciblés sont fonctionnels. Par la suite, les études fonctionnelles des gènes UL25 et UL28 ont été entreprises. Seuls les résultats concernant le gène UL28 sont

présentés dans cette thèse qui se veut publique puisqu'une demande de brevet concernant l'étude du gène UL25 est en préparation.

Le mutant de délétion UL28 est incapable de se répliquer dans la lignée cellulaire RK13 qui ne complémente pas pour le produit du gène UL28 et par conséquent, j'ai pu établir que ce gène est essentiel à la réplication du BHV1. Par la suite, afin de déterminer à quelle étape le cycle réplicatif avorte pour le mutant généré, j'ai analysé si celui-ci a toujours la capacité de synthétiser les protéines virales et de répliquer l'ADN viral. Cela s'est avéré être le cas et j'ai analysé si l'ADN répliqué est clivé et/ou encapsidé à l'aide d'analyses de type Southern et par microscopie électronique.

Les résultats obtenus sont présentés sous forme d'articles scientifiques (Section II - Publications). La section I est une revue bibliographique de la littérature se rapportant au BHV1 et à ses caractéristiques ainsi qu'aux différents types de vaccins permettant de lutter contre cette infection. La section III est une discussion analysant l'ensemble des résultats obtenus et présentant les perspectives qui se rattachent à ce projet.

SECTION I

REVUE BIBLIOGRAPHIQUE

1. GÉNÉRALITÉS SUR LE VIRUS DE L'HERPÈS BOVIN 1

1.1 Historique

Le virus de l'herpès bovin 1 (BHV1) est l'agent étiologique responsable de deux syndromes cliniques majeurs du bétail, à savoir la rhinotrachéite infectieuse bovine (IBR) et la vulvovaginite pustulaire infectieuse (IPV, balanoposthite chez les taureaux). En 1841, Rychner, un vétérinaire suisse, a été l'un des premiers auteurs à décrire les symptômes cliniques de l'IPV et sa nature de maladie vénérienne. L'IPV a été par la suite nommée « Bläschenausschlag », un terme qui a été latinisé pour « exanthema vesiculosum/pustulosum coïtale ». Déjà en 1928, Reisinger et Reimann ont réussi à démontrer la nature virale et la transmissibilité de la maladie par leurs expériences de filtration. Le virus aurait été isolé par des groupes de recherche canadiens et américains dans les années cinquante (Wyler *et al.*, 1989).

Selon McKercher, l'IBR aurait été observé pour la première fois en 1950 dans certains troupeaux du Colorado aux États-Unis. En 1953, la maladie est retrouvée à grande échelle en Californie d'où elle sera disséminée dans les autres états américains et aux autres pays. En 1956, Madin et ses collègues ont réussi à isoler le virus de l'IBR (Madin *et al.*, 1956). Le premier cas d'IBR en Europe a été signalé en Allemagne en 1960 et quelques années plus tard dans les autres pays européens. De nos jours, l'IBR est une maladie ayant une distribution géographique mondiale et se retrouve sur tous les continents (Wyler *et al.*, 1989).

1.2 Taxonomie

Le BHV1 a été classé dans la famille des *Herpesviridae* selon ses caractéristiques morphologiques et ses propriétés physico-chimiques (Armstrong *et al.*, 1961). Le Groupe d'Étude des virus Herpès (Herpesvirus Study Group) du Comité International sur la Taxonomie des Virus (ICTV; "International Committee on the Taxonomy of Viruses") a divisé les membres de cette famille en trois sous-familles selon leurs propriétés

biologiques: *Alphaherpesvirinae*, *Betaherpesvirinae* et *Gammaherpesvirinae* (Roizman *et al.*, 1981). La caractérisation du spectre d'hôte, la durée du cycle de réplication, l'effet cytopathique *in vitro* et les particularités dans l'établissement de la latence ont conduit à la classification du BHV1 dans la sous-famille des *Alphaherpesvirinae* (Roizman *et al.*, 1981). À cause de l'arrangement de son génome, le BHV1 a été classé dans le genre *Varicellovirus* (Brown, 1989), tout comme les virus de la varicelle-zona (VZV; "Varicella-zoster virus") et de la pseudorage porcine (PRV; "Pseudorabies virus") (Roizman, 1996).

La sous-famille des *Alphaherpesvirinae* inclut également le genre *Simplexvirus* lequel comprend les virus de l'herpès simplex 1 et 2 (HSV1 et HSV2) dont l'hôte est l'humain (Roizman et Sears, 1996). Le HSV1 est le prototype de la sous-famille des *Alphaherpesvirinae*. Il a été particulièrement étudié et les connaissances acquises servent souvent de référence pour les autres virus alphaherpès.

1.3 Morphologie et génome

Similairement aux autres virus herpès, le BHV1 possède une nucléocapside icosaédrale de 95-110 nm de diamètre, constituée de 162 capsomères (150 hexons et 12 pentons). Les pentons sont localisés aux douze sommets alors que les hexons occupent les arêtes et les faces de l'icosaèdre. La nucléocapside est entourée par une zone dense aux électrons appelée le tégument et d'une enveloppe bilipidique, formant ainsi des virions de 150-200 nm de diamètre. Sur la surface de l'enveloppe, plusieurs spicules formés par les glycoprotéines virales sont présents (Armstrong *et al.*, 1961; Valicek et Smith, 1976). Une cellule infectée par le BHV1 présente trois types différents de capsides nommés A (vide), B (intermédiaire) et C (pleine). Les capsides C contiennent l'ADN viral et présentent un noyau central foncé lorsque colorées et observées en microscopie électronique, les capsides B contiennent une structure interne constituée d'un échafaudage protéique alors que les capsides A sont dépourvues d'ADN et de structures internes (Perdue *et al.*, 1976; Homa et Brown, 1997).

Le génome du BHV1 est contenu dans la nucléocapside principalement sous la forme d'une molécule d'ADN linéaire double brins de 135,301 paires de bases (GenBank NC_001847). Néanmoins, une faible proportion des génomes existent sous une forme circulaire dans les capsides (C. Simard, S. Ananvoranich, V. Misra et M. Trudel, Résumé au « 15th Annual meeting of the American Society for Virology », Résumé P9-8, 1996), tout comme cela a été rapporté chez deux autres virus alphaherpès, le HSV1 et le VZV (Poffenberger et Roizman, 1985; Kinchington *et al.*, 1985). Le génome est constitué d'un segment unique long (UL) et d'un segment unique court (US), ce dernier étant flanqué de régions répétées inversées (Farley *et al.*, 1981). Ces dernières permettent au segment US de s'inverser par rapport au segment UL ce qui résulte en l'existence de deux formes isomériques du génome (Mayfield *et al.*, 1983; Wyler *et al.*, 1989). Le génome du BHV1 possède deux origines de réplication de séquences identiques mais inversées qui sont localisées dans les régions répétées. Tout comme les autres virus alphaherpès, le génome du BHV1 coderait pour approximativement 70 protéines.

1.4 Glycoprotéines de surface

Le génome du BHV1 possède 10 gènes qui codent pour des glycoprotéines transmembranaires. Par convention, les glycoprotéines ont été nommées gB, gC, gD, gE, gG, gH, gI, gK, gL et gM, selon la nomenclature existante chez le HSV1 (Schwyzer et Ackermann, 1996; Baranowski *et al.*, 1996; Wu *et al.*, 1998). Les glycoprotéines virales, étant impliquées dans l'attachement du virus au récepteur cellulaire et dans la pénétration de la capside dans la cellule-hôte, sont responsables du tropisme viral. Elles sont également impliquées dans l'enveloppement final des capsides, de leur sortie de la cellule-hôte ainsi que de la transmission de l'infection aux cellules adjacentes par les jonctions cellulaires (Baranowski *et al.*, 1996).

Les glycoprotéines gB, gC et gD, anciennement désignées respectivement gI, gIII et gIV, sont responsables de l'attachement et de la pénétration du virion dans la cellule-hôte. La gC, par sa capacité à lier l'héparan sulfate, est considérée comme la protéine majeure de l'attachement du virion à la cellule (Okazaki *et al.*, 1991; Liang *et al.*, 1993). Les

glycoprotéines gB et gD sont aussi impliquées dans l'attachement mais également dans la fusion des membranes cellulaire et virale et dans la pénétration de la capside dans le cytoplasme de la cellule infectée (Liang *et al.*, 1991; Fitzpatrick *et al.*, 1990; Li *et al.*, 1997; Tikoo *et al.*, 1990). La création de mutants a démontré que les glycoprotéines gB et gD sont essentielles à la réplication du BHV1 (Miethke *et al.*, 1995; Fehler *et al.*, 1992). La gC n'est pas essentielle à la réplication du virus *in vitro* (Liang *et al.*, 1991) et le mutant n'exprimant pas la gC est également capable de se répliquer *in vivo* mais sa virulence est réduite par comparaison avec le BHV1 sauvage (Liang *et al.*, 1992). Les glycoprotéines gB, gC et gD sont capables d'induire individuellement une forte réponse d'anticorps neutralisants (Babiuk *et al.*, 1987). La gB est reconnue par les lymphocytes T CD4+ alors que la gC et la gD sont autant reconnues par les lymphocytes T CD4+ que les CD8+ (Hutchings *et al.*, 1990).

La glycoprotéine gE forme un complexe avec la glycoprotéine gI. Ce complexe protéique est impliqué dans la transmission du BHV1 aux cellules adjacentes par les jonctions cellulaires. Les glycoprotéines gE et gI ne sont pas essentielles à la réplication du BHV1 mais la délétion de l'un ou l'autre gène entraîne une diminution de la virulence *in vivo* (van Engelenburg *et al.*, 1994; Whitbeck *et al.*, 1996).

La glycoprotéine gG n'est pas essentielle à la réplication virale mais cette protéine est associée à la virulence. La contribution de la gG dans la transmission du virus de cellule à cellule est néanmoins plutôt limitée (Denis *et al.*, 1996).

La glycoprotéine gH possède des propriétés fusogéniques et elle coopère avec les glycoprotéines gB et gD dans la pénétration du virus dans la cellule-hôte et dans la transmission virale de cellule à cellule, quoiqu'elle ne serve pas de ligand aux récepteurs cellulaires, contrairement aux glycoprotéines gB et gD (Baranowski *et al.*, 1993).

Les propriétés biologiques des glycoprotéines gK, gL et gM sont encore peu connues. Chez le HSV1, la gK ne fait pas partie des glycoprotéines retrouvées à la surface du virion. Le rôle de cette protéine serait de promouvoir le transport, à la surface cellulaire, de

composantes virales contrôlant le processus de fusion membranaire lors de la sortie des virions de la cellule-hôte (Hutchinson et Johnson, 1995). La glycoprotéine gL du HSV1 agit comme chaperon pour la gH. La formation du complexe gH/gL est essentielle pour le transport intracellulaire et pour la maturation de ces deux glycoprotéines (Hutchinson *et al.*, 1992). Quant à la glycoprotéine gM, elle n'est pas essentielle à la réplication du HSV1 *in vitro* et *in vivo*. La gM pourrait jouer un rôle dans la fusion des membranes de cellules adjacentes (Baranowski *et al.*, 1996; Wu *et al.*, 1998).

2. RÉPLICATION DU BHV1

Le BHV1, tout comme les autres virus herpès, a la capacité d'établir deux types d'infection : lytique et latente. L'infection lytique conduit à la production de nouvelles particules virales et éventuellement à la mort de la cellule infectée. L'infection latente permet au virus de persister chez son hôte en conservant son génome dans le noyau de la cellule infectée.

2.1 Infection lytique

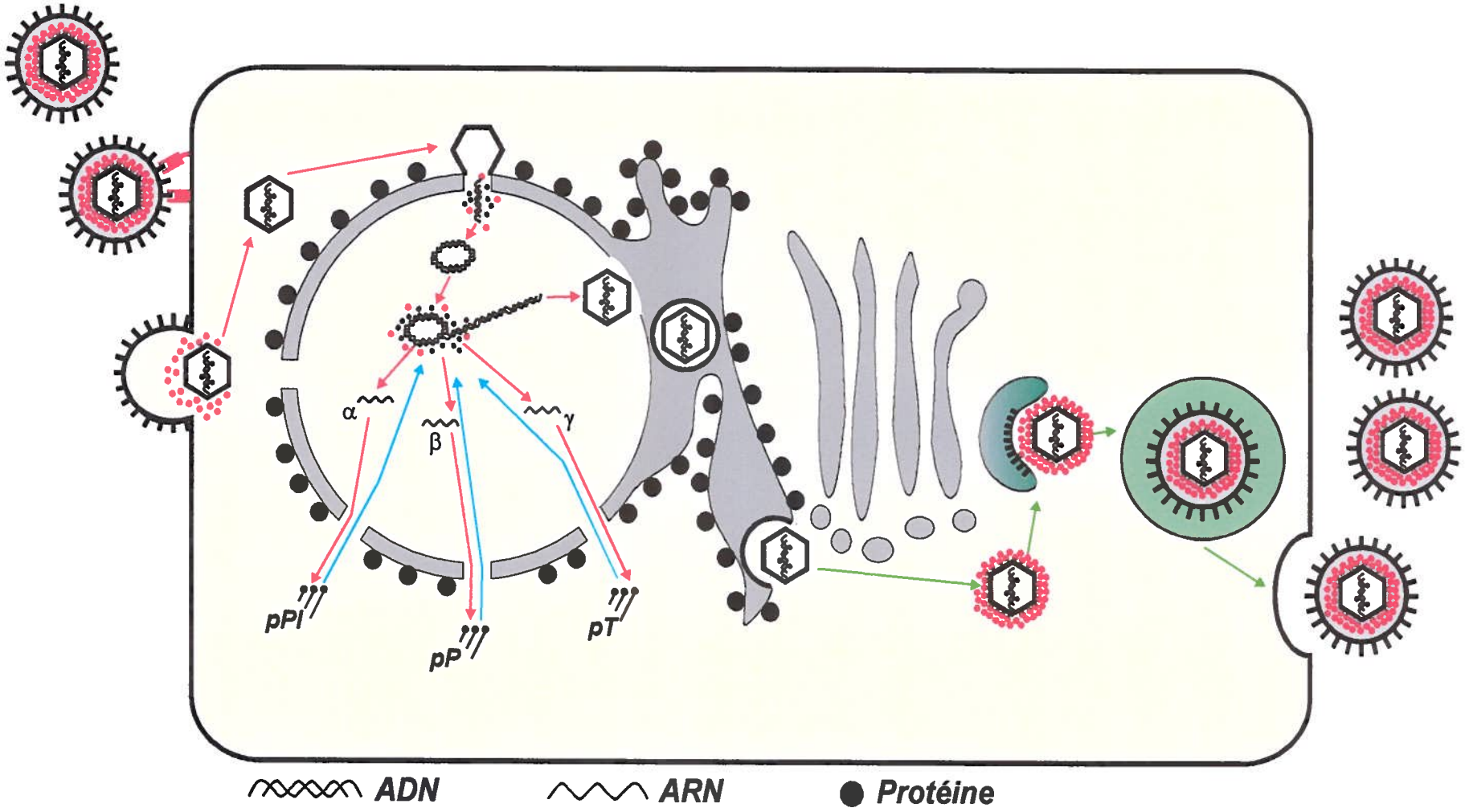
L'infection lytique d'une cellule-hôte par le BHV1 s'initie par l'attachement du virion à l'héparan sulfate de la matrice extracellulaire via les glycoprotéines gB et gC. La glycoprotéine gD interagit avec un second récepteur cellulaire permettant ainsi, avec l'aide des glycoprotéines gB et gH/gL, la fusion des membranes virale et cellulaire. Cette fusion des membranes permet l'entrée de la nucléocapside et des protéines du tégument à l'intérieur du cytoplasme. L'une des protéines du tégument, la Vhs ("virion host shutoff"), demeure dans le cytoplasme où elle induit la dégradation des ARNm cellulaires. La nucléocapside virale s'attache aux microtubules et est transportée au noyau. L'ADN viral, contenu dans la nucléocapside, est libéré dans le noyau et est immédiatement circularisé. La transcription des gènes viraux est régulée de façon séquentielle. La protéine du tégument Btif, facteur de trans-induction des gènes alpha, est transportée au noyau où elle interagit avec des composantes transcriptionnelles de l'hôte afin d'induire la transcription des gènes précoce-immédiats viraux (α) par l'ARN polymérase II cellulaire. Les ARNm α sont

transportés dans le cytoplasme où ils sont traduits, après quoi les protéines précoce-immédiates sont importées au noyau où elles vont activer la transcription des gènes précoces (β) et réguler la transcription des gènes α. Les transcrits des gènes β sont transportés dans le cytoplasme et traduits pour générer les protéines impliquées dans la réplication de l'ADN viral et dans la production de substrats pour la synthèse d'ADN. La réplication de l'ADN permet la production de longues molécules d'ADN concatémériques qui servent entre autres de gabarit pour l'expression des gènes tardifs (γ), lesquels encodent principalement les protéines structurales du virion (Figure 1; Tikoo *et al.*, 1995; Wyler *et al.*, 1989; Roizman et Sears, 1996). La discrimination entre les gènes α et β est basée sur le fait que ces derniers requièrent les protéines α pour leur synthèse et qu'ainsi, en présence d'un inhibiteur de synthèse protéique tel que la cycloheximide, l'expression des gènes β sera inhibée. Au contraire des gènes β, l'expression des gènes γ est inhibée en absence de synthèse d'ADN viral et ainsi, en présence d'un inhibiteur de synthèse d'ADN comme l'acide phosphonoacétique (PAA), les gènes γ ne sont pas exprimés. Les gènes γ ont été séparés en deux groupes γ1 et γ2 même si dans la réalité ils forment un continuum différant dans leur dépendance face à la synthèse de l'ADN viral pour leur expression (Roizman et Sears, 1996).

Une fois que tous les gènes viraux sont exprimés, l'ADN nouvellement synthétisé est clivé et encapsidé. Le passage des virions du noyau à la surface cellulaire n'est pas encore complètement compris. Il semblerait que la nucléocapside bourgeonne de la membrane nucléaire interne mais cette membrane nucléaire est perdue lors de la fusion de la particule virale avec la membrane nucléaire externe, relâchant ainsi la capside dans le cytoplasme. L'addition des protéines du tégument se ferait dans le cytoplasme avant le bourgeonnement final via l'appareil de Golgi permettant ainsi aux virions d'acquérir une enveloppe bilipidique ainsi que les glycoprotéines qui y sont attachées (Figure 1; Flint *et al.*, 2000; Tikoo *et al.*, 1995).

Figure 1. Cycle de réplication du BHV1.

Le virus s'attache et pénètre dans la cellule à l'aide d'interactions spécifiques entre les glycoprotéines virales et les récepteurs cellulaires. Rendue dans le cytoplasme, la nucléocapside est transportée au noyau où l'ADN viral est libéré et circularisé. Les protéines virales sont exprimées en cascade: les gènes α encodent les protéines précoce-immédiates (pPI), lesquelles régulent l'expression des gènes β. Ceux-ci encodent les protéines précoces (pP), principalement impliquées dans la réplication de l'ADN viral. Une fois que l'ADN est répliqué, les gènes γ sont transcrits et les protéines tardives (pT) sont exprimées. Les nucléocapsides, possédant l'ADN nouvellement synthétisé, quittent le noyau par un processus d'enveloppement/désenveloppement à la membrane nucléaire, acquièrent les protéines du tégument dans le cytoplasme et bourgeonnent à la membrane plasmique. Pour plus d'informations, voir le texte.


α
β
γ
pPI
pP
pT
ADN
ARN
Protéine

2.2 Réplication de l'ADN viral

Dès son entrée dans le noyau de la cellule-hôte, le génome viral linéaire des virus alphaherpès se circularise. Un avantage indéniable de la circularisation du génome est qu'il n'est pas nécessaire d'avoir un mécanisme spécialisé pour répliquer les régions terminales. Suite au processus de circularisation, la réplication de l'ADN viral procèderait initialement par un mécanisme thêta. Le génome circularisé agirait comme un gabarit pour ce type de réplication. Jusqu'à maintenant, il n'existe pas d'évidences directes de génomes circulaires ou d'intermédiaires d'une réplication thêta.

Subséquemment, la réplication de l'ADN viral se fait par le mécanisme du cercle roulant afin de générer de longs concatémères où les génomes sont tête-à-queue (Boehmer et Lehman, 1997). L'observation directe d'intermédiaires de réplication de l'ADN du HSV1 par microscopie électronique révèle la présence de molécules possédant de nombreuses régions d'ADN simple brin, de fourches de réplication, de boucles et de structures branchues d'ADN qui seraient la conséquence de recombinaisons homologues, d'isomérisations génomiques et d'autres événements (Shlomai *et al.*, 1976; Friedmann *et al.*, 1977; Hirsch *et al.*, 1977). Il semblerait qu'une désoxyribonucléase alcaline virale encodée par le gène UL12 soit importante pour la maturation de ce réseau d'intermédiaires d'ADN (Martinez *et al.*, 1996a; Goldstein et Weller, 1998a-b).

Quoique l'existence d'intermédiaires de réplication démontre l'utilisation du mécanisme du cercle roulant comme mode de réplication menant à la formation de structures branchues d'ADN, il n'existe pas d'informations sur la transition entre la réplication thêta et la réplication par le mécanisme du cercle roulant ou sur les facteurs requis pour cette transition (Boehmer et Lehman, 1997).

2.3 Clivage et encapsidation de l'ADN viral

Le clivage et l'encapsidation de l'ADN viral nouvellement synthétisé sont des fonctions étroitement liées. Les détails de ce processus proviennent d'études réalisées avec des

mutants du HSV1. Jusqu'à présent, sept gènes essentiels impliqués dans ce processus ont été identifiés, soit UL6, UL15, UL17, UL25, UL28, UL32 et UL33 (Sheaffer *et al.*, 2001). Le produit du gène UL12, la nucléase alcaline, jouerait un rôle important mais non-essentiel en maturant correctement les génomes répliqués en une forme convenable pour l'encapsidation (Martinez *et al.*, 1996a-b; Goldstein et Weller, 1998a-b). Puisque la réplication de l'ADN viral conduit à la formation de longs concatémères contenant plusieurs copies du génome, celles-ci doivent être détachées les unes des autres pour l'encapsidation. Ainsi, les séquences d'encapsidation du HSV1, *pac 1* et *pac 2*, qui sont localisées dans les régions répétées terminales du génome, sont nécessaire pour la reconnaissance et le clivage de l'ADN viral.

Le processus de clivage et d'encapsidation de l'ADN viral du HSV1 présente plusieurs similarités avec le processus retrouvé chez les bactériophages à ADN double brins (Sheaffer *et al.*, 2001). Par analogie avec ces derniers, les protéines impliquées dans le clivage et l'encapsidation de l'ADN du HSV1 doivent inclure un complexe protéique possédant une activité de terminase qui s'assemble à un site spécifique sur une capside préformée et reconnaît l'ADN viral à être encapsidé, clive l'ADN viral au site approprié pour initier l'encapsidation, injecte le génome dans la capside et clive de nouveau pour terminer le processus (Abbotts *et al.*, 2000). Plusieurs données suggèrent que les produits des gènes UL15 et UL28 seraient impliqués dans ce complexe protéique. D'abord, la protéine UL15 du HSV1 possède un motif liant l'ATP, similairement à la grosse sous-unité du complexe terminase du bactériophage T4 (Sheaffer *et al.*, 2001). Ce motif chez la protéine UL15 est requis pour le clivage et l'encapsidation de l'ADN du HSV1 (Yu et Weller, 1998b) et l'encapsidation de l'ADN est inhibée en absence d'ATP (Dasgupta et Wilson, 1999). De plus, il a été démontré qu'une interaction physique existe entre les protéines UL15 et UL28 chez le HSV1 de même que chez le PRV, tout comme une interaction existe entre les deux sous-unités constituant le complexe de terminase des bactériophages (Koslowski *et al.*, 1999; Koslowski *et al.*, 1997; Abbotts *et al.*, 2000). Récemment, il a également été démontré qu'il existe une interaction spécifique entre la protéine UL28 et de l'ADN possédant le motif *pac 1*, accentuant le rôle de UL28 dans la reconnaissance des séquences d'encapsidation (Adelman *et al.*, 2001). La protéine UL6 du

HSV1 est requise pour une association efficace de la protéine UL15 à la capside, suggérant que les unités protéiques UL6 associées à la capside servent de site de liaison pour la machinerie impliquée dans le clivage et l'encapsidation de l'ADN viral (Yu et Weller, 1998a). La protéine codée par le gène UL25 est nécessaire pour l'encapsidation mais non pour le clivage de l'ADN viral. Cette protéine est retrouvée principalement associée aux capsides après l'encapsidation de l'ADN viral, ce qui suggère que son rôle serait de sceller la capside une fois l'ADN viral à l'intérieur, stabilisant ainsi la nucléocapside (Sheaffer *et al.*, 2001; McNab *et al.*, 1998). Enfin, les produits des gènes UL17 et UL32 semblent être requis pour la localisation des capsides et/ou des protéines majeures ou mineures associées aux capsides aux sites intranucléaires où l'ADN viral est clivé et encapsidé (Taus *et al.*, 1998; Lamberti et Weller, 1998). Jusqu'à présent, aucune fonction spécifique n'a été associée au produit du gène UL33.

2.4 Latence

Une infection latente par le BHV1 est caractérisée par quatre propriétés générales: 1) une cellule incapable de se diviser est infectée par le virus, 2) la détection par le système immunitaire de la cellule possédant le génome latent est éliminée ou fortement réduite, 3) l'expression des gènes viraux n'a pas lieu ou est inefficace, 4) le génome viral persiste dans la cellule ce qui permet ultérieurement l'initiation d'une infection lytique et la dissémination du virus (Tikoo *et al.*, 1995).

De façon plus spécifique, le BHV1 peut entrer dans les cellules neurales périphériques qui innervent les cellules infectées et la nucléocapside est transportée par le flux axonal rétrograde aux noyaux des ganglions trijumeaux ou sacraux où il y a établissement de la latence. Le génome latent est maintenu sous la forme d'un chromosome non-réplicatif dans les noyaux de ces cellules et un transcrit viral abondant, nommé transcrit associé à la latence (LR RNA; "latency-related RNA") est épissé alternativement générant entre autres la protéine LRP ("latency-related protein") (Flint *et al.*, 2000; Jiang *et al.*, 1998). La protéine LRP a la capacité d'interagir avec des protéines régulatrices du cycle cellulaire comme cdk2 ("cyclin-dependent kinase 2") pour promouvoir la survie des neurones durant

l'infection aiguë et/ou la réactivation (Jiang *et al.*, 1998). La survie des neurones est également favorisée par l'inhibition de la mort cellulaire programmée par les produits du transcrit associé à la latence (Ciacci-Zanella *et al.*, 1999). Les infections latentes peuvent être réactivées par une grande variété de stimuli comme le stress, le transport de l'animal, le traitement des bêtes avec des glucocorticoïdes et la mise-bas. La réactivation du virus latent est responsable de la perpétuation et de la transmission du virus dans les cheptels bovins (Tikoo *et al.*, 1995).

3. PATHOGÉNICITÉ DU BHV1

3.1 Symptômes cliniques

L'IBR causé par le BHV1 est une maladie virale contagieuse du bétail qui est retrouvée sur tous les continents. L'entrée du virus dans les voies respiratoires survient par des aérosols présents dans l'air ou par contact direct avec les sécrétions nasales. L'IBR est caractérisé par de la fièvre, une augmentation du rythme respiratoire, une toux persistante, de l'anorexie et chez les vaches laitières, une baisse sévère dans la production de lait. Des zones de nécroses de la muqueuse nasale peuvent être observées, occasionnant souvent des sécrétions nasales et oculaires et des conjonctivites. De plus, chez les vaches en gestation, des avortements peuvent survenir, principalement entre le cinquième et le huitième mois de gestation (Tikoo *et al.*, 1995; Wyler *et al.*, 1989).

L'infection par le BHV1 des voies respiratoires supérieures, des bronches, de la trachée et des poumons favorise une augmentation des risques de colonisation de la partie inférieure des poumons par des pathogènes bactériens comme *Pasteurella haemolytica* ou dans une moindre mesure *P. multocida*, menant au développement de pneumonies sévères. De telles pneumonies peuvent entraîner la mort si une thérapie antibiotique n'est pas initiée rapidement. À cause de cette facilitation des infections secondaires, le BHV1 est considéré comme l'un des initiateurs majeurs de la fièvre des transports (Tikoo *et al.*, 1995; van Drunen Little-van den Hurk *et al.*, 1993).

Le BHV1 peut également infecter le système reproducteur causant une vulvovaginite pustulaire infectieuse chez les femelles et une balanoposthite chez les mâles. La transmission au système génital survient invariablement par contact direct ou par de la semence contaminée par le BHV1. Pour cette raison, les taureaux utilisés pour l'insémination artificielle doivent être séronégatifs pour le BHV1 (Wyler *et al.*, 1989).

Occasionnellement, des séquelles neurologiques, telles des encéphalites, peuvent être observées chez les animaux infectés par le BHV1. Les signes neurologiques sont caractérisés par une incoordination des mouvements, des tremblements musculaires, de l'ataxie, des déplacements sans but et éventuellement, la mort de l'animal (Wyler *et al.*, 1989).

3.2 Tests diagnostiques en laboratoire

Un diagnostic rapide d'une infection par le BHV1 se fait par l'examen de fluides vésiculaires ou de frottis par microscopie électronique, par coloration immunofluorescente de frottis ou de sections tissulaires, par la détection d'anticorps spécifiques à l'aide de tests ELISA ("enzyme-linked immunosorbent assay") et par la détection d'acides nucléiques viraux à l'aide d'une réaction de polymérisation en chaîne (PCR; "Polymerase Chain Reaction") (Murphy *et al.*, 1999). Présentement, il est possible de diagnostiquer une infection par le BHV1 à l'aide d'un test ELISA qui permet la détection d'anticorps contre la glycoprotéine E dans des échantillons de lait (Wellenberg *et al.*, 1998) ou par un test PCR qui permet de détecter la présence de BHV1 dans la semence de taureaux naturellement infectés (Rocha *et al.*, 1998).

3.3 Considérations économiques

Étant donné que le BHV1 est associé à différentes pathologies, il représente toujours des frais importants pour l'industrie de la viande bovine et pour l'industrie laitière. Les coûts associés à la maladie respiratoire bovine, dont le BHV1 est l'un des agents initiateurs majeurs, sont estimés à 1 milliard de dollars par année en Amérique du Nord et à 150

millions de florins hollandais aux Pays-Bas où 84% des vaches laitières étaient séropositives en 1994. Les pertes sont dues à la baisse de la production de lait, aux avortements, aux pertes de poids, aux traitements pour soigner les animaux infectés et à la mort de certains animaux (Bowland et Shewen, 2000; Vonk Noordegraaf *et al.*, 1998; Mars *et al.*, 2001; Murphy *et al.*, 1999).

4. RÉPONSES IMMUNITAIRES INDUITES PAR LE BHV1

4.1 Immunité non-spécifique

Suite à l'infection par le BHV1, l'interféron α (IFN-α) est détectable dans les sécrétions nasales, et ce, dès cinq heures post-infection. Les niveaux maximaux sont atteints entre 72 et 96 heures post-infection et la présence de la cytokine persiste jusqu'à huit jours (Straub et Ahl, 1976). L'IFN-α procure une protection locale au début de l'infection en modulant la migration des leucocytes et la phagocytose et en augmentant l'activité des cellules NK ("natural killer") (Babiuk *et al.*, 1985; Lawman *et al.*, 1987; Jensen et Schultz, 1990). De plus, l'exposition des macrophages à l'IFN-α les rend résistants à l'infection par le BHV1 et augmente leur habileté à fonctionner en tant que cellules effectrices dans la lyse des cellules dépendante des anticorps (ADCC; "antibody-dependent cell-mediated cytotoxicity") par une augmentation du nombre de récepteurs Fc présents sur la cellule (Bielefeldt Ohmann *et al.*, 1984). L'activation des macrophages résulte aussi en l'induction d'une activité cytolytique contre les cellules infectées et cela, en utilisant un mécanisme indépendant des anticorps. Les lymphocytes T produisent aussi de l'IFN-α et possiblement d'autres facteurs qui activeraient davantage les macrophages (Campos *et al.*, 1989).

Les cellules NK sont une population hétérogène de cellules effectrices non-adhérentes dont l'activité cytolytique n'est pas restreinte aux molécules du complexe majeur d'histocompatibilité (CMH) de classe I ou II présentes sur les cellules cibles (Hercend et Schmidt, 1988). Chez les bovins, une activité NK a été associée à une population de cellules non-adhérentes ne présentant pas les marqueurs conventionnels des cellules T et B (Cook et Splitter, 1989). Ces cellules NK requièrent une longue période d'incubation avec

les cellules infectées par le BHV1 pour obtenir une lyse optimale (Campos et Rossi, 1986). Comme pour l'activité des macrophages, une activité NK peut jouer un rôle critique dans la phase précoce de l'infection lorsqu'il est nécessaire de limiter la dissémination du virus. Cependant, l'activité cytotoxique peut aussi être un facteur contribuant au développement de la pathologie (Tikoo *et al.*, 1995).

4.2 Immunité spécifique

4.2.1 Immunité humorale

Les réponses immunitaires systémiques humorales dépendent de la production d'anticorps neutralisants contre le virus qui peuvent être détectés de huit à douze jours post-infection (Gibbs et Rweyemamu, 1977). L'analyse de la spécificité de la réponse humorale induite suite à l'infection par le BHV1 a démontré que les glycoprotéines gB, gC et gD sont non seulement les protéines majeures reconnues mais qu'elles sont également importantes pour l'induction d'une réponse d'anticorps neutralisants (van Drunen Littel-van der Hurk et Babiuk, 1986; van Drunen Littel-van der Hurk *et al.*, 1993). En plus de neutraliser le virus, la réponse humorale contre le BHV1 peut aider à lutter contre l'infection par d'autres mécanismes comme la destruction spécifique des cellules infectées avant la transmission du virus aux cellules adjacentes susceptibles, l'ADCC où les IgG peuvent interagir avec les cellules possédant le récepteur Fc comme les neutrophiles et les macrophages et l'ADCC assisté du complément où le C3b lie les IgM et les récepteurs des lymphocytes, des macrophages et des neutrophiles (van Drunen Littel-van der Hurk *et al.*, 1993).

4.2.2 Immunité cellulaire

Une variété de réponses spécifiques aux lymphocytes T a été détectée suite à l'infection de bovins par le BHV1. Ces réponses seraient importantes pour le processus de guérison. Les réponses cytotoxiques et prolifératives générées par les lymphocytes T apparaissent toutes deux, huit jours après l'infection. De plus, les lymphocytes T activés relâchent des

lymphokines qui vont moduler les réponses immunitaires spécifiques et non-spécifiques (van Drunen Littel-van der Hurk *et al.*, 1993).

Des équipes de recherche tentent de définir les protéines du BHV1 qui sont reconnues par les lymphocytes T afin de caractériser leur rôle dans la réponse cellulaire. Jusqu'à présent, il a été montré que quatre protéines du BHV1 (gB, gC, gD et VP8) sont reconnues par les lymphocytes T auxiliaires CD4+ de bovins immuns pour le BHV1 (Hutchings *et al.*, 1990). De plus, des études ont démontré la présence de lymphocytes T cytotoxiques CD8+ reconnaissant les glycoprotéines gC et gD (Denis *et al.*, 1993).

5. VACCINS ANTI-BHV1

5.1 Programmes d'éradication du BHV1

Un certain nombre de pays ont décidé de contrôler l'infection du BHV1 à l'aide d'un programme d'éradication de la maladie. Jusqu'à présent deux pays d'Europe, la Suisse et le Danemark, y ont réussi (Tikoo *et al.*, 1995). En Suisse, le programme national d'éradication a été divisé en 4 phases : 1) la prévention de la transmission de l'infection par des restrictions sur les achats et les ventes du bétail et par l'estimation du nombre d'animaux séropositifs pour le BHV1, 2) l'abattage des animaux séropositifs dans le but d'éradiquer le BHV1 du cheptel suisse, 3) la détection et l'éradication des autres réservoirs du BHV1 et 4) la mise sur pied de programmes et d'actions légales dans le but de maintenir une situation favorable. Au cours de ce programme d'éradication, approximativement 50,000 animaux ont été abattus. Les coûts ont été évalués à 110 millions de francs suisses sur 10 ans. Les coûts pour maintenir cet état d'éradication sont estimés à 5 millions de francs suisses par année, dus aux dépenses de laboratoire nécessaires pour maintenir une surveillance sérologique du cheptel suisse qui représente approximativement 2 millions de têtes (Ackermann *et al.*, 1990).

Malheureusement, à cause du nombre trop élevé de bovins que certains pays possèdent ou bien à cause du transport fréquent des troupeaux d'une région à l'autre, l'éradication de la maladie n'est pas toujours possible.

5.2 Types de réponses immunitaires protectrices

Afin de réduire les pertes économiques causées par le BHV1 dans les pays où les programmes d'éradication sont impossibles, des vaccins anti-BHV1 ont été développés ou sont en voie de l'être. Dans le cas d'une maladie contagieuse comme le BHV1, la vaccination devrait fournir une protection à long terme contre l'infection par le virus sauvage. Trois types de réponses immunitaires protectrices peuvent être induites : une immunité non-spécifique incluant la production de cytokines et l'activation de cellules comme les macrophages et les cellules NK, une immunité cellulaire (CMI; "cell-mediated immunity") qui limite plutôt que prévient l'infection et une immunité humorale conduisant à la neutralisation du virus pour prévenir l'infection virale (van Drunen Littel-van den Hurk *et al.*, 1993). Le vaccin idéal devrait induire une immunité telle que, suite à une infection subséquente avec le virus sauvage, une induction rapide de l'immunité cellulaire et de la production d'anticorps neutralisants survienne. De plus, puisque le BHV1 initie l'infection aux cellules des muqueuses, une immunité locale est particulièrement importante pour la prévention de l'infection.

5.3 Vaccins conventionnels

En 1956, l'année où pour la première fois le BHV1 a été isolé, le premier vaccin atténué anti-BHV1 a été rapporté dans la littérature (Kendrick *et al.*, 1956). Depuis ce temps, une grande variété de vaccins atténués ou inactivés ont été développés.

Des vaccins vivants ont été développés soit par le passage *in vitro* du virus sur différentes lignées cellulaires afin de produire des isolats atténués ou par mutagenèse pour la production de mutants thermosensibles (ts) (Tikoo *et al.*, 1995; van Drunen Littel-van den Hurk *et al.*, 1993). Les vaccins vivants modifiés offrent trois avantages majeurs :

l'induction d'une réponse immunitaire rapide, une durée relativement longue de l'immunité et l'induction d'une immunité mucosale locale. En plus d'induire la production d'interféron, les vaccins administrés intranasalement induisent la sécrétion d'IgA de même que l'immunité cellulaire (van Drunen Littel-van den Hurk *et al.*, 1993). Cependant, l'utilisation de ce type de vaccin présente plusieurs inconvénients. Tout d'abord, si les vaccins ne sont pas entreposés adéquatement, ils peuvent devenir inactifs. En plus de cette instabilité physico-chimique, les souches vaccinales possédant des mutations aléatoires dans leur génome peuvent ne pas être stables génétiquement *in vivo* et une réversion au phénotype sauvage est possible. La vaccination de bovins avec un vaccin vivant modifié occasionne des signes cliniques faibles et n'empêche pas l'excrétion de virus qui peut ainsi se transmettre aux animaux non-vaccinés. De plus, certaines souches vaccinales moins atténuées ne peuvent pas être administrées aux vaches en gestation afin de minimiser les risques d'avortement. Des souches vaccinales peuvent établir la latence et le virus peut être réactivé suite à un traitement des animaux aux corticostéroïdes. Finalement, l'utilisation de vaccins vivants modifiés peut induire une suppression du système immunitaire de l'animal vacciné, résultant en une augmentation de la susceptibilité à d'autres infections (van Drunen Littel-van den Hurk *et al.*, 1993).

Puisque les vaccins vivants modifiés ne sont pas sécuritaires pour les animaux, certains éleveurs préfèrent utiliser des vaccins inactivés. Ces vaccins sont produits soit par une inactivation chimique, comme par traitement à la formaline, l'éthanol ou la β-propiolactone, soit par une inactivation physique, comme par traitement à la chaleur ou aux ultraviolet (Wyler *et al.*, 1989). Selon l'analyse de la production d'anticorps neutralisants, les vaccins inactivés ne sont pas efficaces sans adjuvants. Cependant, en présence d'adjuvants, les vaccins inactivés peuvent induire la production d'anticorps neutralisants à la suite de deux immunisations. La plupart des inconvénients décrits pour les vaccins vivants modifiés ne s'appliquent pas aux vaccins inactivés. En effet, les vaccins inactivés ne causent pas d'avortement, de suppression du système immunitaire ou d'établissement de la latence. Les animaux vaccinés n'excrètent pas de virus et sont par le fait même sans risque pour les animaux non-vaccinés. Un autre avantage de ce type de vaccin est qu'il possède une stabilité d'entreposage relativement bonne. Le désavantage majeur des vaccins

inactivés est que la destruction de certains antigènes majeurs durant la procédure d'inactivation les rend peu immunogènes et ainsi, peu efficaces à induire une réponse immunitaire (van Drunen Littel-van den Hurk *et al.*, 1993).

5.4 Vaccins expérimentaux

Les limitations des vaccins commerciaux ainsi que les progrès de la biologie moléculaire et des techniques de purification des protéines ont conduit au développement de vaccins de nouvelle génération. Ces nouveaux vaccins, contrairement aux vaccins conventionnels, ont la caractéristique d'être marqueurs par l'élimination d'une ou plusieurs (glyco)protéines retrouvées à la surface du virion, permettant ainsi de discriminer les animaux vaccinés des animaux infectés naturellement sur la base de leur réponse sérologique respective. La discrimination peut être faite à l'aide d'un test immunologique qui détecte si les anticorps contre la ou les (glyco)protéines manquantes dans la souche vaccinale sont présents ou non (van Oirschot *et al.*, 1996b).

5.4.1 Les vaccins sous-unitaires

Les vaccins sous-unitaires sont constitués de composants viraux immunogéniques, tels les glycoprotéines retrouvées à la surface du BHV1, qui peuvent être injectés individuellement ou en combinaison à l'animal. Jusqu'à récemment, ces vaccins étaient difficiles à produire. Cependant, il est maintenant possible de synthétiser de grandes quantités de protéines dans des systèmes bactériens, de levures, d'insectes ou de cellules animales en utilisant les techniques de biologie moléculaire. Les vaccins sous-unitaires ne sont pas constitués de virus vivants et ainsi, ne peuvent pas être transmis aux animaux non-vaccinés. Il a également été démontré que les vaccins sous-unitaires peuvent non seulement prévenir la maladie mais également l'infection par le virus, prévenant ainsi l'établissement de la latence (van Drunen Littel-van den Hurk *et al.*, 1993). Toutefois, si l'utilisation de vaccins sous-unitaires présente certains avantages, il n'en demeure pas moins que cette approche comporte plusieurs inconvénients. Deux immunisations sont requises afin d'obtenir la protection désirée et la quantité de protéines par dose est élevée, ce qui en fait une approche

peu économique. De plus, l'efficacité des vaccins sous-unitaires est dépendante de l'utilisation d'un adjuvant approprié, ce qui peut être problématique à formuler pour un vaccin multivalent (van Drunen Littel-van den Hurk *et al.*, 1990; van Drunen Littel-van den Hurk *et al.*, 1993).

5.4.2 Les vaccins à ADN

Il a été démontré que le transfert de gènes fonctionnels peut être accompli en introduisant simplement un plasmide, initialement développé pour la transfection de cellules eucaryotiques en culture, dans les tissus d'un animal vivant (Wolff *et al.*, 1990). Cette découverte a permis le développement de vaccins à ADN anti-BHV1. Récemment, un plasmide exprimant la glycoprotéine gB a servi à immuniser des bovins par voie intravulvomucosale. L'immunisation a induit une forte réponse cellulaire et humorale (Loehr *et al.*, 2000). La vaccination à ADN permet la présentation des antigènes par les molécules du CMH de classe I et II, offre la possibilité d'être utilisée chez les nouveau-nés malgré la présence d'anticorps maternels, est stable et les coûts de production sont faibles (Loehr *et al.*, 2000). Il n'en reste pas moins que les risques à long terme de la vaccination à ADN ne sont pas connus.

5.4.3 Les mutants de délétion

Les souches du BHV1 constituant les vaccins vivants modifiés conventionnels sont obtenues suite à l'introduction de mutations aléatoires et spontanées dans leur génome. Les mutants ainsi produits sont habituellement peu caractérisés en ce qui concerne leurs changements génétiques et ont le potentiel de redevenir virulent. Au contraire, avec le génie génétique, il est possible de cibler un ou plusieurs gène(s) spécifique(s) afin de le ou les éliminer du génome viral. De tels mutants offrent l'avantage d'être beaucoup plus stables génétiquement que les vaccins vivants modifiés conventionnels, réduisant ainsi les risques de réversion au phénotype sauvage. De plus, ils présentent l'avantage d'être mieux caractérisés (van Drunen Littel-van den Hurk *et al.*, 1993; van Oirschot *et al.*, 1996a).

Un ou plusieurs gènes, essentiels ou non à la réplication, peuvent être éliminés du génome viral afin de créer des mutants de délétion. Des groupes de recherche se sont intéressés à créer des mutants de gènes non-essentiels du BHV1 en éliminant entre autres du génome viral, le gène de la thymidine kinase (Whetstone *et al.*, 1992), de la glycoprotéine gC (Liang *et al.*, 1992) et de la glycoprotéine gE (van Engelenburg *et al.*, 1994; Mars *et al.*, 2001). Ces mutants possèdent les caractéristiques d'être stables génétiquement et d'être efficaces à induire une bonne réponse immunitaire. Cependant, même s'il a été démontré que ces souches vaccinales sont moins virulentes *in vivo*, elles possèdent toujours la capacité de se répliquer et le risque que le virus soit transmis aux animaux non-vaccinés existe toujours. Ces mutants peuvent également établir la latence chez l'animal vacciné.

