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Functional Homologies between Avian and Human Alphaherpesvirus VP22 Proteins in Cell-to-Cell Spreading as Revealed by a New *cis*-Complementation Assay▽†

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VP22, encoded by the *UL49* gene of Marek's disease virus (MDV), is indispensable for virus cell-to-cell spreading. We show herein that MDV *UL49* can be functionally replaced with avian and human viral orthologs. Replacement of MDV VP22 with that of avian gallid herpesvirus 3 or herpesvirus of turkey, whose residue identity with MDV is close to 60%, resulted in 73 and 131% changes in viral spreading, respectively. In contrast, VP22 replacement with human herpes simplex virus type 1 resulted in 14% plaque formation. Therefore, heterologous avian and human VP22 proteins share sufficient structural homology to support MDV cell-to-cell spreading, albeit with different efficiencies.

UL49 gene-encoded VP22 is specific to alphaherpesviruses. This 249- to 304-amino-acid protein is a major constituent of the virus tegument layer. *UL49* functional requirements seem to vary from one virus to another and depending on the host cell. In pseudorabies virus, herpes simplex virus type 1 (HSV-1), and bovine herpesvirus type 1 (BoHV-1), *UL49* appears to be not essential for viral replication in cell culture (2, 6–8, 11). In HSV-1 and BoHV-1, however, deletion of *UL49* impairs virus replication, especially in MDBK cells (7, 11). In HSV-1, the absence of VP22 is associated with (i) a decrease in the incorporation of several HSV-1 proteins into virions, (ii) a toxic effect probably due to the uncontrolled RNase activity encoded by *UL41*, and (iii) a decrease in extracellular particle accumulation (6, 7, 15). *UL49* has been shown to be absolutely necessary for the replication of Marek's disease virus (MDV) and varicella-zoster virus in cell culture (5, 16). Despite these differences between alphaherpesviruses, previous amino acid alignments of VP22 unveiled the presence of a conserved central domain suggestive of a conserved function (4, 12, 13). Herein, we tested whether other alphaherpesvirus *UL49* genes, either from the same *Mardivirus* genus or from a more phylogenetically distant human virus, could replace MDV's *UL49* gene by *cis* complementation in an MDV genomic background.

Genus	Species	N ... C	AA Identity (entire VP22)	Sequences used (access n°)
Mardivirus	MDV	1 — 96 [100 %] 173 — 249	100	YP_001033978
Mardivirus	GaHV3	1 — 94 [72 %] 171 — 241	59	NP_066881
Mardivirus	HVT	1 — 136 [64%] 213 — 283	56	AAG45787
Varicellovirus	VZV	1 — 158 [54%] 235 — 302	NA	NP_040132
Simplexvirus	HSV1	1 — 178 [41%] 255 — 301	NA	CAA32299

FIG. 1. Schematic representation of several avian and human VP22 proteins and their percent homologies with MDV VP22. The MDV VP22 polypeptide sequence was aligned pairwise with each ortholog by using Bestfit (GCG package; Accelerys). Grey boxes represent the conserved core of VP22. The percent homologies (amino acid [AA] identities with MDV VP22) were calculated for (i) the conserved core region (written in the boxes) and for (ii) the entire VP22 protein, except for varicella-zoster virus (VZV) and HSV-1, which exhibited low levels of similarity outside the conserved area (see the supplemental material). NA, not appropriate.