Afin de contrer ces inconvénients, des mutants de délétion de gènes essentiels pourraient être développés et utilisés comme souches vaccinales. Ces mutants, inaptes à se répliquer, seraient ainsi sans danger pour l'animal vacciné et pour les animaux qui l'entourent. De plus, ils seraient stables génétiquement et les risques de réversion au phénotype sauvage seraient pratiquement nuls. Les risques d'établir la latence seraient également minimes. Jusqu'à présent, aucune utilisation d'un mutant de délétion d'un gène essentiel du BHV1 comme souche vaccinale n'a été rapportée dans la littérature. Cependant, l'utilisation de mutants inaptes à se répliquer du HSV comme souches vaccinales a été rapportée depuis plusieurs années. De tels mutants, lorsque injectés à des souris, sont capables d'induire des réponses humorale et cellulaire et protègent de l'infection létale suite à une épreuve virale (Nguyen *et al.*, 1992; Brubaker *et al.*, 1996; Morrison et Knipe, 1997). Plus récemment, la construction d'un double mutant de délétion de gènes essentiels impliqués dans la réplication de l'HSV2 a été rapportée dans la littérature. Ce mutant offre une sécurité maximale pour un vaccin vivant, en plus d'induire une bonne réponse immunitaire dans un système animal (Da Costa *et al.*, 2000). Ainsi, des virus de l'herpès génétiquement modifiés pour restreindre leur propagation à une seule ronde d'infection, présentent un excellent potentiel prophylactique.

6. PROJET DE RECHERCHE

6.1 Fonctionnalité des ORFs ciblés

La caractérisation complète du génome du BHV1 a permis d'identifier les différents ORFs par comparaison de séquences avec les autres virus herpès dont le HSV1. Par convention, ces ORFs ont été nommés selon la nomenclature utilisée pour le HSV1 (Roizman et Sears, 1996). Les ORFs viraux UL12, UL25 et UL28, dont les homologues codent pour des protéines potentiellement impliquées dans le processus de clivage et d'encapsidation de l'ADN viral, ont été ciblés pour mes travaux de recherche de doctorat. Le but du projet était d'étudier la fonction de ces gènes dans la réplication du BHV1 et cela, en les supprimant individuellement du génome viral afin de développer des mutants de délétion spécifiques. L'analyse de ces mutants permettrait de déterminer si ces ORFs sont essentiels ou non au cycle réplicatif du BHV1. S'il s'avérait que ces ORFs soient essentiels, les études seraient axées afin de déterminer à quelle étape le cycle réplicatif avorte pour chacun d'eux. Cependant, avant de commencer la construction proprement dite des mutants, il fallait démontrer que les ORFs ciblés représentaient bel et bien des gènes chez le BHV1. D'abord, j'ai identifié les transcrits viraux en utilisant des sondes d'ARN simple brin marquées radioactivement et complémentaires aux séquences codantes des ORFs ciblés. J'ai déterminé également les sites d'initiation de la transcription par des essais de protection à la nucléase S1. J'ai aussi identifié les produits de traduction des ORFs. L'identification des protéines virales potentielles requérant le développement d'antisérums spécifiques, j'ai procédé à l'expression d'une partie des séquences codantes clonées en phase de lecture avec une étiquette T7-Tag chez *E. coli* pour ensuite immuniser des souris avec les protéines recombinantes purifiées. Finalement, j'ai déterminé à quelle classe (précoce-immédiate, immédiate ou tardive) appartiennent les ORFs ciblés en vérifiant si leur expression était inhibée en présence d'un inhibiteur de synthèse d'ADN, le PAA, puisque l'expression des gènes tardifs est inhibée en absence de synthèse d'ADN. Les résultats obtenus, présentés sous forme de trois articles scientifiques dans la prochaine section de la thèse, démontrent clairement que les ORFs UL12, UL25 et UL28 du BHV1 correspondent bien à des gènes.

Par la suite, je me suis intéressée à la construction des mutants de délétion UL25 et UL28 afin d'étudier la fonction de ces gènes au cours du cycle réplicatif du BHV1. Seuls les résultats du mutant de délétion UL28 sont présentés dans cette thèse pour les raisons précisées dans l'introduction de la thèse.

6.2 Création et analyse d'un mutant de délétion

Ayant démontré que l'ORF UL28 est bel et bien un gène chez le BHV1, j'ai pu entreprendre la création du mutant de délétion pour ce gène. Tout d'abord, il a fallu modifier génétiquement une lignée cellulaire permissive au BHV1 sauvage pour lui faire exprimer la protéine UL28. Le développement d'une lignée de complémentation avait pour but de fournir en *trans* cette protéine puisque s'il s'avérait que le gène UL28 soit essentiel à la réplication du BHV1, sa délétion du génome viral entraînerait la non-viabilité du mutant de délétion. Suite à l'obtention de la lignée de complémentation, la construction du mutant désiré a été entreprise en transfectant la lignée avec l'ADN viral sauvage purifié et un plasmide recombinant dont le gène viral d'intérêt, bordé des séquences proximales intactes, a été remplacé en partie par une cassette d'expression de la β-galactosidase (β-gal) de *E. coli*. Les virus régénérés, suite à une recombinaison homologue entre les deux molécules d'ADN, présentaient un phénotype β-gal+ ce qui a facilité leur isolation. Afin de démontrer que le gène UL28 est essentiel à la réplication virale, les cinétiques de rendement viral du mutant dans sa lignée et dans les cellules qui ne complémentent pas ont été comparées aux cinétiques obtenues avec le virus sauvage. Le mutant UL28 est incapable de se reproduire dans la lignée cellulaire qui ne complémente pas, signifiant que ce gène est essentiel à la réplication du BHV1. Par la suite, j'ai déterminé si la délétion du gène UL28 affectait l'expression des protéines virales par l'analyse de l'expression de la protéine tardive Btif encodée par le gène UL48. Des analyses de type Southern ainsi que l'observation en microscopie électronique de cellules infectées ont été effectuées afin d'analyser si la synthèse, le clivage ou l'encapsidation de l'ADN viral sont compromis chez le mutant de délétion.

Les résultats obtenus démontrent que le gène UL28 joue un rôle essentiel dans le processus de clivage et d'encapsidation de l'ADN du BHV1. Ces résultats sont présentés sous la forme d'un article scientifique dans la prochaine section de cette thèse.

SECTION II

PUBLICATIONS

Expression kinetics of the late UL12 gene encoding the bovine herpesvirus 1 alkaline nuclease.

Running title: Bovine herpesvirus 1.1 late UL12 gene

Nathalie Desloges and Claire Simard*

INRS-Institut Armand-Frappier
Centre de microbiologie et biotechnologie
531 Bd des Prairies
Laval-des-Rapides, Québec, Canada, H7V 1B7
Tel. (450) 687-5010 ext 4637
Fax. (450) 686-5626
Email: Claire.Simard@inrs-iaf.uquebec.ca

***Archives of Virology*, 2001; 146(10):1871-1884.**

* Corresponding author

Cinétiques d'expression du gène tardif UL12 encodant la nucléase alcaline du virus de l'herpès bovin 1.

Nous avons caractérisé les cinétiques d'expression du transcrit et de la protéine encodés par l'ORF UL12 du virus de l'herpès bovin 1 (BHV1), un homologue du gène UL12 du virus de l'herpès simplex 1 (HSV1) encodant une nucléase alcaline virale. La séquence codante de l'ORF UL12 du BHV1 est localisée aux positions 82776→84239 du génome viral. Il a déjà été démontré que cette séquence, lorsque exprimée chez *E. coli*, encode une protéine possédant une activité de nucléase. Une analyse Northern d'ARN de cellules infectées par le BHV1, en utilisant une sonde d'ARN simple brin complémentaire à l'ORF UL12, a permis la détection de quatre transcrits viraux spécifiques 3'co-terminaux de 4.2, 3.7, 2.2 et 0.7 kb qui s'accumulent simultanément de 6 heures à 24 heures post-infection (p.i.). La détermination du site d'initiation de la transcription de l'ORF UL12 à la position 82384 du génome viral par un essai de protection à la nucléase S1 ainsi que l'identification d'une séquence consensus d'un signal de polyadénylation aux positions 84490-84495 nous a permis d'établir que le transcrit de 2.2 kb correspond à celui de l'ORF UL12. Un antisérum spécifique à l'ORF UL12 généré contre une protéine de fusion T7-Tag/UL12 exprimée chez *E. coli*, a détecté une protéine de 53 kDa dans des lysats de cellules infectées par le BHV1, dont la grosseur correspond à celle attendue (51,844 Da) et qui s'accumule de 12 à 30 heures p.i.. Les différences observées entre les profils d'expression transcriptionnelle et traductionnelle suggèrent que le produit du gène UL12 du BHV1 est régulé aux niveaux traductionnel et post-traductionnel. Étonnamment, l'expression de la protéine est strictement dépendante de la synthèse de l'ADN viral, démontrant clairement que le gène UL12 du BHV1 appartient aux gènes viraux de la classe γ2 contrairement aux homologues du HSV1 et du virus de la pseudorage porcine qui ont été classifiés comme étant des gènes précoces (β). Des études supplémentaires sont requises pour déterminer si ces différences de profils d'expression ont des implications fonctionnelles.

SUMMARY

We characterized the expression kinetics of the transcript and protein generated from the bovine herpesvirus 1 (BHV1) homologue of the herpes simplex virus 1 (HSV1) UL12 gene that encodes a viral alkaline nuclease. The BHV1 UL12 coding sequence, which was previously shown to express in *E. coli* a protein exhibiting nuclease activity, is located at positions 82776→84239 of the viral genome. Northern blot analysis of RNA from BHV1-infected cells with a single strand RNA probe complementary to UL12 detected four specific 3' coterminal viral transcripts of 4.2, 3.7, 2.2, and 0.7 kb that accumulated simultaneously from 6 to 24 hours post-infection (p.i.). S1 nuclease mapping of the UL12 capping site at position 82384 of the genome as well as the identification of a consensus polyadenylation signal at 84490-84495 allowed us to establish that the 2.2 kb transcript corresponds to that of UL12. A UL12 specific antiserum generated against a T7-Tag/UL12 fusion protein expressed in *E. coli* detected a 53 kDa protein in cell lysates from BHV1-infected cells, whose size correlated with that predicted (51,844 Da), which accumulated from 12 to 30 hours p.i.. Differences observed between the transcriptional and translational expression profiles suggests that the UL12 of BHV1 is regulated at both the translational and post-translational levels. Surprisingly, the protein expression was strictly dependent on viral DNA synthesis, unambiguously demonstrating that BHV1 UL12 belongs to viral genes of the γ2 class. This is in contrast to the HSV1 and pseudorabies homologues that are classified as early (β) genes. Further studies will be required to determine whether these kinetic differences have any functional implications.

INTRODUCTION

Bovine herpesvirus 1 (BHV1) is a member of the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Roizman, 1996). BHV1 is an economically important pathogen of cattle, being associated with many clinical manifestations including respiratory diseases, genital infections, encephalitis, and abortions (Wyler *et al.*, 1989). The viral genome is a 135,301 bp double strand linear DNA molecule composed of a unique long (UL) and a unique short (US) segment, the latter being flanked by inverted repeats (Mayfield *et al.*, 1983). Two isomeric forms of the genome occur naturally as the US segment can invert its orientation relative to the UL segment (Farley *et al.*, 1981).

Infection of host cells by alphaherpesviruses proceeds by the entry of the viral DNA genome into the cell's nucleus, after which a replicative form of the DNA is rapidly generated by circularization of the unit-length virion DNA. Circular molecules then serve as templates for DNA synthesis by the rolling circle model generating endless concatemers that must be cleaved to unit-length DNA for packaging (Roizman, 1996; Wyler *et al.*, 1989). This replicative process is complex requiring several virus-encoded enzymes involved in DNA metabolism. Amongst these is an alkaline pH-dependent deoxyribonuclease termed alkaline nuclease that is encoded by alphaherpesvirus UL12 homologs. This enzyme has been implicated in the processing of viral DNA replication intermediates.

In cells infected with the herpes simplex virus (HSV), the viral specific alkaline nuclease represents a relatively abundant protein with an apparent Mr of 85,000 which is synthesized early upon infection (Banks *et al.*, 1983; Banks *et al.*, 1985). The protein purified from either HSV-infected cells or from *E. coli* transformants expressing the functional enzyme possess both endo- and exo-nuclease activities with an alkaline pH optimum (Banks *et al.*, 1983; Banks *et al.*, 1985; Bronstein and Weber, 1996; Hoffmann and Cheng, 1979; Strobel-Fidler and Francke, 1980). The alkaline nuclease encoded by the HSV1 UL12 is not essential for replication, since a deletion mutant can still replicate in non-complementing cells (Martinez *et al.*, 1996a-b; Shao *et al.*, 1993; Weller *et al.*, 1990). However, the yield of virus progeny is significantly reduced in the mutant as compared to that of wild-type virus, even though the amount of viral DNA replicated by the mutant is comparable to that of wild type virus. The reduced viral yield is attributed to inefficient processing of viral

DNA replication intermediates by the nuclease into a form suitable for encapsidation, thus impairing the egress of capsids from the nucleus to the cytoplasm (Goldstein and Weller, 1998a; Martinez *et al.*, 1996a). It was demonstrated that the deoxyribonuclease activity of the enzyme is required for efficient replication of HSV1 (Goldstein and Weller, 1998b; Henderson *et al.*, 1998).

In contrast to HSV UL12, the only information available on the BHV1 UL12 relates to the nuclease activity of the protein expressed in *E. coli*. A recombinant plasmid harbouring an in-frame fusion between the BHV1 UL12 coding sequence and a histidine tag allowed the synthesis and purification of a 57 kDa protein that exhibited alkaline pH-dependent exonuclease and endonuclease activities (Chung and Hsu, 1996; Chung and Hsu, 1997). To complement the information previously obtained concerning the BHV1 UL12 we undertook the characterization of its transcriptional and translational expression kinetics. The UL12 coding sequence, encoding a 487 aa-long polypeptide with a calculated molecular mass of 51,844 Da, is located at positions 82776→84239 in the rightward orientation of the prototype viral genome. We show that the BHV1 UL12 generates a 2.2 kb transcript that is initiated 392 b upstream from the initiation codon and accumulated in infected cells from 6 to 24 hours p.i.. Western blotting of BHV1-infected protein cell lysates with an antiserum directed against the BHV1 UL12's encoded protein expressed in *E. coli*, specifically detected a 53 kDa protein that was expressed from 12 to 30 hours p.i. Surprisingly, in contrast to HSV UL12, which is expressed as an early gene, we demonstrate that the BHV1 UL12 gene unambiguously belongs to viral genes of the $\gamma 2$ class since the synthesis of the protein in BHV1-infected cells was strictly inhibited in the presence of a DNA synthesis inhibitor. Further studies will be required to determine whether these kinetic differences have any functional implications.

MATERIALS AND METHODS

Cells and virus

Madin Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Strain #34 of BHV1, a field isolate of subtype 1.1, was kindly provided by Dr Mitchell (Gouvernement du Canada, Lethbridge, Alberta) and cultured in confluent monolayer sheets of MDBK cells (Simard *et al.*, 1991).

When needed, following the 90 min adsorption step of the virus to the cells, 300 µg/mL of phosphonoacetic acid (PAA), a DNA synthesis inhibitor, was added to the culture medium. At 18 hours pi, the medium was removed and cells were lysed in 1X SDS sample buffer containing 40 mM Tris-HCl pH 6.8, 2% SDS, 280 mM ß-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue.

Plasmid constructions

The BHV1 UL12 gene is located within the *Hin*d III fragment B of the BHV1 genome, in the rightward orientation, at positions 82776→84239 (Fig. 1). To facilitate its manipulation, we initially subcloned from the recombinant plasmid pKS/Bhd carrying the *Hin*d III fragment B (Simard *et al.*, 1990) the 10,592 bp *Bam* HI-*Hin*d III fragment representing the region 81488-92080 of the viral genome generating pKS/BHV81-92k/Bam-Hd. This recombinant was used to create the following plasmids that were required for different purposes:

pET-28a/UL12. This recombinant procaryotic expression vector was constructed to allow the expression in *E. coli* of a T7-Tag fusion protein representing the UL12 protein product for the development of a monospecific antiserum. For this purpose, the 1314 bp *Sac* I fragment, from pKS/BHV81-92k/Bam-Hd, encoding the amino acids 12-452 of UL12, was inserted in frame into the unique *Sac* I restriction site of the procaryotic translation vector pET-28a (Novagen, Inc.). Plasmid DNA from *E. coli* transformants obtained was analyzed by *Not* I digestion to select those in which the viral DNA was inserted in the proper orientation relative to the vector's T7 promoter (Fig. 1). To ascertain that the reading frame of the insert was properly fused with that of the vector's T7-Tag coding region, the 5'-end of the vector/insert junction of a chosen recombinant was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

pKS/BHV82-84k. This plasmid, representing 636 b of the 5' non-coding region as well as 748 b of the UL12 N-terminal coding region, was constructed to precisely map the UL12 transcription initiation site by S1 nuclease protection assays. For this purpose, plasmid pKS/BHV81-92k/Bam-Hd was digested with *Mlu* I, filled-in by treatment with the Klenow enzyme, digested again with *Bam* HI after which the DNA was fractionated by agarose gel electrophoresis to allow the purification of the generated 2036 bp fragment. After *Sal* I digestion, DNA was fractionated again for the purification of the 1389 bp *Mlu* I(blunted)-

Sal I cohesive end fragment which was finally inserted into compatibles *Eco* RV and *Sal* I restriction sites of pKS-Bluescript (Stratagene)

pSVSPORT/UL12. This recombinant eucaryotic expression vector carrying the complete UL12 coding sequence under the control of the SV40 early/late promoter was constructed for transient expression of the complete UL12 product in Cos-7 eucaryotic cells. For this cloning, we took advantage of the presence of a unique *Bsp* HI restriction site (T/CATGA) containing the ORF's initiating codon (underlined) and of a *Pst* I site located 67 bases downstream from the stop codon (Fig. 1). Hence, pKS/BHV81-92k/Bam-Hd was digested with *Bsp* HI, treated with Klenow then digested again with *Pst* I. The 1524 bp fragment generated was purified by agarose gel electrophoresis then inserted into compatible *Eco* RV and *Pst* I restriction sites of pKS, generating pKS/UL12orf. This recombinant, providing several unique restriction sites bordering the insert, was used for the unidirectional insertion of the complete UL12 coding sequence into the *Kpn* I and *Xba* I restriction sites of pSVSport (Gibco). As usual, DNA sequencing of the 5'-end of the vector/insert junction was used to ascertain the presence of the UL12 iniating codon.

Development of a UL12 specific antiserum

A UL12-specific antiserum was produced in mice immunized with the T7-Tag/UL12 fusion protein expressed in *E. coli* via the recombinant expression vector pET-28a/UL12. Details concerning the induction of *E. coli* cells, the preparation of bacterial protein lysates as well as the identification and purification of the T7-Tag fusion protein were described elsewhere (Desloges *et al.*, in press; Simard *et al.*, 1995). Mice were immunized intraperitoneally with three doses of approximately 30 µg of protein/mouse, at two weeks interval, after which immune sera were collected.

Western blotting

Western blotting of protein lysates from BHV1-infected MDBK cells with the UL12-specific antiserum was performed as previously described with the exception that a multiplicity of infection (m.o.i.) of 2 instead of 1, was used for cell infection (Desloges *et al.*, in press). The polyvinylidene difluoride (PVDF) membrane was incubated overnight at ambient temperature in 50 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) containing 1.5% BSA (TBS-BSA), reacted 2 hours at room temperature with the UL12-specific antiserum diluted 1:250 in TBS-BSA containing 0.05% Tween-20, washed several times in TBS-

Tween 0.2% and then reacted 2 hours with horseradish peroxidase-conjugated (HRP) antimouse immunoglobulin (ICN Immuno-biologicals) diluted 1:2000 in TBS-BSA-Tween 0.05%. After several washes in TBS-Tween 0.2%, immune complexes were revealed by incubation of the membrane in a freshly prepared DAB solution (7.5 mg 3,3'-diaminobenzidine tetrahydrochloride dissolved in 30 mL TBS) containing 0.03% v/v H_2O_2. The colorimetric reaction was finally stopped by soaking the membrane in water.

Transient transfections in Cos-7 cells

The recombinant eucaryotic expression vector pSVSport/UL12 (8 µg), carrying the complete UL12 coding sequence, was used to transiently transfect Cos-7 cells at 80% confluency in 60 mm petri dishes using the calcium phosphate precipitation method (Sambrook *et al.*, 1989). Seventy-two hours post-transfection, the medium was removed and the cell monolayer was lysed by adding 70 µL of 1X SDS sample buffer after which 5 µL were analyzed by Western blotting.

Northern blot hybridization

The UL12 specific transcript was identified by Northern blotting of total RNA (3 µg/slot) isolated from BHV1-infected MDBK cells collected at 0, 1, 3, 6, 9, 12, 15, 18, 21, and 24 hr postinfection (p.i.), as previously described (Simard *et al.*, 1992; Simard *et al.*, 1990). The blot was hybridized with a single strand RNA probe which was generated by *in vitro* transcription of *Cla* I linearized pKS/UL12orf plasmid DNA in the presence of T7 RNA polymerase and [α-^{32}P]dCTP.

S1 nuclease protection assays

The UL12 transcription initiation site was mapped by S1 nuclease protection assays using the conditions previously described (Simard *et al.*, 1992). For this purpose, pKS/BHV82-84k (4 µg) was digested with *Nru* I, dephosphorylated by treatment with the bacterial alkaline phosphatase, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently digested again with *Sal* I (Fig. 1). The resulting 650 bp *Nru* I–*Sal* I fragment, representing the 5' non-coding sequence of UL12, was purified by agarose gel electrophoresis and hybridized with 150 µg of total RNA extracted either from BHV1-infected (21 hours p.i.) or mock-infected MDBK cells. Following digestion with S1 nuclease, samples were analyzed in 8% polyacrylamide sequencing gels in parallel with a sequencing reaction of M13mp18.

RESULTS

Transcript identification and mRNA 5'-end mapping

Northern blotting of total RNA extracted from BHV1-infected MDBK cells collected at different times p.i., was used to examine the expression kinetics of the UL12 specific transcript. The blot was hybridized with a single strand radiolabelled RNA probe complementary to the complete UL12 coding sequence as well as to 67 b of the 3' non-coding sequence generated by *in vitro* transcription of *Cla* I linearized pKS/UL12orf plasmid DNA with T7 RNA polymerase (Fig. 1). As shown in Figure 2, the probe detected five transcripts of 4.6, 4.2, 3.7, 2.2 and 0.7 kb. The presence of the 4.6 kb band in RNA isolated from mock-infected cells (lane 0) demonstrated that it represented a cellular transcript cross-hybridizing with the probe. In contrast, the other four transcripts were found exclusively in RNA isolated from BHV1-infected cells, demonstrating that they represent viral specific mRNA. Hybridization of the probe to multiple viral transcripts suggests that these four transcripts are 3'-coterminal. The expression profile of the four transcripts were very similar, accumulating simultaneously at 6 hours p.i., after which the levels increased up to 9 hours p.i. and then remained constant up to 24 hours p.i. However, even though the expression of the four transcripts was simultaneous, their relative abundance was significantly different with transcript 2.2 > 4.2 > 3.7 > 0.7.

To determine which of the above viral transcripts originates from UL12, the 5'-end of this gene's specific transcript was mapped by S1 nuclease protection assays. For this purpose, we prepared from pKS/BHV82-84k (Fig. 1) a 650 bp *Nru* I–*Sal* I fragment representing 631 nucleotides upstream from the UL12's translation initiation codon, that was end-labeled on the complementary strand at nucleotide 19 of the UL12 coding sequence. The purified fragment was first hybridized with total RNA isolated from either BHV1-infected (21 hours p.i.) or mock-infected MDBK cells and then subjected to S1 nuclease digestion. Sequencing gel analyses of the samples revealed that no DNA fragments had been protected from S1 digestion in the sample containing only cellular RNA (Fig. 3, lane -). In contrast, two S1-resistant DNA fragments in the size range of 650 and 392 bases-long were observed in the sample containing BHV1 mRNA (Fig. 3, lane +). The size of the 650 bases-long DNA band corresponds to the size of the probe, indicating that it represented DNA which was protected from S1-digestion by one or more viral mRNA extending upstream

from the 3'-end of the assayed fragment and overlapping its whole length. Therefore, the size of this fragment most probably does not represent the position of an initiation site, but rather a region of a 3'-coterminal transcript upstream of the UL12 initiation site. The 392 bases-long DNA band most probably corresponds to DNA which was protected by the UL12 specific transcript. These findings established that the strand which is oriented to the right within the region 82140-82776 of the BHV1 genome generates at least two partially overlapping mRNA, one of these corresponding to the UL12 specific transcript. Moreover, the relative abundance of the two S1-resistant DNA bands was reproducibly very similar, suggesting that at 21 hours p.i. the overall abundance of the transcript(s) overlapping the 5'-end of the UL12 transcript is comparable to that of the transcript generated from UL12. Hence, a 392 bases-long fragment from the 650 b 5'-end-labeled probe was protected from S1 digestion by the UL12 specific transcript. This result indicated that UL12 transcription initiated 373 bases upstream from the translation initiation codon of the gene, thus corresponding to position 82384 of the viral genome. This finding, along with the fact that the nearest polyadelylation signal found downstream from the UL12 coding sequence is at 84490-84495 (Fig. 1; see below), identified the 2.2 kb long mRNA as the UL12 specific transcript.

Identification and expression kinetics of the protein encoded by UL12

To identify the viral protein encoded by UL12, a specific antiserum was generated using as antigen a recombinant fusion protein expressed in *E. coli*. IPTG induction of *E. coli* BL21(DE3) recombinant cells harbouring pET-28a/UL12 (Fig. 1), which encodes the amino acids 12-452 of UL12 fused in frame to a T7-Tag peptide encoded by pET28a, expressed an abundant 54 kDa recombinant protein found in the insoluble fractions of cell lysates (results not shown). In contrast, no protein of this size was observed in lysates from BL21(DE3) cells harbouring only the vector (not shown). The observed molecular mass of the recombinant polypeptide was within the size range expected (52,553 Da) for the fusion protein. To ascertain the identity of the 54 kDa protein, Western blotting analyses of extracts from IPTG-induced and non-induced cells were performed using a commercially available anti-T7-Tag monoclonal antibody (MAb). The anti-T7-Tag MAb specifically detected an abundant 54 kDa protein found only in the IPTG-induced samples from cells harbouring pET-28a/UL12, but not pET-28a (results not shown). These results indicated

that the 54 kDa polypeptide included the T7-Tag N-terminal peptide encoded by the translation vector, thus confirming the identity of the T7-Tag/UL12 recombinant protein. The protein was purified by SDS-PAGE and used to immunize mice to generate a UL12 specific antiserum. In contrast to the preimmune serum, the immune serum specifically recognized a 54 kDa protein in Western blots of extracts from IPTG-induced *E. coli* cells harbouring pET-28a/UL12 but not pET-28a (not shown). The specificity of the antiserum was further ascertained by Western blotting of Cos-7 cell lysates transiently transfected with the recombinant eucaryotic expression vector pSVSport/UL12 containing the complete UL12 coding sequence. The UL12 antiserum reproducibly detected a major 53 kDa protein and a minor 30 kDa polypeptide in lysates from Cos-7 cells transfected with pSVSport/UL12 (Fig. 4A, lane +), whereas no polypeptide was detected in lysates transfected with pSVSport (not shown). The 53 kDa protein undoubtedly represented the product of UL12 as its size correlated very well with that predicted (51,844 Da). For its part, the 30 kDa protein most probably represented a truncated product of the UL12 mature protein.

Western blotting of BHV1-infected MDBK protein cell lysates obtained after different periods of infection was used to investigate the expression kinetics of the protein encoded by UL12. The UL12 antiserum specifically detected a 53 kDa polypeptide in lysates from cells infected for 12 until 30 hours with BHV1 (Fig. 4 A, lanes 12-30) but not from mock-infected cells (lane 0). This expression profile was distinct from that observed at the transcriptional level. Detection of the protein was delayed to 12 hours p.i. in contrast to the transcript which could be detected as early as 6 hours. Interestingly, the protein synthesized in BHV1-infected MDBK cells was indistinguishable from that observed following transient transfection of Cos-7 cells with pSVSport/UL12 (lane +). The observed molecular mass of the protein correlated well with that predicted from UL12 amino acid sequence (51,844 Da).

The expression kinetics of the transcript and protein generated by UL12 correlated with that of viral genes of the late γ class. This was further ascertained by examining whether protein expression was dependent on viral DNA synthesis. Western blot analyses of infected cell protein lysates derived from cells infected in the presence or absence of the DNA synthesis inhibitor phosphonoacetic acid (PAA) and collected at 18 hours p.i were performed. The

UL12-specific antiserum failed to detect the UL12 protein in infected PAA-treated cell lysates (Fig. 4B). Similarly, expression of the BHV1 alpha gene trans-inducing factor protein encoded by the UL48 gene, a late gene (Misra *et al.*, 1994; unpublished results), was completely abrogated by PAA treatment. In contrast, the level of expression of the 50 kDa protein encoded by UL54, an early gene (Chalifour *et al.*, 1996; Singh *et al.*, 1996), was unaffected by PAA inhibition of DNA synthesis. These results thus demonstrate that the UL12 gene belongs to the kinetic class of BHV1 late genes.

DISCUSSION

In this study, we characterized the expression kinetics of both the transcript and protein generated by the BHV1 UL12 gene which was previously shown to be functionally expressed in *E. coli* as a 57 kDa protein exhibiting both endo- and exo-nuclease activities (Chung and Hsu, 1996; Chung and Hsu, 1997). The BHV1 UL12 coding sequence is located at positions 82776→84239 in the rightward orientation of the viral genome (Fig. 1). The two proximal left hand side ORFs, located at positions 81301→82779 and 80718→81386, are homologous to the HSV1 UL13 and UL14 genes respectively. Downstream from UL12, at positions 84191→84460, is found an ORF which is homologous to the HSV1 UL11. As with their HSV1 counterparts, ORFs UL11 to UL14 are arranged in tandem in the unique long region of the BHV1 genome and all four are oriented in the same direction in the two viral genomes, considering that the HSV1 prototype genome is conventionally inversed relative to that of BHV1 (Davison and Wilkie, 1983; McGeoch *et al.*, 1988). The search of TATA and CAAT elements in the region comprising 4000 nucleotides upstream of the UL12 initiation codon revealed the presence of a single consensus TATA box at 80358-80364 and two consensus CAAT boxes at 81327-81335 and 81630-81638. The distance separating these elements from the UL12 coding sequence makes it very unlikely that they serve as the promoter for the gene. Downstream from the stop codon of UL12, the nearest polyadenylation signal (AATAAA) found on the rightward oriented strand of the genome is at positions 84490-84495. Upstream from this element, the next consensus polyadenylation signal found on the same DNA strand is at positions 79577-79582.

We showed that a single strand RNA probe, complementary to the UL12 coding sequence, specifically detected four viral mRNA of 4.2, 3.7, 2.2, and 0.7 kb on a Northern blot of RNA isolated from BHV1-infected cells. We postulate that these transcripts are 3'-coterminal. This assumption is supported by the observation that approximately half the amount of a 650 base-long probe spanning 631 bases upstream of the UL12 initiation codon was protected from S1 nuclease digestion by RNA isolated from BHV1-infected cells. Furthermore, sequence analyses of the genomic region surrounding UL12 allow us to predict that the BHV1 UL14, UL13, UL12, and UL11 terminate their transcription near the polyadenylation signal located at positions 84490-84495 of the viral genome to generate 3'-coterminal transcripts in the size range of 4.0, 3.4, 1.9, and 0.5 kb (each including a polyA chain of 200 residues). Our results corroborated this assumption and we thus conclude that the four transcripts detected by the UL12 specific probe represent 3'-coterminal transcripts. Similarly, the corresponding regions of the HSV (Costa *et al.*, 1983; Draper *et al.*, 1986; McGeoch *et al.*, 1986) and pseudorabies (PrV; Hsiang *et al.*, 1996) genomes generate 3'-coterminal transcripts. The four BHV1 transcripts accumulated simultaneously at 6 hours p.i., after which their levels increased up to 9 hours p.i. and then remained constant up to 24 hours p.i.

S1 nuclease protection assays demonstrated that UL12 transcription initiated 373 b upstream from the UL12 start codon, corresponding to position 82384 of the viral genome. Transcription of UL12 from this start site to the polyadenylation signal located downstream should thus yield a transcript in the range of 2.1 kb, excluding the polyA tail. This unambiguously established that the 2.2 kb mRNA detected by Northern blotting indeed represents the transcript generated by UL12. Similarly to the PrV UL12 gene (Dijkstra *et al.*, 1997), but in contrast to that of HSV1 (McGeoch *et al.*, 1986), no typical promoter sequences were found upstream from the BHV1 UL12 cap site.

Surprisingly, earlier transcriptional analyses of the BHV1 genome did not allow the detection of the four transcripts identified in the present report, when a probe representing the *Hin*d III fragment B of the viral genome which contains the UL12 gene (see Fig. 1) was used in Northern blot analyses (Seal *et al.*, 1991; Wirth *et al.*, 1989). Only a 2.2 kb late transcript whose synthesis was completely inhibited in the presence of the DNA synthesis

inhibitor PAA, was identified by Seal *et al.* (1991). It thus appears from the present study that this 2.2 kb late transcript corresponds to that of UL12.

We also showed that the BHV1 UL12 gene generates a 53 kDa protein whose size correlated well with that predicted from the UL12 derived amino acid sequence (51,844 Da), similarly to what was found for PrV UL12 (Hsiang *et al.*, 1996). In contrast, the apparent size of the HSV1 UL12 protein in SDS-PAGE (85 kDa) was found to be significantly different from that expected (67,500 Da; McGeoch *et al.*, 1986), but this discrepancy was attributed to the high proline content of the protein (Draper *et al.*, 1986). The BHV1 UL12 protein accumulated in cells at 12 hours p.i., its levels peaked at 18 hours and then decreased gradually until 30 hours p.i. Interestingly, even though the accumulation of the UL12 transcript was observed as early as 6 hours p.i., that of its product was delayed until 12 hours p.i. This may suggest that UL12 is regulated at the translational level. Moreover, it is noteworthy that the abundance of the transcript remained constant from 9 to 24 hours p.i., whereas that of the protein increased from 12 to 18 hours p.i. and then decreased until 30 hours p.i.. This may suggest that UL12 is post-translationally regulated.

Finally, we demonstrated that the expression of the polypeptide was dependent on viral DNA synthesis since the protein was undetectable in infected-cells maintained in the presence of PAA. This clearly established that the BHV1 UL12 gene belonged to viral genes of the γ2 class. These findings are strikingly different from HSV1 (Banks *et al.*, 1985) and PrV (Hsiang *et al.*, 1996) counterpart genes which were shown to belong to the early (β) class. It will thus be of great interest to determine whether these expression kinetic differences have functional implications.

ACKNOWLEDGMENTS

The authors sincerely thank Dr. Johnny Basso for critically reading the manuscript. This work was funded by Natural Sciences and Engineering Research Council of Canada (NSERC). N.D. was supported by NSERC and by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

REFERENCES

Banks, L., Purifoy, D.J.M., Hurst, P.-F., Killington, R.A. and Powell, K.L. (1983). Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. J. Gen. Virol. 64: 2249-2260.

Banks, L.M., Halliburton, I.W., Purifoy, D.J.M., Killington, R.A. and Powell, K.L. (1985). Studies on the herpes simplex virus alkaline nuclease: detection of type-common and type-specific epitopes on the enzyme. J. Gen. Virol. 66: 1-14.

Bronstein, J.C. and Weber, P.C. (1996). Purification and characterization of herpes simplex virus type 1 alkaline exonuclease expressed in *Escherichia coli*. J. Virol. 70: 2008-2013.

Chalifour, A., Basso, J., Gagnon, N., Trudel, M. and Simard, C. (1996). Transcriptional and translational expression kinetics of the early gene encoding the BICP27 protein of bovine herpesvirus type 1. Virology 224: 326-329.

Chung, Y.-T. and Hsu, W. (1996). Functional expression of the bovine herpesvirus 1 alkaline deoxyribonuclease (UL12) in *Escherichia coli*. Arch. Virol. 141: 2457-2464.

Chung, Y.-T. and Hsu, W. (1997). Purification of the infectious bovine rhinotracheitis virus alkaline deoxyribonuclease expressed in *Escherichia coli*. J. Vet. Med. Sc. 59: 35-38.

Costa, R.H., Draper, K.G., Banks, L., Powell, K.L., Cohen, G., Eisenberg, R. and Wagner, E.K. (1983). High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-Dalton protein tentatively identified as a capsid protein. J. Virol. 48: 591-603.

Davison, A.J. and Wilkie, N.M. (1983). Location and orientation of homologous sequences in the genome of five herpesviruses. J. Gen. Virol. 64: 1927-1942.

Desloges, N., Boucher, H. and Simard, C. Transcriptional and Translational Expression Kinetics of the UL25 Homologue of Bovine Herpesvirus 1.1. Arch. Virol. (in press).

Dijkstra, J.M., Fuchs, W., Mettenleiter, T.C. and Klupp, B.G. (1997). Identification and transcriptional analysis of pseudorabies virus UL6 to UL12 genes. Arch. Virol. 142: 17-35.

Draper, K.G., Devi-Rao, G., Costa, R.H., Blair, E.D., Thompson, R.L. and Wagner, E.K. (1986). Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. 57: 1023-1036.

Farley, J.E., Skare, I.B. and Skare, J. (1981). Inverted repeat sequences in infectious bovine rhinotracheitis virus DNA. In: Nahmias, A.J., Dowdle, W.R. and Schinazi, R.F. (eds) The human herpes viruses: an interdisciplinary perspective. Elsevier/North Holland Publishing New York, p 590.

Goldstein, J.N. and Weller, S.K. (1998a). In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. J. Virol. 72: 8772-8781.

Goldstein, J.N. and Weller, S.K. (1998b). The exonuclease activity of HSV-1 UL12 is required for *in vivo* function. Virology 244: 442-457.

Henderson, J.O., Ball-Goodrich, L.J. and Parris, D.S. (1998). Structure-fonction analysis of the herpes simplex virus type 1 UL12 gene: correlation of deoxyribonuclease activity *in vitro* with replication function. Virology 243: 247-259.

Hoffmann, P.J. and Cheng, Y.-C. (1979). DNase induced after infection of KB cells by herpes simplex virus type 1 or type 2. II. Characterization of an associated endonuclease activity. J. Virol. 32: 449-457.

Hsiang, C.-Y., Ho, T.-Y. and Chang, T.-J. (1996). Identification of a pseudorabies virus UL12 (deoxyribonuclease) gene. Gene 177: 109-113.

Martinez, R., Sarisky, R.T., Weber, P.C. and Weller, S.K. (1996a). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J. Virol. 70: 2075-2085.

Martinez, R., Shao, L., Bronstein, J.C., Weber, P.C. and Weller, S.K. (1996b). The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. Virology 215: 152-164.

Mayfield, J.E., Good, P.J., Vanoort, H.J., Campbell, A.R. and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus (Cooper strain). J. Virol. 47: 259-264.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69: 1531-1574.

McGeoch, D.J., Dolan, A. and Frame, M.C. (1986). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. Nucleic Acids Res. 14: 3435-3448.

Misra, V., Bratanich, A.C., Carpenter, D. and Ohare, P. (1994). Protein and DNA elements involved in transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. J. Virol. 68: 4898-4909.

Roizman, B. (1996). Herpesviridae. In: Fields, B.N., Knipe, D.M., Howley, P.M. (eds) Fields Virology, 3rd edn, Lippincott-Raven publishers, Philadelphia, New York, pp 2221-2230.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, a laboratory manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sc. USA 74: 5463-5467.

Seal, B.S., Irving, J.M. and Whetstone, C.A. (1991). Transcriptional analysis of bovine herpesvirus 1 Cooper isolate. Temporal analysis and characterization of immediate-early, early, and late RNA. Arch. Virol. 121: 55-73.

Shao, L., Rapp, L.M. and Weller, S.K. (1993). Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology 196: 146-162.

Simard, C., Bastien, N. and Trudel, M. (1992). Sequencing and 5'-and 3'-end transcript mapping of the gene encoding the small subunit of ribonucleotide reductase from bovine herpesvirus type-1. Virology 190: 689-701.

Simard, C., LaBoissière, S., Séguin, C. and Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: a major variant selected from a field isolate. Intervirol. 32: 117-126.

Simard, C., Nadon, F., Séguin, C., LaBoissière, S. and Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch. Virol. 110: 63-75.

Simard, C., Langlois, I., Styger, D., Vogt, B., Vlcek, C., Chalifour, A., Trudel, M. and Schwyzer, M. (1995). Sequence analysis of the UL39, UL38, and UL37 homologues of bovine herpesvirus 1 and expression studies of UL40 and UL39, the subunits of ribonucleotide reductase. Virology 212: 734-740.

Singh, M., Fraefel, C., Bello, L.J., Lawrence, W.C. and Schwyzer, M. (1996). Identification and characterization of BICP27, an early protein of bovine herpesvirus 1 which may stimulate mRNA 3' processing. J. Gen. Virol. 77: 615-625.

Strobel-Fidler, M. and Francke, B. (1980). Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. Virology 103: 493-501.

Weller, S.K., Seghatoleslami, M.R., Shao, L., Rowse, D. and Carmichael, E.P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a *lacZ* insertion mutant. J. Gen. Virol. 71: 2941-2952.

Wirth, U.V., Gunkel, K., Engels, M. and Schwyzer, M. (1989). Spatial and temporal distribution of bovine herpesvirus 1 transcripts. J. Virol. 63: 4882-4889.

Wyler, R., Engels, M. and Schwyzer, M. (1989). Infectious bovine rhinotracheitis/vulvovaginitis (BHV1). In: Wittmann, G. (ed) Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic Publishers, Boston/Dordrecht/London, pp 1-72.

Figure 1. Subcloning strategies of BHV1 UL12.

The *Hin*d III restriction map of the 135,301 bp BHV1 genome is illustrated at the top. The UL12 gene is located within the fragment B at positions 82776→84239 in the rightward orientation. The 10,592 bp *Bam* HI-*Hin*d III fragment shown in an expanded view, was first subcloned from pKS/Bhd (Simard *et al.*, 1990) to generate pKS/BHV81-92k/Bam-Hd. The open arrow (at 82384) identifies the transcription initiation site of UL12. The polyadenylation site (AATAAA) is indicated. Restriction sites which are pertinent to this study are illustrated. pKS/BHV81-92k/Bam-Hd was used as described to create the other plasmids illustrated for the purposes indicated.

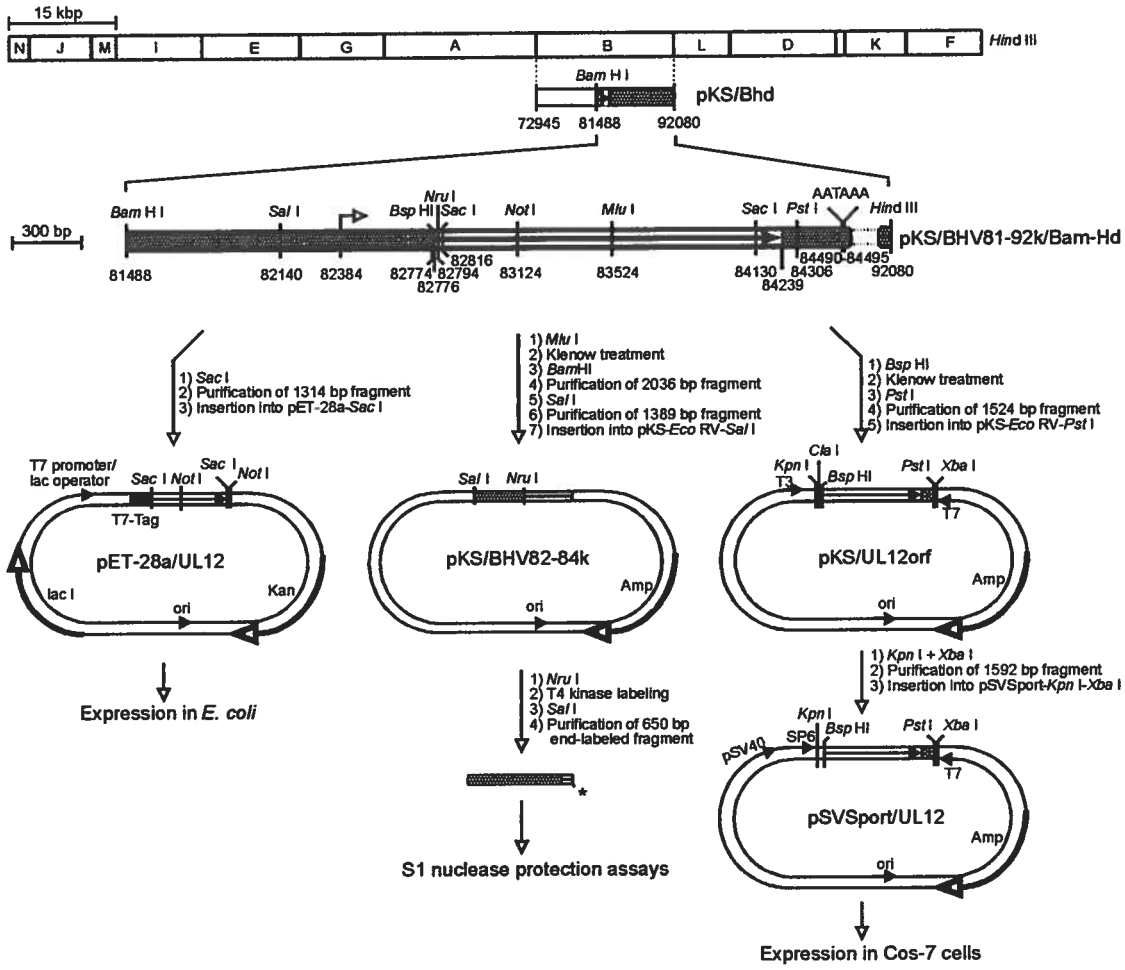

15 kbp
N J M I E G A B L D K F
Hind III
Bam H I
pKS/Bhd
72945 81488 92080
300 bp
Bam H I
Sal I
Nru I
Bsp HI Sac I
Not I
Mlu I
Sac I
Pst I
AATAAA
Hind III
pKS/BHV81-92k/Bam-Hd
81488 82140 82384 82774 82794 82776 82816 83124 83524 84130 84306 84239 84490-84495 92080
1) Sac I
2) Purification of 1314 bp fragment
3) Insertion into pET-28a-Sac I
1) Mlu I
2) Klenow treatment
3) BamHI
4) Purification of 2036 bp fragment
5) Sal I
6) Purification of 1389 bp fragment
7) Insertion into pKS-Eco RV-Sal I
1) Bsp HI
2) Klenow treatment
3) Pst I
4) Purification of 1524 bp fragment
5) Insertion into pKS-Eco RV-Pst I
T7 promoter/ lac operator
Sac I Not I
Sac I Not I
T7-Tag
pET-28a/UL12
lac I
Kan
ori
Sal I Nru I
pKS/BHV82-84k
Amp
ori
Cla I
Kpn I
T3
Bsp HI
Pst I Xba I
T7
pKS/UL12orf
Amp
ori
Expression in E. coli
1) Nru I
2) T4 kinase labeling
3) Sal I
4) Purification of 650 bp end-labeled fragment
S1 nuclease protection assays
1) Kpn I + Xba I
2) Purification of 1592 bp fragment
3) Insertion into pSVSport-Kpn I-Xba I
Kpn I
pSV40
SP6
Bsp HI
Pst I Xba I
T7
pSVSport/UL12
Amp
ori
Expression in Cos-7 cells

Figure 2. Transcriptional kinetics of UL12.

A Northern blot of total RNA (3 μg/slot) extracted from BHV1-infected MDBK cells at 0, 1, 3, 6, 9, 12, 15, 18, 21, and 24 hr postinfection was hybridized with a radiolabeled single strand RNA probe complementary to the UL12 coding sequence which was generated by *in vitro* transcription of *Cla* I linearized pKS/UL12orf plasmid DNA with T7 RNA polymerase (Fig. 1). Sizes in kb of RNA standards are indicated.

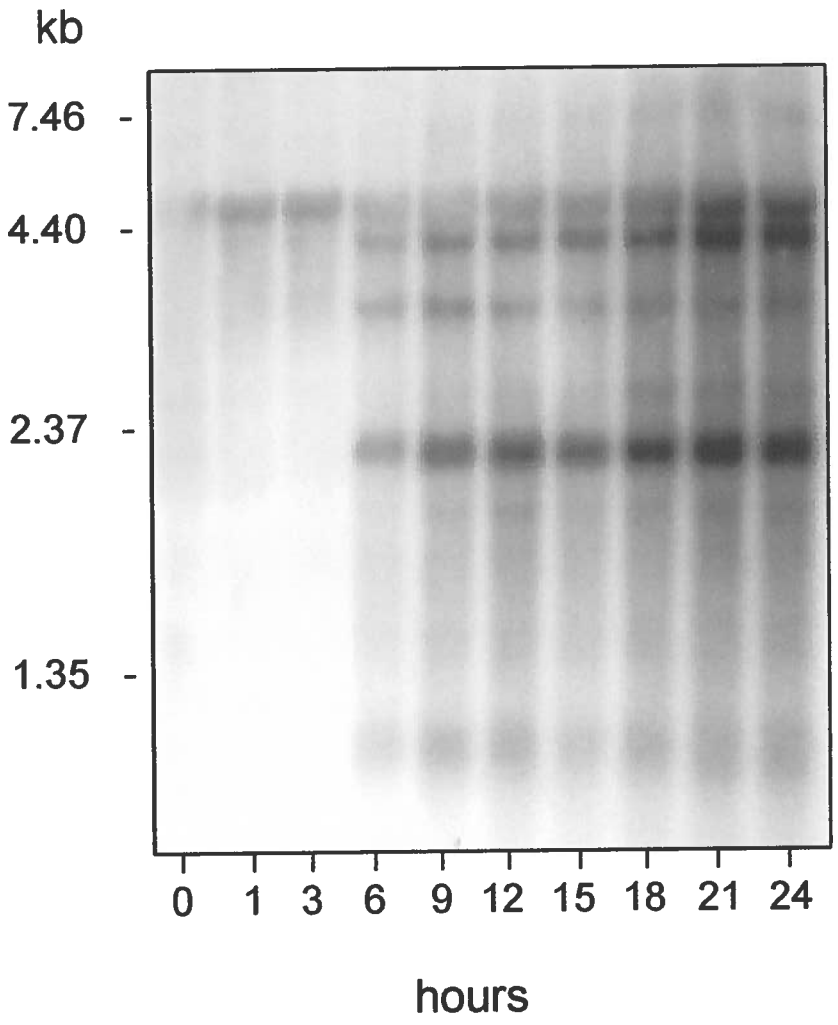

kb
7.46
4.40
2.37
1.35
0
1
3
6
9
12
15
18
21
24
hours

Figure 3. 5'-end mapping of the UL12's transcript.

A 650 bp *Nru* I–*Sal* I fragment representing 631 nucleotides upstream from the UL12 initiation codon, end-labeled on the complementary strand at nucleotide 19 of the UL12 coding sequence, was hybridized with total RNA isolated from either BHV1-infected (lane +) or mock-infected MDBK cells (lane -) and then digested with S1 nuclease. The samples were analyzed on sequencing gels in parallel with a dideoxy sequencing reaction of M13mp18 (lanes G, A, T, C).

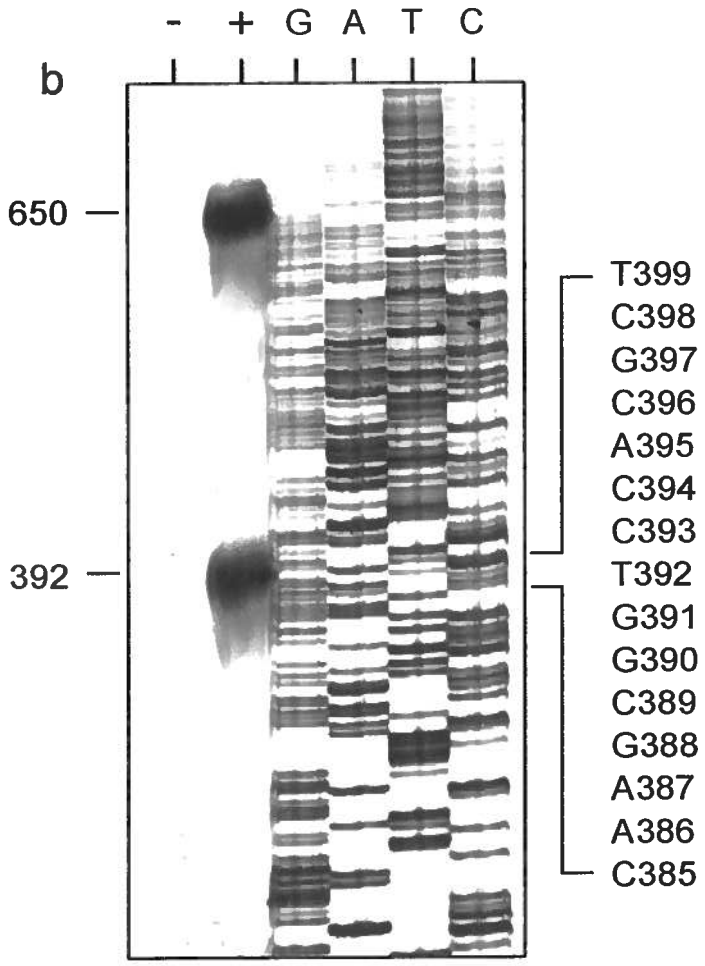
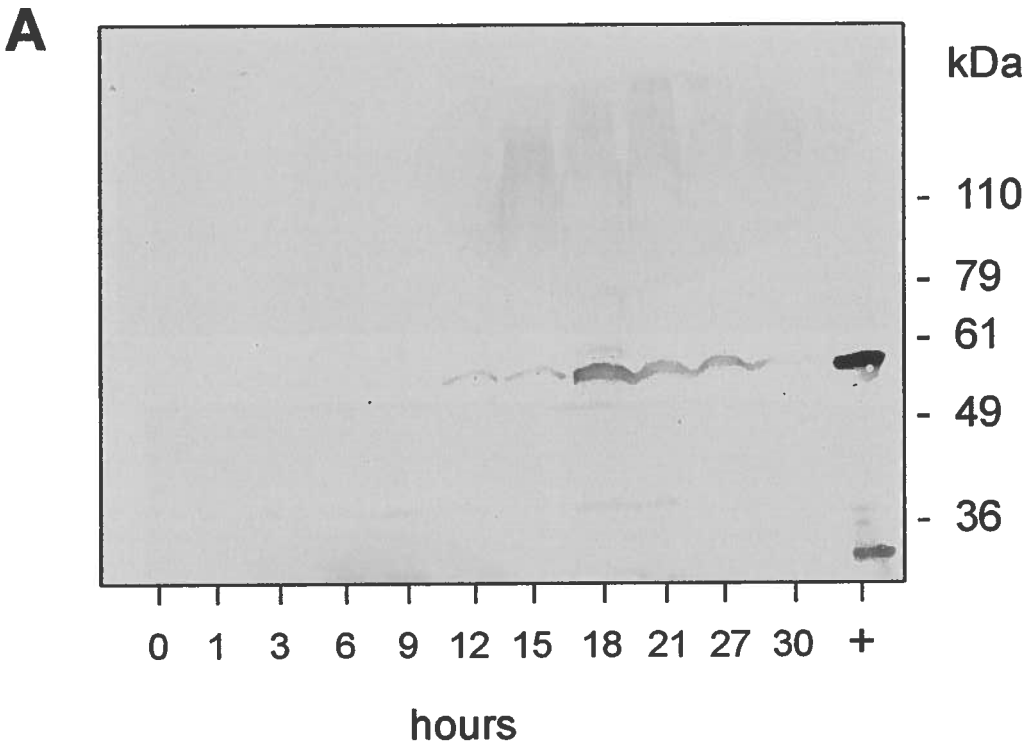

b
-
+
G
A
T
C
650
392
T399
C398
G397
C396
A395
C394
C393
T392
G391
G390
C389
G388
A387
A386
C385

Figure 4. Translational kinetics of UL12.

A) Confluent monolayers of MDBK cells were infected with BHV1 at an m.o.i. of 2 for 0, 1, 3, 6, 9, 12, 15, 18, 21, 27, and 30 hours. Protein cell lysates were fractionated by SDS-PAGE in parallel with a Cos-7 cell lysate collected 72 hours following transient transfection with pSVSport/UL12 (lane +). Fractionated proteins were electrotransferred onto a PVDF membrane and then reacted with the UL12-specific antiserum. Sizes in kDa of standards are indicated.

B) MDBK cells infected with BHV1 at an m.o.i. of 2 were incubated for 18 hours in the presence (lanes +) or the absence (lanes -) of 300 μg of phosphonoacetic acid (PAA) per mL of culture medium. Western blots of protein cell lysates, prepared as above, were reacted with monospecific antisera either directed against the products of the UL54 (Chalifour *et al.*, 1996), UL48 or UL12 genes.


A
kDa
110
79
61
49
36
0 1 3 6 9 12 15 18 21 27 30 +
hours
PAA: - + - + - +
B
Serum: UL54 UL48 UL12

Transcriptional and Translational Expression Kinetics of the UL25 Homologue of Bovine Herpesvirus 1.1.

Running title: Bovine herpesvirus 1.1 UL25 gene

Nathalie Desloges, Hélène Boucher, and Claire Simard*

INRS-Institut Armand-Frappier
Centre de microbiologie et biotechnologie
531 Bd des Prairies
Laval-des-Rapides, Québec, Canada, H7V 1B7
Tel. (450) 687-5010 ext 4637
Fax. (450) 686-5626
Email: Claire.Simard@inrs-iaf.uquebec.ca

***Archives of Virology*, 2001; 146(9):1693-1704**

* Corresponding author

Cinétiques d'expression transcriptionnelle et traductionnelle de l'homologue UL25 du virus de l'herpès bovin 1.1.

Nous avons examiné si l'ORF UL25 du virus de l'herpès bovin 1.1 (BHV1), un homologue du gène UL25 du virus de l'herpès simplex 1 (HSV1), représente un gène fonctionnel. L'ORF UL25 du BHV1, localisé aux positions 60602←62398 du génome viral, génère un transcrit de 4.5 kb qui commence à s'accumuler aussitôt que 3 heures post-infection (p.i.), après quoi les niveaux augmentent jusqu'à 12 heures p.i. et restent constants jusqu'à 24 heures p.i.. La transcription s'initie 303 bases en amont du codon d'initiation de la traduction, correspondant à 26 et 354 nucléotides en aval respectivement de boîtes TATA et CAAT potentielles. L'identification de ces éléments aux positions désignées suggère qu'ils font partie du promoteur du gène UL25. L'immunobuvardage de type Western de lysats de cellules infectées par le BHV1, à l'aide d'un antisérum spécifique à l'UL25 du BHV1 généré contre une protéine recombinante de fusion exprimée chez *E. coli*, a permis la détection d'une protéine de 63 kDa dont la grosseur correspond à celle attendue (63,083 Da) et dont le profil d'expression suit celui du transcrit. La synthèse de la protéine de 63 kDa a été inhibée par un inhibiteur de synthèse d'ADN, démontrant clairement que la protéine virale spécifique encodée par l'ORF UL25 du BHV1 appartient à la classe $\gamma 2$.

SUMMARY

We investigated whether the bovine herpesvirus 1.1 (BHV1) ORF UL25, a homologue of the herpes simplex virus 1 (HSV1) UL25 gene, represented a functional gene. The BHV1 UL25 ORF, which is located at positions 60602←62398 of the viral genome, generated a 4.5 kb transcript accumulating at low abundance as soon as 3 hours p.i., after which the levels increased up to 12 hours p.i. and remained constant up to 24 hours p.i. In addition, UL25 transcription initiated at 303 bases upstream from the translation initiation codon, corresponding to 26 and 354 b downstream from putative TATA and CAAT boxes, respectively, thus providing evidence that these elements function as the UL25 promoter. Western blotting of BHV1-infected cell lysates, using a BHV1-UL25 specific antiserum generated against a T7-Tag/UL25 fusion recombinant protein expressed in *E. coli,* detected a 63 kDa protein of the expected size (63,083 Da) whose expression profile followed that of its transcript. The synthesis of the 63 kDa protein was abrogated by a DNA synthesis inhibitor, unambiguously demonstrating that the viral specific protein expressed from the BHV1 UL25 ORF belongs to the γ2 class.

INTRODUCTION

Bovine herpesvirus 1.1 (BHV1), a member of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*, is an important pathogen of cattle causing severe respiratory tract diseases and genital illnesses that result in substantial economic losses each year (Kit, 1989; Tikoo *et al.*, 1985). The viral genome is a 135,301 bp double stranded linear DNA composed of a unique long (UL) and a unique short (US) segments, the latter being flanked by inverted repeats (Wyler *et al.*, 1989). Two isomeric forms of the genome occur naturally as the US can invert its orientation relative to the UL (Farley *et al.*, 1981; Mayfield *et al.*, 1983).

The recently completed characterization of the BHV1 genome suggests the presence of more than 70 different open reading frames (ORFs), conventionally designated according to the herpes simplex virus 1 (HSV1) nomenclature (Roizman and Sears, 1996), of which approximately 50 have never been further investigated. The possible functions of these putative genes were only assessed by amino acid sequence homology to previously identified gene's products of other alphaherpesviruses. For instance, based on HSV1 findings (McNab *et al.*, 1998), the BHV1 ORF, which is homologous to the HSV1 UL25 gene, is thought to be required for packaging of viral DNA within virion capsids but not for cleavage of replicated viral DNA.

Ali *et al.* (1996) identified a 60 kDa protein as the product of the HSV1 UL25 gene and concluded that it was expressed as a late (γ) or a leaky late (γ1) viral protein, based on the observation that its accumulation in HSV1 infected cells cultured in the presence of phosphonoacetic acid, an inhibitor of DNA synthesis, was slightly reduced. In pseudorabies virus (PrV), the corresponding UL25 gene encodes a 57 kDa protein which is expressed with truly late kinetics as its synthesis was strictly inhibited in the absence of viral DNA replication (Kaelin *et al.*, 2000).

Being interested in defining the contribution of the BHV1 UL25 ORF towards viral replication and pathogenesis, we initially examined whether it represents a functional gene by identifying its putative transcript and protein product. The BHV1 UL25 ORF, encoding a 598 aa-long polypeptide with a calculated molecular mass of 63,083, is located at positions 60602←62398 in the leftward orientation of the prototype viral genome. Our results show that the BHV1 UL25 ORF generates a 4.5 kb transcript and a 63 kDa protein

accumulating at low levels as soon as 3 and 6 hrs postinfection (p.i.), respectively, after which the levels increased up to 12 hours p.i. and then remained constant up to 24 hours p.i. We also demonstrate that synthesis of the protein was strictly inhibited in the presence of a DNA synthesis inhibitor. Finally, S1 nuclease protection assays allowed us to determine that transcription initiates 303 bases upstream from the translation initiation codon, corresponding to position 62701 of the viral genome. This finding provides evidence that the putative TATA and CAAT boxes located 26 and 354 nucleotides upstream from the transcription initiation site, respectively, most probably act as the UL25 promoter. We conclude that the BHV1 UL25 ORF represents a functional gene whose expression kinetics correlate with that of a late (γ) gene.

MATERIALS AND METHODS

Cells and virus

Madin Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Strain #34 of BHV1, a field isolate of subtype 1.1, was kindly provided by Dr Mitchell (Gouvernement du Canada, Lethbridge, Alberta) and cultured in confluent monolayer sheets of MDBK cells (Simard *et al.*, 1991). When needed, following the 90 min adsorption step of the virus to the cells, 300 μg/mL of phosphonoacetic acid (PAA), a DNA synthesis inhibitor, were added to the culture medium (Ali *et al.*, 1996).

Plasmid constructions

The BHV1 UL25 ORF is located within the large (21,234 bp) *Hin*d III fragment A of the BHV1 genome, in the leftward orientation, at positions 60602←62398 (Fig. 1). The complete ORF is contained within a 4,299 bp fragment bordered by two *Apa* I and a single *Bam* HI restriction sites (Fig. 1). To clone this *Apa* I-*Bam* HI fragment, we first subcloned the two 11,409 and 9,825 bp *Hin*d III-*Cla* I fragments from pKS/Ahd (Simard *et al.*, 1990), generating pKS/BHV51-61k/Hd-Cla and pKS/BHV61-72k/Hd-Cla. Then, the purified 2,619 bp *Apa* I-*Cla* I fragment from pKS/BHV51-61k/Hd-Cla was ligated with the purified 1,680 bp *Cla* I-*Bam* HI fragment from pKS/BHV61-72k/Hd-Cla, in the presence of *Apa* I-*Bam* HI digested pKS vector (Stratagene), generating pKS/BHV58-63k/Apa-Bam. This

recombinant was used to create three additional plasmids that were required for different purposes.

First, to allow the development of a monospecific antiserum, the 924 bp *Sal* I fragment, encoding the amino acids 86-395 of the UL25 ORF, was expressed in *E. coli* as a T7-Tag fusion protein following the in frame insertion of the DNA fragment into the unique *Sal* I restriction site of the procaryotic translation vector pET-28b (Novagen, Inc.). Plasmid DNA from some of the *E. coli* transformants obtained was analyzed by *Cla* I digestion to select those in which the viral DNA was inserted in the proper orientation relative to the vector's T7 promoter. Moreover, in order to ascertain that the reading frame of the insert was properly fused with that of the vector's T7-Tag coding region, the 5' end of the vector/insert junction in an appropriate recombinant was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). This recombinant was identified pET-28b/UL25.

Second, the 1,860 bp *Nco* I fragment, representing the complete coding sequence of the UL25 ORF and where the initiating codon was included within the *Nco* I site (CCATGG) located at the ORF's 5'end, was purified from pKS/BHV58-63k/Apa-Bam, filled-in by treatment with the Klenow enzyme, then inserted into the unique *Sma* I restriction site of the eucaryotic expression vector pSVSport (Gibco/BRL); this strategy would thus allow the regeneration of the two *Nco* I sites. *E. coli* transformants carrying recombinant DNA in which the insert was in the proper orientation relative to the SV40 early promoter of the vector were selected as above by analyzing asymmetrical digests of purified plasmid DNA. In addition, the preservation of the UL25's initiating codon in a chosen pSVSport/UL25 recombinant was initially confirmed by demonstrating the presence of the two regenerated *Nco* I sites and then, by DNA sequencing.

Finally, pKS/BHV58-63k/Apa-Bam was modified by eliminating the *Nco* I fragment representing the UL25 coding sequence to engineer pKS/BHV58-63KΔUL25. This recombinant, representing the 5' non-coding region of the UL25 ORF as well as the first 4 bases of the coding sequence (ATGG), was used in S1 nuclease protection assays to precisely map the putative UL25's transcription initiation site.

Development of a UL25 specific antiserum

To identify the protein putatively encoded by the UL25 ORF, we set out to generate a UL25-specific antiserum using as an antigen a T7-Tag/UL25 fusion protein expressed in

E. coli via the recombinant expression vector pET-28b/UL25. Exponential cultures of *E. coli* BL21(DE3) cells, harboring either pET-28b or pET-28b/UL25 plasmid DNA, were induced with 0.4 mM IPTG. Cells from aliquots collected at 0, 1, 2, 3, or 4 hr post-induction were recovered by centrifugation, resuspended in 0.1 volume of TEN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl) containing 1 mg/mL lysozyme, and sonicated. Lysates were centrifuged and the protein contents of both the soluble and insoluble (inclusion bodies) fractions were analyzed by SDS-PAGE to identify the protein of interest. Following preparative SDS-PAGE, the band corresponding to the T7-Tag/UL25 fusion protein was isolated and finely ground in sterile phosphate buffer saline (PBS) by several and successive passages through lower gauge needles. Aliquots of the resulting acrylamide/protein suspension were empirically quantified by comparing the intensity of the Coomassie blue stained bands observed with that of known quantities of bovine serum albumin fractionated by SDS-PAGE. The slurry was used to immunize mice intraperitoneally. Mice were given three doses of approximately 30 µg of protein/mouse, at two weeks interval, after which immune sera were collected.

Western blotting

Confluent monolayers of MDBK cells (46.5 X 10^6 total cells) in T150 flasks were infected with BHV1, using an m.o.i. of 1, for 0, 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 hr. Cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free) then scraped in ice-cold PBS. After centrifugation at 4 °C, the cell pellet was resuspended in 1.0 mL PBS containing 1 mM phenylmethylsulfonyl fluoride, quickly freezed in liquid nitrogen then conserved à -70 °C. After thawing on ice, 100 µL of cell lysates were added to 25 µL of 5X SDS sample buffer (1X = 40 mM Tris-HCl pH 6.8, 2% SDS, 280 mM ß-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). The resulting mixture was boiled for 3 min, then 25 µL were analyzed by SDS-PAGE. Fractionated proteins were electrotransferred onto a PVDF membrane (Bio-Rad) after which the membrane was incubated overnight at ambiant temperature in 50 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) containing 1.5% BSA, reacted 2 hours at room temperature with the UL25-specific antiserum diluted 1:500 in TBS containing 0.05% Tween-20, washed several times in TBS-Tween 0.2% and then reacted 2 hours with horse radish peroxidase-conjugated (HRP) antimouse immunoglobulin (ICN Immuno-biologicals) diluted 1:2000 in TBS-Tween 0.05%. After several washes in

TBS-Tween 0.2%, immune complexes were revealed by incubation of the membrane in a freshly prepared DAB solution (7.5 mg 3,3'-diaminobenzidine tetrahydrochloride dissolved in 30 mL TBS) containing 0.03% v/v H_2O_2. The colorimetric reaction was finally stopped by soaking the membrane in water.

Transient transfections in Cos-7 cells

The recombinant eucaryotic expression vector pSVSport-UL25 (10 µg), encoding the complete UL25 ORF, was used to transiently transfect Cos-7 cells at 80% confluency in 60 mm petri dishes, using the calcium phosphate precipitation method (Sambrook *et al.*, 1989). Seventy-two hours post-transfection, the medium was removed and the cell monolayer was lysed by adding 70 µL of 1X SDS sample buffer after which 15 µL were analyzed by Western blotting.

Northern blot hybridization

The putative transcript of the UL25 ORF was identified by Northern blotting of total RNA (3 µg/slot) isolated from BHV1-infected MDBK cells collected at 0, 1, 3, 6, 9, 12, 18, 21, and 24 hr postinfection (p.i.), as previously described (Simard *et al.*, 1992; Simard *et al.*, 1990). The blot was hybridized with a single stranded RNA probe which was generated by *in vitro* transcription of *Kpn* I linearized pSVSport/UL25 plasmid DNA in the presence of T7 RNA polymerase and [α-^{32}P]dCTP.

S1 nuclease protection assays

The putative transcription initiation site of the UL25 ORF was mapped by S1 nuclease protection assays using the conditions previously described (Simard *et al.*, 1992). The plasmid pKS/BHV58-63KΔUL25 (4 µg) was digested with *Nco* I, dephosphorylated, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently digested again with *Bam* HI. The resulting 821 bp *Nco* I–*Bam* HI fragment, representing the 5' non coding sequence of the ORF (Fig. 1), was purified by agarose gel electrophoresis and hybridized with 150 µg of total RNA extracted either from BHV1-infected (21 hours p.i.) or mock-infected MDBK cells. Following digestion with S1 nuclease, samples were analyzed in 8% polyacrylamide sequencing gels in parallel with a sequencing reaction of M13mp18.

RESULTS

In this study, we investigated whether the BHV1 ORF, a homologue to the HSV UL25 gene, represents a functional gene. The ORF of interest, encoding a polypeptide of 598 amino acids with a calculated molecular mass of 63,083, is located at positions 60602←62398 (map units 0.4479-0.4612) in the leftward orientation of the prototype viral genome (Fig. 1). Sequence analyses of the surrounding regions revealed the presence of putative TATA and CAAT promoter elements, at positions 62734-62728 and 63064-63056, respectively. Downstream from the ORF, the nearest polyadenylation signal found on the genome's strand oriented leftward is at positions 58569-58564. The location of these elements would thus predict a transcript in the range of 4.2 kb, excluding the polyA tail.

Transcript identification and mRNA 5'end mapping

Northern blotting of total RNA extracted from BHV1-infected MDBK cells collected at different times postinfection (p.i.), was used to examine the expression kinetics of the putative UL25's transcript. For this purpose, a single stranded radiolabelled RNA probe, complementary to the complete UL25 coding sequence, was generated by *in vitro* transcription of *Kpn* I linearized pSVSport/UL25 (Fig. 1) using T7 RNA polymerase. The probe detected a major 4.5 and a minor 4.8 kb transcript in RNA isolated from BHV1 infected cells from 6 to 24 hours p.i. (Fig. 2, lanes 6-24). However, overexposure of the blot revealed the presence of the 4.5 kb transcript in RNA isolated from cells at 3 hours p.i. (results not shown). The absence of a hybridization signal in RNA extracted from mock-infected cells (lane 0) indicated that the two RNA species represented viral specific transcripts. Interestingly, the size of the 4.5 kb transcript correlated well with the size predicted from the location of UL25's putative promoter and polyadenylation controlling sequences. This clearly suggests that the 4.5 kb transcript corresponded to the UL25 mRNA. In agreement with previous studies (Dezélée *et al.*, 1996; Haanes *et al.*, 1997; Holland *et al.*, 1984; Jacobson *et al.*, 1989; Whitbeck *et al.*, 1994), the 4.8 kb band represented a 3' co-terminal transcript from the UL24 ORF which is located upstream from UL25 and whose transcription initiates at position 63412 of the BHV1 genome, generating a 4.8 kb long mRNA (Whitbeck *et al.*, 1994).

To confirm the identity of the 4.5 kb mRNA, as well as to provide further evidence that the putative TATA and CAAT boxes identified above represent the UL25 ORF's promoter elements, the 5' end of the UL25's specific transcript was mapped by S1 nuclease protection assays. For this purpose, a 821 bp *Nco* I–*Bam* HI fragment representing 818 nucleotides upstream from the ORF's initiation codon end-labeled on the complementary strand at the third base of the translation initiation codon was prepared from pKS/BHV58-63KΔUL25 (Fig. 1). The purified fragment was hybridized with total RNA isolated from either BHV1-infected (21 hours p.i.) or mock-infected MDBK cells. DNA-RNA hybrids were then subjected to S1 nuclease digestion. Sequencing gel analyses of the samples revealed the presence of a thick DNA band co-migrating with 306-310 bases-long fragments of the sequencing ladder in the sample containing BHV1 mRNA (Fig. 3A, lane +). No DNA fragments were protected in the sample that contained only cellular RNA (lane -), thus demonstrating the specificity of the assays. These results indicate that UL25 transcription initiated 303-307 bases upstream from the translation initiation codon. To more accurately map the transcription initiation site, we took advantage of the presence of a *Mlu* I site at 62 nucleotides upstream from the *Nco* I site of pKS/BHV58-63KΔUL25 (Fig. 1) and repeated the assays using a 759 bp *Mlu* I-*Bam* HI fragment end-labeled at the *Mlu* I site. Following hybridization with mRNA and S1 nuclease digestion, a specific 244 bases-long DNA fragment that was protected from digestion by BHV1 mRNA (Fig. 3B, lane +), but not by cellular mRNA (lane -), was detected in sequencing gels. This result indicates that UL25 transcription initiated 303 bases upstream from the translation initiation codon.

UL25 protein identification

To verify whether the UL25 ORF encoded a viral protein, a UL25 specific antiserum was generated using as an antigen a recombinant fusion protein expressed in *E. coli*. IPTG induction of *E. coli* BL21(DE3) recombinant cells harboring pET-28b/UL25 (Fig. 1), which encodes the ORF's amino acids 86-395 fused in frame with a T7-Tag peptide encoded by pET28b, expressed an abundant 40 kDa recombinant protein found in the insoluble fractions of cell lysates (results not shown). In contrast, no protein of this size was observed in lysates from BL21(DE3) cells harboring only the vector (not shown). The observed molecular mass of the recombinant polypeptide was within the size range expected (37,961

Da) for the fusion protein. To ascertain proper identity of the 40 kDa protein, Western blotting analyses of extracts from IPTG-induced and non-induced cells were performed using a commercially available anti-T7-Tag monoclonal antibody (MAb). The anti-T7-Tag Mab specifically detected an abundant 40 kDa protein which was found only in the IPTG-induced samples from cells harboring pET-28b/UL25, but not pET-28b (results not shown). These results indicate that the 40 kDa polypeptide included the T7-Tag N-terminal region provided by the translation vector, thus confirming the identity of the T7-Tag/UL25 recombinant protein. The protein was purified by SDS-PAGE and used to immunize mice to generate a UL25 specific antiserum. In contrast to the preimmune serum, the immune serum specifically recognized a 40 kDa protein in Western blots of extracts from IPTG-induced *E. coli* cells harboring pET-28b/UL25 but not pET-28b (not shown). The specificity of the antiserum was further ascertained by Western blotting of Cos-7 cell lysates transiently transfected with the recombinant eucaryotic expression vector pSVSport/UL25 containing the complete UL25 coding sequence. The UL25 antiserum detected a 63 kDa polypeptide in lysates from Cos-7 cells transfected with pSVSport/UL25 (see below) but not with pSVSport (not shown).

Western blotting of BHV1-infected MDBK cell lysates infected for different periods of time was used to investigate the expression kinetics of the putative protein encoded by the UL25 ORF. The UL25 antiserum specifically detected a 63 kDa polypeptide in lysates from cells infected for 6 until 30 hours with BHV1 (Fig. 4 A and B, lanes 6-30) but not in mock-infected cells (lane 0). This expression profile is consistent with that observed at the transcriptional level. Interestingly, the protein synthesized in BHV1-infected MDBK cells was indistinguishable from that observed following transient transfection of Cos-7 cells with pSVSport/UL25 (lane +). The observed molecular mass of the protein correlated very well with that predicted from the UL25 ORF's amino acid sequence (63,083).

The expression kinetics of the UL25 transcript and protein might correlate with that of viral genes of either the early (ß) or late (γ) classes. To identify the class to which UL25 belongs, we examined whether the synthesis of the polypeptide was dependent on viral DNA synthesis or not. For this purpose, we performed Western blotting of protein cell lysates from BHV1-infected MDBK cells which had been maintained for 18 hours in the presence

or the absence of phosphonoacetic acid (PAA), a DNA synthesis inhibitor. Figure 4C shows that the UL25-specific antiserum detected the UL25 protein in lysates prepared in the absence but not in the presence of PAA. Similarly, expression of the BHV1 alpha gene trans-inducing factor protein encoded by the UL48 gene, a late gene (Misra *et al.*, 1994; unpublished results), was completely abrogated in PAA-treated cells. In contrast, the level of expression of the 50 kDa protein (BHV1 ICP27) encoded by UL54, an early gene (Chalifour *et al.*, 1996; Singh *et al.*, 1996), was unaffected by the inhibition of DNA synthesis by PAA. These results thus demonstrated that the UL25 gene definitely belongs to the kinetic class of late genes.

DISCUSSION

The present study demonstrates that the BHV1 ORF, a homologue of the HSV UL25 gene, represents a functional gene generating a viral specific protein. The BHV1 UL25 gene directed the expression of a 4.5 kb transcript that accumulates at low abundance as soon as 3 hours p.i., after which the levels increase up to 12 hours p.i. and then remain constant up to 24 hours p.i. (Fig. 2). UL25 transcription initiates 303 bases upstream from the translation initiation codon, corresponding to position 62701 of the viral genome. Interestingly, this site is located 26 and 354 b downstream from putative TATA (positions 62734-62728) and CAAT (63064-63056) boxes, respectively. These findings strongly suggest that these elements function as the gene's promoter. Moreover, the identification of the transcription initiation site at 62701 and of a polyadenylation signal consensus sequence at 58569-58564 correlates very well with the size of the transcript detected by Northern blotting. This strongly suggests that the latter signal is used to terminate transcription. Our results are consistent with those reported by Haanes and coll. (1997) who estimated the size of the BHV1 UL25 transcript to be 4.1-kb-long.

We have also shown that the BHV1 UL25 gene encodes a protein with an observed molecular mass which correlated very well with that predicted from the amino acid sequence of the UL25 ORF (63,083), suggesting that the protein is not post-translationally modified. Similarly, no significant difference was observed between the apparent and expected MW of the HSV1 and the PrV specific UL25 proteins (Ali *et al.*, 1996; Kaelin *et al.*, 2000). The BHV1 UL25 protein accumulated, albeit at a very low abundance, as soon

as 6 hours p.i., after which the levels increased up to 12 hours and then remained constant up to 30 hours p.i. (Fig. 4 A and B). This expression profile is somewhat different from those observed in HSV1 and PrV where the level of the corresponding protein was found to peak at 6-12 hours p.i. and then decrease to minimal levels at 14-16 hours p.i. (Ali *et al.*, 1996; Kaelin *et al.*, 2000). However, these differences may reflect differences in the experimental conditions used since in the present study the accumulation of the protein was monitored by immunoblotting of protein lysates derived from cells infected with BHV1 at an moi of 1, whereas HSV1 and PrV studies used immunoprecipitation of proteins obtained from pulse-chase labelled cells infected at an moi of 5.

Interestingly, the expression of the UL25 protein showed a strict dependence on viral DNA synthesis as the polypeptide was undetectable in infected-cells maintained in the presence of a DNA synthesis inhibitor. This clearly indicated that the BHV1 UL25 gene belongs to viral genes of the γ2 class, similarly to its counterpart in PrV (Kaelin *et al.*, 2000). However, this is in contrast to the HSV1 UL25 gene which was classified as a γ1 gene (Ali *et al.*, 1996). At present, we cannot postulate whether these differences have any functional implications.

In summary, the present study demonstrated that the BHV1 UL25 ORF represents a functional gene generating a specific viral protein. Experiments whereby the activity of the UL25 protein was reduced suggest that UL25 is involved in the viral replicative process.

ACKNOWLEDGMENTS

The authors sincerely thank Dr. Johnny Basso for critically reading the manuscript. This work was funded by Natural Sciences and Engineering Research Council of Canada (NSERC) and by the Conseil des recherches en pêche et en agroalimentaire du Québec (CORPAQ). N.D. was supported by NSERC and by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). H.B. was supported by the FCAR.

REFERENCES

Ali, M.A., Forghani, B. and Cantin, E.M. (1996). Characterization of an essential HSV-1 protein encoded by the UL25 gene reported to be involved in virus penetration and capsid assembly. Virology 216: 278-283.

Chalifour, A., Basso, J., Gagnon, N., Trudel, M. and Simard, C. (1996). Transcriptional and translational expression kinetics of the early gene encoding the BICP27 protein of bovine herpesvirus type 1. Virology 224: 326-329.

Dezélée, S., Bras, F., Vende, P., Simonet, B., Nguyen, X., Flamand, A. and Masse, M.J. (1996). The BamHI fragment 9 of pseudorabies virus contains genes homologous to the UL24, UL25, UL26, and UL26.5 genes of herpes simplex virus type 1. Virus Res. 42: 27-39.

Farley, J.E., Skare, I.B. and Skare, J. (1981). Inverted repeat sequences in infectious bovine rhinotracheitis virus DNA. In: Nahmias, A.J., Dowdle, W.R., Schinazi, R.F. (eds) The human herpes viruses: an interdisciplinary perspective. Elsevier/North Holland Publishing New York, p 590.

Haanes, E.J., Chen, C.C. and Lowery, D.E. (1997). Nucleotide sequence and transcriptional analysis of a portion of the bovine herpesvirus genome encoding genes homologous to HSV-1 UL25, UL26 and UL26.5. Virus Res. 48: 19-26.

Holland, L.E., Sandri-Goldin, R.M., Goldin, A.L., Glorioso, J.C. and Levine, M. (1984). Transcriptional and genetic analyses of the herpes simplex virus type 1 genome coordinates 0.29 to 0.45. J. Virol. 49: 947-959.

Jacobson, J.G., Martin, S.L. and Coen, D.M. (1989). A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. 63: 1839-1843.

Kaelin, K., Dezélée, S., Masse, M.J., Bras, F. and Flamand, A. (2000). The UL25 protein of pseudorabies virus associates with capsids and localizes to the nucleus and to microtubules. J. Virol. 74: 474-482.

Kit, S. (1989). Recombinant-derived modified-live herpesvirus vaccines. Adv. Exp. Med. Biol. 251: 219-236.

Mayfield, J.E., Good, P.J., Vanoort, H.J., Campbell, A.R. and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus (Cooper strain). J. Virol. 47: 259-264.

McNab, A.R., Desai, P., Person, S., Roof, L.L., Thomsen, D.R., Newcomb, W.W., Brown, J.C. and Homa, F.L. (1998). The product of the herpes simplex virus type 1 UL25 gene is required for encapsidation but not for cleavage of replicated viral DNA. J. Virol. 72: 1060-1070.

Misra, V., Bratanich, A.C., Carpenter, D. and Ohare, P. (1994). Protein and DNA elements involved in transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. J. Virol. 68: 4898-4909.

Roizman, B. and Sears, A.E. (1996). Herpes simplex viruses and their replication. In: Fields, B.N., Knipe, D.M. and Howley, P.M. (eds) Fields Virology, 3rd edn, Lippincott-Raven publishers, Philadelphia, New York, pp 2231-2295.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, a laboratory manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sc. USA 74: 5463-5467.

Simard, C., Bastien, N. and Trudel, M. (1992). Sequencing and 5'-and 3'-end transcript mapping of the gene encoding the small subunit of ribonucleotide reductase from bovine herpesvirus type-1. Virology 190: 689-701.

Simard, C., LaBoissière, S., Séguin, C. and Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: a major variant selected from a field isolate. Intervirol. 32: 117-126.

Simard, C., Nadon, F., Séguin, C., LaBoissière, S. and Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch. Virol. 110: 63-75.

Singh, M., Fraefel, C., Bello, L.J., Lawrence, W.C. and Schwyzer, M. (1996). Identification and characterization of BICP27, an early protein of bovine herpesvirus 1 which may stimulate mRNA 3' processing. J. Gen. Virol. 77: 615-625.

Tikoo, S.K., Campos, M. and Babiuk, L.A. (1995). Bovine herpesvirus 1 (BHV1-1): biology, pathogenesis and control. Adv. Virus. Res. 45: 191-223.

Whitbeck, J.C., Lawrence, W.C. and Bello, L.J. (1994). Characterization of the bovine herpesvirus 1 homolog of the herpes simplex virus 1 UL24 open reading frame. Virology 200: 263-270.

Wyler, R., Engels, M. and Schwyzer, M. (1989). Infectious bovine rhinotracheitis/ vulvovaginitis (BHV1). In: Wittmann, G. (ed) Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic Publishers, Boston/Dordrecht/London, pp 1-72.

Figure 1. Location of the UL25 homologue on the BHV1 genome.

The *Hin*d III restriction map of the 135,301 bp BHV1 genome is illustrated at the top. The UL25 ORF is located within the fragment A, in the leftward orientation, at positions 60602←62398. The region of interest was subcloned from pKS/Ahd (Simard *et al.*, 1990) to generate pKS/BHV58-63k/Apa-Bam (see Materials and Methods). From this recombinant, three other plasmids were constructed as illustrated for the purposes indicated.

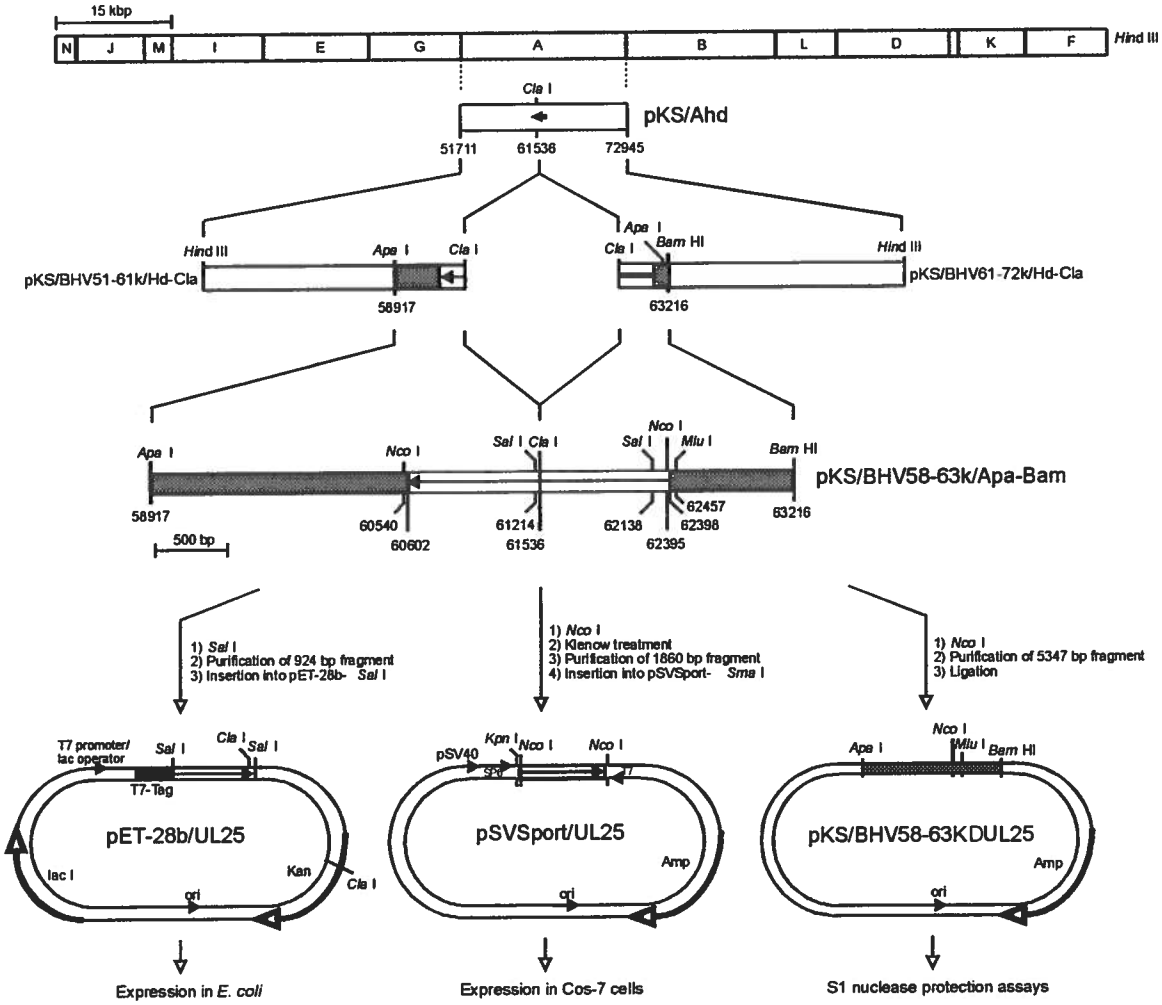

15 kbp
N
J
M
I
E
G
A
B
L
D
K
F
Hind III
Cla I
pKS/Ahd
51711
61536
72945
Hind III
Apa I
Cla I
pKS/BHV51-61k/Hd-Cla
58917
Apa I
Cla I
Bam HI
Hind III
pKS/BHV61-72k/Hd-Cla
63216
Apa I
Nco I
Sal I
Cla I
Sal I
Nco I
Mlu I
Bam HI
pKS/BHV58-63k/Apa-Bam
58917
60540
60602
61214
61536
62138
62457
62398
62395
63216
500 bp
1) Sal I
2) Purification of 924 bp fragment
3) Insertion into pET-28b- Sal I
1) Nco I
2) Klenow treatment
3) Purification of 1860 bp fragment
4) Insertion into pSVSport- Sma I
1) Nco I
2) Purification of 5347 bp fragment
3) Ligation
T7 promoter/ lac operator
Sal I
Cla I
Sal I
T7-Tag
pET-28b/UL25
lac I
Kan
Cla I
ori
pSV40
Kpn I
Nco I
Nco I
pSVSport/UL25
Amp
ori
Nco I
Apa I
Mlu I
Bam HI
pKS/BHV58-63KDUL25
Amp
ori
Expression in E. coli
Expression in Cos-7 cells
S1 nuclease protection assays

Figure 2. Transcriptional kinetics of UL25.