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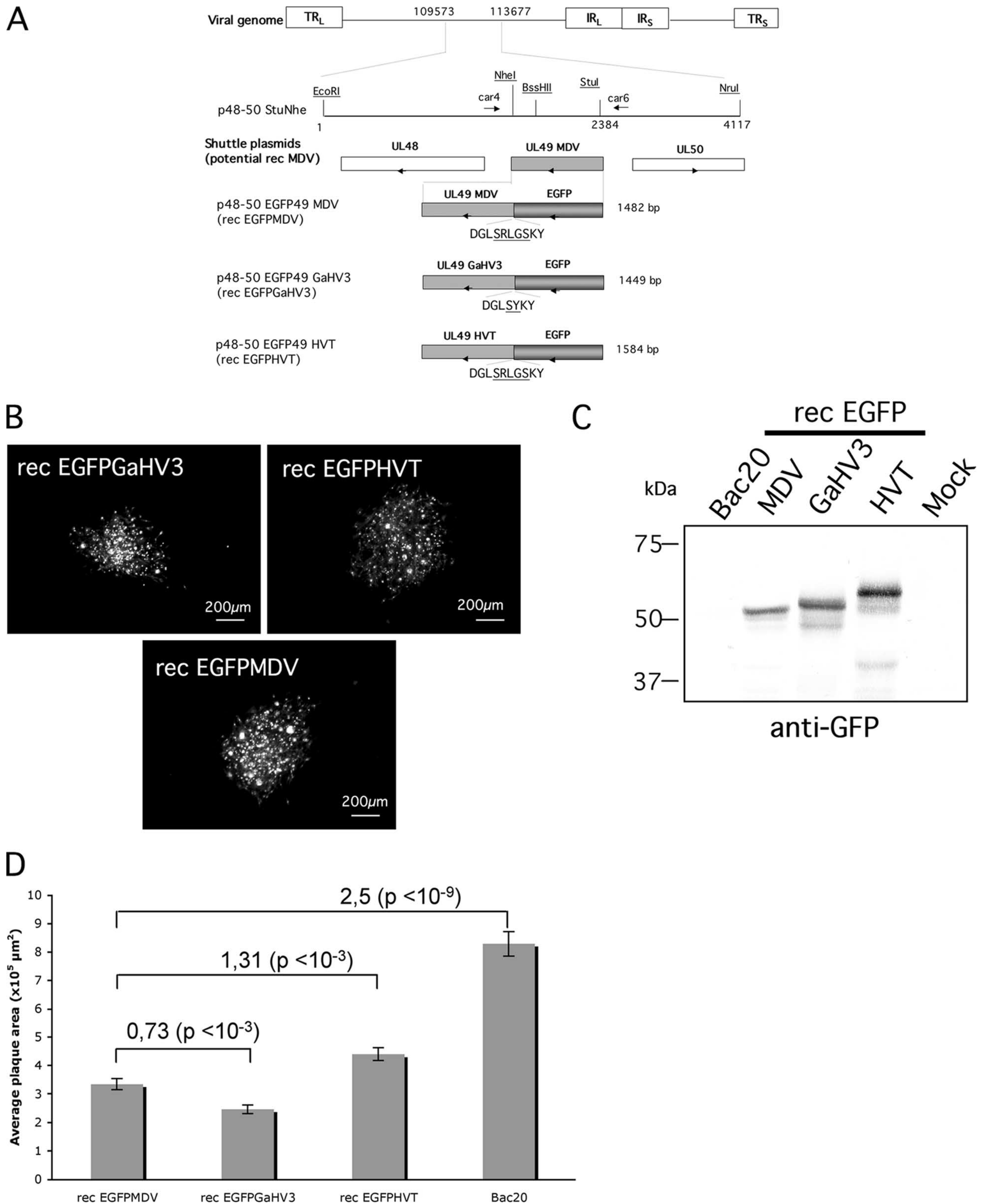

FIG. 2. Construction and cell-to-cell spreading of the recombinant MDVs containing EGFP-tagged *UL49* genes derived from three *Mardivirus* species. (A) Schematic representation of the three shuttle plasmids used for homologous recombination by cotransfection in CESC with the Bac20delUL49 DNA bacmid. (B) Picture of a plaque (one for each virus) with EGFP fluorescence. (C) Analysis of EGFPVP22 protein expression by immunoblotting revealed with a rabbit anti-GFP antibody. Mock, noninfected cells. (D) Plaques size comparison after plaque staining. Fifty plaques were analyzed with the cell observer system (Zeiss, Göttingen, Germany) on the red channel, and plaque size was measured with the Axiovision software. Statistical analysis (analysis of variance) showed a significant difference among the three viruses ($P < 10^{-3}$). The ratio between the average plaque area for each virus and that of recEGFPMDV is given, as well as the P values (Student's t test).

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TABLE 1. Characteristics of the MDV genome *cis* complemented with several avian and human UL49 open reading frames

ORF[a] inserted at MDV UL49 position	Homologous recombination in CESC with Bac20ΔUL49		Homologous recombination in *E. coli* with Bac20	
	Progeny	% Cell-to-cell spreading efficiency[b]	Progeny	% Cell-to-cell spreading efficiency[c]
None/Kan[r]	No	0	No	0
MDV UL49	Yes	250	Yes	100
MDV EGFPUL49	Yes	100	Yes (NS[d])	49 (NS)
GaHV-3 EGFPUL49	Yes	73	ND[e]	ND
HVT EGFPUL49	Yes	131	ND	ND
HSV-1 UL49	ND	ND	Yes	14
HSV-1 EGFPUL49	ND	ND	No	0

[a] ORF, open reading frame.
[b] Versus recEGFPMDV.
[c] Versus Bac20.
[d] NS, not shown.
[e] ND, not done.