A Northern blot of total RNA (3 μg/slot) extracted from BHV1-infected MDBK cells at 0, 1, 3, 6, 9, 12, 18, 21, and 24 hr postinfection was hybridized with a radiolabeled single stranded RNA probe which was generated by *in vitro* transcription of pSVSport/UL25 with T7 RNA polymerase (Fig. 1) and was thus complementary to the UL25 coding sequence. Sizes in kb of RNA standards are indicated.

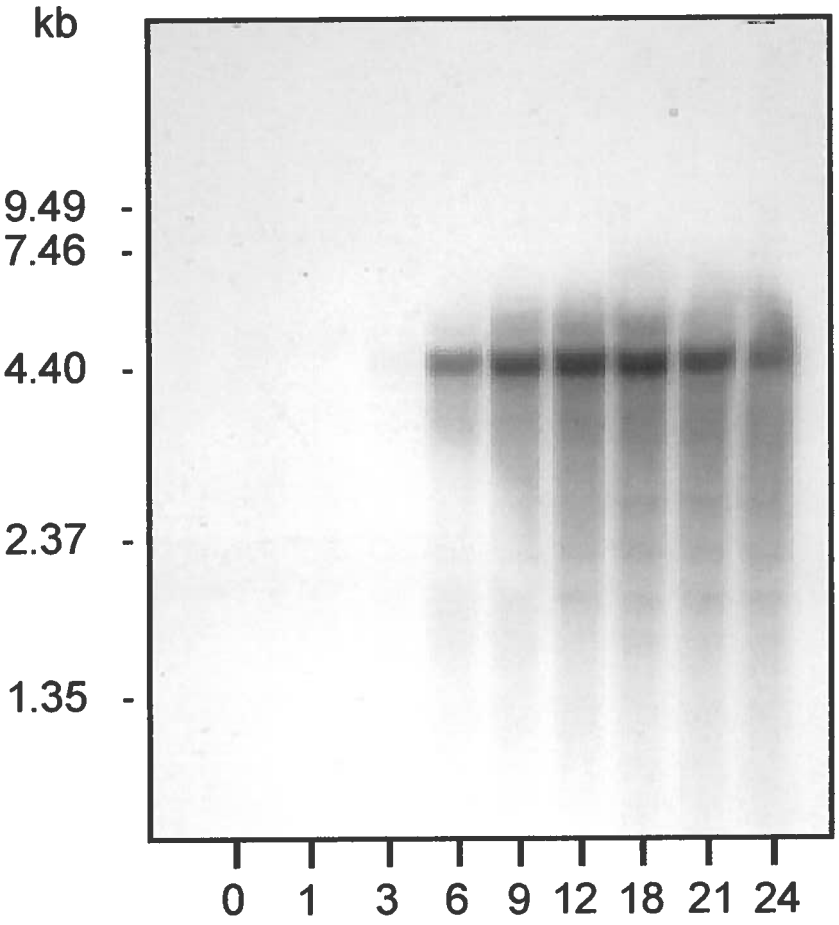

kb
9.49
7.46
4.40
2.37
1.35
0
1
3
6
9
12
18
21
24

Figure 3. 5'-end mapping of the UL25's transcript.

A 821 bp *Nco* I–*Bam* HI fragment (A) and a 759 bp *Mlu* I-*Bam* HI fragment (B) representing the 5' non-coding region of the UL25 ORF, and which were end-labeled on the complementary strand, were hybridized with total RNA isolated from either BHV1-infected (lanes +) or mock-infected MDBK cells (lanes -), then digested with S1 nuclease. DNA that was resistant to digestion was analyzed on sequencing gels in parallel with dideoxy sequencing reactions of M13mp18 (lanes G, A, T, C).

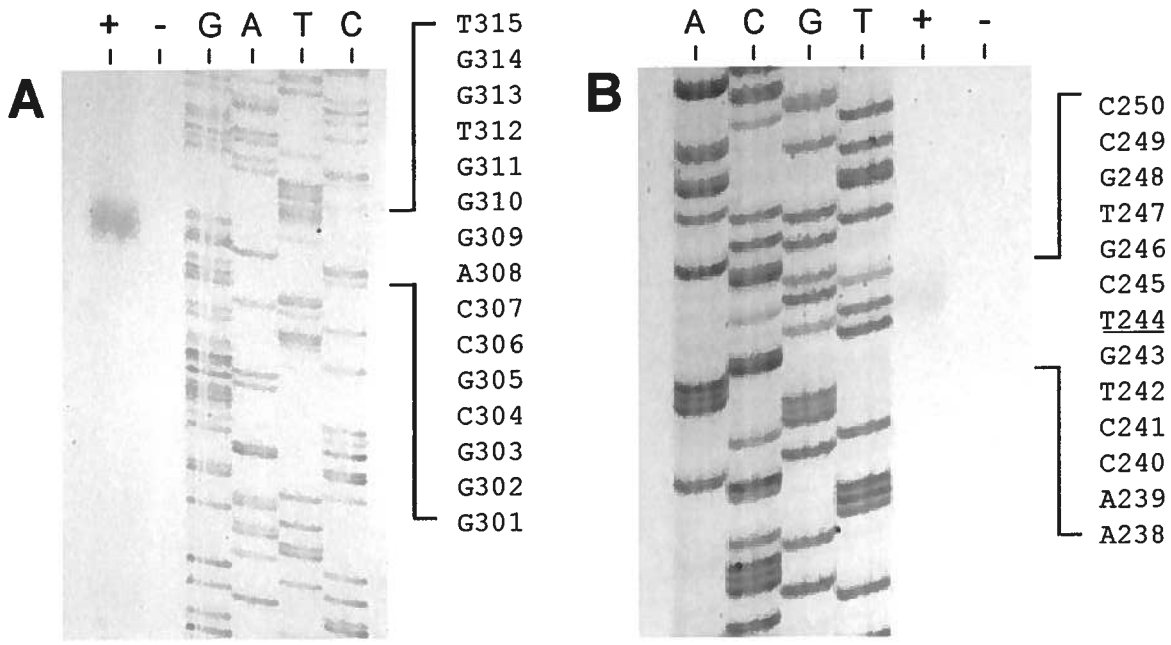
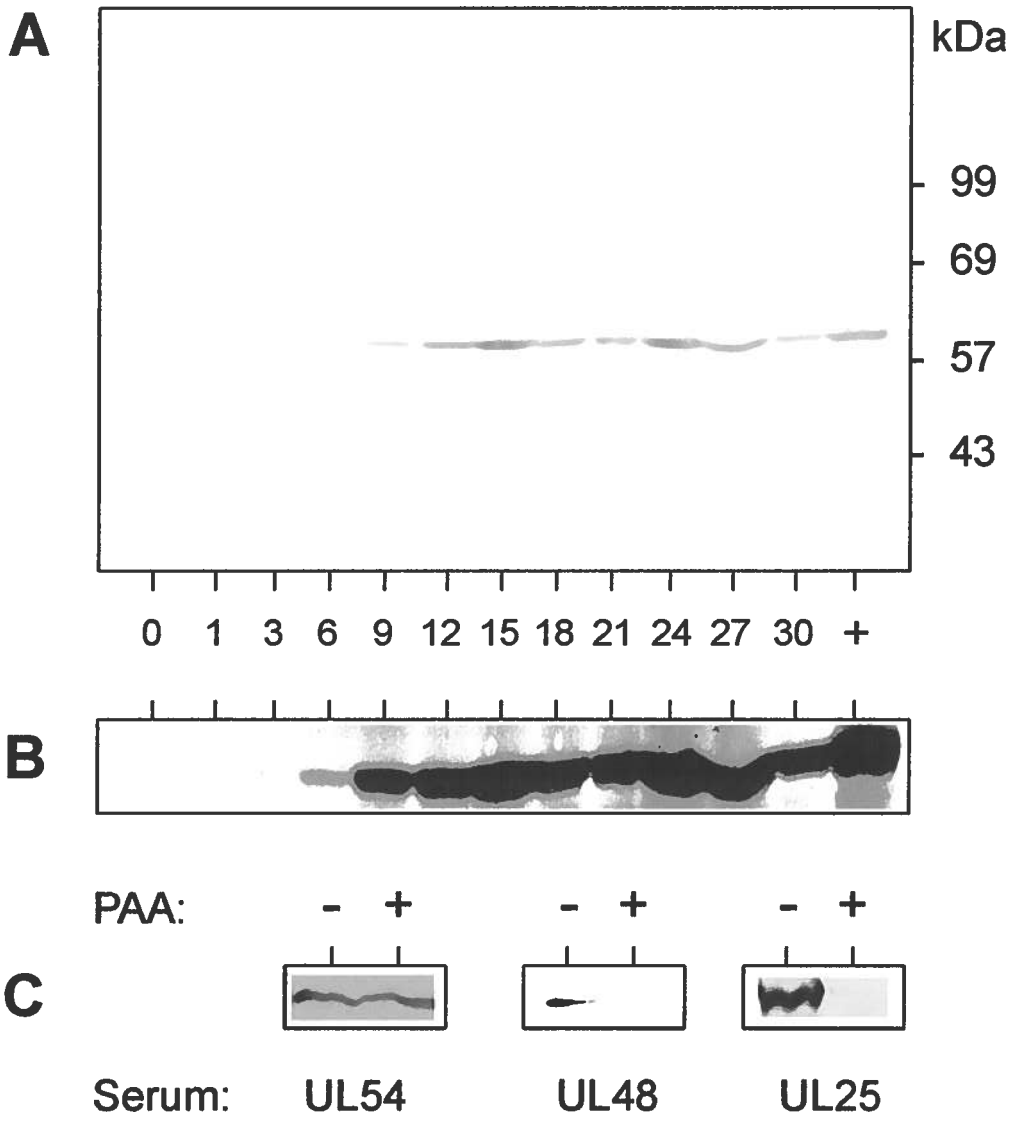

A
+ - G A T C
T315
G314
G313
T312
G311
G310
G309
A308
C307
C306
G305
C304
G303
G302
G301
B
A C G T + -
C250
C249
G248
T247
G246
C245
T244
G243
T242
C241
C240
A239
A238

Figure 4. Translational kinetics of UL25.

Confluent monolayers of MDBK cells were infected with BHV1 at an m.o.i. of 1 for 0, 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 hours. Protein cell lysates were fractionated by SDS-PAGE in parallel with a Cos-7 cell lysate collected 72 hours following transient transfection with pSVSport/UL25 (lane +). Fractionated proteins were electrotransferred onto a PVDF membrane and then reacted with the UL25-specific antiserum. The blotting membrane was then scanned to generate panels A and B; the latter panel was overexposed to allow the visualisation of the UL25 polypeptide band at 6 hours p.i. Sizes in kDa of standards are indicated.

(C) MDBK cells infected with BHV1 at an m.o.i. of 2 were incubated for 18 hours in the presence (lanes +) or the absence (lanes -) of 300 μg of phosphonoacetic acid (PAA) per mL of culture medium. Western blots of protein cell lysates, prepared as above, were reacted with monospecific antisera either directed against the products of the UL54 (Chalifour *et al.*, 1996), UL48 (unpublished results) or UL25 genes.


A
kDa
99
69
57
43
0 1 3 6 9 12 15 18 21 24 27 30 +
B
PAA: - + - + - +
C
Serum: UL54 UL48 UL25

Expression kinetics of the transcript and product of the UL28 homologue of bovine herpesvirus 1.

Nathalie Desloges and Claire Simard*

INRS-Institut Armand-Frappier
Centre de microbiologie et biotechnologie
531 Bd des Prairies
Laval-des-Rapides, Québec, Canada, H7V 1B7
Tel. (450) 687-5010 ext. 4637
Fax (450) 686-5626
E-mail: Claire.Simard@inrs-iaf.uquebec.ca

***Virus Research*, 2001; 80(1-2):23-31**

* Corresponding author

Cinétiques d'expression du transcrit et du produit de l'homologue UL28 du virus de l'herpès bovin 1.

Nous rapportons que l'ORF UL28 du virus de l'herpès bovin 1, un homologue du gène UL28 du virus de l'herpès simplex (HSV), représente un gène fonctionnel encodant une protéine virale spécifique. L'ORF UL28 du BHV1, localisé aux positions 53058→55538 du génome viral, encode un transcrit spécifique de 3.4 kb détecté à 6 heures post-infection (p.i.) après quoi les niveaux s'accumulent jusqu'à 12 heures p.i. et restent constants jusqu'à 24 heures p.i.. La transcription de UL28 s'initie 95 bases en amont du codon initiateur de l'ORF, ce qui correspond à 33 nucléotides en aval d'une boîte TATA potentielle. Un antisérum spécifique à l'ORF UL28 du BHV1, généré contre une protéine de fusion T7-Tag/UL28 produite chez *E. coli*, a réagit spécifiquement contre une protéine de 100 kDa lors de l'immunobuvardage de type Western de lysats de cellules infectées par le BHV1. L'expression de la protéine est retardée de 6 heures par comparaison avec le transcrit, suggérant que le gène est régulé au niveau traductionnel. Le gène UL28 du BHV1 a été clairement classifié comme un gène γ2, contrairement aux homologues du virus HSV et du virus de la pseudorage porcine, lesquels appartiennent à la classe des gènes viraux précoces (β). Des études supplémentaires seront requises pour déterminer si ces différences ont une implication fonctionnelle.

SUMMARY

We report that the bovine herpesvirus 1 (BHV1) UL28 ORF, a homologue of the herpes simplex virus (HSV) UL28 gene, represents a functional gene encoding a viral specific protein. The BHV1 UL28 ORF, located at positions 53058 → 55538 of the viral genome, encodes a viral specific transcript of 3.4 kb detected at 6 hours post-infection (p.i.) after which its levels accumulated up to 12 hours p.i. and then remained constant up to 24 hours p.i.. Transcription of the BHV1 UL28 was determined to initiate 95 bases upstream from the ORF's initiating codon, which corresponds to 33 nucleotides downstream from a putative TATA box. A BHV1 UL28 specific antiserum, generated against a T7-Tag/UL28 fusion protein expressed in *E. coli*, specifically reacted with a 100 kDa protein in Western blots of BHV1-infected protein cell lysates. The expression kinetics of the protein was delayed by 6 hours relative to that of its transcript, suggesting that the gene is regulated at the translational level. In contrast to the HSV and pseudorabies virus UL28 genes which belong to viral genes of the early (β) class, that of BHV1 was unambiguously classified as a γ2 gene. Further studies will be required to determine whether these kinetic differences have any functional implications.

Key words: bovine herpesvirus 1, UL28, expression kinetics, late gene

INTRODUCTION

Bovine herpesvirus 1 (BHV1), a member of the *Alphaherpesvirinae* (Roizman, 1996), is an important pathogen of cattle causing two major clinical syndromes, namely infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (Kit, 1989; Wyler *et al.*, 1989; Tikoo *et al.*, 1995). The BHV1 genome, a 135,301 bp linear double-strand DNA molecule, is divided into a unique long (UL) and a unique short (US) segment, the latter being flanked by inverted repeats (Farley *et al.*, 1981). Two isomeric forms of the genome occur naturally as the US can invert its orientation relative to the UL (Mayfield *et al.*, 1983; Wyler *et al.*, 1989). As in herpes simplex virus (HSV; Roizman and Sears, 1996), BHV1 viral genes are sequentially expressed in three temporal phases designated α (immediate-early), β (early) and γ (late) (Wyler *et al.*, 1989).

The recent complete sequencing of the BHV1 genome suggests the presence of more than 70 different open reading frames (ORFs). These ORFs are conventionally designated according to the HSV nomenclature (Roizman and Sears, 1996). One of these ORFs is homologous to the HSV1 UL28 gene which encodes the ICP18.5 assembly protein (Meyer *et al.*, 1997). This ORF appears to be highly conserved in herpesviruses as homologues were identified in pseudorabies virus (PRV), HSV2, Epstein-Barr virus, human and murine cytomegaloviruses, varicella-zoster virus, equine herpesvirus, Marek's disease virus and herpesvirus saimiri (Pederson and Enquist, 1991).

The HSV1 UL28 gene encodes a 87 kDa protein, as determined by Western blotting of infected protein cell lysates with a rabbit polyclonal antiserum directed against an *E. coli*-expressed Cro-UL28 fusion protein (Tengelsen *et al.*, 1993). In PRV, the size of the corresponding protein was estimated at 79 kDa (Pederson and Enquist, 1989; Pederson and Enquist, 1991). Both HSV1 and PRV UL28 proteins are expressed early upon infection and their accumulation do not require viral DNA synthesis. The two proteins play an important role in the cleavage and encapsidation of viral DNA (Addison *et al.*, 1990; Tengelsen *et al.*, 1993; Mettenleiter *et al.*, 1993).

In the present study, we identified the putative transcript and protein encoded by the BHV1 UL28 ORF, characterized the kinetics of expression, and identified the transcription initiation site.

MATERIALS AND METHODS

Cells, virus, and RNA isolations

Madin Darby bovine kidney (MDBK, ATCC CCL-22) cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Strain #34 of BHV1, a field isolate of subtype 1.1, was kindly provided by Dr Mitchell (Gouvernement du Canada, Lethbridge, Alberta) and cultured in confluent monolayer sheets of MDBK cells (Simard *et al.*, 1991). The purification of total RNA from BHV1- and mock-infected cells was performed as previously described (Simard *et al.*, 1990). To determine the kinetic class of UL28, BHV1-infected MDBK cells were maintained for 18 hours in the presence or absence of 300 μg/mL of phosphonoacetic acid (PAA; ICN Biomedicals Inc.) added one hour after the onset of infection.

Plasmid constructions

The BHV1 UL28 ORF is located within the *Hind* III fragment A of the BHV1 genome in the rightward orientation, at positions 53058 → 55538 (Fig. 1). We initially subcloned from the recombinant plasmid pKS/Ahd carrying the *Hind* III fragment A (Simard *et al.*, 1990) the 9825 bp *Hind* III-*Cla* I fragment representing the region 51711-61536 of the viral genome to generate pKS/BHV51-61kHd-Cla. This recombinant was next used to create a plasmid strictly representing the complete UL28 ORF. First, the 1469 bp *Bsm* FI fragment from pKS/BHV51-61kHd-Cla, representing codons 2-490 of the UL28 ORF, was ligated with the phosphorylated and auto-complementary oligonucleotide 5'-CGCCATTCTAGA-GGTACCTCTAGAAT-3' which was specifically designed to regenerate the ORF's initiation codon (see the boxed ATG in Fig. 1) as well as to create *Kpn* I (doubly underlined) and *Xba* I (underlined) unique restriction sites. Following digestion with *Kpn* I and *Nru* I, the resulting 900 bp-long fragment encoding the first 302 amino acids of the ORF was inserted into compatibles *Kpn* I and *Hind* II restriction sites of the pKS vector (Stratagene), generating pKS/UL28(1-302). To ascertain the in-frame presence of the initiating codon, the 5'-end of the vector/insert junction was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). To insert the remaining of the UL28 coding sequence into pKS/UL28(1-302), we took advantage of the presence of a unique *Nhe* I restriction site (GCTAGC) encompassing the ORF's stop codon (underlined). For this purpose, the 2067 bp *Nco* I (cohesive) - *Nhe* I (filled-in) fragment,

encoding amino acids 128-826 and the stop codon of the UL28 ORF, was purified from pKS/BHV51-61kHd-Cla then inserted into the unique *Nco* I-*Eco* RV restrictions sites of pKS/UL28(1-302), generating pKS/UL28orf. This recombinant was used to generate a single-strand RNA specific probe of UL28 as well as to construct pET28b/UL28 (see below).

To allow the expression in *E. coli* of a T7-Tag/UL28 fusion protein for the development of a monospecific antiserum directed against the product encoded by the BHV1 UL28 ORF, we constructed the recombinant procaryotic expression vector pET28b/UL28. For this purpose, the 999 bp *Nru* I-*Sal* I fragment from pKS/UL28orf, corresponding to codons 304-637 of the UL28 ORF, was inserted into compatible *Bam* HI (filled-in)-*Sal* I restriction sites of pET-28b (Novagen) (Fig. 1). To ascertain that the reading frame of the insert was properly fused with that of the vector's T7-Tag coding region, the 5'-end of the vector/insert junction was sequenced as above.

To precisely map the UL28 transcription initiation site by S1 nuclease protection assays, we constructed pKS/BHV52-53kSacI-PvuII. For this purpose, the 583 bp *Sac* I-*Pvu* II fragment of pKS/BHV51-61kHd-Cla was inserted into *Sac* I and *Sma* I digested pKS vector (Stratagene). The recombinant pKS/BHV52-53kSacI-PvuII represents 422 bp of the UL28 ORF's 5' non-coding region as well as 161 bp of the 5' coding region.

Development of a UL28 specific antiserum

To allow the identification of the protein putatively encoded by the UL28 ORF, we set out to generate a UL28-specific antiserum using as antigens a T7-Tag/UL28 fusion protein expressed in *E. coli* via the recombinant expression vector pET-28b/UL28. Exponential cultures of *E coli* BL21(DE3) cells, either harbouring pET-28b/UL28 or pET-28b plasmids, were induced with 0.4 mM IPTG. Cells from aliquotes collected at 0, 1, 2, 3, and 4 hr post-induction were recovered by centrifugation, resuspended in 0.1 volume of TEN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl) containing 1 mg/mL lysozyme, and then sonicated. Lysates were centrifuged and the protein contents of both the soluble and insoluble fractions were analysed by SDS-PAGE to identify the protein of interest. Coomassie blue staining of the gel revealed the presence of an abundant 40 kDa protein of the expected size (39,960) in insoluble fractions of lysates from cells harbouring pET-28b/UL28 but not pET-28b. Following preparative SDS-PAGE, the band corresponding to

the T7-Tag/UL28 fusion protein was isolated and finely ground in sterile phosphate buffer saline (PBS) by several passages in syringes connected to successively lower gauge needles. Aliquotes of the resulting acrylamide/protein suspension were empirically quantified by SDS-PAGE analyses by comparison of the intensity of the Coomassie blue stained bands observed with that of known quantities of bovine serum albumine (BSA) analyzed in parallel. The slurry was finally used to immunize mice intraperitoneally, given three doses of approximately 30 μg of protein/mouse, at two weeks interval, after which immune sera were collected.

Western blotting

Confluent monolayers of MDBK cells (4.6 X 10^7 total cells) in T150 flasks were infected with BHV1, using an m.o.i. of 2, for 0, 1, 3, 6, 9, 12, 18, 21, 27, and 30 hr. Cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free) then scraped in ice-cold PBS. After centrifugation at 4°C, the cell pellet was resuspended in 1.0 mL PBS containing 1 mM phenylmethylsulfonyl fluoride, quickly frozen in liquid nitrogen then stored at -70°C. After thawing on ice, 100 μL of cell lysates were added to 25 μL of 5X SDS sample buffer (1X = 40 mM Tris-HCl pH 6.8, 2% SDS, 280 mM β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). The resulting mixture was boiled for 3 min and then 25 μL were analyzed by SDS-PAGE. Fractionated proteins were electrotransferred onto a PVDF membrane (Bio-Rad) after which the membrane was incubated overnight at ambient temperature in 50 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) containing 1.5% BSA (blocking solution), reacted 2 hours at room temperature with the UL28-specific antiserum diluted 1:300 in the blocking solution, washed several times in TBS-Tween 0.2% and then reacted 2 hours with horse radish peroxidase-conjugated (HRP) antimouse immunoglobulin (ICN Immunobiologicals) diluted 1:2000 in the blocking solution. After several washes in TBS-Tween 0.2%, immune complexes were revealed by incubating the membrane in a freshly prepared DAB solution (7.5 mg 3,3'-diaminobenzidine tetrahydrochloride dissolved in 30 mL TBS) containing 0.03% v/v H_2O_2. The colorimetric reaction was finally stopped by soaking the membrane in water.

Northern blot hybridization

The putative transcript of the UL28 ORF was identified by Northern blotting of total RNA (20 μg/slot) isolated from BHV1-infected MDBK cells collected at 0, 1, 3, 6, 9, 12, 18, 21, and 24 hr p.i., as previously described (Simard *et al.*, 1992). The blot was hybridized with a single strand RNA probe which was generated by *in vitro* transcription of *Nco* I linearized pKS/UL28orf plasmid DNA in the presence of T7 RNA polymerase and [α-^{32}P]dCTP.

S1 nuclease protection assays

The putative transcription initiation site of the UL28 ORF was mapped by S1 nuclease protection assays using the conditions previously described (Simard *et al.*, 1992). For this purpose, pKS/BHV52-53kSacI-PvuII (4 μg; Fig. 1) was digested with *Not* I, dephosphorylated, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and then, digested again with *Sac* I. The resulting end-labelled 450 bp *Not* I-*Sac* I fragment, representing the 5' non coding sequence of the ORF, was purified by agarose gel electrophoresis and hybridized with 150 μg of total RNA extracted either from BHV1-infected (21 hours p.i.) or mock-infected MDBK cells. Following digestion with S1 nuclease, samples were analyzed in 8% polyacrylamide sequencing gels in parallel with a sequencing reaction of M13mp18.

RESULTS

In this study, we investigated whether the BHV1 UL28 ORF, a homologue of the HSV1 UL28 gene, represents a functional gene which generates a viral specific protein. The UL28 ORF, encoding a 826 amino-acids long protein, is located at positions 53058 → 55538 in the rightward orientation of the prototype viral genome (Fig. 1). Sequence analyses of the region preceding the ORF revealed the presence of a putative TATA box (5'-TATATAc-3') at positions 52923-52929 which could be an element of the gene's promoter.

Expression kinetics and 5'end mapping of the UL28's transcript

The putative transcript of the UL28 ORF was identified by Northern blotting of total RNA isolated from BHV1-infected MDBK cells collected at different times p.i.. For this purpose, T7 directed *in vitro* transcription of *Nco* I linearized pKS/UL28orf was used to generate a sense specific single strand radiolabelled RNA probe complementary to the UL28 coding

sequence (Fig. 1). The probe specifically hybridized to a 3.4 kb mRNA detected at low abundance 6 hours p.i. after which point the levels increased up to 12 hours p.i. and subsequently remained constant up to 24 hours p.i. (Fig. 2A).

To provide evidence that the above described TATA box represented an element of the UL28's promoter, the 5' end of the transcript was mapped by S1 nuclease protection assays. For this purpose, pKS/BHV52-53kSacI-PvuII (Fig. 1) was used to generate a 450 bp *Not* I-*Sac* I fragment, which spans 426 nucleotides upstream of the UL28 ORF's initiation codon as well as the first 24 bases of the coding sequence, end-labeled on the complementary strand. The purified fragment was hybridized with total RNA extracted from either BHV1-infected (Fig. 3, lane +) or mock-infected (lane -) MDBK cells. Following S1 nuclease digestion, samples were analyzed in 8% polyacrylamide sequencing gels in parallel with a sequencing reaction of M13mp18 (Fig. 3, lanes G, A, T, C). A 119 bases-long S1-resistant DNA band was present in the sample containing viral RNA (lane +) whereas no DNA fragment was protected in the sample only containing cellular RNA (lane -), thus demonstrating the specificity of the assays. These results show that transcription initiation of UL28 occurs 95 bases upstream from the initiating codon, corresponding to 33 b downstream from the TATA box.

Identification and kinetics of expression of the protein encoded by UL28

To identify and characterize the UL28 ORF viral specific protein, a specific antiserum was generated using as antigen a recombinant fusion protein expressed in *E. coli* BL21(DE3) harbouring pET-28b/UL28. This plasmid encodes the amino acids 304-637 of UL28 fused in frame with a T7-Tag peptide encoded by pET-28b. IPTG induction of *E. coli* cells harbouring this plasmid expressed an abundant 40 kDa recombinant protein present in the insoluble fractions of cell lysates (Fig. 4A, lane 1). In contrast, no protein of this size was observed in either lysates from non-induced cells (Fig. 4A, lane 0) or from BL21(DE3) cells harbouring only the vector (not shown). The observed molecular mass of the recombinant protein was within the size range expected (39,960 Da) for the fusion protein. To ascertain the proper identity of the 40 kDa protein, Western blotting analyses of extracts from IPTG-induced and non-induced cells were performed using a commercially available anti-T7-Tag monoclonal antibody (Mab). The anti-T7-Tag Mab specifically detected a 40 kDa protein found exclusively in the IPTG-induced samples from cells harbouring pET-

28b/UL28 (Fig. 4A, lane 4), but not from those harbouring only the vector (not shown). These results demonstrated that the 40 kDa protein included the T7-Tag N-terminal region provided by the translation vector, thus confirming the identity of the T7-Tag/UL28 recombinant protein. The protein was purified by SDS-PAGE and used to immunize mice for the development of a UL28 specific antiserum. In contrast to the preimmune serum, the immune serum specifically recognized a 40 kDa protein in Western blots of extracts from IPTG-induced *E. coli* cells harbouring pET-28b/UL28 (Fig. 4A, lane 6) but not pET-28b (not shown).

The expression kinetics of the putative protein encoded by the BHV1 UL28 ORF was investigated by Western blotting analyses of lysates from MDBK cells infected with BHV1 for different time periods. The UL28 antiserum specifically detected a 100 kDa polypeptide which accumulated at very low abundance at 12 hours p.i. after which its level increased significantly at 18 hours p.i. and then remained constant until 30 hours p.i. (Fig. 4B, lanes 12-30). In contrast, no protein of this size was detected in the mock-infected MDBK cell lysate sample (Fig. 4B, lane 0), thus demonstrating the specificity of the assay. The size of the protein correlated well with that expected from the derived amino acid sequence of the ORF (86,919 Da).

The expression profile of the UL28 protein correlated well with that of viral genes of the late class, whereas that of the transcript could correlate with that of viral genes of either the early (β) or late (γ) classes. To unambiguously identify the class to which UL28 belongs, we examined whether the synthesis of the transcript was dependent on viral DNA synthesis. For this purpose, we performed Northern blotting of total RNA obtained 12 and 18 hours p.i. of BHV1-infected MDBK cells grown in the presence or absence of the DNA synthesis inhibitor phosphonoacetic acid (PAA). As shown in Figure 2B, a sense specific radiolabelled RNA probe complementary to the UL28 coding sequence detected the UL28 transcript in the samples obtained from cells infected in the absence but not in the presence of PAA. Similarly, PAA also inhibited transcription of BHV1 UL48, a late gene (Misra *et al.*, 1994) encoding the alpha gene trans-inducing factor protein (Fig. 2B). These results thus unambiguously demonstrated that the BHV1 UL28 belongs to the kinetic class of late genes.

DISCUSSION

In this study, we demonstrated that the BHV1 ORF which is homologous to the HSV UL28 gene represents a functional gene which encodes a viral specific protein. The BHV1 UL28 gene directed the expression of a 3.4 kb mRNA accumulating at low abundance at 6 hours p.i. after which the levels increased up to 12 hours p.i. and then remained constant up to 24 hours p.i.. As determined by S1 nuclease protection assays, UL28 transcription initiated 95 nucleotides upstream from the translation initiation codon, corresponding to position 52963 of the viral genome. Downstream from the UL28 coding sequence, the first consensus polyadenylation signal (AATAAA) found on the genome's strand oriented rightward is located downstream from the UL27 ORF (55394-58192), at positions 58223-58228. If this signal was used to terminate UL28 transcription, a transcript in the size range of 5.2 kb (excluding the polyA tail) would be expected. The discrepancy between the transcript's expected and observed sizes suggests that a non-classical polyadenylation signal is used to terminate UL28 transcription. Indeed, the use of alternative polyadenylation signals to terminate viral gene transcription (McLauchlan *et al.*, 1989) or that of other eukaryotic genes has already been observed. For instance, it was recently shown that less than 73% of human genes use a consensus polyadenylation signal (AWTAAA) to terminate their transcription and as a result, several signal sequence variants were identified (Beaudoing *et al.*, 2000). Our results thus reveal that the 3'-end of the BHV1 UL28 transcript maps within the UL27 ORF. This is in contrast to the HSV1 UL28 transcript which was found to be 3' co-terminal with the UL27 mRNA (Holland *et al.*, 1984). However, it is of interest to note that the homologous UL28 gene of murine cytomegalovirus, a member of the *Betaherpesvirinae*, terminates its transcription within the corresponding UL27 gene (Messerle *et al.*, 1992), similarly to BHV1.

The transcription initiation site of BHV1 UL28 was located 33 bases downstream from a consensus TATA box identified at positions 52923-52929. These findings suggest that this sequence represents an element of the UL28's promoter since its position relative to that of the transcription initiation site correlated well with that of eucaryotic gene promoters.

We have also shown that the BHV1 UL28 encodes a viral specific protein in infected cells, with an observed molecular mass of 100 kDa, which correlates well with that predicted from the derived amino acid sequence of the UL28 coding sequence (86,919 Da). The

slight discrepancy between the estimated and expected sizes of the protein may be attributed to errors associated with SDS-PAGE size estimation. The protein accumulated at very low abundance at 12 hours p.i., its level significantly increased at 18 hours p.i. and then remained constant until 30 hours p.i.. Hence, the expression profile of BHV1 UL28 at the translational level was significantly different from that at the transcriptional level since the accumulation of the protein relative to that of its transcript was delayed by 6 hours. This suggests that protein expression is regulated at the translational level. However, we cannot exclude the possibility that at earlier time points, the protein levels were below the detection limit.

The expression of BHV1 UL28 was strictly dependent on viral DNA synthesis since the synthesis of the transcript was completely inhibited in infected-cells maintained in the presence of a DNA synthesis inhibitor. These results unambiguously established that the BHV1 UL28 gene belonged to viral genes of the γ2 class. This is in contrast to the HSV1 and PRV counterpart genes which belong to the early (β) genes class. Specifically, the PRV UL28 encoded protein was found to accumulate in infected cells within 2 hours p.i. even in the presence of a DNA synthesis inhibitor (Pederson and Enquist, 1991). Similarly, the abundance of the HSV1 UL28 transcript was not affected by the presence of a DNA synthesis inhibitor in infected cells (Holland *et al.*, 1984). Nevertheless, it is of interest to note that the corresponding murine cytomegalovirus gene belongs to the γ class (Messerle *et al.*, 1992). It will thus be of interest to determine whether these differences in the expression kinetics have any functional implications.

ACKNOWLEDGMENTS

The authors are grateful to Dr Johnny Basso for reviewing the manuscript and wish to thank Mrs Nicole Gagnon for her excellent technical assistance. This work was funded by Natural Sciences and Engineering Research Council of Canada (NSERC). N.D. was supported by NSERC, by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) and by the Fondation Armand-Frappier.

REFERENCES

Addison, C., Rixon, F.J. and Preston, V.G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. 71: 2377-2384.

Beaudoing, E., Freier, S., Wyatt, J.R., Claverie, J.-M. and Gautheret, D. (2000). Patterns of variant polyadenylation signal usage in human genes. Genome Res. 10: 1001-1010.

Farley, J.E., Skare, I.B. and Skare, J. (1981). Inverted repeat sequences in infectious bovine rhinotracheitis virus DNA. In: Nahmias, A.J., Dowdle, W.R. and Schinazi, R.F. (Eds), The human herpes viruses: an interdisciplinary perspective. Elsevier/North Holland Publishing New York, p. 590.

Holland, L.E., Sandri-Goldin, R.M., Goldin A.L., Glorioso, J.C. and Levine, M. (1984). Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. 49: 947-959.

Kit, S. (1989). Recombinant-derived modified-live herpesvirus vaccines. Adv. Exp. Med. Biol. 251: 219-236.

Mayfield, J.E., Good, P.J., Vanoort, H.J., Campbell, A.R. and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus (Cooper strain). J. Virol. 47: 259-264.

McLauchlan, J., Simpson, S. and Clements, J.B. (1989). Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. Cell 59: 1093-1105.

Messerle, M., Keil, G.M., Schneider, K. and Koszinowski, U.H. (1992). Characterization of the murine cytomegalovirus genes encoding the major DNA binding protein and the ICP18.5 homolog. Virology 191: 355-367.

Mettenleiter T.C., Saalmüller, A. and Weiland, F. (1993). Pseudorabies virus protein homologous to herpes simplex virus type 1 ICP18.5 is necessary for capsid maturation. J. Virol. 67: 1236-1245.

Meyer, G., Vlcek, C., Paces, V., O'Hara, M.K., Pastoret, P.-P., Thiry, E. and Schwyzer, M. (1997). Sequence analysis of the bovine herpesvirus type 1 genes homologous to the DNA polymerase (UL30), the major DNA-binding protein (UL29) and ICP18.5 assembly protein (UL28) genes of herpes simplex virus. Arch. Virol. 142: 89-102.

Misra, V., Bratanich, A.C., Carpenter, D. and Ohare, P. (1994). Protein and DNA elements involved in transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. J. Virol. 68: 4898-4909.

Pederson, N.E. and Enquist, L.W. (1989). The nucleotide sequence of a pseudorabies virus gene similar to ICP18.5 of herpes simplex virus type 1. Nucleic Acids Res. 17: 3597.

Pederson, N.E. and Enquist, L.W. (1991). Overexpression in bacteria and identification in infected cells of the pseudorabies virus protein homologous to the herpes simplex virus type 1 ICP18.5. J. Virol. 65: 3746-3758.

Roizman, B. (1996). Herpesviridae. In: Fields, B.N., Knipe, D.M. and Howley, P.M. (Eds), Fields Virology, 3rd edn. Lippincott-Raven publishers, Philadelphia, New York, pp. 2221-2230.

Roizman, B. and Sears, A.E. (1996). Herpes simplex viruses and their replication. In: Fields, B.N., Knipe, D.M. and Howley, P.M. (Eds), Fields Virology, 3rd edn. Lippincott-Raven publishers, Philadelphia, New York, pp. 2231-2295.

Sanger F., Nicklen S. and Coulson A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sc. USA 74: 5463-5467.

Simard, C., Bastien, N. and Trudel, M. (1992). Sequencing and 5'-and 3'-end transcript mapping of the gene encoding the small subunit of ribonucleotide reductase from bovine herpesvirus type-1. Virology 190: 689-701.

Simard, C., LaBoissière, S., Séguin, C. and Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: a major variant selected from a field isolate. Intervirology 32: 117-126.

Simard, C., Nadon, F., Séguin, C., LaBoissière, S. and Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch. Virol. 110: 63-75.

Tengelsen, L.A., Pederson, N.E., Shaver, P.R., Wathen, M.W. and Homa, F.L. (1993). Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J. Virol. 67: 3470-3480.

Tikoo, S.K., Campos, M. and Babiuk, L.A. (1995). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis and control. Adv. Virus Res. 45: 191-323.

Wyler, R., Engels, M. and Schwyzer, M. (1989). Infectious bovine rhinotracheitis/ vulvovaginitis (BHV1). In: Wittmann, G. (Ed), Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic Publishers, Boston/Dordrecht/London, pp 1-72.

Figure 1. Cloning strategies of BHV1 UL28.

The *Hin*d III restriction map of the 135,301 bp BHV1 genome is illustrated at the top. The UL28 ORF is located within the A fragment at positions 53058 → 55538 in the rightward orientation. The 9825 bp *Hin*d III-*Cla* I fragment, representing the region 51711-61536 of the viral genome and shown in an expanded view, was first subcloned from pKS/Ahd to generate pKS/BHV51-61kHd-Cla. This recombinant was used as illustrated (see Materials and Methods) to create the other plasmids that were required for this study.

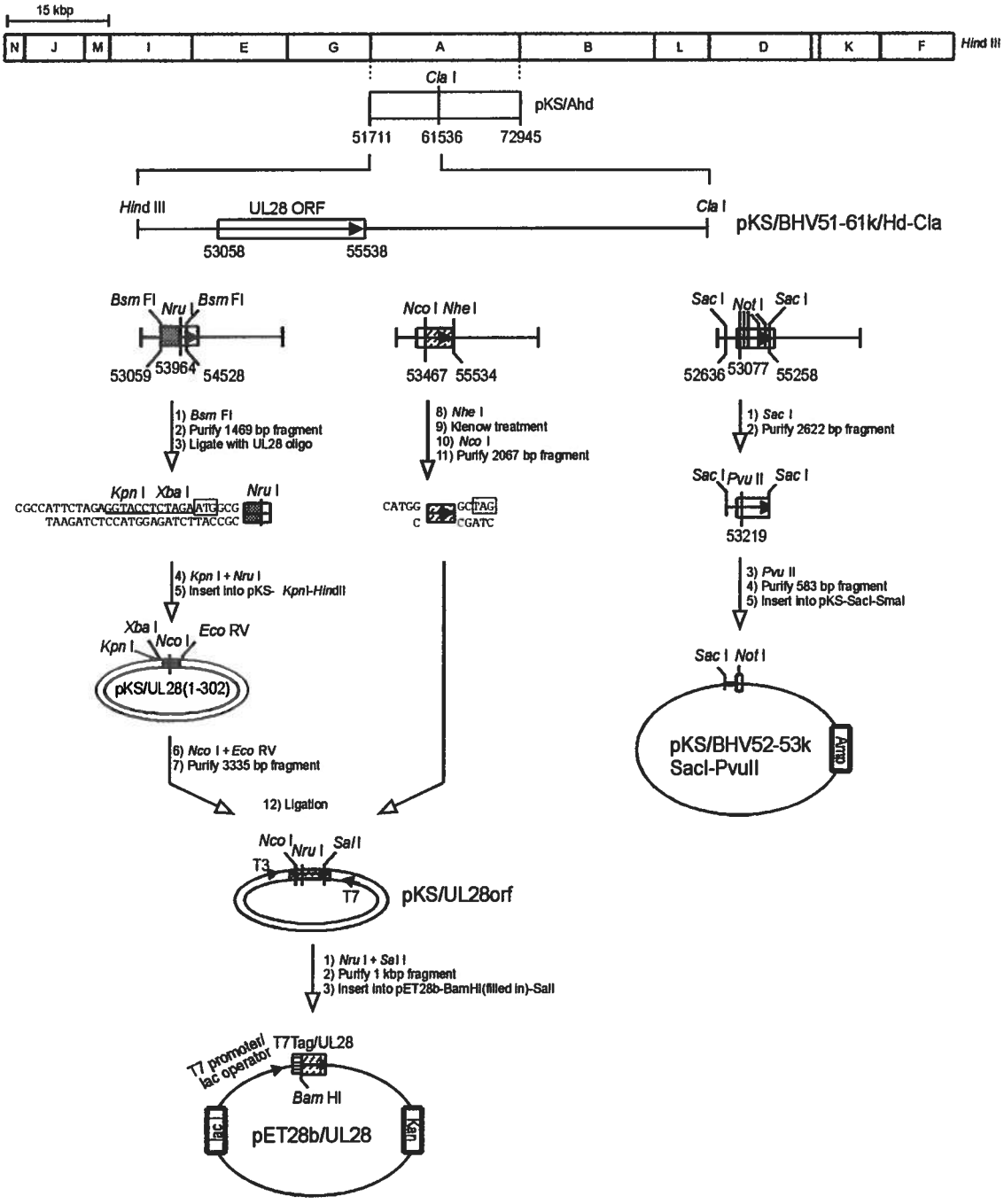

15 kbp
N
J
M
I
E
G
A
B
L
D
K
F
Hind III
Cla I
pKS/Ahd
51711
61536
72945
Hind III
UL28 ORF
Cla I
pKS/BHV51-61k/Hd-Cla
53058
55538
Bsm FI
Nru I
Bsm FI
53059
53964
54528
Nco I
Nhe I
53467
55534
Sac I
Not I
Sac I
52636
53077
55258
1) Bsm FI
2) Purify 1469 bp fragment
3) Ligate with UL28 oligo
8) Nhe I
9) Klenow treatment
10) Nco I
11) Purify 2067 bp fragment
1) Sac I
2) Purify 2622 bp fragment
Kpn I
Xba I
Nru I
CGCCATTCTAGAGGTACCTCTAGAATGCGCG
TAAGATCTCCATGGAGATCTTACCGC
CATGG
C
GCTAG
CGATC
Sac I
Pvu II
Sac I
53219
4) Kpn I + Nru I
5) Insert into pKS- KpnI-HindII
3) Pvu II
4) Purify 583 bp fragment
5) Insert into pKS-SacI-SmaI
Xba I
Kpn I
Nco I
Eco RV
pKS/UL28(1-302)
Sac I
Not I
pKS/BHV52-53k
SacI-PvuII
Amp
6) Nco I + Eco RV
7) Purify 3335 bp fragment
12) Ligation
Nco I
Nru I
Sal I
T3
T7
pKS/UL28orf
1) Nru I + Sal I
2) Purify 1 kbp fragment
3) Insert into pET28b-BamHI(filled in)-SalI
T7 promoter/
lac operator
T7Tag/UL28
Bam HI
lac I
pET28b/UL28
Kan

Figure 2. Transcriptional expression kinetics of UL28.

A) A Northern blot of total RNA (20 μg/slot) extracted from BHV1-infected MDBK cells at 0, 1, 3, 6, 9, 12, 18, 21, and 24 hr postinfection was hybridized with a radiolabeled single strand RNA probe complementary to the UL28 coding sequence which was generated by *in vitro* transcription of pKS/UL28orf with T7 RNA polymerase (Fig. 1). Sizes in kb of RNA standards are indicated.

B) Northern blotting of total RNA isolated from MDBK cells infected with BHV1 at an m.o.i. of 2, and grown for either 12 or 18 hours in the presence (lanes +) or the absence (lanes -) of 300 μg of phosphonoacetic acid (PAA) per ml of culture medium. The blots were hybridized with either a UL28 or UL48 BHV1 specific probes.

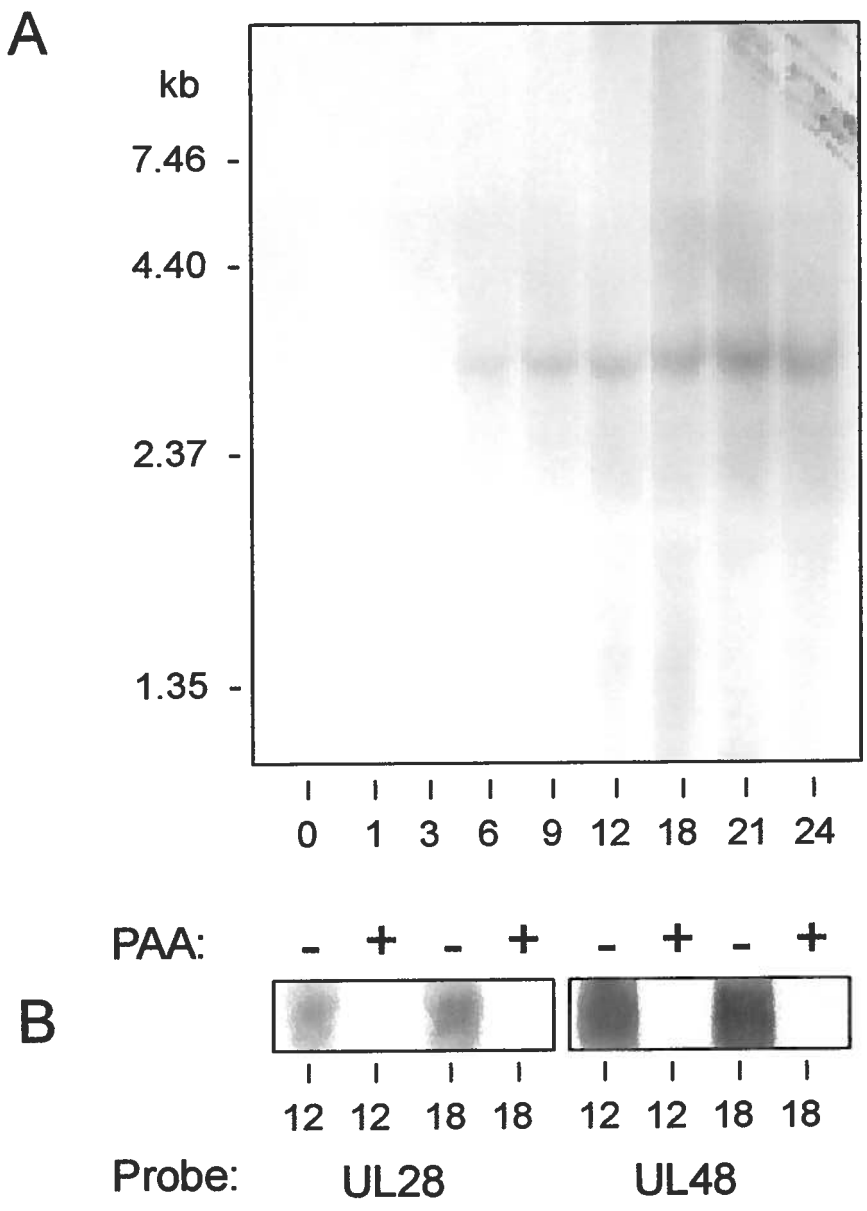

A
kb
7.46
4.40
2.37
1.35
0 1 3 6 9 12 18 21 24
PAA: - + - + - + - +
B
12 12 18 18 12 12 18 18
Probe: UL28 UL48

Figure 3. 5'-end mapping of the UL28 mRNA.

A 450 bp *Not* I-*Sac* I fragment representing 426 nucleotides upstream from the UL28 ORF's initiation codon and which was end labelled on the complementary strand at nucleotide 24 of the coding sequence was hybridized with total RNA isolated from either BHV1-infected (lane +) or mock-infected (lane -) MDBK cells and then digested with S1 nuclease. The samples were analyzed on sequencing gels in parallel with dideoxy sequencing reactions of M13mp18 (lanes G, A, T, C).

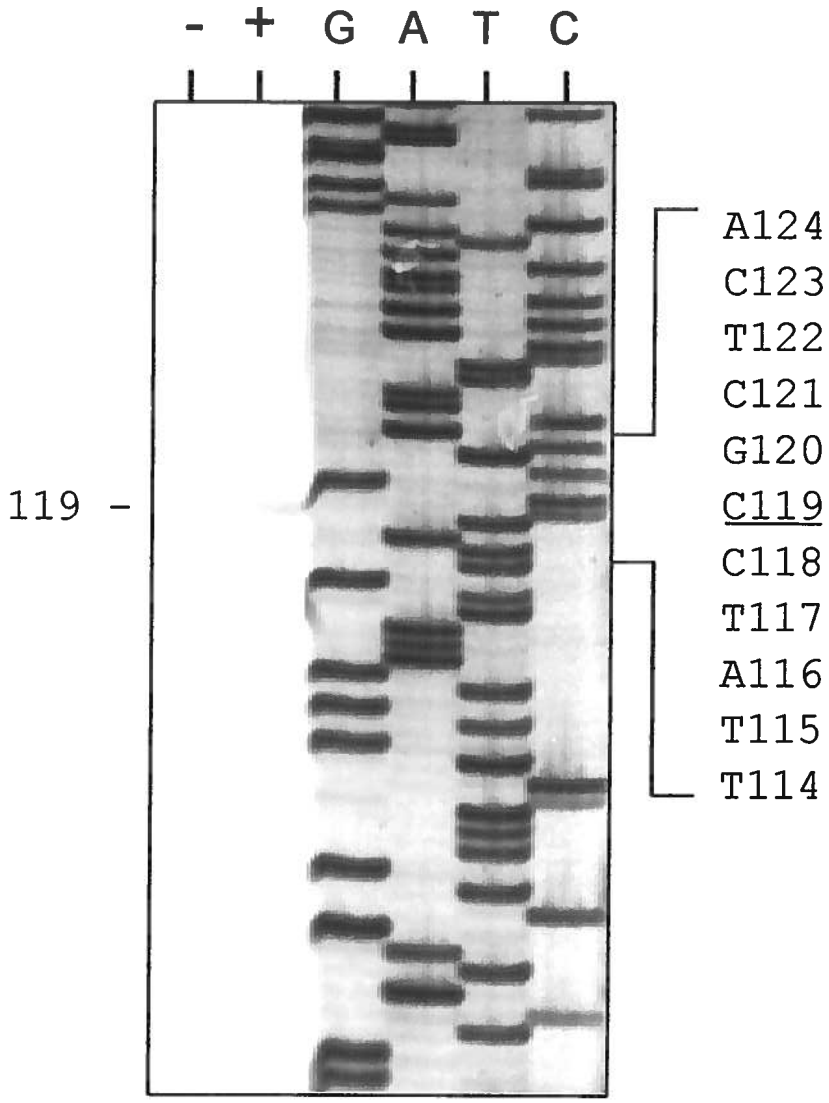
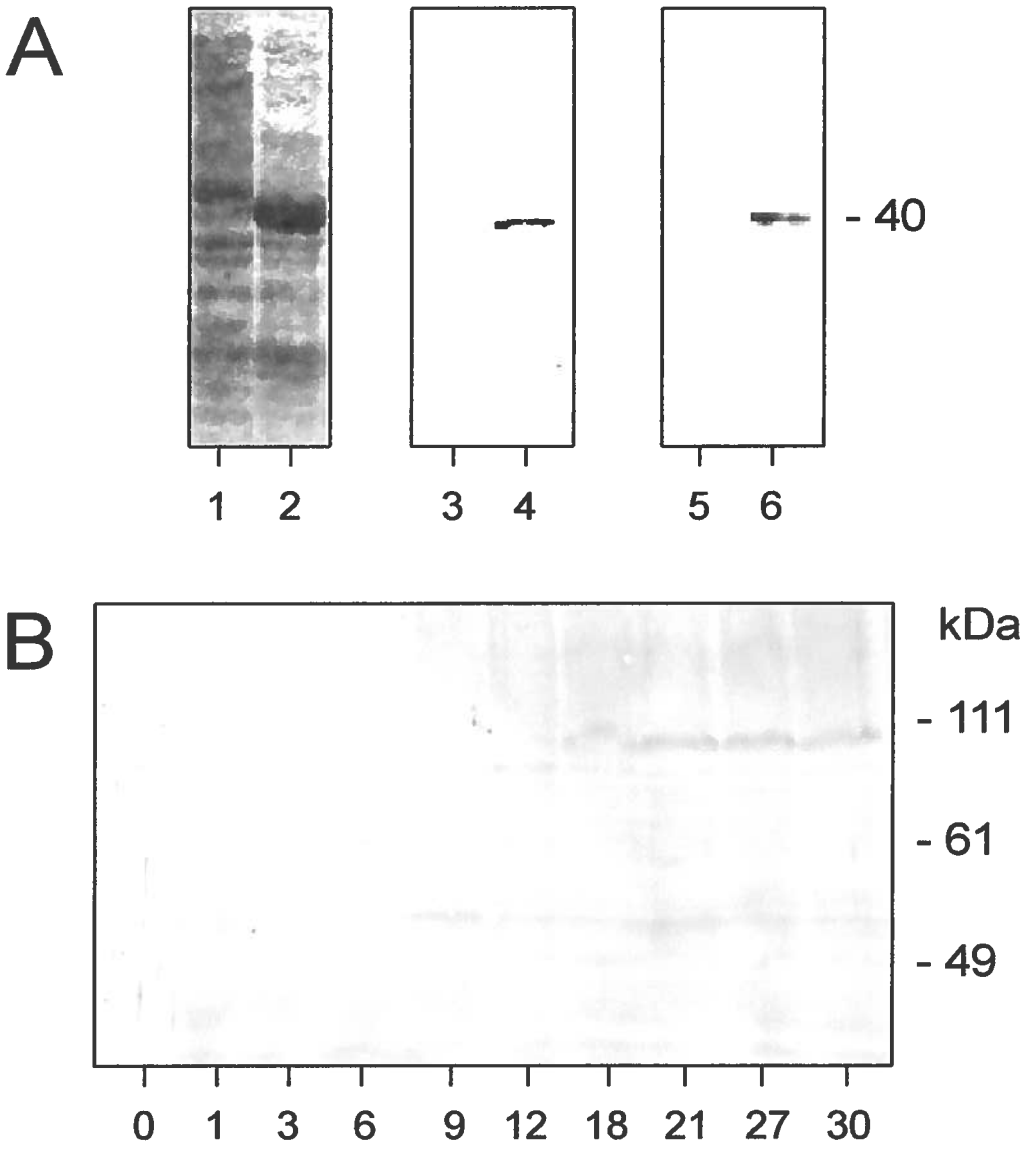

- + G A T C
119 -
A124
C123
T122
C121
G120
C119
C118
T117
A116
T115
T114

Figure 4. Development of a BHV1-specific UL28 antiserum and translational expression kinetics of UL28.

A) Insoluble protein fractions from either IPTG-induced (lanes 2, 4, 6) or non-induced (1, 3, 5) *E. coli* cells harbouring pET-28b/UL28 were fractionated by SDS-PAGE. Proteins were stained with Coomassie blue (1, 2) or immunodetected with either an anti-T7-Tag MAb (3, 4) or a UL28-specific antiserum generated in mice immunized with the T7-Tag/UL28 recombinant protein (5, 6). The protein content in lanes 3-6 represents only 4% of that present in lanes 1 and 2. The size in kDa of the recombinant protein is indicated.

B) Confluent monolayers of MDBK cells were infected with BHV1 at an m.o.i. of 2 for 0, 1, 3, 6, 9, 12, 18, 21, 27, and 30 hours. Protein cell lysates were fractionated by SDS-PAGE, electrotransferred onto a PVDF membrane and then reacted with the UL28-specific antiserum using the conditions described in the materials and methods section. Sizes of standards in kDa are indicated.


A
- 40
1 2 3 4 5 6
B
kDa
- 111
- 61
- 49
0 1 3 6 9 12 18 21 27 30

UL28 deficiency in bovine herpesvirus 1 does not abolish DNA packaging even though processing of newly synthesized DNA concatemers is abrogated

Running title: Role of BHV1 UL28 in virus replication

Nathalie Desloges and Claire Simard*

INRS-Institut Armand-Frappier
Centre de Microbiologie et Biotechnologie
531 Bd des Prairies
Laval, Québec, Canada, H7V 1B7
Tel. (450) 687-5010 ext. 4637
Fax (450) 686-5626
E-mail : Claire.Simard@inrs-iaf.uquebec.ca

*Corresponding author

La déficience du gène UL28 chez le virus de l'herpès bovin 1 n'abolit pas l'encapsidation de l'ADN viral même si la maturation des concatémères d'ADN nouvellement synthétisés est abrogée.

Nous avons caractérisé un mutant de délétion du gène UL28 du virus de l'herpès bovin 1 (BHV1), BHV1ΔUL28. La réplication productive du BHV1ΔUL28 n'a été observée que dans une lignée cellulaire spécifiquement conçue exprimant la protéine UL28, démontrant que le gène UL28 est essentiel à la réplication du virus. La déficience du gène UL28 ne compromet pas la synthèse des protéines virales, ni la réplication de l'ADN dans les cellules infectées qui ne complémentent pas. Des analyses de type Southern d'ADN isolé de cellules normales infectées et qui a été traité ou non à la DNase, révèlent que l'ADN viral nouvellement synthétisé est effectivement encapsidé à 6 heures post-infection (p.i.). Cependant, même si la quantité d'ADN synthétisé augmente continuellement de 6 à 24 heures p.i., celle d'ADN encapsidé reste constante, démontrant une interruption de l'encapsidation. Les résultats ont été confirmés par l'analyse en microscopie électronique de cellules normales infectées par le BHV1ΔUL28 où un petit nombre de capsides C contenant de l'ADN et pouvant maturer ont été observées. Ceci est différent des résultats obtenus avec les mutants homologues des virus de l'herpès simplex et de la pseudorage porcine qui n'encapsident pas d'ADN. De plus, nos résultats suggèrent que le substrat d'ADN qui est encapsidé chez le BHV1ΔUL28 est des génomes simples et linéaires synthétisés tôt lors de l'infection de la cellule. Nous concluons que le gène UL28 du BHV1 n'est pas essentiel pour l'encapsidation de l'ADN mature mais est requis pour le clivage et l'encapsidation des concatémères d'ADN. À notre connaissance, cette étude est la première à rapporter que l'ADN d'un virus herpès peut être encapsidé en absence de maturation des concatémères d'ADN. Nos résultats amènent de nouvelles connaissances se rapportant à la réplication de l'ADN des virus alphaherpès.

SUMMARY

We characterized a UL28 deletion mutant of bovine herpesvirus 1 (BHV1), BHV1ΔUL28. Productive growth of BHV1ΔUL28 was only observed in a specifically engineered complementing cell line expressing the native UL28 protein, demonstrating that UL28 is essential for virus replication. UL28 deficiency neither compromised viral protein synthesis nor DNA replication in non-complementing infected cells. Interestingly, Southern blotting analyses of DNase I-treated and untreated DNA from infected normal cells revealed that newly synthesized viral DNA was effectively packaged into capsids at 6 hours post-infection (p.i.). However, although the levels of synthesized DNA increased continuously from 6 to 24 hours p.i, that of packaged DNA remained steady, demonstrating packaging interruption. Our findings were further corroborated by electron microscopy analyses of BHV1ΔUL28-infected normal cells where a low number of C capsids containing DNA which could mature, were observed. This is in contrast to homologous mutants from both herpes simplex and pseudorabies viruses that do not package DNA. We present evidences that packaging DNA substrates in BHV1ΔUL28 are linear unit-length genomes synthesized early upon cell infection. We conclude that BHV1 UL28 is dispensable for packaging of mature DNA but required for the cleavage and packaging of DNA concatemers. To our knowledge, this is the first study reporting that DNA from a herpesvirus can be packaged in the absence of maturation of DNA concatemers. Our findings provide clues pertaining to alphaherpesviruses DNA replication.

INTRODUCTION

Bovine herpesvirus 1 (BHV1) is a member of the *Alphaherpesvirinae*, similarly to herpes simplex virus 1 and 2 (HSV1 and HSV2), pseudorabies virus (PrV), equine herpes virus 1 (EHV1), and varicella-zoster virus (VZV; Roizman, 1996). The BHV1 genome is a 135,301 bp double-strand linear DNA molecule composed of a unique long (UL) and a unique short (US) segments, the latter being flanked by inverted repeats (Mayfield *et al.*, 1983). Two isomeric forms of the genome occur naturally as US can invert its orientation relative to the UL segment (Farley *et al.*, 1981). The viral genome contains two origins of replication of identical but inverted sequences, being located within the inverted repeats. The morphology of the BHV1 virion is largely comparable to that of other alphaherpesviruses, consisting of a bilayer envelope, an amorphous layer known as the tegument, a capsid shell and a DNA-containing core (Ludwig, 1983). As in other alphaherpesviruses, BHV1-infected cells contain three different capsid types designated A (empty), B (intermediate) and C (full). C capsids contain DNA and exhibit a densely staining core, B capsids contain an internal structure constituted of a proteinaceous scaffold whereas type A capsids are devoid of DNA and of internal structure (Homa and Brown, 1997).

Infection of host cells by alphaherpesviruses begins with the entry of the virion's nucleocapsid into the cell after which the linear viral DNA is translocated into the nucleus where it circularizes rapidly to generate a replicative form (Roizman and Sears, 1996). DNA replication is believed to proceed in two phases (reviewed in Boehmer and Lehman, 1997): an initial phase of origin-dependent bidirectional or θ (theta) replication yielding daughter progeny circular rings, followed by a rolling-circle mode of replication generating long head-to-tail concatemers. To date however, there is no direct evidence of unit-length circular DNA or theta replication intermediates. Direct examination of HSV1 DNA replication intermediates by electron microscopy revealed molecules that contain extensive regions of single-strand DNA, DNA replication forks, loops, and highly branched DNA structures presumably arising from homologous recombination, genome isomerization and other events (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977; Jacob and Roizman, 1977; Shlomai *et al.*, 1976). These structures are resolved into unit-length genomes for DNA packaging into B capsids, a process resulting in the release of the scaffold and the

formation of C capsids which eventually mature to become infectious virions (Roizman and Sears, 1996). Occasionnally, the packaging reaction aborts presumably resulting in the formation of A capsids in which the internal proteins are lost but DNA is not inserted (Perdue *et al.*, 1976). The elements within the genome that are responsible for DNA cleavage and packaging are designated pac1 and pac2; in BHV1, sequence analysis suggests that these signals are located at opposite termini of the genome, flanking the cleavage site (Deiss *et al.*, 1986).

Studies with HSV1 specific mutants have shown that the cleavage/packaging process of viral DNA requires at least seven virus-encoded proteins generated by UL6, UL15, UL17, UL25, UL28, UL32, and UL33 genes (Addison *et al.*, 1990; Al-Kobaisi *et al.*, 1991; Baines *et al.*, 1997; Goshima *et al.*, 2000; Lamberti and Weller, 1996; Lamberti and Weller, 1998; McNab *et al.*, 1998; Patel *et al.*, 1996; Poon and Roizman, 1993; Taus *et al.*, 1998; Tengelsen *et al.*, 1993; Yu *et al.*, 1997). In addition, a viral-specific alkaline deoxyribonuclease encoded by UL12, although not essential to virus replication, appears to be important in resolving branched DNA structures that are generated during DNA replication (Goldstein and Weller, 1998; Martinez *et al.*, 1996). The function of each of the seven essential genes in the cleavage/packaging process remains poorly understood. However, similarly to double-strand DNA bacteriophages such as Lambda (Catalano, 2000; Catalano *et al.*, 1995), it is likely that the cleavage/packaging process involves a terminase complex that recognizes the DNA to be packaged, assembles a packaging complex at a specific DNA entry site (*pac* site) on a preformed capsid, cleaves the DNA at a proper site to initiate encapsidation, inserts a unit-length genome into the capsid and excises again the DNA to terminate the process (Abbotts *et al.*, 2000). By circumstantial analogy with phage terminases which constitute a complex of 2 hetero-subunits transiently associated with procapsids during packaging but absent from the mature DNA-containing capsid, it has been suggested that the products of UL15 and UL28 gene homologs might constitute the terminase of herpesviruses based on the following data (Sheaffer *et al.*, 2001; Yu and Weller, 1998a-b). First, UL15 homologs exhibit sequence similarity, including a consensus ATP-binding site that is required for UL15 function (Yu and Weller, 1998b), with the large catalytic subunit of bacteriophage T4 terminase (Davison, 1992). Second, whereas HSV and PrV UL28 proteins localize to the cytoplasm in the absence of other viral proteins, they

are directed to the nucleus in the presence of UL15-encoded proteins, thus indicating that the two proteins interact physically as do the two subunits constituting the terminase complex in bacteriophage systems (Abbotts *et al.*, 2000; Koslowski *et al.*, 1999; Koslowski *et al.*, 1997). Third, the products of HSV1 UL15 and UL28 are both found predominantly in precursor procapsids and B capsids, and the levels of the two proteins are reduced in C capsids (Salmon and Baines, 1998; Sheaffer *et al.*, 2001; Yu and Weller, 1998a). Fourth, human cytomegalovirus (HCMV) UL56, the homolog of HSV UL28, binds to the viral DNA packaging signal (*pac* site), an activity associated with the small subunits of several terminases, and furthermore it possesses nuclease activity (Bogner *et al.*, 1998). Finally, a direct and specific interaction between the HSV1 UL28-encoded protein and oligonucleotides bearing pac elements either derived from the HSV1 or murine cytomegalovirus (MCMV) genome sequences was recently demonstrated, further emphasizing the role of UL28 in the recognition of DNA packaging sequences (Adelman *et al.*, 2001). Although these similarities with the terminase activity of bacteriophage systems are promising, further studies are required to definitely establish that the proteins encoded by UL15 and UL28 effectively constitute a herpesvirus terminase.

To our knowledge, mutations in one or the other of the seven identified essential genes (listed above) in DNA cleavage/packaging of alphaherpesviruses were invariably found to prevent the production of C capsids. In the present study, we show that a UL28 deletion mutant of BHV1, BHV1ΔUL28, does not compromise packaging of newly synthesized viral DNA early in infection of non-complementing host cells. Later on however, packaging is interrupted even though higher levels of viral DNA accumulate in infected cells.

MATERIALS AND METHODS

Cells, virus and preparation of viral DNA for cell transfection

Rabbit kidney cells (RK13; ATCC CCL-377) were grown in equal parts of Earle's minimal essential medium and Hanks' medium 199 supplemented with 10% fetal bovine serum (FBS). Madin-Darby bovine kidney (MDBK; ATCC CCL22) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. Strain #34 of BHV1, a field isolate of subtype 1.1, was kindly provided by Dr. Mitchell (Government of

Canada, Lethbridge, Alberta) and cultured on RK13 cells. To prepare viral DNA for transfection, confluent monolayer sheets of MDBK cells were infected with BHV1 using a multiplicity of infection (m.o.i.) of 2, in a minimal volume of serum-free medium and incubated at 37°C for 90 minutes in a CO_2 incubator. Following this incubation step, fresh culture medium supplemented with 1% FBS was added and the incubation continued. Virus was harvested 48 hours post-infection (p.i.) and sedimented by ultracentrifugation as previously described (Simard *et al.*, 1990). The viral pellet was resuspended in TEN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) and the viral DNA was purified as reported (Simard *et al.*, 1990), with the exception that the DNase I treatment was omitted.

Plasmid constructions

The BHV1 UL28 ORF is located within the *Hind* III fragment A of the BHV1 genome in the rightward orientation, at positions 53058→55538 (Fig. 1; Meyer *et al.*, 1997). The 9825 bp *Hind* III-*Cla* I fragment representing the region 51711-61536 of the viral genome was subcloned from the recombinant plasmid pKS/Ahd carrying the *Hind* III fragment A (Simard *et al.*, 1990) to generate pKS/BHV51-61kHd-Cla. This plasmid was used as previously described (Desloges and Simard, 2001) to create pKS/UL28orf, a plasmid strictly containing the complete coding sequence of UL28 flanked by *Xba* I restriction sites. Briefly the *Bsm* FI fragment of pKS/BHV51-61kHd-Cla, representing codons 2-490 of the UL28 coding sequence, was ligated with an oligonucleotide designed to regenerate the ORF initiation codon and to create unique flanking restriction sites. Following further digestion, the fragment carrying the first 302 codons was inserted into the pKS vector, generating pKS/UL28(1-302). Taking advantage of a unique *Nhe* I restriction site encompassing the ORF stop codon, the fragment from pKS/BHV51-61kHd-Cla extending from codon 128 to the stop codon was finally used to replace codons 128-302 carried in pKS/UL28(1-302), generating pKS/UL28orf. The latter plasmid was used to create pRetroTET-OFF/UL28 by inserting the filled-in 2525 bp *Xba* I fragment carrying the complete UL28 ORF into pRetroTET-OFF (Clontech), under the control of the *tet*O-minimal CMV promoter (Fig. 1). This eucaryotic expression vector, developed by Paulus *et al.* (1996), integrates the tetracycline dependent high level gene expression system developed by Gossen and Bujard (1992): the vector encodes the tetracycline-controlled transactivator (tTA) regulated by the SV40 promoter and provides the

puromycin resistance gene as a selectable marker. This vector was used to allow tight control of UL28 expression in stably transfected cells. A recombinant in which the inserted DNA was in the proper orientation relative to the vector's inducible promoter was selected by analyzing asymmetrical digests of purified plasmid DNA.

The recombinant vector pKS/51-61kΔUL28/ß-gal$^+$ was constructed from pKS/BHV51-61kHd-Cla by replacing the 1,049 bp *Nru* I-*Hpa* I subfragment representing codons 303-652 of UL28 (Fig. 1), by a functional ß-galactosidase (ß-gal) reporter gene cassette derived from pEC10, a plasmid graciously provided by Dr. L. J. Bello (University of Glasgow, Glasgow). In pEC10, the ß-gal cassette is contained within a 3.8 kbp *Hin*d III fragment carrying the complete ß-gal coding sequence from *E. coli,* which is under the control of the BHV1 gB promoter and is followed by a consensus polyadenylation signal to direct termination of transcription. We initially flanked the cassette with blunt-ended unique restriction sites by subcloning the 3.8 kbp *Hin*d III fragment representing the cassette with the aid of a specially designed linker allowing the addition of *Nru* I flanking sites while preserving the *Hin*d III sites. Ligation of the 11,727 bp *Nru* I-*Hpa* I fragment from pKS/BHV51-61kHd-Cla with the 3.8 kbp *Nru* I fragment representing the ß-gal cassette, allowed the isolation of recombinants in which the ß-gal gene cassette's orientation was either the same or opposite to that of the UL28 gene. The recombinant pKS/51-61kΔUL28/ß-gal$^+$ used in the present study had its ß-gal cassette in the same orientation as that of the UL28 ORF.

Development of a UL28 specific complementing cell line

The recombinant eucaryotic expression vector pRetroTET-OFF/UL28, containing the complete UL28 coding sequence under the control of the *tet*O-minimal CMV promoter (Fig. 1), was used for the development of a stable cell line inducibly expressing the native BHV1 UL28 protein. For this purpose, RK13 monolayer cells at 80% confluency in 60 mm petri dishes were transfected with 2 µg of S*ca* I-linearized plasmid (see Fig. 1) in the presence of 25 µL of Lipofectamine Reagent (Gibco BRL) according to the supplier's instructions. Twenty-four hours post-transfection, cells were trypsinized, counted, and then 100 000 cells per 150 mm petri dish were seeded and grown in fresh culture medium supplemented with 600 ng/mL of puromycin as well as with 200 ng/mL of doxycycline, a tetracycline derivative compound (Gossen *et al.*, 1995); these drugs respectively allowed to

apply a selective pressure on transfected cells and to turn off the expression of the UL28 protein. Every 48 hours, the culture medium was replaced with freshly prepared medium. Two weeks later, several puromycin-resistant colonies were visible. Isolated colonies were trypsinized by using cloning rings and the cells were grown in successively larger flasks. Individual cell lines were next tested by Western blot analyses for their ability to inducibly express the UL28 protein in the absence of doxycycline. A suitable cell line was chosen and named RK13/UL28$^+$.

Construction of the BHV1ΔUL28 mutant

Subconfluent RK13/UL28$^+$ cells grown in tetracycline-free FBS (Clontech) and in the absence of doxycycline in 60 mm petri dishes were co-transfected with purified BHV1 DNA and pKS/BHV51-61kΔUL28/ßgal$^+$ by the calcium phosphate precipitation method (Sambrook *et al.*, 1989). Specifically, 15 µg of BHV1 DNA and 10 µg of *Sca* I-linearized pKS/BHV51-61kΔUL28/ßgal$^+$ in a total volume of 379 µL of HEBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 20 mM HEPES, pH 7.1) were coprecipitated by adding 21 µL of 2.5 M $CaCl_2$ followed by a 15 min incubation step at ambient temperature. The mixture was then added drop by drop directly onto the cells and the petri dish incubated for 30 minutes at 37°C. Culture medium containing 10% FBS was then added and the incubation continued for 5 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Cells were washed once with FBS-free medium and then treated for 2 min with 2 mL of 30% dimethyl sulfoxide prepared in HEBS. Cells were washed twice with FBS-free medium, supplemented with medium containing 10% FBS and incubated at 37°C in the CO_2-incubator until complete cytopathic degeneration was observed (5-7 days). Following a freeze-thaw cycle, the viral supernatant was harvested, briefly sonicated, titrated and then used to infect confluent monolayers of RK13/UL28$^+$ cells in 150 mm petri dishes at an m.o.i. of 0.005. As soon as plaques were visible, the cell monolayers were overlaid with a layer of 1% agarose in phenol red free Dulbecco's modified Eagle medium (DMEM) supplemented with 1% FBS. Two to 12 hours later, a second layer of 1% agarose in DMEM containing 300 µg/mL of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal; Roche Diagnostics) was added. After further incubation at 37°C, plaques staining blue were collected by aspiration with a sterile Pasteur

pipet, replated and plaque purified four additional times before viral stocks of the designated BHV1ΔUL28 mutant were made.

Rescue of the BHV1ΔUL28 mutant

Subconfluent RK13 cells in 60 mm petri dishes were co-transfected with purified viral DNA from BHV1ΔUL28 and the recombinant plasmid DNA pKS/BHV51-61k/Hd-Cla using essentially the conditions described above. Following the DMSO treatment, cells were superinfected for 1 hour at 37°C with BHV1ΔUL28 at an m.o.i. of 0.06 in a minimal volume of serum-free medium. Culture medium supplemented with 1% FBS was then added and the incubation continued until complete cytopathic degeneration (7-8 days). Virus progeny was cloned by serial dilutions of the viral supernatant in 96-wells flasks before viral stocks were made.

One-step growth analyses

One-step growth curves were performed as follows. Several flasks of non-complementing RK13 as well as complementing RK13/UL28$^+$ cells were synchronously infected with either wild-type (WT) BHV1 or BHV1ΔUL28 at an m.o.i. of 5. After a 60 min adsorption period, the inoculum was removed and the cells were washed three times with 1X phosphate buffered saline (PBS). Fresh culture medium supplemented with 1% FBS was added to each flask. A flask/cell line was immediately transferred to a -70°C freezer to represent the 0 hours p.i. samples. The other flasks were incubated for different times p.i.. When all the flasks had been collected, they were submitted to three freeze-thaw cycles after which cell lysates were transferred to 15 mL conical tubes, briefly sonicated, and then centrifuged at 2000 rpm at 4°C for 15 min to remove cellular debris. Clarified supernatants of BHV1ΔUL28-infected samples were titered on RK13/UL28$^+$ cells, whereas RK13 cells were used for those infected with BHV1.

Analysis of viral DNA replication and maturation by Southern blotting

Confluent monolayers of RK13 as well as RK13/UL28$^+$ cells in several T75 flasks were infected for different times with BHV1ΔUL28 using the conditions described above. Frozen flasks were thawed and cell lysates harvested in 50 mL conical tubes after which cell debris were eliminated by centrifugation at 2000 rpm for 15 minutes at 4°C. Total-infected cell DNA as well as viruses and nucleocapsids present in the supernatants were

precipitated overnight at 4°C after the addition of 0.17 volumes of 50% polyethylene-glycol-8000 (PEG) prepared in TEN. The following day, the precipitate was recovered by centrifugation at 4000 rpm for 30 minutes at 4°C (Sorvall SS-34 rotor), resuspended in 1 mL of TEN, sonicated 5 seconds. Each sample was then split into two equal parts (one part from a duplicated sample representing total cell DNA extracts collected at 24 hours p.i. received 0.05 volume of a solution containing 10% dodecyl lithium sulfate). To the first series of tubes was added 0.1 volumes of a solution containing 100 mM $MgCl_2$, 1 mg DNase I/mL and 1 mg RNase A/mL, whereas the second series received 0.1 volumes of 100 mM EDTA pH 8.0 containing 1 mg RNase A/mL. The two series of tubes were incubated overnight at 37°C, after which the DNase I-containing tubes received 0.02 volumes of 500 mM EDTA to inactivate the enzyme. After the addition of 0.05 volumes of 20% SDS, each sample was treated for 2 hours at 37°C with 200 µg of proteinase K/mL. The mixture was then extracted once with 1 volume of phenol and once with 1 volume of phenol:$CHCl_3$:isoamylalcohol (24:24:1). The DNA was ethanol precipitated, washed with 70% ethanol, and briefly dried. DNA pellets from DNase I-treated and non-treated samples were dissolved in 40 µL and 125 µL of sterile water, respectively.

Purified viral DNA (5 µL) was digested with *Hin*d III and fractionated on two 0.4% agarose gels prepared in TBE buffer (Simard *et al.*, 1991). DNA profiles were then transferred onto positively charged nylon membranes (Roche Diagnostics) using a VacuGene vacuum blotting system (LKB Bromma). Following covalent mobilisation of the DNA to the membrane by UV exposure for 4 min, the blot was prehybridized at 42°C for 3-4 hours in a solution containing 50% formamide, 5X SSC, 0.1% N-lauroyl-sarcosine, 0.02% SDS and 2% blocking reagent (Roche Diagnostics). The blot was then hybridized overnight in a freshly prepared solution containing the probe of interest which had been previously DIG-labeled using the DIG random-primed DNA labeling kit from Roche Diagnostics. The following day, the blot was washed twice in 2X SSC, 0.1% SDS at room temperature, twice in 0.5X SSC, 0.1% SDS at 68°C, and twice in 0.1X SSC, 0.1% SDS at 68°C. DIG-labeled hybrids were detected by chemiluminescence according to the supplier's instructions (Roche Diagnostics), using the chemiluminescent substrate CSPD and the DIG DNA labeling and detection kit. To optimize the detection of fragments of

very low abundance, the blots were overexposed onto Konica X-Ray Medical Films (Medical Tronik Ltd) by using 16 hours exposure time.

Northern blot hybridization

Total RNA from BHV1ΔUL28-infected RK13 and RK13/UL28$^+$ cells was isolated using the TRIzol Reagent (Gibco BRL). Twenty μg of RNA/sample were used to prepare a Northern blot as previously described (Simard *et al.*, 1992). The blot was hybridized with a UL28 specific DIG-labeled probe under the same conditions as those used for Southern blotting.

Western blotting

Western blotting of protein cell lysates onto polyvinylidene difluoride (PVDF) membranes was done as previously described (Desloges and Simard, 2001; Desloges *et al.*, 2001).

Transmission electron microscopy

Monolayers of RK13 and RK13/UL28$^+$ cells were infected with either BHV1 or BHV1ΔUL28 at a m.o.i. of 2 in a minimal volume of serum-free medium. Following a 60 min adsorption period, the inoculums were removed and the cells were then washed three times with 1X PBS after which fresh culture medium supplemented with 1% FBS was added and the incubation continued. At 18 hours p.i., cells were fixed for 15 min with 2.5% glutaraldehyde prepared in 50 mM sodium cacodylate buffer pH 7.2. Cell monolayers were then scrapped with a policeman, centrifuged and washed 3 times for 15 min with 3% sucrose in cacodylate buffer. Samples were held overnight at 4°C in the latter solution and then post-fixed for 2 hours at ambient temperature with 1.33% osmium tetroxide prepared in 200 mM collidine buffer pH 7.4 containing 5% sucrose. Samples were dehydrated step by step from acetone 25% to 100% after which they were embedded in Spurr and incubated at 60°C for 24 hours. Finally, samples were thin sectioned, stained with lead citrate and uranyl acetate, and then examined with a Hitachi H-7100 electron microscope.

RESULTS

Creation of a UL28-complementing cell line

To propagate a potentially non-viable BHV1 UL28 deletion mutant, we initially set out to develop a specific cell line that could complement in *trans* a UL28 deficiency. For

this purpose, RK13 cells were stably transfected with the recombinant eucaryotic expression vector pRetroTET-OFF/UL28 (Fig. 1), encoding the complete UL28 ORF under the control of a tetracycline-regulated promoter as well as a puromycin selectable marker. Several puromycin-resistant cell colonies were isolated, amplified, and used to prepare cell lysates for Western blot analysis to verify whether these clones possessed the ability to express the UL28 protein in a tetracycline dependent regulated fashion. Figure 2 shows that a selected cell line grown in the absence of doxycycline, a tetracycline derivative compound (Gossen *et al.*, 1995), expressed a predominant 90-100 kDa protein as well as two minor 58 and 48 kDa polypeptides that specifically reacted with a UL28 antiserum directed against an *E. coli*-expressed T7-tag-UL28 fusion protein (lane 2; Desloges and Simard, 2001). Undoubtedly, the larger polypeptide represents the full length product of UL28 since its size correlated well with that predicted (86,919 Da) and with that of the protein observed in BHV1-infected cells (Desloges and Simard, 2001). The two smaller proteins most probably represented truncated products derived from the mature UL28 protein. This interpretation is further confirmed by the observation that the addition of doxycycline to the culture medium almost completely abrogated the expression of UL28 (lane 1). This cell line, which tightly controls UL28 expression, was designated RK13/UL28$^+$ and was used as a complementing host cell for the construction of a UL28 deletion viral mutant.

Isolation of a UL28 deletion mutant and characterization of the resulting viral genome

To genetically engineer a BHV1 UL28 deletion mutant, we constructed the plasmid pKS/51-61kΔUL28/ß-gal$^+$ from pKS/BHV51-61k/Hd-Cla (Fig. 1), by replacing the *Nru* I-*Hpa* I subfragment encoding amino acids 303-652 of the UL28 protein by a ß-gal reporter gene cassette. RK13/UL28$^+$ cells grown in the absence of doxycycline (on-state) were then co-transfected with purified BHV1 viral DNA and pKS/51-61kΔUL28/ß-gal$^+$. Mutants arising as a consequence of homologous recombination were phenotypically identified and isolated from WT virus by plating the virus progeny on RK13/UL28$^+$ cells followed by screening for ß-gal activity in the presence of the chromogenic compound X-Gal. ß-gal$^+$ mutant virus was plaque purified 5 times on RK13/UL28$^+$ cells before viral production.

Southern blot hybridization using specific probes representing either the complete UL28 or ß-gal coding sequences against purified viral DNA digested with *Hin*d III was used to examine whether the deletion/insertion mutation was located at the appropriate position within the mutant genome. The ß-gal probe did not detect any fragments within WT BHV1 DNA (Fig. 3B, lane 2), but hybridized with a 3.8 kbp *Hin*d III fragment present in both mutant viral DNA (lane 1) and pKS/51-61kΔUL28/ß-gal$^+$ plasmid DNA (lane 3). These findings show that the complete 3.8 kbp *Hin*d III fragment carrying the ß-gal cassette was inserted within the mutant viral DNA genome. Hybridization of the blot with the UL28-specific probe revealed the presence of a large *Hin*d III fragment of 21 kbp in WT BHV1 DNA (Fig. 3A, lane 2). This fragment, corresponding to the *Hin*d III fragment A of the BHV1 genome where the insertion was introduced (see the restriction map shown in Fig. 1), was absent from the mutant DNA (Fig. 3A, lane 1). Instead, three hybridizing bands of 18, 3.8 and 2.2 kbp were observed. Similarly, the *Hin*d III profile of pKS/51-61kΔUL28/ß-gal$^+$ possessed three fragments of 9.5, 3.8 and 2.2 kbp that hybridized with the UL28-specific probe (Fig. 3A, lane 3). As demonstrated above with the ß-gal probe, the 3.8 kbp *Hin*d III band common to the latter two profiles represents the ß-gal gene cassette. This 3.8 kbp fragment, containing the BHV1 gB promoter regulating the ß-gal ORF within the cassette, was recognized by the UL28-specific probe since the 3'-end of the UL28 coding sequence overlaps with the gB gene's promoter within the viral genome (the gB gene coding sequence is located at positions 55394-58192 of the viral genome; see Fig. 1). For their part, the UL28 probe specific 2.2 kbp band corresponds to the newly created *Hin*d III fragment located to the left of the *Nru* I insertion site of the ß-gal reporter gene cassette (region 51711-53964, see Fig. 1), whereas the 18 and 9.5 kbp bands represent the regions 55013-72945 (*Hpa* I-*Hin*d III) and 55013-61536 (*Hpa* I-*Cla* I), respectively, located on the right hand side of the *Hpa* I mutation insertion site. These findings thus demonstrate that the ß-gal reporter gene cassette interrupts as intended the UL28 coding sequence of the viral mutant, which was designated BHV1ΔUL28.

To verify that the engineered mutant was indeed incapable of expressing the UL28 gene product, Northern blot analysis was performed on total RNA isolated 18 hours after BHV1ΔUL28 infection of RK13 and RK13/UL28$^+$ cells with a probe corresponding to the 1049 bp *Nru* I-*Hpa* I fragment deleted from the mutant genome. As expected, no transcript

could be detected in RNA isolated from BHV1ΔUL28-infected RK13 cells (Fig. 3C, lane 1). In contrast, the probe detected a 3.8 kb specific transcript in RNA from mutant-infected (lane 2) and non-infected (lane 3) RK13/UL28$^+$ cells. The size of this transcript correlated well with that expected (approximately 4 kb) from the UL28 expression cassette of the recombinant pRetroTET-OFF/UL28, thus confirming that this mRNA originates from the UL28-expressing cell line. These results demonstrate that the mutant genome of BHV1ΔUL28 had lost the 1049 bp *Nru* I-*Hpa* I fragment.

Growth properties of BHV1ΔUL28

The influence of the UL28 defect on viral growth was assessed by comparing the viral yields obtained following a single BHV1ΔUL28 infectious cycle in both non-complementing RK13 and complementing RK13/UL28$^+$ cells to that of a single infectious cycle of WT BHV1 infected cells. For this purpose, the total number of virus particles, either extracellularly or intracellularly, recovered at different times p.i. from cells synchronously infected with either virus was determined by titration assays. Figure 4A shows that WT BHV1 was efficiently replicated in either RK13 and RK13/UL28$^+$ cells, reaching similar maximal viral yields at 30 hours p.i.. However, WT BHV1 growth kinetics were different in the two cell lines. Infectious viral particles were reproducibly recovered from RK13 infected cells as soon as 8 hours p.i., whereas no viable virus could be detected from RK13/UL28$^+$ infected cells until 12 hours p.i. This delay in the onset of the synthetic phase is likely responsible for the lower viral yield observed at 16 and 24 hours p.i. in RK13/UL28$^+$ as compared to that from RK13 cells. These observations suggest that the presence of the UL28 protein in RK13/UL28$^+$ interferes with the onset of WT BHV1 virus production.

In contrast, BHV1ΔUL28 could only replicate in RK13/UL28$^+$ cells, as no virus progeny was produced in normal RK13 cells (Fig. 4A). These results demonstrate that the UL28 gene product is absolutely required for viral replication in non-complementing cells. Interestingly, the onset of mutant virus production in RK13/UL28$^+$ was similar to that observed in WT BHV1 infected RK13 cells, initiating at 8 hours p.i.. In addition, the maximal BHV1ΔUL28 viral yield obtained in RK13/UL28$^+$ cells was similar to that of WT

virus yield recovered from either cell line, indicating that the RK13/UL28^{+} cell line was capable to fully complement the UL28 deficiency.

To verify that the inability of BHV1ΔUL28 to replicate in normal RK13 cells was a direct consequence of the UL28 deletion and not the result of an arbitrary mutation inadvertently introduced outside of the UL28 coding sequence during the recombination event, the mutant virus was rescued by co-transfecting RK13 cells with pKS/BHV51-61k/Hd-Cla and purified viral DNA from BHV1ΔUL28. Since BHV1ΔUL28 is incapable of propagating itself in RK13, it was expected that 100% of the virus progeny recovered from these experiments would represent WT revertant virus. Effectively, none of the viral plaques obtained on RK13 developed blue coloration in the presence of X-Gal. Serial dilution of rescued virus on RK13 cells seeded in 96-wells flasks was used to purify a ß-gal-minus revertant virus which was designated BHV1rUL28. To verify whether BHV1rUL28 represented a true revertant, Southern blotting was performed on *Hind* III digests of viral DNA using a probe representing the complete UL28 coding sequence. This probe detected as expected a single large fragment, which co-migrated with the *Hind* III fragment A of WT BHV1 DNA (results not shown). In addition, Northern blotting analyses of total RNA from either BHV1rUL28-infected or WT BHV1-infected RK13 cells with a probe representing the delimited *Nru* I-*Hpa* I fragment from UL28 revealed a common 3.5 kb transcript (results not shown). Finally, as seen in Figure 4B, the growth profile of BHV1rUL28 in RK13 essentially followed that observed with WT BHV1. These findings thus unequivocally demonstrate that the inability of BHV1ΔUL28 to replicate in non-complementing RK13 cells is only a consequence of the deletion introduced within the UL28 gene.

The UL28 defect does not impair viral protein synthesis nor viral DNA replication but severely compromises DNA maturation

To determine at which step replication of BHV1ΔUL28 within RK13 cells aborts, we initially examined whether the defect affected viral gene expression. For this purpose, Western blotting of BHV1- and BHV1ΔUL28-infected RK13 cell protein lysates obtained at different times p.i. in the presence or absence of the DNA synthesis inhibitor phosphonoacetic acid (PAA) was performed. The blots were reacted with a BHV1-specific antiserum directed against an *E. coli*-expressed T7-tag-UL48 fusion protein representing

the viral alpha gene trans-inducing factor protein encoded by UL48, a gene of the γ2 class (Desloges *et al.*, 2001, Misra *et al.*, 1994). The antiserum reacted with a 50 kDa protein present in lysates of cells infected with either BHV1 or BHV1ΔUL28 collected at 12 and 18 hours p.i. (Fig. 5, lanes 12 and 18, - PAA). In contrast, no protein was detected in the mock-infected cell lysates (lanes 0), demonstrating the specificity of the antiserum. Furthermore, PAA completely abrogated expression of this polypeptide at 18 hours p.i., confirming the kinetic class of UL48 (lanes 18, + PAA). These findings demonstrate that the UL28 defect does not impair the expression of viral genes of the γ2 class. These results also indirectly demonstrate that the mutation does not compromise viral DNA synthesis.

To obtain direct evidence that the UL28 defect does not compromise viral DNA synthesis, Southern blotting analysis of *Hind* III digests of total cell DNA isolated from BHV1ΔUL28-infected RK13 and RK13/UL28$^+$ cells was performed, using an internal fragment of the BHV1 genome, the *Hind* III fragment M, as a probe (see Fig. 1). The probe hybridized to a low abundance 3.7 kbp fragment present in DNA from BHV1ΔUL28-infected RK13 cells obtained at 6 hours p.i. (Fig. 6A, lane 11) whose level significantly increased up to 12 hours p.i. (lane 12) and then remained constant up to 24 hours p.i. (lane 13). In contrast, no hybridization signal was observed at 0 hours p.i. (lane 10) demonstrating that the bands observed represented newly synthesized viral DNA. This is consistent with the results from the one-step growth curve analyses performed with the viral mutant. The band detected at 6-24 hours p.i. co-migrated with a *Hind* III fragment from pKS/Mhd (lane 9) harbouring the *Hind* III fragment M of the viral genome (Simard *et al.*, 1990), thus confirming its identity. In comparison, DNA from BHV1ΔUL28-infected RK13/UL28$^+$ cells also contained a probe specific 3.7 kbp fragment present at similar abundance at 12 and 24 hours p.i. (lanes 14 and 15, respectively). Interestingly, no significant difference was observed in the intensity of the bands generated at 12 and 24 hours p.i. in RK13/UL28$^+$, as compared to that in the corresponding RK13 samples. Indeed, the slight increase in band intensity observed in RK13/UL28$^+$ samples as compared to that observed in RK13 is most likely attributable to experimental variations of the assays. These findings indicate that DNA replication of BHV1ΔUL28 is as effective in normal RK13 than in complementing RK13/UL28$^+$ cells. In addition, our results suggest that

mutant viral DNA synthesis in one or the other cell line is complete as soon as 12 hours p.i., similarly to that of WT BHV1 in RK13 (results not shown). These results are consistent with those obtained in the one-step growth curve analyses which established that infectious virion progeny was actively produced at 8 hours p.i. of RK13/UL28^{+} and RK13 cells with BHV1ΔUL28 and WT BHV1, respectively.

To assess whether viral DNA synthesized by the mutant virus is packaged into nucleocapsids, total infected-cell DNA samples were rigorously treated with DNase I prior to DNA purification and Southern blot analyses. Very little DNase I-resistant viral DNA was recovered from BHV1ΔUL28-infected RK13 cells at either 12 or 24 hours p.i. as evidenced by the drastic decrease in the intensity of the hybridizing signals observed (Fig. 6A, lanes 3-4) as compared to that from the corresponding DNase I-untreated samples (lanes 12-13). This is further emphasized by the fact that the DNA pellet from DNase I-treated samples had been dissolved in less than one third of the water volume used for the samples containing total cell DNA, such that the expected amount of DNA present in the former should be proportionally three times higher than that in the latter (see the materials and methods section). The absence of hybridizing DNA at 0 hours p.i. (lane 1), which correlates with the results obtained with the one-step growth curves (see Fig. 4) demonstrates that the hybridizing DNA observed at 6 to 24 hours p.i. (Fig. 6A, lanes 2-4) did not originate from residual particles of the viral inoculum used for cell infection but rather from newly synthesized DNA. In contrast to these observations, no significant difference in the abundance of the hybridizing DNA fragment was observed in DNase I-treated samples from infected RK13/UL28^{+} cells at 12 and 24 hours p.i. (lanes 5 and 6, respectively), as compared to that observed in the corresponding non-treated samples (lanes 14 and 15). These results demonstrate that viral DNA synthesized in infected RK13/UL28^{+} cells is mostly if not completely packaged into capsids, since this DNA appeared to be completely resistant to DNase I digestion. In contrast, most of the viral DNA present at 12 and 24 hours p.i. of normal RK13 cells is in the free form, since it was sensitive to DNase I treatment. Nevertheless, the presence of DNase I-resistant DNA in infected RK13 cell extracts clearly suggests that a low level of synthesized viral DNA is effectively packaged into capsids. To exclude the possibility that this represented residual DNA resulting from a partial DNase I digestion, total cell DNA extracts collected at 24 hours p.i. were pretreated

with 0.5% dodecyl lithium sulfate (LiDS) to provoke capsid lysis prior to DNase I treatment and DNA purification. Following this treatment, no DNase I-resistant DNA could be detected from either BHV1ΔUL28-infected RK13 (lane 7) or RK13/UL28$^+$ cells (lane 8). However, pretreatment with LiDS had no effect on the amount of DNA that could be detected in the DNase I-untreated samples (lanes 16 and 17). These results thus demonstrate that the DNA detected in the DNase I-treated samples from infected RK13 cells represents viral DNA that was packaged into viral capsids. Moreover, our results show that although very little DNA is effectively encapsidated, packaging is completed as soon as 6 hours p.i., since no increase in the level of DNase I-resistant DNA was observed following this time point, even though more viral DNA was synthesized.

The evidence of free viral DNA in BHV1ΔUL28-infected RK13 cells raised the question as to whether this DNA was in the form of unit-length genomic DNA or of immature DNA concatemers. To resolve this, Southern blot analysis of total infected-cell DNA extracts was performed with a probe representing the leftward terminal region of the BHV1 linear viral genome, the *Hin*d III fragment N (see Fig. 1). Probe N hybridized to a predominant DNase I-resistant 2.4 kbp and a minor 15 kbp band present in extracts from BHV1ΔUL28-infected RK13 cells at 6, 12 and 24 hours p.i. (Fig. 6B, lanes 2-4). In contrast, no DNA was detected at 0 hours p.i., demonstrating that DNA detected at 6-24 hours p.i. represents newly synthesized viral DNA. The abundance of DNase I-resistant DNA fragments hybridizing with probe N correlated quite well with that observed with probe M (Fig. 6A, lanes 2-4). In comparison, DNase I-treated DNA obtained from BHV1ΔUL28-infected RK13/UL28$^+$ cells at 12 and 24 hours p.i. contained three hybridizing bands of 2.4, 15 and at least 30 kbp (Fig. 6B, lanes 5 and 6). As previously observed, pretreatment with LiDS of total infected-cell DNA extracts obtained at 24 hours p.i. resulted in the complete disappearance of the DNA bands (lanes 7 and 8), demonstrating that the DNase I treatment carried out prior to DNA purification was complete. The common 2.4 kbp bands observed in DNA extracts from the two cell lines represent the terminal 2.4 kbp *Hin*d III fragment N of the BHV1 linear genome, since they co-migrated with the *Kpn* I-*Hin*d III insert of the recombinant plasmid pKS/Nhd harbouring the *Hin*d III fragment N (lane 9; Simard *et al.*, 1990). These findings unambiguously demonstrate that BHV1ΔUL28 viral DNA in the form of linear unit-length genome is

packaged into viral capsids in infected RK13 cells. In addition, the size of the common 15 kbp bands is correlated to that expected for head-to-tail *Hin*d III genomic fragments (see Fig. 1; N-F and N-H head-to-tail fragments are in the range of 14 and 15 kbp, respectively), clearly suggesting the presence of viral DNA that is packaged in the form of a circular unit-length genome in infected cells. The identity of the third and larger band detected in extracts from BHV1ΔUL28-infected RK13/UL28$^+$ cells at 12 and 24 hours p.i. (lanes 5 and 6) is less obvious. This band, whose presence was also revealed in total DNA extracts from infected RK13/UL28$^+$ cells (see below), was reproducibly detected in two independent experiments, although in the second assay its relative abundance as compared to that of the 15 and 2.4 kbp bands was reduced (results not shown). Since the 30 kbp band hybridizes with N and represents DNA which was packaged, it must contain at least part of the terminal fragment N as well as functional *pac* signals and at least a DNA replication origin of the viral genome. It is thus likely that the 30 kbp band carries head-to-tail junction fragments and we postulate that it originates from defective virus genomes. However, the reason for which the size of the band is much larger than that of *Hin*d III digested head-to-tail fragments remains unknown. Finally, we hypothesize that a band of similar size, but whose abundance was below detection levels, was also present in extracts from infected normal RK13 cells.

Probe N hybridization profiles obtained with total cell DNA extracts (Fig. 6B, lanes 10-15) were significantly different from those observed with packaged DNA (lanes 1-6). In particular, with the exception of lane 11, bands revealed in DNase I-treated samples were strongly masked by the presence of long hybridization smears. These smears suggest the presence of DNA fragments of variable sizes and conformations, which most likely fractionated abnormally due to complex branched DNA structures. Interestingly, total cell DNA from infected RK13 cells obtained at 6 hours p.i. was essentially free of DNA smears as the probe only revealed the presence of a 2.4 kbp fragment (lane 11). Whereas, at 12 and 24 hours p.i. (lanes 12 and 13, respectively), the terminal fragment of the linear viral genome was barely discernible, due to the presence of hybridizing DNA smears. These results show that most of the viral DNA synthesized in RK13 cells at 12 and 24 hours p.i. lacks terminal fragments, demonstrating that this DNA is in the form of immature concatemers. In comparison, even though DNA smears were also present, a significant

amount of the 2.4 kbp terminal fragment was detected in total cell DNA extracts isolated from infected RK13/UL28$^+$ cells at 12 and 24 hours p.i. (lanes 14 and 15). These findings demonstrate that a large amount of the viral DNA present in complementing cells is in the form of unit-length genomic DNA, although some DNA concatemers do remain. Finally, it is of interest to note the presence of the two 15 and 30 kbp hybridizing DNA bands in lanes 14 and 15 as well as that of the 15 kbp band in lanes 12 and 13 that were previously observed in preparations of packaged DNA (lanes 2-6).

To further corroborate the data obtained in the Southern blot analyses, thin sections of BHV1ΔUL28-infected RK13 and RK13/UL28$^+$ cells and of WT BHV1-infected RK13 cells collected at 18 hours p.i. were examined by transmission electron microscopy in order to assess the presence of full (type C), intermediate (type B), as well as empty (type A) capsids. Type B capsids are free of DNA but do contain an electron-translucent core representing scaffolding protein. Type C and A capsids are the result of successful and aborted DNA packaging, respectively. Sections from both WT BHV1-infected RK13 and BHV1ΔUL28-infected RK13/UL28$^+$ cells contained a significant number of all three capsid types (Table 1). Each of these infected cell samples (Fig. 7A and B, respectively) contained several C capsids surrounded by a bilayer envelope, thus demonstrating that they were mature infectious virions. In contrast, most of the capsids in BHV1ΔUL28-infected RK13 cells were of type B (Table 1) as illustrated in Fig. 7C showing an accumulation of type B capsids in infected cells. However, careful examination of these infected cells revealed the presence of a few electron-dense cored C capsids (Table 1). This is demonstrated in Fig. 7D showing a complete enveloped type C capsid found in the extracellular compartment of BHV1ΔUL28-infected RK13 cells. Moreover, a significant level of empty capsids were observed (Table 1) as illustrated in Fig. 7E and F showing non-enveloped and enveloped type A capsids found in the perinuclear and extracellular compartments of BHV1ΔUL28-infected RK13 cells, respectively. The presence within infected cells of type A capsids indicates that the full capsids which were visualized were the result of active DNA packaging. These results thus confirm that BHV1ΔUL28 can package DNA in non-complementing RK13 cells.

DISCUSSION

In this study, a UL28-deletion mutant of BHV1, designated BHV1ΔUL28, was engineered to assess the role of UL28 in the replicative cycle of the virus. Analyses of the BHV1ΔUL28 mutant genome by Southern blot confirmed that part of the UL28 coding sequence had been effectively replaced by a ß-gal reporter gene cassette as evidenced by the observation that two novel *Hin*d III sites, which flank the cassette, were introduced within the viral genome. These two restriction sites gave rise to three new genomic *Hin*d III fragments of the expected size, demonstrating that the ß-gal cassette had been introduced at the intended location within the UL28 coding sequence. Inactivation of the UL28 coding sequence was further confirmed by Northern blot analyses of viral transcripts expressed in BHV1ΔUL28-infected RK13 cells which revealed that UL28 derived transcripts lacked the targeted *Nru* I-*Hpa* I fragment present within the WT BHV1 UL28 ORF. These findings demonstrate that the recombination event giving rise to BHV1ΔUL28 was accurate to within a few base pairs.

As observed with UL28 deletion mutants of HSV1 (Tengelsen *et al.*, 1993) and PrV (Mettenleiter *et al.*, 1993), analyses of the growth characteristics of BHV1ΔUL28 revealed that the UL28 deficiency prevented the production of progeny virus particles in non-complementing normal host cells capable of generating a detectable cytopathic effect when plated on the complementing cell line. In addition we demonstrate in the present study that the replication-deficient phenotype observed was a direct consequence of the UL28 deletion since following reversion of the mutation, rescued virus successfully replicated to wild-type levels in normal cells. Nevertheless, the replication kinetics of BHV1ΔUL28 in a complementing cell line expressing the BHV1 UL28 product in *trans* were indistinguishable from those of wild type virus. These findings thus unequivocally demonstrate that BHV1 UL28 is absolutely required for productive and continued viral replication.

As is the case in HSV1 and PrV, the UL28 deficiency in BHV1ΔUL28 compromised neither viral protein synthesis nor DNA replication in normal cells. However, in contrast to the HSV1 and PrV UL28 deletion mutants, which do not seem to package DNA upon infection of normal host cells, Southern blot analyses of DNase I-resistant DNA extracted from infected normal cells revealed that BHV1ΔUL28 retained its ability to direct

the packaging of newly synthesized viral DNA in nucleocapsids soon after cell infection, albeit at low levels. This was further confirmed by examination of thin sections of infected normal tissues by electron microscopy (EM), which revealed the presence of a few full C capsids. Latter experiments also revealed that C capsids retained their ability to mature as evidenced by the presence in the extracellular compartment of infected cells of an electron-dense cored capsid surrounded by a bilayer envelope. However, the enveloped particles produced were either non-infectious or their number was too low for cytopathogenesis as no virus was detected in one-step growth curve analyses following infection of RK13/UL28^{+} cells with lysates from BHV1ΔUL28-infected RK13 cells. Furthermore, no cytopathic effect was observed following infection of normal cells with BHV1ΔUL28. Results from EM analyses thus suggest that UL28 is not directly involved in the maturation of C capsids since its deficiency did not appear to interfere with the process.

It is unlikely that the phenotypic divergence observed between BHV1 and its two viral counterparts be attributed to residual activity resulting from the expression of the first 302 amino acid residues of the UL28 coding sequence remaining in BHV1ΔUL28 (the UL28 ORF in BHV1ΔUL28 was truncated at codon 303) since the HSV1 and PrV mutants retained longer 5'-end UL28 coding sequences in their genomes [in the HSV1 gCΔ7B mutant, codons 528-706 of the UL28 ORF are deleted (Tengelsen *et al.*, 1993) whereas in PrV 332-31 mutant, the ORF is interrupted approximately halfway through (Mettenleiter *et al.*, 1993)]. Nevertheless, the observed differences could be accounted for by a higher sensitivity of detection of Southern blots of DNase I-resistant DNA isolated from infected normal host cells. Rather than using a ^{32}P-labeled probe, as was done with HSV1 (no analysis of DNase I-resistant DNA was performed in PrV), not only have we used a highly sensitive chemiluminescent detection assay but additionally our blots were overexposed to allow detection of packaged DNA. Obviously, the results obtained prompted us to bear particular attention to the EM preparations of infected cells. If the sensitivity of the assay is not an issue, our findings would suggest that at least one of the functions exerted by both HSV1 and PrV UL28 genes in the viral replicative cycle is fundamentally different from that of BHV1. The fact that the two former genes belong to viral genes of the ß class (Holland *et al.*, 1984; Pederson *et al.*, 1991), whereas BHV1 UL28 is a γ2 gene (Desloges and Simard, 2001), could support this difference in functionality. However, it is of interest

to mention that although none of the HSV mutants harbouring a deletion in one or the other of the 7 genes known to be essential to the cleavage/packaging process had initially been found to package DNA (see the introduction section), it was very recently shown by Stow (2001) that the HSV1 UL25-deletion mutant KUL25NS was indeed capable to package DNA in normal host cells, in contrast to what had been previously reported by McNab *et al.* (1998). In light of the results obtained in the present study, it will thus be of great interest to reexamine whether HSV and PrV UL28 deletion mutants retain some ability to package DNA.

Southern blot analyses of total DNA extracted from BHV1ΔUL28-infected cells revealed several noteworthy features of newly replicated viral DNA (Fig. 6A and B). Probe N, which represents the leftward terminal fragment of the linear viral genome, did not hybridize with viral fragments of specific sizes (i.e. with the expected fragment N and/or head-to-tail N-F and N-H junction fragments; see Fig. 1) in DNase I-untreated DNA at 12 and 24 hours p.i. of either RK13 and RK13/UL28^{+} cells but instead revealed the presence of extensive DNA smears. In contrast, when a second blot containing the same DNA samples as those analyzed with N was hybridized with probe M, which represents an internal fragment of the BHV1 genome, a single band of the size expected for fragment M was revealed. These results lead us to conclude that the smears, detected with probe N but not M, must represent head-to-tail junction fragments generated from newly synthesized DNA concatemers following their digestion with *Hin*d III. Since the BHV1 genome occurs naturally in two isomeric forms of similar abundance, one would have expected that the probe N would only hybridize with two fragments of approximately 14 and 15 kbp, corresponding to N-F and N-H head-to-tail fragments of the two possible isomers. Our interpretation of the results obtained is that the smears observed denote the presence within head-to-tail fragments of complex structures affecting the migration of the DNA such that it does not fractionate solely as a function of its size. The observation that probe M did not reveal DNA smears indicates that the unique long segment of the viral genome is not involved in the formation of such complex structures, suggesting that these are restricted to regions harbouring repeated sequences of the viral genome. Our interpretation is consistent with the presence of highly branched DNA networks in HSV replicating DNA (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977; Severini *et al.*, 1996; Shlomai *et al.*, 1976). As mentioned

in the introduction, branched structures are thought to arise from homologous recombination events, genome isomerization, as well as other events (Boehmer and Lehman, 1997). Under these circumstances, our results indicate that UL28 is not involved in these processes since the hybridization profiles of probe N to DNA replicated in the absence or presence of UL28 expression (RK13 and RK13/UL28$^+$ cells samples, respectively) were essentially identical. Finally, it is of interest to note that the smears revealed by probe N at 12 and 24 hours p.i. of normal RK13 cells were absent from DNA isolated at 6 hours p.i., suggesting that at this time point very little or no DNA concatemers are present (see below).

Other noteworthy features of newly replicated viral DNA are drawn from Southern blot analyses of DNase I-treated DNA derived from non-complementing RK13 cells infected with BHV1ΔUL28 (Fig. 6 A and B, lanes 0-4) which revealed the presence of DNA which had been packaged within viral capsids. Results obtained with either probe revealed that the levels of packaged DNA remained steady from 6 to 24 hours p.i. even though the amount of newly synthesized DNA increased continuously, as shown in total infected-cell DNA samples (lanes 10-13). These results, which were reproduced in a second independent experiment, demonstrate that newly synthesized viral DNA was successfully packaged very early upon cell infection after which the whole process was interrupted. These findings added to the observation that very little or no DNA concatemers are present at 6 hours p.i. strongly suggest that the DNA substrate which was used for packaging did not originate from DNA concatemers (see below). In addition, although probe M only hybridized with fragment M in DNase I-treated DNA samples from both BHV1ΔUL28-infected RK13 and RK13/UL28$^+$ cells (Fig. 6A, lanes 2-6), thus demonstrating the completion of the *Hin*d III digestion, probe N revealed the presence of two bands whose sizes correlated with that expected for terminal fragment N derived from packaged linear viral genomes and for joined head-to-tail fragments derived from packaged circular viral genomes (Fig. 6B, lanes 2-6). These results are consistent with our previous findings that submolar amounts of WT BHV1 DNA exists in the circular form within virion capsids (C. Simard, S. Ananvoranich, V. Misra, and M. Trudel, Abstr. 15th Annual meeting of the American Society for Virology, Abstract P9-8, 1996). In our earlier work with WT BHV1 DNA purified from DNase I-treated MDBK cell lysates (Simard *et al.*, manuscript in

preparation), head-to-tail N-F and N-H *Hin*d III fragments of the two possible viral isomers were resolved into two probe N-specific hybridizing bands of the expected 14 and 15 kbp size which were of identical abundance and whose size shifted to the expected 10 kbp length following further digestion with *Eco* RI, thus unambiguously confirming their identity (see the Fig. 1 and observe the *Eco* RI site present in both F and H *Hin*d III terminal fragments). The presence within viral capsids of submolar amounts of circular genome DNA has also been reported in two other alphaherpesviruses, HSV1 (Poffenberger and Roizman, 1985) and VZV (Kinchington *et al.*, 1985). In the present study, the 14-15 kbp band revealed by probe N indubitably represented co-migrating N-F and N-H head-to-tail fragments, demonstrating the presence of submolar amounts of circular genomes within viral capsids. It thus ensues that UL28 is not involved in the circularization of packaged DNA since the presence or absence of the protein had no effect on the relative proportion of packaged circular genomes observed as compared with that of packaged linear genomes. Moreover, our results suggest that DNA circularization occurs concomitantly to or early after DNA packaging (see below).

Interestingly, Southern blot hybridization with probe N of total infected-cell DNA derived from BHV1ΔUL28-infected RK13 cells at 6 hours p.i. revealed the presence of a significant amount of fragment N but failed to detect head-to-tail fragments, even though the abundance of fragment N was at least twice that observed in packaged DNA where head-to-tail fragments were evidenced (Fig. 6B, compare lanes 11 and 2-4). Strictly speaking, the failure to detect a band representing head-to-tail fragments in lane 11 is not particularly surprising, considering that the corresponding band was barely discernible in lanes 2-4, and that as mentioned earlier the amount of DNA in DNase I untreated total cell DNA samples was proportionally three times lower than that in samples treated with DNase I. Based on these considerations, it is thus very likely that if the source of head-to-tail fragments in lane 11 originated solely from the amount of DNA which was packaged at this time point, fragment levels would be below the detection limit. Our interpretation is that the levels of fragment N which are observed in lane 11 added to our inability to detect head-to-tail fragments are indicative of the presence within infected cells of free (DNase I sensitive) viral DNA in the form of linear unit-length genomes (remember that the amount of DNA in DNase I treated samples is proportionally three times higher than in samples not

treated with DNase I). Moreover, the failure to detect head-to-tail fragments in lane 11 demonstrates that at 6 hours p.i. the levels of DNA concatemers as well as of circular progeny rings, putatively resulting from an initial phase of DNA replication via the theta mode (see the introduction) if any, were both below the detection limit. These observations added to the evidence that DNA packaging in BHV1ΔUL28-infected normal RK13 cells is complete at 6 hours p.i., suggest that the substrate DNA which is used for packaging indeed corresponds to linear unit-length genomes which are synthesized early upon cell infection. Based on this assumption, it is thus very likely that circularization of packaged DNA occurs concomitantly to or early after DNA packaging.

Our results suggest that replication of BHV1 DNA proceeds in two distinguishable phases: an early mode during which mature DNA in the form of linear unit-length genomes is synthesized followed by a second phase during which long head-to-tail concatemers are generated. At present, we cannot exclude the possibility that the synthesis of mature DNA occurs after the generation, albeit at undetectable levels, of circular progeny rings by theta replication of the initial cell infecting DNA following nuclear translocation and circularization (see the introduction). Should this arise, three instead of two phases of DNA replication would be implicated. We postulate that mature DNA is synthesized via a rolling circle-type mechanism where, rather than growing to multigenomic lengths, linear unit-length genomes are initially spun off from the circular template whenever a round of template replication is completed. Our results suggest that UL28 is dispensable for DNA packaging during this phase. Later on, a shift to a classical rolling circle mode occurs, presumably following induction by a viral factor, giving rise to long DNA concatemers, which must then be processed prior to or concomitantly to packaging. Alternatively, similarly to the mechanism used by phage T4 DNA replication (Broker and Doermann, 1975), it is possible that the second phase of replication is a recombination-dependent process requiring a sufficiently high copy number of infecting progeny genomes for initiation. Our data indicate that during this second phase, UL28 activity is essential for DNA cleavage and packaging. This is consistent with the hypothesis that UL28 participates in the formation of a terminase complex that recognizes the DNA to be packaged (Sheaffer *et al.*, 2001; Yu and Weller, 1998a-b).

ACKNOWLEDGMENTS

The authors sincerely thank Dr. Johnny Basso for numerous helpful discussions and for critically reading the manuscript. The authors are grateful to Mrs Nicole Gagnon for her excellent technical assistance, to Mr Robert Alain for his greatly appreciated help in electron microscopy experiments and to Dr. Bello for kindly providing the pEC10 plasmid. This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC). N.D. was supported by NSERC, by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) and by the Fondation Armand-Frappier.

REFERENCES

Abbotts, A. P., Preston, V.G., Hughes, M., Patel, A.H. and Stow, N.D. (2000). Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. J. Gen. Virol. 81: 2999-3009.

Addison, C., Rixon, F.J. and Preston, V.G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. 71: 2377-2384.

Adelman K., Salmon, B. and Baines, J.D. (2001). Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc. Natl. Acad. Sci. USA 98: 3086-3091.

Al-Kobaisi, M. F., Rixon, F.J., McDougall, I. and Preston, V.G. (1991). The herpes simplex virus UL33 gene product is required for the assembly of full capsids. Virology 180: 380-388.

Baines, J. D., Cunningham, C., Nalwanga, D. and Davison, A. (1997). The UL15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the UL15 gene product. J. Virol. 71: 2666-2673.

Boehmer, P. E. and Lehman, I.R. (1997). Herpes simplex virus DNA replication. Annu. Rev. Biochem. 66: 347-384.

Bogner, E., Radsak, K. and Stinski, M.F. (1998). The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity. J. Virol. 72: 2259-2264.

Broker, T. R. and Doermann, A.H. (1975). Molecular and genetic recombination of bacteriophage T4. Annu. Rev. Genet. 9: 213-244.

Catalano, C. E. (2000). The terminase enzyme from bacteriophage lambda: a DNA-packaging machine. Cell. Mol. Life Sci. 57: 128-148.

Catalano, C. E., Cue, D. and Feiss, M. (1995). Virus DNA packaging: the strategy used by phage l. Mol. Microbiol. 16: 1075-1086.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. Virology 186: 9-14.

Deiss, L. P., Chou, J. and Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. 59: 605-618.

Desloges, N. and Simard, C. (2001). Expression kinetics of the transcript and product of the UL28 homologue of bovine herpesvirus 1. Virus Res. 80: 23-31.

Desloges, N., Boucher, H. and Simard, C. (2001). Transcriptional and translational expression kinetics of the UL25 homologue of bovine herpesvirus 1. Arch. Virol. 146: 1693-1704.

Farley, J. E., Skare, I.B. and Skare, J. (1981). Inverted repeat sequences in infectious bovine rhinotracheitis virus DNA, p. 590. *In* A. J. Nahmias, W. R. Dowdle, and R. F. Schinazi (ed), The human herpes viruses: an interdisciplinary perspective. Elsevier/North Holland Publishing New York.

Friedmann, A., Shlomai, J. and Becker, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. J. Gen. Virol. 34: 507-522.

Goldstein, J. N. and Weller, S.K. (1998). In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. J. Virol. 72: 8772-8781.

Goshima, F., Watanabe, D., Takakuwa, H., Wada, K., Daikoku, T., Yamada, M. and Nishiyama, Y. (2000). Herpes simplex virus UL17 protein is associated with B capsids and colocalizes with ICP35 and VP5 in infected cells. Arch. Virol. 145: 417-426.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science 268: 1766-1769.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89: 5547-5551.

Hirsch, I., Cabral, G., Patterson, M. and Biswal, N. (1977). Studies on the intracellular replicating DNA of herpes simplex virus type 1. Virology 81: 48-61.

Holland, L. E., Sandri-Goldin, R.M., Goldin, A.L., Glorioso, J.C. and Levine, M. (1984). Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. 49: 947-959.

Homa, F. L. and Brown, J.C. (1997). Capsid assembly and DNA packaging in herpes simplex virus. Rev. Med. Virol. 7: 107-122.

Jacob, R. J. and Roizman, B. (1977). Anatomy of herpes simplex virus DNA. VIII. Properties of the replicating DNA. J. Virol. 23: 394-411.

Kinchington P. R., Reinhold, W.C., Casey, T.A., Straus, S.E., Hay, J. and Ruyechan, W.T. (1985). Inversion and circularization of the varicella-zoster virus genome. J. Virol. 56: 194-200.

Koslowski, K. M., Shaver, P.R., Casey II, J.T., Wilson, T., Yamanaka, G., Sheaffer, A.K., Tenney, D.J. and Pederson, N.E. (1999). Physical and functional interactions between the herpes simplex virus UL15 and UL28 DNA cleavage and packaging proteins. J. Virol. 73: 1704-1707.

Koslowski, K. M., Shaver, P.R., Wang, X.-Y., Tenney, D.J. and Pederson, N.E. (1997). The pseudorabies virus UL28 protein enters the nucleus after coexpression with the herpes simplex virus UL15 protein. J. Virol. 71: 9118-9123.

Lamberti, C. and Weller, S.K. (1996). The herpes simplex virus 1 UL6 protein is essential for cleavage and packaging but not for genomic inversion. Virology 226: 403-407.

Lamberti, C. and Weller, S.K. (1998). The herpes simplex virus type 1 cleavage/ packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. J. Virol. 72: 2463-2473.

Ludwig, H. (1983). Bovine herpesviruses, p. 135-214. *In* B. Roizman (ed.), The Herpesviruses, vol 2. Plenum Press, New York and London.

Martinez, R., Sarisky, R.T., Weber, P.C. and Weller, S.K. (1996). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J. Virol. 70: 2075-2085.

Mayfield, J. E., Good, P.J., Vanoort, H.J., Campbell, A.R. and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus (Cooper strain). J. Virol. 47: 259-264.

McNab, A. R., Desai, P., Person, S., Roof, L.L., Thomsen, D.R., Newcomb, W.W., Brown, J.C. and Homa, F.L. (1998). The product of the herpes simplex virus type 1 UL25 gene is required for encapsidation but not for cleavage of replicated viral DNA. J. Virol. 72: 1060-1070.

Mettenleiter T. C., Saalmuller, A. and Weiland, F. (1993). Pseudorabies virus protein homologous to herpes simplex virus type 1 ICP18.5 is necessary for capsid maturation. J. Virol. 67: 1236-1245.

Meyer, G., Vlcek, C., Paces, V., O'Hara, M.K., Pastoret, P.-P., Thiry, E. and Schwyzer, M. (1997). Sequence analysis of the bovine herpesvirus type 1 genes homologous to the DNA polymerase (UL30), the major DNA-binding protein (UL29) and ICP18.5 assembly protein (UL28) genes of herpes simplex virus. Arch. Virol. 142: 89-102.

Misra, V., Bratanich, A.C., Carpenter, D. and Ohare, P. (1994). Protein and DNA elements involved in transactivation of the promoter of the bovine herpervirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. J. Virol. 68: 4898-4909.

Patel, A. H., Rixon, F.J., Cunningham, C. and Davison, A.J. (1996). Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL6 gene. Virology 217: 111-123.

Paulus, W., Baur, I., Boyce, F.M., Breakefield, X.O. and Reeves, S.A. (1996). Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. J. Virol. 70: 62-67.

Pederson, N. E. and Enquist, L.W. (1991). Overexpression in bacteria and identification in infected cells of the pseudorabies virus protein homologous to the herpes simplex virus type 1 ICP18.5. J. Virol. 65: 3746-3758.

Perdue, M. L., Cohen, J.C., Randall, C.C. and O'Callaghan, D.J. (1976). Biochemical studies of the maturation of herpes virus nucleocapsid species. Virology 74: 194-208.

Poffenberger K. L. and Roizman, B. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head to tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. 53: 587-595.

Poon, A. P. and Roizman, B. (1993). Characterization of a temperature-sensitive mutant of the UL15 open reading frame of herpes simplex virus 1. J. Virol. 67: 4497-4503.

Roizman, B. (1996). Herpesviridae, p. 2221-2230. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed), Fields Virology, 3rd ed. Lippincott-Raven publishers, Philadelphia, New York.

Roizman, B. and Sears, A.E. (1996). Herpes simplex viruses and their replication, p. 2231-2295. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed), Fields Virology, 3rd ed. Lippincott-Raven publishers, Philadelphia, New York.

Salmon, B. and Baines, J.D. (1998). Herpes simplex virus DNA cleavage and packaging: association of multiple forms of UL15-encoded proteins with B capsids requires at least the UL6, UL17, and UL28 genes. J. Virol. 72: 3045-3050.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, a laboratory manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Severini, A., Scraba, D.G. and Tyrrell, D.L. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. J. Virol. 70: 3169-3175.

Sheaffer, A. K., Newcomb, W.W., Gao, M., Yu, D., Weller, S.K., Brown, J.C. and Tenney, D.J. (2001). Herpes simplex virus DNA cleavage and packaging proteins associate with the procapsid prior to its maturation. J. Virol. 75: 687-698.

Shlomai, J., Friedmann, A. and Becker, Y. (1976). Replicative intermediates of herpes simplex virus DNA. Virology 69: 647-659.

Simard, C., Nadon, F., Séguin, C., LaBoissière, S. and Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch. Virol. 110: 63-75.

Simard, C., Bastien, N. and Trudel, M. (1992). Sequencing and 5'-and 3'-end transcript mapping of the gene encoding the small subunit of ribonucleotide reductase from bovine herpesvirus type-1. Virology 190: 689-701.

Simard, C., LaBoissière, S., Séguin, C. and Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: A major variant selected from a field isolate. Intervirology 32: 117-126. 53.

Stow, N.D. (2001). Packaging of genomic and amplicon DNA by the herpes simplex virus type 1 UL25-null mutant KUL25NS. J. Virol. 75: 10755-10765.

Taus, N. S., Salmon, B. and Baines, J.D. (1998). The herpes simplex virus 1 UL17 gene is required for localization of capsids and major and minor capsid proteins to intranuclear sites where viral DNA is cleaved and packaged. Virology 252: 115-125.

Tengelsen, L. A., Pederson, N.E., Shaver, P.R., Wathen, M.W. and Homa, F.L. (1993). Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J. Virol. 67: 3470-3480.

Yu, D., Sheaffer, A.K., Tenney, D.J. and Weller, S.K. (1997). Characterization of ICP6-lacZ insertion mutants of the UL15 gene of herpes simplex virus type 1 reveals the translation of two proteins. J. Virol. 71: 2656-2665.

Yu, D. and Weller, S.K. (1998a). Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. J. Virol. 72: 7428-7439.

Yu, D. and Weller, S.K. (1998b). Genetic analysis of the UL 15 gene locus for the putative terminase of herpes simplex virus type 1. Virology 243: 32-44.

Figure 1. Cloning strategies.

The 135,301 bp linear genome of BHV1 is schematically represented at the top with its unique long (UL) and unique short segments (US) the latter being flanked by internal and terminal inverted repeats (IR and TR, respectively). Below is shown the *Hin*d III restriction maps of the two possible viral isomers. The UL28 ORF is located in the rightward orientation within the *Hin*d III fragment A at positions 53058→55538. The 9825-bp *Hin*d III-*Cla* I fragment, representing the region 51711-61536 of the viral genome, was first subcloned from the plasmid pKS/Ahd carrying the *Hin*d III fragment A (Simard *et al.*, 1990) to generate pKS/BHV51-61kHd-Cla. This recombinant plasmid was used as previously described (Desloges and Simard, 2001) to create pKS/UL28orf, a plasmid strictly harbouring the UL28 coding sequence flanked by *Xba* I sites. Plasmid pKS/UL28orf was used as illustrated to create the recombinant eucaryotic expression vector pRetroTET-OFF/UL28 (see the Materials and Methods section for a brief description of the elements carried by this vector) for the development of a cell line expressing an inducible UL28. The right of the figure describes the strategy used to generate the recombinant pKS/51-61kΔUL28/β-gal^{+} plasmid that was used to create BHV1ΔUL28. The ß-gal gene cassette is flanked by the promoter of the gene encoding the BHV1 gB glycoprotein (pgB) and a consensus polyadenylation signal (pA). The illustrations below the vertical arrows are not drawn to scale.

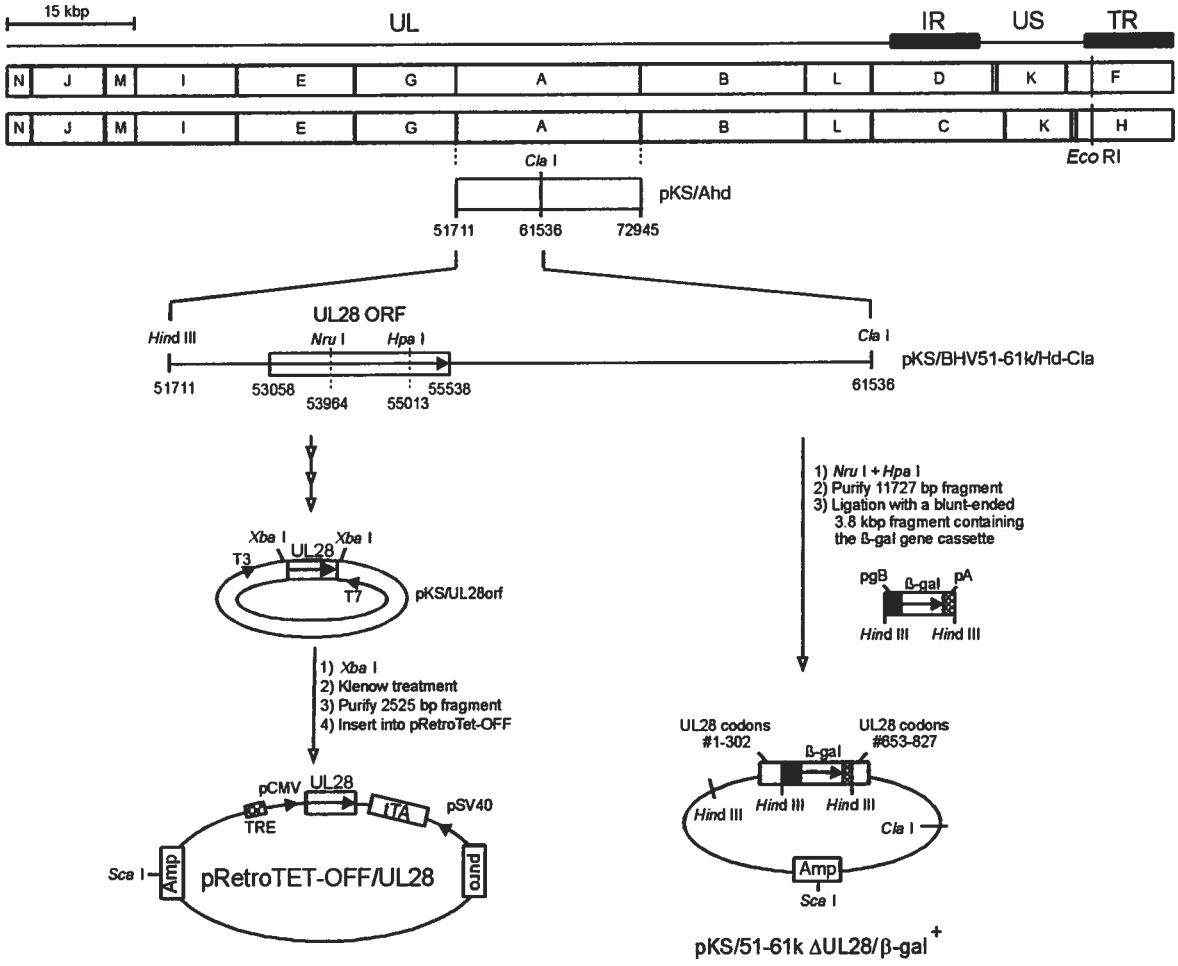

15 kbp
UL
IR
US
TR
N J M I E G A B L D K F
N J M I E G A B L C K H
Eco RI
Cla I
pKS/Ahd
51711
61536
72945
UL28 ORF
Hind III
Nru I
Hpa I
Cla I
pKS/BHV51-61k/Hd-Cla
51711
53058
53964
55013
55538
61536
Xba I
Xba I
T3
UL28
T7
pKS/UL28orf
1) Xba I
2) Klenow treatment
3) Purify 2525 bp fragment
4) Insert into pRetroTet-OFF
pCMV
UL28
TRE
tTA
pSV40
Sca I
Amp
pRetroTET-OFF/UL28
puro
1) Nru I + Hpa I
2) Purify 11727 bp fragment
3) Ligation with a blunt-ended
3.8 kbp fragment containing
the ß-gal gene cassette
pgB
ß-gal
pA
Hind III
Hind III
UL28 codons
#1-302
UL28 codons
#653-827
ß-gal
Hind III
Hind III
Hind III
Cla I
Amp
Sca I
pKS/51-61k ΔUL28/β-gal+

Figure 2. Expression of UL28 in the complementing RK13/UL28$^+$ cell line.

Protein lysates of RK13/UL28$^+$ cells grown in the presence (lane 1) or the absence (lane 2) of 200 ng/mL of doxycycline were fractionated by SDS-PAGE, electrotransferred onto a PVDF membrane, and then reacted with a BHV1-specific UL28 antiserum directed against an *E. coli*-expressed T7-tag-UL28 fusion protein (Desloges and Simard, 2001). Sizes in kDa of standards are indicated.

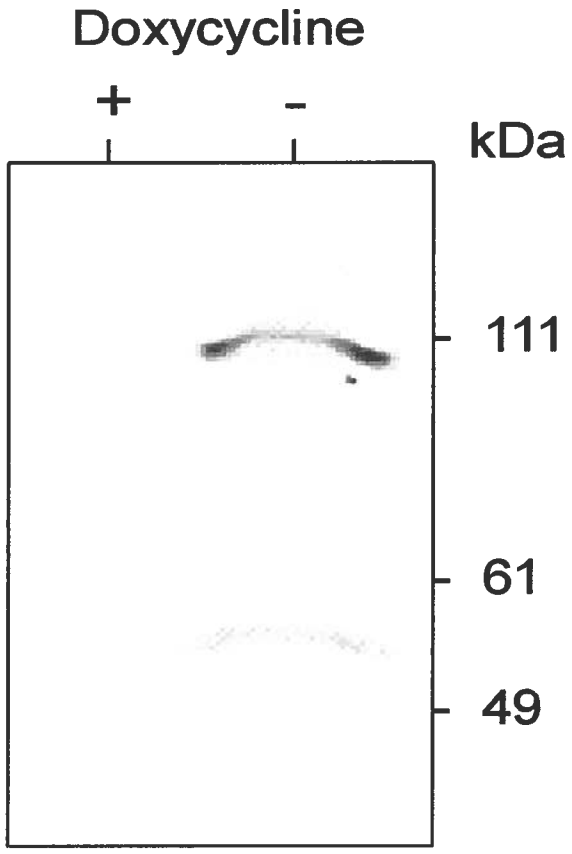

Doxycycline
+
-
kDa
111
61
49

Figure 3. Characterization of BHV1ΔUL28.

Southern blots of *Hin*d III digests of DNA from BHV1ΔUL28 (lanes 1), WT BHV1 (lanes 2), and pKS/51-61kΔUL28/β-gal$^+$ (lanes 3) were hybridized with a probe representing either the complete coding sequence of UL28 (A) or that of ß-gal (B). The size in kbp of fragments hybridizing with the probes are indicated to the left.

C) Northern blot hybridization of total RNA (20 µg/slot) isolated at 18 hours p.i. of RK13 (lane 1) and RK13/UL28$^+$ (lane 2) cells with BHV1ΔUL28, as well as from mock-infected RK13/UL28$^+$ cells (lane 3), with a DIG-labeled probe corresponding to the 1049 bp *Nru* I-*Hpa* I fragment deleted from the mutant genome. The size in kb of the hybridizing transcript is indicated at the right.

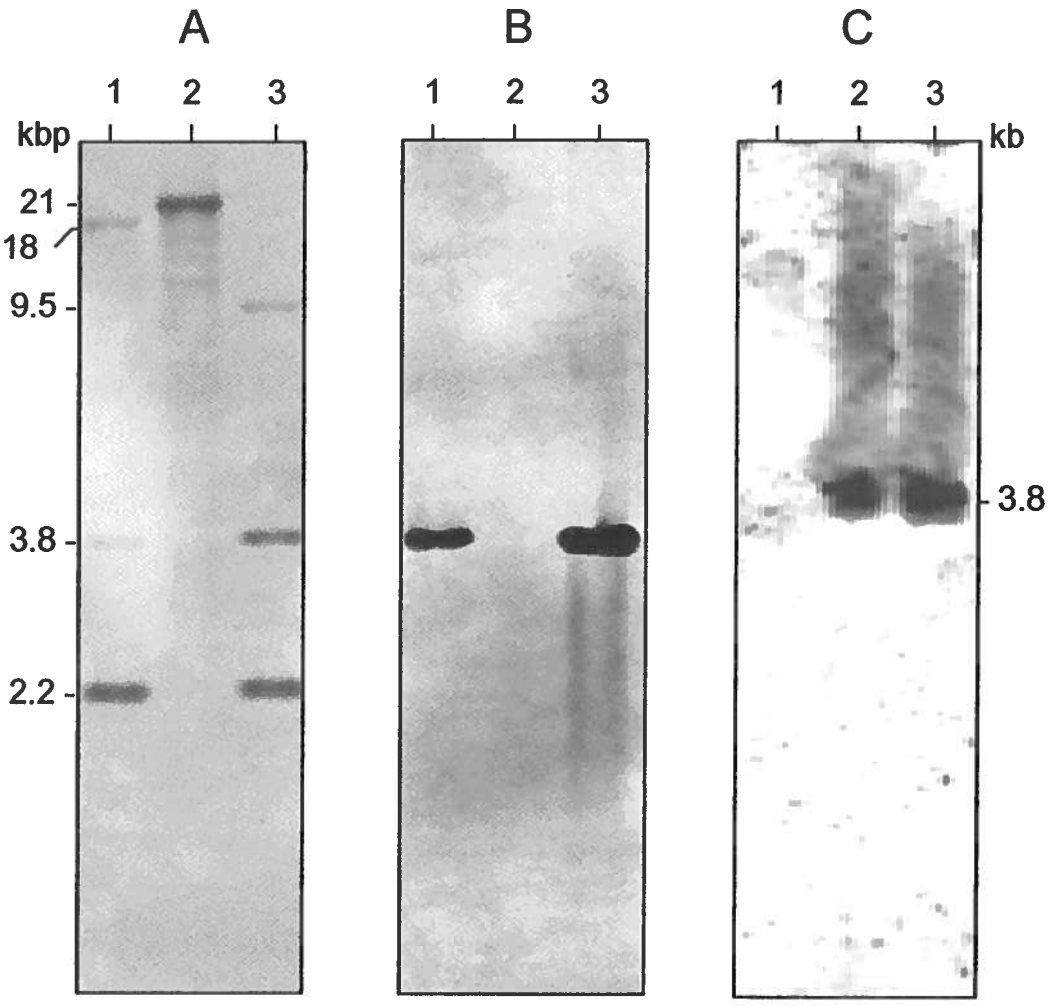
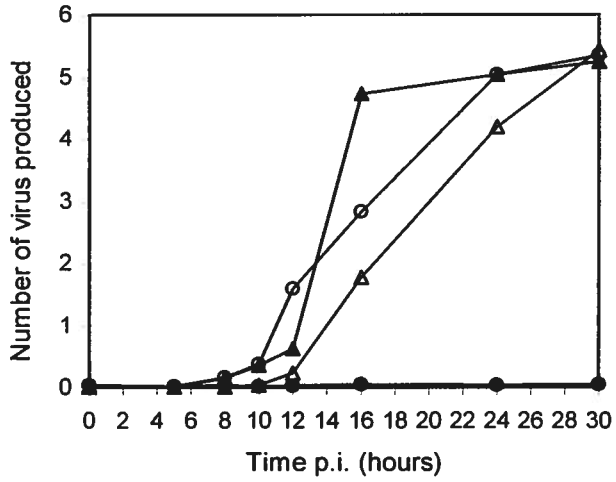

A
B
C
1
2
3
kbp
21
18
9.5
3.8
2.2
kb
3.8

Figure 4. One-step growth curves of BHV1ΔUL28, WT BHV1 and BHV1rUL28.

A) Normal RK13 cells (closed symbols) and complementing RK13/UL28^{+} cells (open symbols) were synchronously infected at an m.o.i. of 5 with either BHV1ΔUL28 (circles) or WT BHV1 (triangles). The cultures were harvested at the indicated times p.i. and the mean yield of virus/cell generated in either BHV1ΔUL28-infected or WT BHV1-infected samples was determined by plaque assays on RK13/UL28^{+} and RK13 cells, respectively.

B) Normal RK13 cells were infected at an m.o.i. of 5 with either BHVrUL28 (squares) or WT BHV1 (triangles). Virus generated at the indicated times p.i. was titered on RK13 cells.

A


Number of virus produced
0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time p.i. (hours)

B

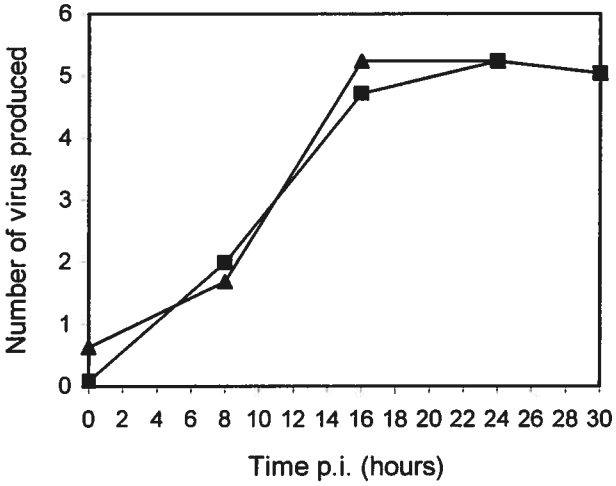

Number of virus produced
0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time p.i. (hours)

Figure 5. Expression of the alpha gene trans-inducing factor protein in non-complementing RK13 cells infected with either BHV1ΔUL28 or WT BHV1.

Protein lysates from either BHV1ΔUL28-infected or WT BHV1-infected RK13 cells grown in the presence (+) or the absence (-) of PAA and collected at 0, 6, 12 and 18 hours p.i. were fractionated on SDS-PAGE then electrotransferred onto a PVDF membrane. The blot was reacted with a monospecific antiserum directed against the product of the BHV1 UL48 gene. Sizes in kDa of protein standards are indicated.

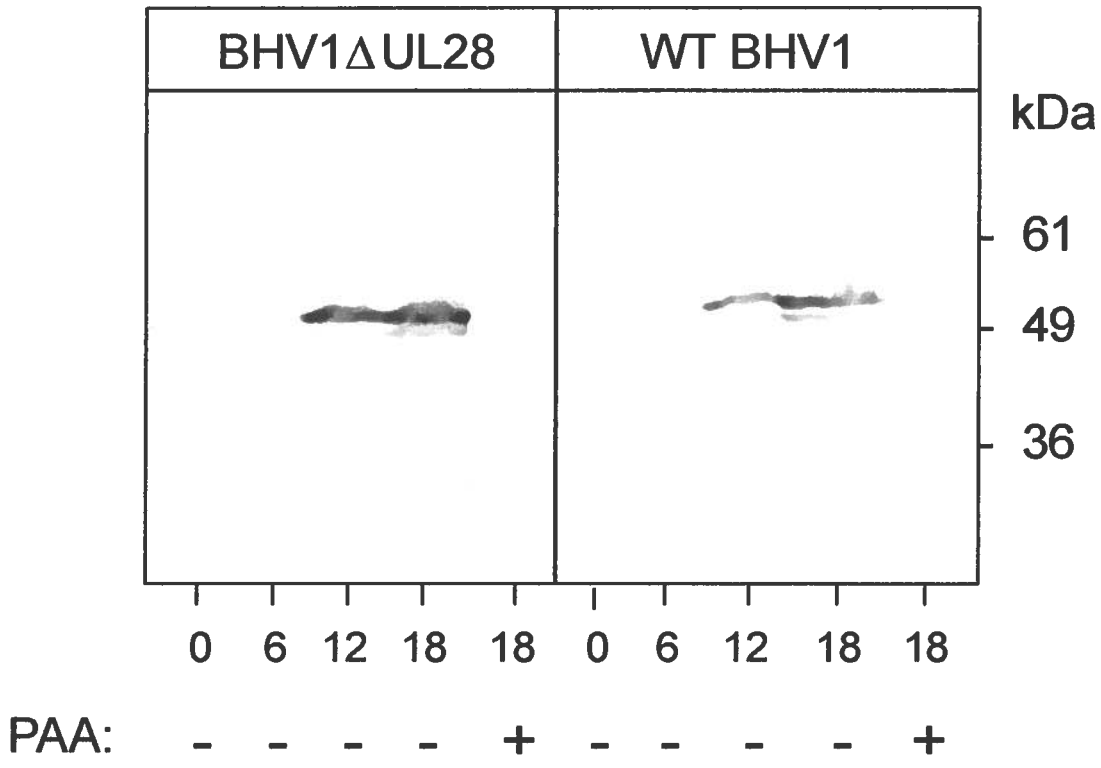

BHV1ΔUL28
WT BHV1
kDa
61
49
36
0 6 12 18 18 0 6 12 18 18
PAA: - - - - + - - - - +

Figure 6. Synthesis, processing and packaging of BHV1ΔUL28 DNA in normal RK13 cells.

Total DNA as well as viruses and nucleocapsids from RK13 and RK13/UL28^{+} cell lysates infected with BHV1ΔUL28 for different times p.i. were treated with (+) or without (-) LiDS and DNase I after which DNA was purified, digested with *Hin*d III, and then analyzed by Southern blotting. The blots were hybridized with DIG-labeled probes representing either the internal *Hin*d III fragment M (A) or the leftward *Hin*d III terminal fragment N (B) of the BHV1 linear viral genome (see Fig. 1). In A, lane 9 contains a *Hin*d III digest of the recombinant plasmid pKS/Mhd carrying the *Hin*d III fragment M of the BHV1 genome (Simard *et al.*, 1990) whereas in B, the corresponding lane contains a *Kpn* I-*Hin*d III digest of pKS/Nhd which carries the terminal *Hin*d III fragment N. The blots were reacted with a chemiluminescent substrate then exposed to X-Ray films for 16 hours, with the exception of lane 9 in panel B which was only exposed for 30 min. Downward arrows at the right of each blot indicate the start and direction of DNA migration.

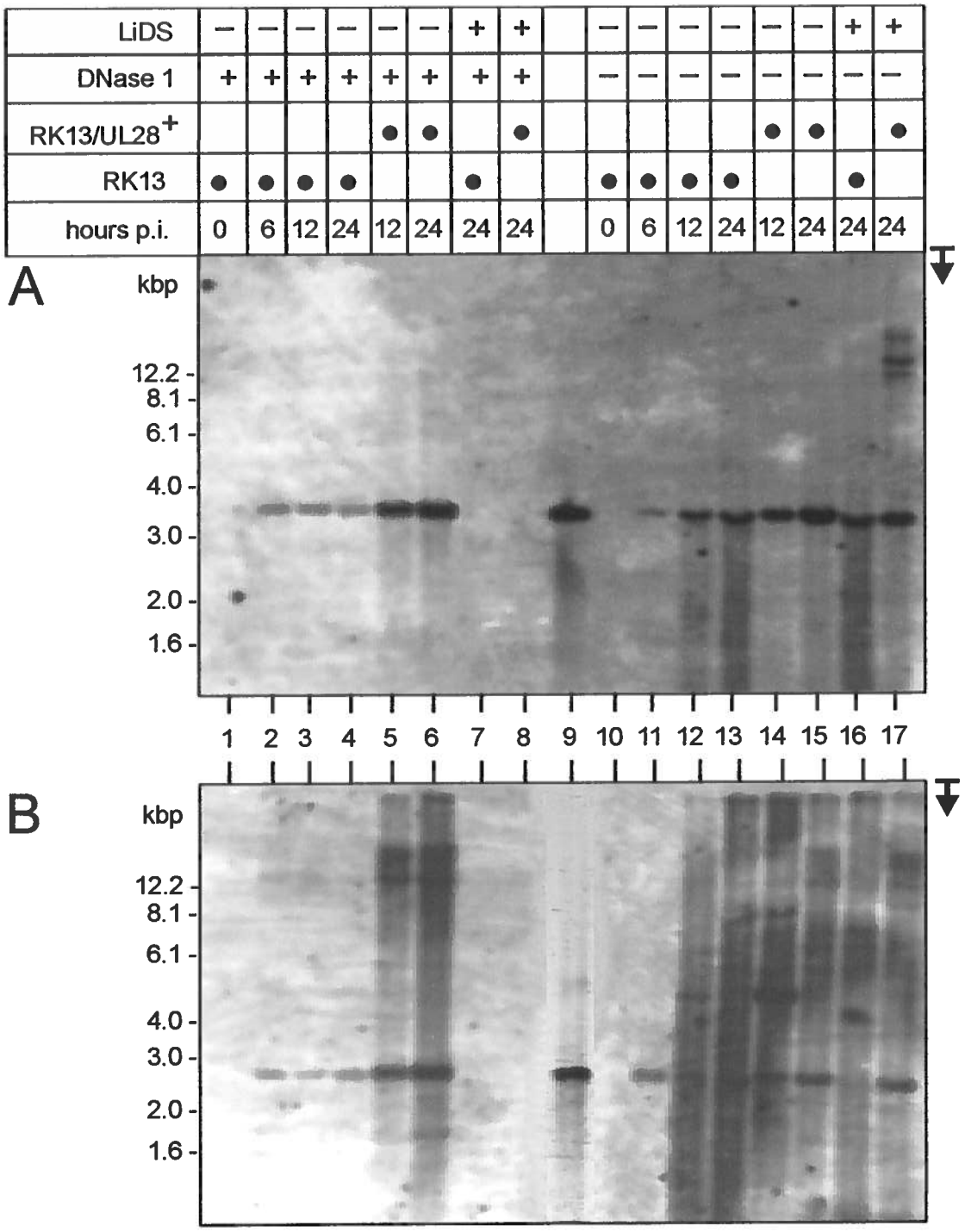

LiDS
DNase 1
RK13/UL28+
RK13
hours p.i.
0 6 12 24 12 24 24 24 0 6 12 24 12 24 24 24
A
kbp
12.2
8.1
6.1
4.0
3.0
2.0
1.6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
kbp
12.2
8.1
6.1
4.0
3.0
2.0
1.6

Figure 7. Transmission electron micrographs of infected cells.

Thin sections of WT BHV1-infected RK13 (A), BHV1ΔUL28-infected RK13/UL28^{+} (B) and BHV1ΔUL28-infected RK13 (C-F) cells were prepared at 18 hours p.i. and examined by electron microscopy. The arrows in panels C and D point to type B and a complete enveloped type C capsids, respectively. The arrows in panels E and F point to non-enveloped and enveloped type A capsids, respectively. Each illustration is magnified 70,000X. The bars represent 100 nm.

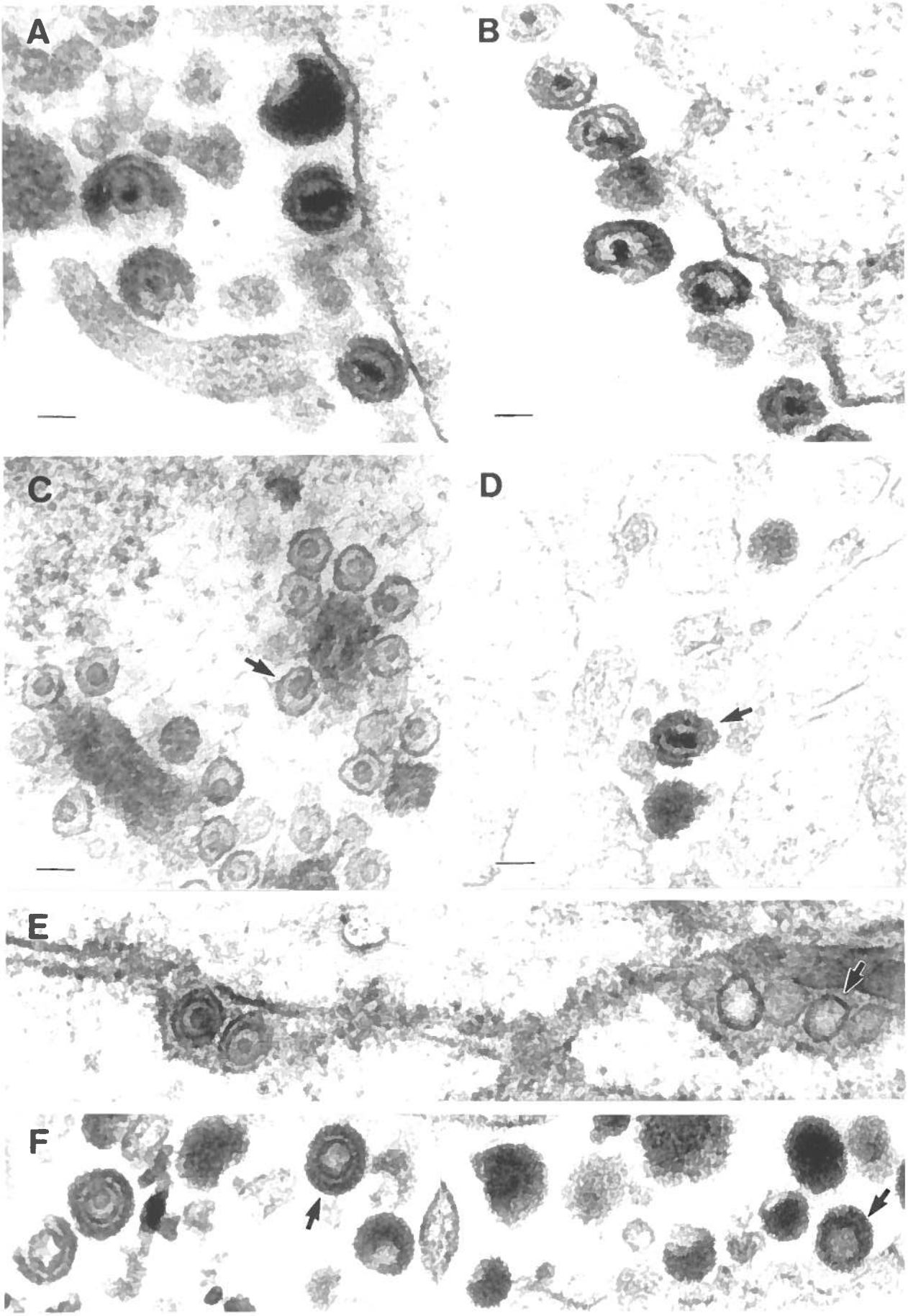

A
B
C
D
E
F

Table 1. Relative amount of A, B and C capsids among 400 capsids counted in virus-infected cells.

		Capsid types		
Virus	Cell host	A	B	C
WT BHV1	RK13	35.7%	16.7%	47.6%
BHV1ΔUL28	RK13/UL28^{+}	41.2%	37.0%	21.8%
BHV1ΔUL28	RK13	13.0%	86.3%	0.7%

SECTION III

DISCUSSION

1. L'À-PROPOS D'UN PROJET DE RECHERCHE SUR LE BHV1

La plupart des informations disponibles concernant les virus alphaherpès proviennent d'études réalisées avec le HSV1 qui est le prototype de cette sous-famille. Certains scientifiques ont tendance à généraliser les données obtenues aux autres virus alphaherpès comme le VZV, le PRV ou bien le BHV1. Pourtant, même si les caractéristiques de base sont les mêmes chez ces virus, des différences significatives existent entre eux. Que ce soit au niveau de l'hôte, du tropisme cellulaire, de l'expression des gènes ou même de la présence ou de l'absence de certains gènes, chaque virus alphaherpès a ses propres caractéristiques.

Afin de lutter efficacement contre les virus alphaherpès, tel que le BHV1 qui occasionne des pertes économiques sévères aux éleveurs de bovins, il est essentiel de bien connaître leurs caractéristiques typiques. Jusqu'à présent, le BHV1 a été peu étudié comparativement au HSV1. Pourtant, des travaux de recherche sur des virus alphaherpès autres que le HSV, et spécialement sur des virus vétérinaires, peuvent apporter d'innombrables informations sur cette sous-famille de virus puisque les travaux peuvent être effectués directement chez l'hôte naturel. Par la suite, ces travaux peuvent servir de modèle pour l'étude du HSV.

La recherche effectuée dans le cadre de mes études doctorales a permis d'éclaircir certains aspects du BHV1. Le premier objectif était de démontrer que les ORFs UL12, UL25 et UL28 du BHV1 représentent bel et bien des gènes. Puisque les différents ORFs du BHV1 ont été localisés sur le génome par comparaison de séquences avec les autres virus herpès, il fallait s'assurer de leur fonctionnalité. J'ai donc commencé par démontrer que les ORFs UL12, UL25 et UL28 constituent bien des gènes. Cette conclusion est basée sur la détection spécifique de leurs transcrits et des produits des gènes qui y sont associés. De plus, l'analyse des cinétiques transcriptionnelles et traductionnelles ainsi que la détermination des sites d'initiation de la transcription ont permis respectivement de caractériser l'expression de ces gènes au cours du cycle réplicatif du BHV1 ainsi que de situer approximativement les régions promotrices.

Un point fort intéressant qui ressort de cette étude est les différences existant entre les gènes UL12, UL25 et UL28 et leurs homologues chez le HSV1 et le PRV en ce qui concerne leur expression au cours de la réplication. Il a été surprenant de constater l'appartenance des trois gènes ciblés dans la classe des gènes tardifs γ2 contrairement aux homologues UL12 et UL28 chez le HSV1 et le PRV classés comme gènes précoces (β) (Banks *et al.*, 1985; Hsiang *et al.*, 1996; Holland *et al.*, 1984; Pederson et Enquist, 1991) et à l'homologue UL25 chez le HSV1 classé comme gène tardif γ1 (Ali *et al.*, 1996). Au moment où les articles scientifiques rapportant l'expression de ces gènes au cours de la réplication du BHV1 ont été écrits, il était impossible de savoir si ces différences avaient des implications fonctionnelles. L'étude du mutant de délétion UL28 nous a démontré que celui-ci conserve une partie de son habileté à encapsider l'ADN viral nouvellement synthétisé contrairement au HSV1 et au PRV qui, en l'absence de la protéine UL28, n'encapsident pas d'ADN (Tengelsen *et al.*, 1993; Mettenleiter *et al.*, 1993). L'ADN qui est encapsidé semble être préalablement sous forme de génomes linéaires dans le noyau. Ce processus d'encapsidation survient précocement (dès 6 heures p.i.) et ne requiert pas la présence de UL28. Cependant, la protéine UL28 est nécessaire par la suite pour cliver et encapsider l'ADN concatémérique synthétisé. Si la sensibilité de la méthode utilisée pour la détection de la présence ou non d'ADN encapsidé chez les mutants UL28 du HSV1 et du PRV n'est pas en cause, nos résultats suggèreraient qu'au moins une des fonctions exercées par les protéines UL28 des virus HSV1 et PRV dans le cycle réplicatif est différente de celles du BHV1. L'appartenance du gène UL28 du BHV1 à la classe γ2, contrairement aux homologues du HSV1 et du PRV classés comme gènes β, supporterait cette différence de fonction. Quant à l'UL12, une analyse de sa fonction serait nécessaire pour arriver à une conclusion. Il existe dans la littérature, une étude qui démontre que la protéine UL12 du BHV1 exprimée chez *E. coli* possède des activités d'exonucléase et d'endonucléase à pH alcalin (Chung et Hsu, 1996; Chung et Hsu, 1997), tout comme son homologue chez le HSV1 qui démontre le même type d'activité lorsque exprimé chez *E. coli* (Bronstein et Weber, 1996). Il est probable que la protéine UL12 du BHV1, possédant une activité de nucléase, joue un rôle similaire dans la réplication comparativement à son homologue chez le HSV1. Cependant, le délai dans l'expression de l'UL12 chez le BHV1 comparativement

à ses homologues chez le HSV1 et le PRV pourrait indiquer une absence de fonctions précoces de cette protéine chez le BHV1 mais non pas chez les deux autres virus.

Un second point intéressant qui ressort de l'étude des gènes UL12, UL25 et UL28 du BHV1 est la présence ou non de séquences typiques pour la régulation de ces gènes. En général, le HSV1 utilise des signaux typiques de régulation (Mavromara-Nazos et Roizman, 1989) mais il semble commun chez d'autres virus herpès d'utiliser des éléments de séquences non-classiques comme c'est le cas pour le VZV où l'utilisation de boîtes TATA non-typiques ne semble pas être inhabituelle (Cohen et Straus, 1996). Lors de mes travaux de doctorat, nous avons constaté que le gène UL12 du BHV1 ne possède pas de boîte TATA typique similairement à son homologue chez le PRV (Dijkstra *et al.*, 1997) mais contrairement au gène UL12 du HSV1 qui possède des séquences promotrices typiques (McGeoch *et al.*, 1986). Quant au gène UL28, il utilise un signal de polyadénylation non-classique puisque aucune séquence typique n'a été trouvée à l'endroit où le transcrit doit se terminer, au contraire de l'ARNm UL28 du HSV1 qui est 3' co-terminal avec celui de UL27 et utilise un signal typique (Holland *et al.*, 1984). Pour l'instant, trop peu d'études sur les séquences régulatrices du BHV1 ont été effectuées pour conclure si l'utilisation de séquences typiques est habituelle ou non.

Les résultats que j'ai obtenus au cours de mes études doctorales sur le BHV1 ont permis d'améliorer les connaissances que nous avons de ce virus. La comparaison de mes résultats à ceux préalablement publiés pour les virus du HSV1 et du PRV nous démontre clairement que le BHV1 a certaines caractéristiques qui lui sont typiques. Lorsque davantage d'informations seront disponibles pour les différents virus alphaherpès, il sera possible de mieux comprendre cette sous-famille de virus incluant leur réplication et leur interaction avec l'hôte. De plus, il sera possible de mieux comprendre l'évolution de ces virus entre eux.

2. RÉPLICATION, CLIVAGE ET ENCAPSIDATION DE L'ADN DES VIRUS ALPHAHERPÈS

Il existe de plus en plus d'informations dans la littérature concernant la réplication, le clivage et l'encapsidation de l'ADN des virus alphaherpès. Avec l'accumulation de données sur le sujet, il est apparu que l'encapsidation de l'ADN de cette sous-famille de virus ressemble à celle observée pour les bactériophages à ADN double brins tels que P22, T7 et Lambda. L'assemblage de ces bactériophages débute par la formation de capsides précurseurs sphériques appelées procapsides. Un vertex unique est retrouvé sur ces procapsides; il est constitué d'un anneau de 12 protéines par lequel l'ADN entre et sort de la capside (Bazinet et King, 1985). Le vertex est un site d'ancrage pour les protéines impliquées dans l'encapsidation comme le complexe de terminase ainsi que les protéines responsables du clivage des concatémères d'ADN en unités monomériques et de l'injection de l'ADN dans la capside selon un mécanisme dépendant de l'ATP (Catalano, 2000; Catalano *et al.*, 1995). Les protéines du vertex sont retrouvées comme partie intégrante des procapsides et des capsides matures alors que les protéines du complexe de terminase sont seulement associées aux procapsides et sont absentes des capsides matures ou des virions (Murialdo et Becker, 1978).

Les différentes données existantes supportent une relation entre les virus herpès et les bactériophages à ADN double brins au cours de l'évolution. Le mécanisme de base de l'encapsidation de l'ADN semble avoir été conservé au cours de cette période qui serait d'environ 2 milliards d'années ou plus, ce qui lie les bactéries aux organismes vertébrés qui sont les hôtes des virus herpès (Newcomb *et al.*, 2001). Par analogie avec les systèmes phagiques, des fonctions approximatives ont été assignées aux protéines impliquées dans le clivage et l'encapsidation de l'ADN des virus herpès selon la conservation de leurs séquences d'acides aminés avec les séquences des protéines phagiques et par le comportement de virus herpès déficients en ces protéines. La plupart des travaux ont été réalisés avec le HSV1. Cependant, il est connu que des différences dans les mécanismes existent parmi les virus herpès. Par exemple, au cours de mes études doctorales, nous avons créé un mutant de délétion du gène UL28 du BHV1 qui est incapable de se répliquer dans

des cellules qui ne complémentent pas pour la délétion. Nous avons constaté que la synthèse des protéines virales ainsi que la réplication de l'ADN ne sont pas compromises chez ce mutant. Il conserve une partie de son habileté à encapsider l'ADN viral nouvellement synthétisé contrairement aux virus HSV1 et PRV (Tengelsen *et al.*, 1993; Mettenleiter *et al.*, 1993) mais les processus de clivage et d'encapsidation des longs concatémères d'ADN sont compromis. En combinant les données disponibles sur la réplication, le clivage et l'encapsidation de l'ADN du HSV1 à celles obtenues avec le mutant UL28 du BHV1, j'ai pu tracer un schéma présentant sommairement ces processus (Fig. 1).

Peu après l'infection et en absence de synthèse protéique, l'ADN viral se retrouve dans le noyau de la cellule infectée et adopte une conformation qui est cohérente avec sa circularisation puisque aucune extrémité libre n'est retrouvée (Fig. 1, étape 1; Poffenberger et Roizman, 1985). Quoique le mécanisme de circularisation du génome n'ait pas été établi, il devrait impliquer un événement de recombinaison. Il a été démontré que la réplication du HSV1 est inhibée à une température non-permissive dans une lignée cellulaire possédant un régulateur de condensation chromosomique thermolabile, RCC1. Dans ces cellules, le génome viral ne réussit pas à se circulariser, suggérant que le RCC1 est impliqué dans la circularisation du génome et que la circularisation est une condition préalable pour la réplication de l'ADN (Umene et Nishimoto, 1996). Le génome circularisé agirait vraisemblablement comme gabarit pour la réplication thêta (Fig. 1, étape 2). Cette réplication s'initierait à l'une ou l'autre des deux origines de réplication localisées dans les régions répétées. Jusqu'à présent, il n'existe pas de preuves directes d'intermédiaires de réplication thêta mais il a été démontré que la réplication de l'ADN viral est dépendante de la circularisation du génome. De plus, la réplication requiert les origines de réplication et l'action du produit du gène UL9 qui code pour une protéine liant les origines de réplication ("replication origin binding protein"), laquelle possède toutes les propriétés d'une protéine initiatrice (Weir et Stow, 1990; Hernandez *et al.*, 1991; Martin *et al.*, 1991). Ces observations suggèrent fortement que la réplication de l'ADN du HSV1 s'initie par un mécanisme thêta et rendent peu probable d'autres mécanismes comme la recombinaison

inter- ou intramoléculaire ou la transcription, lesquels pourraient potentiellement survenir sur des génomes linéaires (Boehmer et Lehman, 1997).

L'utilisation de la réplication thêta bidirectionnelle pour générer de nouveaux génomes préalablement au début de la réplication de l'ADN par le mécanisme du cercle roulant n'est pas un phénomène inhabituel. En effet, le bactériophage Lambda utilise cette stratégie pour répliquer son ADN (Catalano, 2000). Cependant, il n'existe pas pour l'instant d'informations sur la transition de la réplication thêta au mécanisme du cercle roulant (réplication sigma), ni sur les facteurs impliqués dans cette transition, autant pour les virus alphaherpès que pour les bactériophages.

Les résultats obtenus par l'analyse du mutant de délétion UL28 du BHV1 suggèrent l'existence d'un mode précoce de réplication sigma de l'ADN où des génomes linéaires seraient synthétisés (Fig. 1, étape 3A; Desloges et Simard, soumis). Nous avons postulé que cet ADN serait synthétisé par le mécanisme du cercle roulant où, plutôt que de générer de longs concatémères d'ADN, des génomes linéaires seraient détachés du gabarit circulaire chaque fois qu'une ronde de réplication est complétée. Ces génomes seraient encapsidés mais le mécanisme par lequel ce processus est accompli nous est inconnu. Néanmoins, les résultats obtenus indiquent que la protéine virale UL28 n'est pas essentielle pour l'encapsidation de ces génomes linéaires puisqu'une déficience de cette protéine n'empêche pas l'encapsidation précoce d'ADN viral. Ultérieurement, un changement pour la réplication par le mécanisme du cercle roulant classique a lieu, permettant la synthèse de longs concatémères d'ADN (Fig.1, étape 3B).

Le clivage et l'encapsidation de l'ADN concatémérique est un processus complexe qui requiert plusieurs protéines. Les procapsides doivent tout d'abord être libérées de leur structure interne constituée d'un échafaudage protéique, et cela par l'action de la protéase VP24 (Fig. 1, étape 4A; Flint *et al.*, 2000). Sur ces procapsides, un complexe protéique est retrouvé au site d'entrée de l'ADN viral. Il a été démontré récemment que la protéine UL6 en fait partie et constitue le canal d'entrée pour l'ADN (Newcomb *et al.*, 2001). Par l'observation de la protéine UL6 en microscopie électronique à balayage, il est apparu que

Figure 1. Représentation schématique de la réplication, du clivage et de l'encapsidation de l'ADN viral.

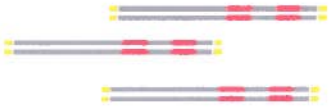
Le génome du BHV1 est représenté schématiquement en haut de la figure avec ses segments UL et US ainsi que ses deux régions répétées inversées IR et TR. Celles-ci possèdent chacune une origine de réplication qui sont indiquées en rouge. De plus, le génome possède à ses extrémités les séquences d'encapsidation *pac* qui sont représentées en jaune. Initialement, le génome se circulariserait (1) et se répliquerait selon le mode thêta (2). Ces molécules circulaires serviraient à la production de génomes linéaires simples par un mécanisme du cercle roulant (réplication sigma) non-typique et ces génomes linéaires seraient encapsidés par un processus qui est encore inconnu (3A). Par la suite, l'ADN serait répliqué selon le mécanisme du cercle roulant classique générant de longs concatémères d'ADN (3B). Au même moment, l'échafaudage protéique retrouvé à l'intérieur des capsides B serait dégradé par la protéase VP24 (4A). Sur ces capsides est retrouvé un complexe protéique constitué de la protéine UL6 (en bleu) qui agirait comme site d'ancrage de la protéine UL15 (en orange) et de la protéine UL28 (en vert foncé). Ces dernières constitueraient le complexe de terminase et ce serait la protéine UL28 qui reconnaîtrait les séquences *pac* sur le génome (4B). À l'aide de l'énergie dégagée par l'hydrolyse d'ATP, une unité de génome serait injectée à l'intérieur de la capside (4C). Le complexe de terminase se dissocierait et la protéine UL25 (en vert pâle) fermerait le canal d'entrée de l'ADN (4D). Finalement, la capside acquerrait les protéines du tégument et une enveloppe bilipidique (4E). Pour plus d'informations, voir le texte.

1. Circularisation de l'ADN viral

2. Réplication par mode thêta

3. Réplication par mode sigma

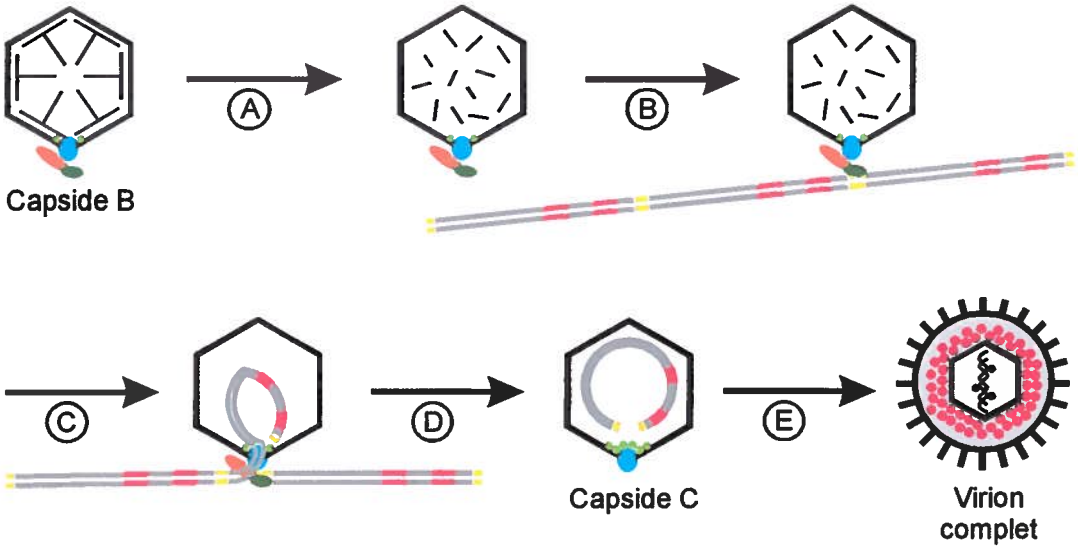
A. Production de génomes linéaires simples



Processus d'encapsidation?

B. Production de concatémères d'ADN

4. Clivage et encapsidation



cette protéine a une forme d'anneau et ainsi, ressemble aux protéines qui constituent les vertex retrouvés sur les procapsides des bactériophages comme ϕ29, T4, P22 et Lambda (Newcomb *et al.*, 2001). La protéine UL6 servirait également de site d'ancrage pour les protéines UL15 et UL28. Ces deux protéines formeraient le complexe de terminase nécessaire pour cliver l'ADN (Sheaffer *et al.*, 2001). Cette hypothèse est basée sur les données suivantes. D'abord, la protéine UL15 possède des similarités de séquence avec la grosse sous-unité catalytique du complexe de terminase du bactériophage T4, incluant un motif liant l'ATP (Yu and Weller, 1998b). Il semblerait que l'hydrolyse de l'ATP soit nécessaire pour procurer l'énergie pour la translocation de l'ADN dans les capsides (Sheaffer *et al.*, 2001). De plus, les protéines UL15 et UL28 interagissent physiquement comme le font les sous-unités du complexe de terminase des bactériophages (Koslowski *et al.*, 1999; Koslowski *et al.*, 1997). Il a également été démontré que la protéine UL28 interagit spécifiquement avec de l'ADN possédant le motif *pac 1* (Adelman *et al.*, 2001) suggérant que cette protéine est nécessaire pour la reconnaissance des séquences spécifiques pour l'encapsidation de l'ADN. Ainsi, le complexe constitué des protéines UL15 et UL28 reconnaîtrait l'ADN viral, en injecterait une longueur de génome dans la capside et cliverait l'ADN (Fig. 1, étapes 4B, C, D).

La protéine UL25 est présente en faibles quantités dans les capsides avant l'encapsidation de l'ADN (Sheaffer *et al.*, 2001). Son rôle n'est pas encore complètement élucidé mais il a été démontré que cette protéine a la capacité de lier l'ADN viral (Ogasawara *et al.*, 2001). Il se pourrait que l'UL25 se lie à l'ADN condensé dans les capsides suite à l'encapsidation, dissociant du même coup le complexe de terminase, ce qui est en accord avec la démonstration récente que l'UL25 accroît la dissociation de ce complexe (Yu et Weller, 1998a). Étant donné que la protéine UL25 est plus abondante dans les capsides C que dans les B, il a été suggéré que la fermeture du canal d'entrée, suite à l'encapsidation de l'ADN, se ferait par l'addition de nouvelles molécules UL25 (Fig. 1, étape 4D; Sheaffer *et al.*, 2001). Par la suite, la capside poursuivrait sa maturation en acquérant les protéines du tégument ainsi qu'une enveloppe bilipidique (Fig. 1, étape 4E).

La représentation schématique de la réplication, du clivage et de l'encapsidation de l'ADN des virus herpès présentée à la figure 1 donne un bon aperçu de ces processus. Néanmoins, des informations clés sont toujours manquantes comme une preuve irréfutable de l'existence d'une réplication thêta du génome suite à sa circularisation, la découverte du mécanisme et des facteurs impliqués dans la transition de la réplication thêta au mécanisme du cercle roulant, par quel mécanisme les génomes linéaires simples sont encapsidés précocement chez le BHV1 et si ce mécanisme existe chez d'autres virus herpès. De plus, les fonctions des différentes protéines virales impliquées dans les processus de clivage et d'encapsidation (UL6, UL12, UL15, UL17, UL25, UL28, UL32 et UL33) ne sont pas encore complètement élucidées. L'encapsidation de l'ADN viral s'avère d'une grande complexité, requérant non seulement l'interaction entre l'ADN nouvellement synthétisé et les capsides préformées mais aussi la synchronisation dans les événements de maturation des capsides pour une encapsidation stable de l'ADN viral. Malgré tout, les processus impliqués sont de mieux en mieux connus et mes travaux de doctorat ont contribué à l'accroissement des connaissances dans ce domaine.

3. PERSPECTIVES

Malgré l'utilisation intensive de vaccins anti-BHV1 au cours des dernières décennies, des pertes économiques considérables sont toujours associées à l'infection par le BHV1, démontrant l'incapacité des vaccins actuels à protéger efficacement le bétail. Il est donc pertinent de développer une nouvelle génération de vaccins efficaces et sécuritaires. Les mutants de délétion inaptes à se répliquer, en plus de servir d'outils afin d'apporter de nouvelles connaissances sur la fonction des gènes, pourraient servir éventuellement de base pour le développement d'un vaccin anti-BHV1.

Afin de générer un mutant du BHV1 inapte à se répliquer, un gène essentiel à sa réplication doit être éliminé du génome viral. L'absence d'un seul gène impliqué dans la régulation de l'expression des gènes, dans la réplication, le clivage et l'encapsidation de l'ADN viral ou dans l'assemblage des nouveaux virions conduirait à l'avortement du cycle réplicatif. L'avortement de la réplication procéderait à un stade plus ou moins avancé dépendamment

de l'étape où le gène éliminé est impliqué. Plusieurs gènes essentiels pourraient être supprimés du génome viral pour la création d'un mutant inapte à se répliquer mais les gènes impliqués dans le clivage et l'encapsidation de l'ADN viral représenteraient des cibles optimales. En effet, leur délétion conduirait à l'interruption de l'assemblage des virions, provoquant un avortement tardif de la réplication du BHV1.

L'utilisation d'un mutant inapte à se répliquer comme souche vaccinale serait très sécuritaire puisque chez l'animal, la réplication du mutant serait irrémédiablement compromise et avorterait abruptement lorsque la protéine du gène supprimé serait requise pour la continuation du cycle réplicatif. L'infection resterait donc strictement confinée aux cellules initialement infectées suite à l'immunisation et ainsi, les risques de transmission des animaux vaccinés à ceux non-vaccinés seraient éliminés. L'efficacité de ces vaccins à induire une bonne réponse immunitaire dans l'animal vacciné reste à être prouvée mais comme l'expression des gènes viraux de la classe tardive pourrait ne pas être compromise chez le mutant de délétion, tous les antigènes majeurs du BHV1, comme les glycoprotéines, seraient synthétisés. Plusieurs évidences suggèrent qu'une fois synthétisées, les glycoprotéines sont transportées à la membrane plasmique, ce qui en ferait des cibles majeures pour la réponse immunitaire (Roizman et Sears, 1996). De plus, la délétion permanente d'un gène viral sur le génome du mutant lui conférerait une grande stabilité génétique en comparaison avec les vaccins vivants modifiés conventionnels, de sorte que les risques de réversion au phénotype sauvage seraient virtuellement éliminés.

Pour permettre la différenciation sérologique des animaux vaccinés de ceux infectés naturellement, le mutant utilisé pourrait être davantage modifié en supprimant de son génome un gène codant pour une ou plusieurs (glyco)protéines retrouvées à la surface du virion. Les glycoprotéines impliquées dans l'attachement et/ou la pénétration du virus à l'intérieur de la cellule-hôte, telles que la gB, la gC, la gD, la gH et la gL, ne sont pas des cibles de choix puisque le mutant créé doit conserver la capacité de s'attacher et de pénétrer les cellules-hôtes. Cependant, les glycoprotéines gE, gI, gG et gM pourraient être des cibles intéressantes puisqu'elles ne sont pas essentielles à la réplication du BHV1 et qu'elles sont impliquées dans la transmission du virus aux cellules adjacentes lors de la seconde ronde de

réplication. Le choix final devrait tenir compte de la capacité de ces glycoprotéines à induire une production d'anticorps lors d'une infection par le BHV1. En effet, la présence ou l'absence d'anticorps spécifiques à la glycoprotéine supprimée permettrait de discriminer facilement les animaux vaccinés de ceux infectés naturellement par la souche sauvage. Cette différenciation sérologique permettrait la certification des animaux vaccinés, facilitant ainsi la gestion des troupeaux. De plus, les producteurs impliqués dans le commerce des animaux ainsi que de leurs semences et de leurs embryons auraient la possibilité d'attester que leurs produits sont exempts d'infections par le BHV1 et qu'ils en sont protégés.

Les bénéfices d'un double mutant seraient davantage exploités en l'utilisant comme vecteur viral pour délivrer des gènes codant des antigènes majeurs de d'autres pathogènes importants du bétail comme le virus respiratoire syncytial bovin (BRSV), le virus de la diarrhée virale bovine (BVDV), le virus parainfluenza-3 (PI-3V) ou de bactéries de type *Pasteurella* (Bowland et Shewen, 2000; Van Oirschot *et al.*, 1996a). Il a déjà été rapporté dans la littérature que le BHV1 sauvage peut être utilisé comme vecteur viral pour exprimer la glycoprotéine E2 du BVDV qui est normalement associée à l'enveloppe de ce pestivirus. Lorsque exprimée par le BHV1, la glycoprotéine E2 peut s'incorporer dans l'enveloppe des particules virales du BHV1 recombinant sous la forme d'un dimère, conformation qui est retrouvée dans des cellules infectées par le BVDV et dans les virions du BVDV. De plus, l'incorporation de cette glycoprotéine dans les particules du BHV1 ne requiert pas de séquences signal spécifiques aux virus herpès. Cependant, la présence de la protéine E2 interfère avec l'entrée du BHV1 recombinant dans les cellules susceptibles et la sortie des nouveaux virions des cellules infectées (Schmitt *et al.*, 1999). En ce qui concerne le BRSV, des études ont démontré l'importance de la glycoprotéine de surface G, impliquée dans l'attachement du virus à la cellule-hôte, dans l'établissement d'une protection contre l'infection par ce pneumovirus (Olmsted *et al.*, 1986; Stott *et al.*, 1987). En sachant ce fait, Taylor *et al.* (1998) ont inséré le gène encodant la glycoprotéine G du BRSV sous le contrôle du promoteur de la glycoprotéine gE du BHV1 produisant ainsi un mutant négatif pour la gE mais positif pour la protéine G du BRSV. Ce chimère induit une réponse immunitaire contre le BRSV dans la partie inférieure des voies respiratoires et également,

mais dans une moindre mesure, dans la partie supérieure des voies respiratoires. Il induit aussi une réponse protectrice contre le BHV1 sauvage. Cependant, l'expression de la protéine G du BRSV augmente la virulence du mutant du BHV1 négatif pour la gE dans les voies respiratoires inférieures (Taylor *et al.*, 1998; Taylor *et al.*, 1997; Schrijver *et al.*, 1997). L'utilisation d'un mutant du BHV1 inapte à se répliquer comme vecteur viral pour exprimer la protéine G du BRSV permettrait d'éviter une augmentation de la virulence par l'expression de cette protéine puisque par sa nature, le mutant ne pourrait effectuer qu'un seul cycle de réplication et serait donc considéré comme avirulent.

Il a également été démontré que des recombinants du BHV1, possédant dans leur génome les ORFs encodant les cytokines IL-1β, IL-2, IL-4 ou IFN-γ sécrètent ces cytokines qui sont actives biologiquement dans le milieu de culture (Raggo *et al.*, 1996; Kühnle *et al.*, 1996). La production de cytokines aux sites de réplication virale pourrait avoir un potentiel significatif à moduler la réponse immunitaire qui de ce fait, contrôlerait la réplication du virus, préviendrait la latence et possiblement, préviendrait les infections bactériennes secondaires. Raggo *et al.* (2000) ont voulu approfondir la question et ont donc infecté des bovins avec un mutant du BHV1 qui exprime l'IFN-γ (BHV-1/IFN-γ). Ils ont établi que le BHV1 est un vecteur stable pour la production de cytokines. En effet, suite à la réactivation d'une infection latente causée par le mutant BHV-1/IFN-γ, celui-ci possède toujours un gène fonctionnel de l'IFN-γ qui produit une protéine active biologiquement. D'autres vecteurs viraux n'ont pas toujours démontré une telle stabilité. Par exemple, lorsqu'un vecteur recombinant du virus de l'immunodéficience simienne (SIV) exprimant l'IFN-γ est utilisé pour infecter des macaques, il y a une délétion du gène de l'IFN-γ au cours des 12 premières semaines p.i. (Giavedoni *et al.*, 1997). Le groupe de Raggo (2000) a également établi que l'expression d'IFN-γ recombinant n'a pas atténué la croissance du virus ou modulé significativement les réponses immunitaires humorale et cellulaire. Ce résultat pourrait être expliqué par la production élevée d'IFN-γ endogène au cours d'une infection aiguë par le BHV1. Il s'agirait maintenant d'analyser l'effet de l'expression de d'autres cytokines lors de l'utilisation d'un vecteur BHV1. Ces travaux serviraient par la suite à déterminer quelle(s) cytokine(s) pourrai(en)t être incorporée(s) dans le génome d'un mutant

du BHV1 inapte à se répliquer pour moduler adéquatement la réponse immunitaire afin de développer un vaccin ayant la capacité de protéger efficacement l'animal.

L'utilisation d'un mutant du BHV1 inapte à se répliquer, dont un gène codant pour l'une des glycoprotéines retrouvées à sa surface est éliminé du génome viral et exprimant des antigènes spécifiques de d'autres pathogènes majeurs du bétail, est une avenue prometteuse pour le développement d'un vaccin polyvalent pour les bovins. En effet, ce type de vaccin serait sécuritaire puisqu'il ne pourrait se propager chez l'animal vacciné et qu'ainsi, il serait intransmissible aux autres animaux. Il serait efficace puisqu'il préserverait les propriétés immunes naturelles du BHV1. De plus, ce vaccin permettrait la vaccination contre plus d'un pathogène du bétail en une seule immunisation et permettrait de différencier les animaux vaccinés de ceux infectés naturellement. Finalement, ce vecteur viral pourrait également servir à exprimer des cytokines bovines afin de moduler la réponse immunitaire.

CONCLUSION

Mes travaux de recherche de doctorat ont contribué à améliorer la compréhension que nous avons de l'expression des gènes viraux et du cycle de réplication des virus alphaherpès, et plus particulièrement du BHV1. Tout d'abord, en identifiant les transcrits et les produits de traduction des ORFs UL12, UL25 et UL28, j'ai pu établir que ces ORFs constituent bel et bien des gènes chez le BHV1 et qu'ils appartiennent à la classe γ2. La détermination des sites d'initiation de la transcription m'a permis de localiser les régions 5' non-codantes et certains éléments des régions promotrices de ces gènes.

En deuxième lieu, l'élimination du gène UL28 du génome du BHV1 a permis d'étudier sa fonction dans la réplication virale et particulièrement dans les processus de synthèse, de clivage et d'encapsidation de l'ADN viral. Il s'est avéré que le produit du gène UL28 est essentiel à la réplication virale. Il n'est pas nécessaire pour l'expression des protéines virales, ni pour la réplication de l'ADN mais sa présence est requise pour le clivage et l'encapsidation de l'ADN concatémérique nouvellement synthétisé.

Mes résultats permettent aussi de mettre en lumière l'existence possible d'un mode précoce de réplication de l'ADN viral. En effet, au début de la réplication par le mécanisme du cercle roulant, préalablement à la synthèse de concatémères d'ADN, des génomes linéaires seraient détachés dès la fin de leur synthèse et encapsidés, cela du moins chez le BHV1. Pour l'instant, il est impossible de conclure si ce mécanisme n'est restreint qu'au BHV1 ou s'il est retrouvé chez d'autres virus herpès. Le mécanisme par lequel ces génomes linéaires matures seraient encapsidés n'est pas connu. Il semble seulement que le produit du gène UL28 ne soit pas requis pour ce processus.

La compréhension du processus de réplication du BHV1 ainsi que la connaissance de la fonction des gènes viraux sont essentielles afin de lutter efficacement contre ce virus qui occasionne à chaque année d'importantes pertes économiques. Les vaccins conventionnels contre le BHV1, incluant les vaccins inactivés et les vaccins vivants modifiés, ont été et sont toujours largement utilisés pour contrôler l'infection par le BHV1 mais ce virus est toujours présent dans les cheptels bovins (Liang *et al.*, 1997). Ainsi, il existe un besoin pour développer de nouveaux vaccins plus efficaces et sécuritaires contre le BHV1. Une avenue

des plus prometteuses consisterait en l'utilisation de mutants de délétion inaptes à se répliquer comme vaccin anti-BHV1 et/ou comme vecteur viral. Puisque le gène supprimé du génome viral doit être absolument indispensable à la réplication du virus afin de provoquer l'avortement prématuré du cycle réplicatif, la connaissance de la fonction des gènes viraux est primordiale pour la sélection du gène à supprimer.

Les virus herpès vétérinaires, par leur frappante similarité aux virus humains, sont des modèles valables autant pour étudier la biologie des virus herpès que pour développer des stratégies destinées à créer de nouveaux vaccins tels que les mutants inaptes à se répliquer. Puisque les vaccins anti-BHV1 peuvent être testés chez l'hôte naturel, le BHV1 constitue sans aucun doute un modèle valable pour le développement de nouveaux vaccins humains ou vétérinaires.

REMERCIEMENTS

Tout d'abord, je tiens à remercier ma directrice de recherche, la Dre Claire Simard, pour l'intérêt qu'elle a porté à mes travaux de recherche ainsi que pour les nombreux conseils qu'elle a pu me prodiguer tout au long des années passées dans son laboratoire.

Je remercie également Mme Nicole Gagnon pour son excellente assistance technique, Dre Francine Hamel et Hélène Boucher pour les discussions constructives ainsi que Dr Johnny Basso pour la correction des manuscrits anglais. Je remercie le Service de microscopie électronique, et plus particulièrement M. Robert Alain, ainsi que le Service de séquençage de l'INRS-Institut Armand-Frappier pour l'aide estimée qu'ils m'ont prodiguée. De plus, je tiens à mentionner mon appréciation du soutien et des encouragements que parents et amis m'ont apportés au cours de ces années.

Finalement, je tiens à souligner l'appui financier du Conseil de Recherches en Sciences Naturelles et en Génie du Canada (CRSNG), du Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) et de la Fondation Armand-Frappier pour mes études doctorales.

BIBLIOGRAPHIE

Abbotts, A.P., Preston, V.G., Hughes, M., Patel, A.H. et Stow, N.D. (2000). Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. J. Gen. Virol. 81: 2999-3009.

Ackermann, M., Müller, H.K., Bruckner, L. et Kihm, U. (1990). Eradication of infectious bovine rhinotracheitis in Switzerland: review and prospects. Vet. Microbiol. 23: 365-370.

Addison, C., Rixon, F.J. et Preston, V.G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. 71: 2377-2384.

Adelman, K., Salmon, B. et Baines, J.D. (2001). Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc. Nat. Acad. Sci. USA 98: 3086-3091.

Ali, M.A., Forghani, B. et Cantin, E.M. (1996). Characterization of an essential HSV-1 protein encoded by the UL25 gene reported to be involved in virus penetration and capsid assembly. Virology 216: 278-283.

Al-Kobaisi, M. F., Rixon, F.J., McDougall, I. et Preston, V.G. (1991). The herpes simplex virus UL33 gene product is required for the assembly of full capsids. Virology 180: 380-388.

Armstrong, J.A., Pereira, H.G. et Andrews, C.H. (1961). Observations of the virus of infectious bovine rhinotracheitis, and its affinity with the herpesvirus group. Virology 14: 276-285.

Babiuk, L.A., Bielefeldt Ohamnn, H., Gifford, H., Czarniecki, Ch.W., Scialli, V.T. et Hamilton, E.B. (1985). Effect of bovine alpha 1 interferon on bovine herpesvirus type 1 induced respiratory disease. J. Gen. Virol. 66: 2383-2394.

Babiuk, L.A., Italien, J.L., van Drunen Littel-van den Hurk, S., Zamb, T., Lawman, M.P.J., Hughes, G. et Gifford, G.A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual viral glycoproteins. Virology 159: 57-66.

Baines, J. D., Cunningham, C., Nalwanga, D. et Davison, A. (1997). The UL15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the UL15 gene product. J. Virol. 71: 2666-2673.

Banks, L.M., Halliburton, I.W., Purifoy, D.J.M., Killington, R.A. et Powell, K.L. (1985). Studies on the herpes simplex virus alkaline nuclease: detection of type-commun and type-specific epitopes on the enzyme. J. Gen. Virol. 66: 1-14.

Banks, L., Purifoy, D.J.M., Hurst, P.-F., Killington, R.A. et Powell, K.L. (1983). Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. J. Gen. Virol. 64: 2249-2260.

Baranowski, E., Dubuisson, J., Pastoret, P.-P. et Thiry, E. (1993). Identification of 108 K, 93 K and 42 K glycoproteins of bovine herpesvirus-1 by monoclonal antibodies. Arch. Virol. 133: 97-111.

Baranowski, E., Keil, G., Lyaku, J., Rijsewijk, F.A.M., Van Oirschot, J.T., Pastoret, P.-P et Thiry, E. (1996). Structural and functional analysis of bovine herpesvirus 1 minor glycoproteins. Vet. Microbiol. 53: 91-101.

Bazinet, C. et King, J. (1985). The DNA translocating vertex of dsDNA bacteriophage. Annu. Rev. Microbiol. 39: 109-129.

Beaudoing, E., Freier, S., Wyatt, J.R., Claverie, J.-M. et Gautheret, D. (2000). Patterns of variant polyadenylation signal usage in human genes. Genome Res. 10: 1001-1010.

Bielefeldt Ohmann, H., Gilchrist, J.E. et Babiuk, L.A. (1984). Effect of recombinant alpha 1 interferon (BoIFN-alpha 1) on interaction between bovine alveolar macrophages and bovine herpesvirus-1. J. Gen. Virol. 65: 1487-1495.

Boehmer, P. E. et Lehman, I.R. (1997). Herpes simplex virus DNA replication. Annu. Rev. Biochem. 66: 347-384.

Bogner, E., Radsak, K. et Stinski, M.F. (1998). The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity. J. Virol. 72: 2259-2264.

Bowland, S.L. et Shewen, P.E. (2000). Bovine respiratory disease: commercial vaccines currently available in Canada. Can. Vet. J. 41: 33-48.

Broker, T. R. et Doermann, A.H. (1975). Molecular and genetic recombination of bacteriophage T4. Annu. Rev. Genet. 9: 213-244.

Bronstein, J.C. et Weber, P.C. (1996). Purification and characterization of herpes simplex virus type 1 alkaline exonuclease expressed in *Escherichia coli*. J. Virol. 70: 2008-2013.

Brown, F. (1989). The classification and nomenclature of viruses: summary of results of meetings of the International Committee on Taxonomy of Viruses in Edmonton, Canada, 1987. Intervirology 30: 181-186.

Brubaker, J.O., Thompson, C.M., Morrison, L.A., Knipe, D.M., Siber, G.R. et Finberg, R.W. (1996). Th1-associated immune responses to β-galactosidase expressed by a replication-defective herpes simplex virus. J. Immunol. 157: 1598-1604.

Campos, M., Bielefeldt Ohmann, H., Hutchings, D., Rapin, N. et Babiuk, L.A. (1989). Role of interferon gamma in inducing cytotoxicity of peripheral blood mononuclear leukocytes to bovine herpesvirus type 1 (BHV-1)-infected cells. Cell. Immunol. 120: 259-269.

Campos, M. et Rossi, C.R. (1986). Cytotoxicity of bovine lymphocytes after treatment with lymphokines. Am. J. Vet. Res. 47: 1524-1528.

Catalano, C. E. (2000). The terminase enzyme from bacteriophage lambda: a DNA-packaging machine. Cell. Mol. Life Sci. 57: 128-148.

Catalano, C. E., Cue, D. et Feiss, M. (1995). Virus DNA packaging: the strategy used by phage l. Mol. Microbiol. 16: 1075-1086.

Chalifour, A., Basso, J., Gagnon, N., Trudel, M. et Simard, C. (1996). Transcriptional and translational expression kinetics of the early gene encoding the BICP27 protein of bovine herpesvirus type 1. Virology 224: 326-329.

Chung, Y.-T. et Hsu, W. (1996). Functional expression of the bovine herpesvirus 1 alkaline deoxyribonuclease (UL12) in *Escherichia coli*. Arch. Virol. 141: 2457-2464.

Chung, Y.-T. et Hsu, W. (1997). Purification of the infectious bovine rhinotracheitis virus alkaline deoxyribonuclease expressed in *Escherichia coli*. J. Vet. Med. Sc. 59: 35-38.

Ciacci-Zanella, J., Stone, M., Henderson, G. et Jones, C. (1999). The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. J. Virol. 73: 9734-9740.

Cohen, J.H. et Straus, S.E. (1996). Varicella-zoster virus and its replication. Dans: Fields, B.N., Knipe, D.M. et Howley, P.M. (Eds), Fields Virology, 3^{ième} édition. Lippincott-Raven publishers, Philadelphia, New York, pp. 2525-2545.

Cook, C.G. et Splitter, G.A. (1989). Characterization of bovine mononuclear cell population with natural cytotoxic activity against bovine herpesvirus-1 infected cells. Cell. Immunol. 120: 240-249.

Costa, R.H., Draper, K.G., Banks, L., Powell, K.L., Cohen, G., Eisenberg, R. et Wagner, E.K. (1983). High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-Dalton protein tentatively identified as a capsid protein. J. Virol. 48: 591-603.

Da Costa, X., Kramer, M.F., Zhu, J., Brockman, M.A. et Knipe, D.M. (2000). Construction, phenotypic analysis, and immunogenicity of a UL5/UL29 double deletion mutant of herpes simplex virus 2. J. Virol. 74: 7963-7971.

Dasgupta, A. et Wilson, D.W. (1999). ATP depletion blocks herpes simplex virus DNA packaging and capsid maturation. J. Virol. 73: 2006-2015.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. Virology 186: 9-14.

Davison, A.J. et Wilkie, N.M. (1983). Location and orientation of homologous sequences in the genome of five herpesviruses. J. Gen. Virol. 64: 1927-1942.

Deiss, L. P., Chou, J. et Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. 59: 605-618.

Denis, M., Hanon, E., Rijsewijk, F.A., Kaashoek, M.J., van Oirschot, J.T., Thiry, E. et Pastoret, P.-P. (1996). The role of glycoproteins gC, gE, gI, and gG in the induction of cell-mediated immune responses to bovine herpesvirus 1. Vet. Microbiol. 53: 121-132.

Denis, M., Slaoui, M., Keil, G., Babiuk, L.A., Ernst, E., Pastoret, P.-P. et Thiry, E. (1993). Identification of different target glycoproteins for bovine herpesvirus-1-specific cytotoxic T lymphocytes depending on the method of in vitro stimulation. Immunology 78: 7-13.

Desloges, N., Boucher, H. et Simard, C. (2001). Transcriptional and translational expression kinetics of the UL25 homologue of bovine herpesvirus 1. Arch. Virol. 146: 1693-1704.

Desloges, N. et Simard, C. (2001). Expression kinetics of the transcript and product of the UL28 homologue of bovine herpesvirus 1. Virus Res. 80: 23-31.

Desloges, N. et Simard, C. UL28 deficiency in bovine herpesvirus 1 does not abolish DNA packaging even though processing of newly synthesized DNA concatemers is abrogated. Soumis.

Dezélée, S., Bras, F., Vende, P., Simonet, B., Nguyen, X., Flamand, A. et Masse, M.J. (1996). The BamHI fragment 9 of pseudorabies virus contains genes homologous to the UL24, UL25, UL26, and UL26.5 genes of herpes simplex virus type 1. Virus Res. 42: 27-39.

Dijkstra, J.M., Fuchs W., Mettenleiter T.C. et Klupp, B.G. (1997). Identification and transcriptional analysis of pseudorabies virus UL6 to UL12 genes. Arch. Virol. 142: 17-35.

Draper, K.G., Devi-Rao, G., Costa, R.H., Blair, E.D., Thompson, R.L. et Wagner, E.K. (1986). Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. 57: 1023-1036.

Farley, J.E., Skare, I.B. et Skare, J. (1981). Inverted repeat sequences in infectious bovine rhinotracheitis virus DNA. Dans: Nahmias, A.J., Dowdle, W.R., Schinazi, R.F. (Eds) The human herpes viruses: an interdisciplinary perspective. Elsevier/North Holland Publishing New York, p 590.

Fehler, F., Herrmann, J.M., Saalmuller, A., Mettenleiter, T.C. et Keil, G.M. (1992). Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. J. Virol. 66: 831-839.

Fitzpatrick, D.R., Zamb, T.J. et Babiuk, L.A. (1990). Expression of bovine herpesvirus type 1 glycoprotein gI in transfected bovine cells induces spontaneous cell fusion. J. Gen. Virol. 71: 1215-1219.

Flint, S.J., Enquist, L.W., Krug, R.M., Racaniello, V.R. et Skalka, A.M. (2000). Principles of virology, Molecular biology, pathogenesis, and control. ASM Press, Washington, USA.

Friedmann, A., Shlomai, J. et Becker, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. J. Gen. Virol. 34: 507-522.

Giavedoni, L., Ahmad, S., Jones, L. et Yilma, T. (1997). Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces postchallenge virus load in vaccinated rhesus macaques. J. Virol. 71: 866-872.

Gibbs, E.P.J. et Rweyemamu, M.M. (1977). Bovine herpesviruses, part 1. Vet. Bull. 47: 317-343.

Goldstein, J.N. et Weller, S.K. (1998a). In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. J. Virol. 72: 8772-8781.

Goldstein, J.N. et Weller, S.K. (1998b). The exonuclease activity of HSV-1 UL12 is required for *in vivo* function. Virology 244: 442-457.

Goshima, F., Watanabe, D., Takakuwa, H., Wada, K., Daikoku, T., Yamada, M. et Nishiyama, Y. (2000). Herpes simplex virus UL17 protein is associated with B capsids and colocalizes with ICP35 and VP5 in infected cells. Arch. Virol. 145: 417-426.

Gossen, M. et Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89: 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. et Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science 268: 1766-1769.

Haanes, E.J., Chen, C.C. et Lowery, D.E. (1997). Nucleotide sequence and transcriptional analysis of a portion of the bovine herpesvirus genome encoding genes homologous to HSV-1 UL25, UL26 and UL26.5. Virus Res. 48: 19-26.

Henderson, J.O., Ball-Goodrich, L.J. et Parris, D.S. (1998). Structure-fonction analysis of the herpes simplex virus type 1 UL12 gene: correlation of deoxyribonuclease activity *in vitro* with replication function. Virology 243: 247-259.

Hercend, T. et Schmidt, R.E. (1988). Characteristics and uses of natural killer cells. Immunol. Today 9: 291-295.

Hernandez, T.R., Dutch, R.E., Lehman, I.R., Gustafsson, C. et Elias, P. (1991). Mutations in a herpes simplex virus type 1 origin that inhibit interaction with origin-binding protein also inhibit DNA replication. J. Virol. 65: 1649-1652.

Hirsch, I., Cabral, G., Patterson, M. et Biswal, N. (1977). Studies on the intracellular replicating DNA of herpes simplex virus type 1. Virology 81: 48-61.

Hoffmann, P.J. et Cheng, Y.-C. (1979). DNase induced after infection of KB cells by herpes simplex virus type 1 or type 2. II. Characterization of an associated endonuclease activity. J. Virol. 32: 449-457.

Holland, L.E., Sandri-Goldin, R.M., Goldin A.L., Glorioso, J.C. et Levine, M. (1984). Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. 49: 947-959.

Homa, F.L. et Brown, J.C. (1997). Capsid assembly and DNA packaging in herpes simplex virus. Rev. Med. Virol. 7: 107-122.

Hsiang, C.Y., Ho, T.Y. et Chang, T.J. (1996). Identification of a pseudorabies virus UL12 (deoxyribonuclease) gene. Gene 177: 109-113.

Hutchings, D.L., van Drunen Littel-van den Hurk, S. et Babiuk, L.A. (1990). Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. J. Virol. 64: 5114-5122.

Hutchinson, L. et Johnson, D.C. (1995). Herpes simplex virus glycoprotein K promotes egress of virus particles. J. Virol. 69: 5401-5413.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A.C. et Johnson, D.C. (1992). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. 66: 2240-2250.

Jacob, R. J. et Roizman, B. (1977). Anatomy of herpes simplex virus DNA. VIII. Properties of the replicating DNA. J. Virol. 23: 394-411.

Jacobson, J.G., Martin, S.L. et Coen, D.M. (1989). A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. 63: 1839-1843.

Jensen, J. et Schultz, R.D. (1990). Bovine natural cell mediated cytotoxicity (NCMC): Activation by cytokines. Vet. Immunol. Immunopathol. 24: 113-129.

Jiang, Y., Hossain, A., Winkler, M.T., Holt, T., Doster, A. et Jones, C. (1998). A protein encoded by the latency-related gene of bovine herpesvirus 1 is expressed in trigeminal ganglionic neurons of latently infected cattle and interacts with cyclin-dependant kinase 2 during productive infection. J. Virol. 72: 8133-8142.

Kaelin, K., Dezélée, S., Masse, M.J., Bras, F. et Flamand, A. (2000). The UL25 protein of pseudorabies virus associates with capsids and localizes to the nucleus and to microtubules. J. Virol. 74: 474-482.

Kendrick, J.W., York, C.J. et McKercher, D.G. (1956). A controlled field trial of a vaccine for infectious bovine rhinotracheitis. Proc. U.S. Livestock San. Assoc. 60: 155-158.

Kinchington, P.R., Reinhold, W.C., Casey, T.A., Strauss, S.E., Hay, J. et Ruyechan, W.T. (1985). Inversion and circularization of the varicella-zoster virus genome. J. Virol. 56: 194-200.

Kit, S. (1989). Recombinant-derived modified-live herpesvirus vaccines. Adv. Exp. Med. Biol. 251: 219-236.

Koslowski, K.M., Shaver, P.R., Casey II, J.T., Wilson, T., Yamanaka, G., Sheaffer, A.K., Tenney, D.J. et Pederson, N.E. (1999). Physical and functional interactions between the herpes simplex virus UL15 and UL28 DNA cleavage and packaging proteins. J. Virol. 73: 1704-1707.

Koslowski, K.M., Shaver, P.R., Wang, X.-Y., Tenney, D.J. et Pederson, N.E. (1997). The pseudorabies virus UL28 protein enters the nucleus after coexpression with the herpes simplex virus UL15 protein. J. Virol. 71: 9118-9123.

Kühnle, G., Collins, R.A., Scott, J.E. et Keil, G.M. (1996). Bovine interleukins 2 and 4 expressed in recombinant bovine herpesvirus 1 are biologically active secreted glycoproteins. J. Gen. Virol. 77: 2231-2240.

Lamberti, C. et Weller, S.K. (1996). The herpes simplex virus 1 UL6 protein is essential for cleavage and packaging but not for genomic inversion. Virology 226: 403-407.

Lamberti, C. et Weller, S.K. (1998). The herpes simplex virus type 1 cleavage/packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. J. Virol. 72: 2463-2473.

Lawman, M.J.P., Gifford, G., Gyongyossy-Issa, M., Dragan, R., Heise, J. et Babiuk, L.A. (1987). Activity of polymorphonuclear (PMN) leukocytes diseases bovine herpesvirus-1 induced respiratory disease: Effect of recombinant bovine interferon alpha 1. Antiviral Res. 8: 225-238.

Li, Y., van Drunen Littel-van den Hurk, S., Liang, X. et Babiuk, L.A. (1997). Functional analysis of the transmembrane anchor region of bovine herpesvirus 1 glycoprotein gB. Virology 228: 39-54.

Liang, X., Babiuk, L.A., van Drunen Little-van den Hurk, S., Fitzpatrick, D.R. et Zamb, T.J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. J. Virol. 65: 1124-1132.

Liang, X., Babiuk, L.A. et Zamb, T.J. (1992). An in vitro study of a glycoprotein gIII-negative bovine herpesvirus 1 (BHV-1) mutant expressing β-galactosidase: evaluation of the role of gIII in virus infectivity and its use as a vector for mucosal immunization. Virology 189: 629-639.

Liang, X., Babiuk, L.A. et Zamb, T.J. (1993). Mapping of heparin-binding structures on bovine herpesvirus 1 and pseudorabies virus gIII glycoproteins. Virology 194: 233-243.

Liang, X., Chow, B. et Babiuk, L.A. (1997). Study of immunogenicity and virulence of bovine herpesvirus 1 mutants deficient in the UL49 homolog, UL49.5 homolog and dUTPase genes in cattle. Vaccine 15(10): 1057-1064.

Loehr, B.I., Willson, P., Babiuk, L.A. et van Drunen Little-van den Hurk, S. (2000). Gene gun-mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus 1 in cattle. J. Virol. 74: 6077-6086.

Ludwig, H. (1983). Bovine herpesviruses, p. 135-214. Dans: B. Roizman (ed.), The Herpesviruses, vol 2. Plenum Press, New York and London.

Madin, S.H., York, C.J. et McKercher, D.G. (1956). Isolation of infectious bovine rhinotracheitis virus. Science 124: 721-722.

Mars, M.H., de Jong, M.C.M., Franken, P. et van Oirschot, J.T. (2001). Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field. Vaccine 19: 1924-1930.

Martin, D.W., Deb, S.P., Klauer, J.S. et Deb, S. (1991). Analysis of the herpes simplex virus type 1 OriS sequence: mapping of functional domains. J. Virol. 65: 4359-4369.

Martinez, R., Sarisky, R.T., Weber, P.C. et Weller, S.K. (1996a). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J. Virol. 70: 2075-2085.

Martinez, R., Shao, L., Bronstein, J.C., Weber, P.C. et Weller, S.K. (1996b). The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. Virology 215: 152-164.

Mavromara-Nazos, P. et Roizman, B. (1989). Delineation of regulatory domains of early (beta) and late (gamma 2) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes. Proc. Nat. Acad. Sci. USA 86: 4071-4075.

Mayfield, J.E., Good, P.J., Vanoort, H.J., Campbell, A.R. et Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus (Cooper strain). J. Virol. 47: 259-264.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. et Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69: 1531-1574.

McGeoch, D.J., Dolan, A. et Frame, M.C. (1986). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. Nucleic Acids Res. 14: 3435-3448.

McLauchlan, J., Simpson, S. et Clements, J.B. (1989). Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. Cell 59: 1093-1105.

McNab, A.R., Desai, P., Person, S., Roof, L.L., Thomsen, D.R., Newcomb, W.W., Brown, J.C. et Homa, F.L. (1998). The product of the herpes simplex virus type 1 UL25 gene is required for encapsidation but not for cleavage of replicated viral DNA. J. Virol. 72: 1060-1070.

Messerle, M., Keil, G.M., Schneider, K. et Koszinowski, U.H. (1992). Characterization of the murine cytomegalovirus genes encoding the major DNA binding protein and the ICP18.5 homolog. Virology 191: 355-367.

Mettenleiter, T.C., Saalmüller, A. et Weiland, F. (1993). Pseudorabies virus protein homologous to herpes simplex virus type 1 ICP18.5 is necessary for capsid maturation. J. Virol. 67: 1236-1245.

Meyer, G., Vlcek, C., Paces, V., O'Hara, M.K., Pastoret, P.-P., Thiry, E. et Schwyzer, M. (1997). Sequence analysis of the bovine herpesvirus type 1 genes homologous to the DNA polymerase (UL30), the major DNA-binding protein (UL29) and ICP18.5 assembly protein (UL28) genes of herpes simplex virus. Arch. Virol. 142: 89-102.

Miethke, A., Keil, G.M., Weiland, F. et Mettenleiter, T.C. (1995). Unidirectional complementation between glycoprotein B homologues of pseudorabies virus and bovine herpesvirus 1 is determined by the carboxy-terminal part of the molecule. J. Gen. Virol. 76 : 1623-1635.

Misra, V., Bratanich, A.C., Carpenter, D. et Ohare, P. (1994). Protein and DNA elements involved in transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. J. Virol. 68: 4898-4909.

Morrison, L.A. et Knipe, D.M. (1997). Contributions of antibody and T cell subsets to protection elicited by immunization with a replication-defective mutant of herpes simplex virus type 1. Virology 239: 315-326.

Murialdo, H. et Becker, A. (1978). Head morphogenesis of complex double-stranded deoxyribonucleic acid bacteriophages. Microbiol. Rev. 42: 529-576.

Murphy, F.A., Gibbs, E.P.J., Horzinek, M.C. et Studdert, M.J. (1999). Veterinary virology, 3^{ième} édition. Academic Press, San Diego, California, USA, pp 162-163, 301-311.

Newcomb, W.W., Juhas, R.M., Thomsen, D.R., Homa, F.L., Burch, A.D., Weller, S.K. et Brown, J.C. (2001). The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. J. Virol. 75: 10923-10932.

Nguyen, L.H., Knipe, D.M. et Finberg, R.W. (1992). Replication-defective mutants of herpes simplex virus (HSV) induce cellular immunity and protect against lethal HSV infection. J. Virol. 66: 7067-7072.

Ogasawara, M., Suzutani, T., Yoshida, I. et Azuma, M. (2001). Role of the UL25 gene product in packaging DNA into the herpes simplex virus capsid: location of UL25 product in the capsid and demonstration that it binds DNA. J. Virol. 75: 1427-1436.

Okazaki, K., Matsuzaki, T., Sugahara, Y., Okada, J., Hasebe, M., Iwamura, Y., Ohnishi, M., Kanno, T., Shimizu, M., Honda, E. et Kono, Y. (1991). BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. Virology 181: 666-70.

Olmsted, R.A., Elango, N., Prince, G.A., Murphy, B.R., Johnson, P.R., Moss, B., Chanock, R.M. et Collins, P.L. (1986). Expression of the glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc. Nat. Acad. Sci. USA 83: 7462-7466.

Patel, A. H., Rixon, F.J., Cunningham, C. et Davison, A.J. (1996). Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL6 gene. Virology 217: 111-123.

Paulus, W., Baur, I., Boyce, F.M., Breakefield, X.O. et Reeves, S.A. (1996). Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. J. Virol. 70: 62-67.

Pederson, N.E. et Enquist, L.W. (1989). The nucleotide sequence of a pseudorabies virus gene similar to ICP18.5 of herpes simplex virus type 1. Nucleic Acids Res. 17: 3597.

Pederson, N.E. et Enquist, L.W. (1991). Overexpression in bacteria and identification in infected cells of the pseudorabies virus protein homologous to the herpes simplex virus type 1 ICP18.5. J. Virol. 65: 3746-3758.

Perdue, M.L., Cohen, J.C., Randall, C.C. et O'Callaghan, D.J. (1976). Biochemical studies of the maturation of herpes virus nucleocapsid species. Virology 74: 194-208.

Poffenberger, K.L. et Roizman, B. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. 53: 587-595.

Poon, A. P. et Roizman, B. (1993). Characterization of a temperature-sensitive mutant of the UL15 open reading frame of herpes simplex virus 1. J. Virol. 67: 4497-4503.

Raggo, C., Fitzpatrick, D.R., Babiuk, L.A. et Liang, X. (1996). Expression of bovine interleukin-1β in a bovine herpesvirus-1 vector: in vitro analysis. Virology 221: 78-86.

Raggo, C., Habermehl, M., Babiuk, L.A. et Griebel, P. (2000). The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-γ. J. Gen. Virol. 81: 2665-2673.

Rocha, M.A., Barbosa, E.F., Guimaraes, S.E.F., Dias Neto, E. et Gouveia, A.M.G. (1998). A high sensitivity-nested PCR assay for BHV-1 detection in semen of naturally infected bulls. Vet. Microbiol. 63: 1-11.

Roizman, B. (1996). Herpesviridae. Dans: Fields, B.N., Knipe, D.M. et Howley, P.M. (Eds), Fields Virology, 3ième édition. Lippincott-Raven publishers, Philadelphia, New York, pp. 2221-2230.

Roizman, B. et Sears, A.E. (1996). Herpes simplex viruses and their replication. Dans: Fields, B.N., Knipe, D.M. et Howley, P.M. (Eds), Fields Virology, 3ième édition. Lippincott-Raven publishers, Philadelphia, New York, pp. 2231-2295.

Roizman, B., Carmichael, L.E., Deinhardt, F., de-The, G., Nahmias, A.J., Plowright, W., Rapp, F., Sheldrick, P., Takahashi, M. et Wolf, K. (1981). Herpesviridae. Definition, provisional nomenclature, and taxonomy. The herpesvirus study group, the international committee on Taxonomy of viruses. Intervirology 16: 201-217.

Salmon, B. et Baines, J.D. (1998). Herpes simplex virus DNA cleavage and packaging: association of multiple forms of UL15-encoded proteins with B capsids requires at least the UL6, UL17, and UL28 genes. J. Virol. 72: 3045-3050.

Sambrook, J., Fritsch, E.F. et Maniatis, T. (1989). Molecular cloning, a laboratory manual, 2ième édition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger F., Nicklen S. et Coulson A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sc. USA 74: 5463-5467.

Schmitt, J., Becher, P., Thiel, H.-J. et Keil, G.M. (1999). Expression of bovine viral diarrhoea virus glycoprotein E2 by bovine herpesvirus-1 from a synthetic ORF and incorporation of E2 into recombinant virions. J. Gen. Virol. 80: 2839-2848.

Schrijver, R.S., Langedijk, J.P.M., Keil, G.M., Middel, W.G.J., Maris-Veldhuis, M., van Oirschot, J.T. et Rijsewijk, F.A.M. (1997). Immunization of cattle with a BHV1 vector vaccine or a DNA vaccine both coding for the G protein of BRSV. Vaccine 15: 1908-1916.

Schwyer, M. et Ackermann, M. (1996). Molecular virology of ruminant herpesviruses. Vet. Microbiol. 53: 17-29.

Seal, B.S., Irving, J.M. et Whetstone, C.A. (1991). Transcriptional analysis of bovine herpesvirus 1 Cooper isolate. Temporal analysis and characterization of immediate-early, early, and late RNA. Arch. Virol. 121: 55-73.

Severini, A., Scraba, D.G. et Tyrrell, D.L. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. J. Virol. 70: 3169-3175.

Shao, L., Rapp, L.M. et Weller, S.K. (1993). Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology 196: 146-162.

Sheaffer, A.K., Newcomb, W.W., Gao, M., Yu, D., Weller, S.K., Brown, J.C. et Tenney, D.J. (2001). Herpes simplex virus DNA cleavage and packaging proteins associate with the procapsid prior to its maturation. J. Virol. 75: 687-698.

Shlomai, J., Friedmann, A. et Becker, Y. (1976). Replicative intermediates of herpes simplex virus DNA. Virology 69: 647-659.

Simard, C., Bastien, N. et Trudel, M. (1992). Sequencing and 5'-and 3'-end transcript mapping of the gene encoding the small subunit of ribonucleotide reductase from bovine herpesvirus type-1. Virology 190: 689-701.

Simard, C., LaBoissière, S., Séguin, C. et Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: a major variant selected from a field isolate. Intervirol. 32: 117-126.

Simard, C., Langlois, I., Styger, D., Vogt, B., Vlcek, C., Chalifour, A., Trudel, M. et Schwyzer, M. (1995). Sequence analysis of the UL39, UL38, and UL37 homologues of bovine herpesvirus 1 and expression studies of UL40 and UL39, the subunits of ribonucleotide reductase. Virology 212: 734-740.

Simard, C., Nadon, F., Séguin, C., LaBoissière, S. et Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. Arch. Virol. 110: 63-75.

Singh, M., Fraefel, C., Bello, L.J., Lawrence, W.C. et Schwyzer, M. (1996). Identification and characterization of BICP27, an early protein of bovine herpesvirus 1 which may stimulate mRNA 3' processing. J. Gen. Virol. 77: 615-625.

Stott, E.J., Taylor, G., Ball, L.A., Anderson, K., Young, K.K.Y., King, A.M.Q. et Wertz, G.W. (1987). Immune and histological responses in animals vaccinated with recombinant vaccinia viruses that express individual genes of human respiratory syncytial virus. J. Virol. 61: 3855-3861.

Stow, N.D. (2001). Packaging of genomic and amplicon DNA by the herpes simplex virus type 1 UL25-null mutant KUL25NS. J. Virol. 75: 10755-10765.

Straub, O.C. et Ahl, R. (1976). Lokale interferonbildung beim rind nach intranasaler infektion mit avirulentem IBR-IPV-virus und deren wirkung auf eine anschliebende infektion mit maul und klauenseuche-virus. Zbl. Vet. Med. B. 23: 470-482.

Strobel-Fidler, M. et Francke, B. (1980). Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. Virology 103: 493-501.

Taus, N.S., Salmon, B. et Baines, J.D. (1998). The herpes simplex virus 1 UL17 gene is required for localization of capsids and major and minor capsid proteins to intranuclear sites where viral DNA is cleaved and packaged. Virology 252: 115-125.

Taylor, G., Rijsewijk, F.A.M., Thomas, L.H., Wyld, S.G., Gaddum, R.M., Cook, R.S., Morrison, W.I., Hensen, E., van Oirschot, J.T. et Keil, G. (1998). Resistance to bovine respiratory syncytial virus (BRSV) induced in calves by a recombinant bovine herpesvirus-1 expressing the attachment glycoprotein of BRSV. J. Gen. Virol. 79: 1759-1767.

Taylor, G., Thomas, L.H., Furze, J.M., Cook, R.S., Wyld, S.G., Lerch, R., Hardy, R. et Wertz, G.W. (1997). Recombinant vaccinia viruses expressing the F, G and N, but not the M2, protein of bovine respiratory syncytial virus (BRSV) induce resistance to BRSV challenge in the calf and protect against the development of pneumonic lesions. J. Gen. Virol. 78: 3195-3206.

Tengelsen, L. A., Pederson, N.E., Shaver, P.R., Wathen, M.W. et Homa, F.L. (1993). Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J. Virol. 67: 3470-3480.

Tikoo, S.K., Campos, M. et Babiuk, L.A. (1995). Bovine herpesvirus 1 (BHV1-1): biology, pathogenesis and control. Adv. Virus Res. 45: 191-223.

Tikoo, S.K., Fitzpatrick, D.R., Babiuk, L.A. et Zamb, T.J. (1990). Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. 64: 5132-5142.

Umene, K. et Nishimoto, T. (1996). Replication of herpes simplex virus type 1 DNA is inhibited in a temperature-sensitive mutant of BHK-21 cells lacking RCC1 (regulator of chromosome condensation) and virus DNA remains linear. J. Gen. Virol. 77: 2261-2270.

Valicek, L. et Smith, B. (1976). Envelopment and the envelopes of infectious bovine rhinotracheitis virus in ultrathin sections. Arch. Virol. 51: 131-40.

Van Drunen Littel-van den Hurk, S. et Babiuk, L.A. (1986). Polypeptide specificity of the antibody response after primary and recurrent infection with bovine herpesvirus-1. J. Clin. Microbiol. 23: 274-282.

Van Drunen Littel-van den Hurk, S., Gifford, G.A. et Babiuk, L.A. (1990). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 11: 358-367.

Van Drunen Littel-van den Hurk, S., Tikoo, S.K., Liang, X. et Babiuk, L.A. (1993). Bovine herpesvirus-1 vaccines. Immunol. Cell Biol. 71: 405-420.

Van Engelenburg, F.A.C., Kaashoek, M.J., Rijsewijk, F.A.M., van den Burg, L., Moerman, A., Gielkens, A.L.J. et van Oirschot, J.T. (1994). A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. J. Gen. Virol. 75: 2311-2318.

Van Oirschot, J.T., Kaashoek, M.J. et Rijsewijk, F.A.M. (1996a). Advances in the development and evaluation of bovine herpesvirus 1 vaccines. Vet. Microb. 53: 43-54.

Van Oirschot, J.T., Kaashoek, M.J., Rijsewijk, F.A.M. et Stegeman, J.A. (1996b). The use of marker vaccines in eradication of herpesviruses. J. Biotech. 44: 75-81.

Vonk Noordegraaf, A., Buijtels, J.A., Dijkhuizen, A.A., Franken, P., Stegeman, J.A. et Verhoeff, J. (1998). An epidemiological and economic simulation model to evaluate the spread and control of infectious bovine rhinotracheitis in the Netherlands. Prev. Vet. Med. 36: 219-238.

Weir, H.M. et Stow, N.D. (1990). Two binding sites for the herpes simplex virus type 1 UL9 protein are required for efficient activity of the oriS replication origin. J. Gen. Virol. 71: 1379-1385.

Wellenberg, G.J., Verstraten, E.R.A.M., Mars, M.H. et Van Oirschot, J.T. (1998). Detection of bovine herpesvirus 1 glycoprotein E antibodies in individual milk samples by enzyme-linked immunosorbent assays. J. Clin. Microbiol. 36: 409-413.

Weller, S.K., Seghatoleslami, M.R., Shao, L., Rowse, D. et Carmichael, E.P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a *lacZ* insertion mutant. J. Gen. Virol. 71: 2941-2952.

Whetstone, C.A., Miller, J.M., Seal, B.S., Bello, L.J. et Lawrence, W.C. (1992). Latency and reactivation of a thymidine kinase-negative bovine herpesvirus 1 deletion mutant. Arch. Virol. 122: 207-214.

Whitbeck, J.C., Knapp, A.C., Enquist, L.W., Lawrence, W.C. et Bello, L.J. (1996). Synthesis, processing, and oligomerization of bovine herpesvirus 1 gE and gI membrane proteins. J. Virol. 70: 7878-7884.

Whitbeck, J.C., Lawrence, W.C. et Bello, L.J. (1994). Characterization of the bovine herpesvirus 1 homolog of the herpes simplex virus 1 UL24 open reading frame. Virology 200: 263-270.

Wirth, U.V., Gunkel, K., Engels, M. et Schwyzer, M. (1989). Spatial and temporal distribution of bovine herpesvirus 1 transcripts. J. Virol. 63: 4882-4889.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. et Felgner, P.L. (1990). Direct gene transfer into mouse muscle in vivo. Science 247: 1465-1468.

Wu, S.X., Zhu, X.P. et Letchworth, G.J. (1998). Bovine herpesvirus 1 glycoprotein M forms a disulfide-linked heterodimer with the UL49.5 protein. J. Virol. 72: 3029-3036.

Wyler, R., Engels, M. et Schwyzer, M. (1989). Infectious bovine rhinotracheitis/ vulvovaginitis (BHV1). Dans: Wittmann, G. (Ed) Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic Publishers, Boston/Dordrecht/London, pp 1-72.

Yu, D., Sheaffer, A.K., Tenney, D.J. et Weller, S.K. (1997). Characterization of ICP6-lacZ insertion mutants of the UL15 gene of herpes simplex virus type 1 reveals the translation of two proteins. J. Virol. 71: 2656-2665.

Yu, D. et Weller, S.K. (1998a). Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. J. Virol. 72: 7428-7439.

Yu, D. et Weller, S.K. (1998b). Genetic analysis of the UL 15 gene locus for the putative terminase of herpes simplex virus type 1. Virology 243: 32-44.

ANNEXES

ANNEXE 1

LISTE DES PUBLICATIONS ET DES COMMUNICATIONS

LISTE DES PUBLICATIONS

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

- Desloges, N. et Simard, C. (2001). Expression kinetics of the late UL12 gene encoding the bovine herpesvirus 1 alkaline nuclease. Arch. Virol. 146(10): 1871-1884. DOI: 10.1007/s007050170039

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

- Desloges, N. Boucher, H., et Simard, C. (2001). Transcriptional and translational expression kinetics of the UL25 homologue of bovine herpesvirus 1. Arch. Virol. 146(9): 1693-1704. DOI:10.1007/s007050170057

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

- Desloges, N. et Simard, C. (2001). Expression kinetics of the transcript and product ofthe UL28 homologue ofbovine herpesvirus 1.1. Virus Res. 80(1-2): 23-31. DOI:10.1016/S0168-1702(01)00338-0

- Desloges, N. et Simard, C. UL28 deficiency in bovine herpesvirus 1 does not abolish DNA packaging even though processing of newly synthesized DNA concatemers is abrogated. Soumis.

LISTE DES COMMUNICATIONS

- Desloges, N. et Simard, C. (2001). The bovine herpesvirus 1.1 UL28 gene is involved in cleavage and packaging of viral DNA. 26th International Herpesvirus Workshop, Regensburg, Allemagne.

- Desloges, N. et Simard, C. (2001). Role of the bovine herpesvirus 1 UL28 gene in cleavage and packaging of viral DNA. 2nd Veterinary Herpesvirus Satellite Workshop, Regensburg, Allemagne.

- Desloges, N. et Simard, C. (2000). Expression kinetics of the UL28 homologue of bovine herpesvirus 1. 25th International Herpesvirus Workshop, Portland, Oregon, USA.

- Desloges, N. et Simard, C. (1999). Transcriptional and translational expression kinetics of the UL25 homologue of bovine herpesvirus 1. 24th International Herpesvirus Workshop, Boston, USA.

- Desloges, N. et Simard, C. (1999). Transcriptional and translational expression kinetics of the UL25 homologue of bovine herpesvirus 1. 6th International Bovine Herpesvirus Workshop, Boston, USA.

- Desloges, N. et Simard, C. (1998). Étude des cinétiques de transcription et de traduction des gènes UL12, UL25 et UL28 du virus herpès bovin 1. Journées de recherche et Colloque en productions animales du Québec, Québec, Québec, Canada.

ANNEXE2

COPIE DES PUBLICATIONS