Efficient *cis* complementation of MDV cell-to-cell spreading with *UL49* genes from other mardiviruses. The homologies (percent amino acid identities) between MDV VP22 and its orthologs from the two other *Mardivirus* species, herpesvirus of turkey (HVT) and gallid herpesvirus 3 (GaHV-3), are 56 and 59%, respectively (Bestfit software; Fig. 1). This percent homology was even over 60% in the core region. To assess the ability of the *UL49* genes from HVT and GaHV-3 to *cis* complement the MDV genome lacking *UL49*, we generated recombinant viruses by homologous recombination in chicken embryonic skin cells (CESC). For this purpose, we cotransfected the nonreplicative Bac20delUL49 clone (MDV lacking *UL49*) with the p48-50 shuttle plasmid containing the *UL49* ortholog in its MDV genetic environment as previously described (3). As no antibody was available to detect either HVT or GaHV-3 VP22, both the HVT and GaHV-3 *UL49* orthologs were tagged with enhanced green fluorescent protein (EGFP) at the 3′ end of the construct (Fig. 2A). After two blind passages of the cotransfected cells on CESC, replicative viruses designated recEGFPHVT and recEGFPGaHV-3 were obtained with both *UL49* orthologs (Table 1). Expression of the HVT or the GaHV-3 EGFPVP22 protein was monitored on infected cells by the green fluorescence (Fig. 2B) and by immunoblotting with a rabbit anti-EGFP antibody as previously described (3) (Fig. 2C). The apparent molecular masses, 53 kDa for GaHV-3 and 57 kDa for HVT, closely matched the predicted sizes. Moreover, sequencing of the PCR products obtained with primers car4 and car6 (Fig. 2A shows the position) on extracted viral DNA showed correct recombinations and sequences (not shown). To estimate the functional efficiencies of the HVT and GaHV-3 VP22 proteins, the cell-to-cell spreading of recombinant viruses recEGFPHVT and recEGFPGaHV-3 was compared to that of the previously described virus MDVEGFPVP22 (3), renamed herein recEGFPMDV. To this aim, viral plaques obtained after infection of CESC for 4 days were fixed and stained for MDV antigens VP5, ICP4, and gB as already described (1). Plaque size was determined by measuring 50 plaques after fluorescent red staining and image analysis with the axiovision software (Zeiss, Göttingen, Germany). There were significant differences in plaque size among the three viruses ($P < 10^{-3}$ by analysis of variance after square root transformation) (Fig. 2D), showing a difference in the efficacy of cell-to-cell spreading. Interestingly, while GaHV-3 VP22 led to a 1.4-fold decrease in plaque size, HVT VP22 increased plaque size by 1.4-fold, as measured in two independent experiments.

Substantial *cis* complementation of MDV cell-to-cell spreading with the divergent human HSV-1 *UL49* gene. We next assessed whether avian MDV VP22 could be replaced with VP22 from a distant genus. We chose herein to test HSV-1 VP22 because it is very divergent from MDV VP22 (only 41% homology in the core region) and has been described as not mandatory for HSV-1 cell-to-cell spreading. We constructed two recombinant MDV strains by homologous recombination with the HSV-1 *UL49* gene, one with and one without a 5′ EGFP tag sequence (EGFPHSV and HSV, respectively). The procedure we used was two-step red recombination in *Escherichia coli* as described by Tischer et al. (17). For the first recombination step, we constructed the p48-50 kana IsceI "en passant" plasmids as schematically represented in Fig. 3 (for details, see the supplemental material). The first recombination was obtained after the transformation of EL250 bacteria containing the Bac20 bacmid with either the 2,911-bp or the 3,634-bp XmnI/HpaI restriction fragment from the HSV or EGFPHSV en passant plasmid, respectively. After the second recombination step, the mutant bacmids were verified by sequencing between the XmnI/HpaI restriction sites. The bacmids recHSV and recEGFPHSV were next transfected into CESC by the calcium phosphate method. For the recHSV bacmid, a viral progeny was obtained after one passage, demonstrating successful *cis* complementation. Expression of the heterologous VP22 protein was verified by fluorescence on infected cells and by immunoblotting with AGVO31, an anti-HSV-1 polyclonal antibody (9) (Fig. 3B). The cell-to-cell spreading efficacy of the recHSV virus, evaluated by measuring plaque size in three independent experiments, was reduced by 6.25- to 8.25-fold compared to that of parental Bac20. However, even though the spreading of this virus was limited, it was still able to propagate to neighboring cells, leading to small plaques, in contrast to parental Bac20 lacking *UL49* (Bac20delUL49) leading only to single cells expressing late viral antigens (5). For the recHSV strain, the sequence integrity of MDV *UL41* was verified to ensure that compensation did not occur elsewhere, as was previously reported for replicative *UL49*-null HSV-1 (15).

No replicative virus could be obtained after the transfection of the recEGFPHSV bacmid despite several passages. However, an EGFP signal was observed in single cells after transfection, indicating that the fusion protein is well expressed but the signal is lost after a few passages. Moreover, this bacmid was readily rescued after cotransfection into CESC with the p48-50 StuNhe plasmid containing MDV *UL49*. The rescued virus produced plaques similar in size to those of Bac20 (not shown), showing that the recEGFPHSV replication defect was due not to an unexpected mutation elsewhere in the genome but to the HSV EGFPUL49 gene insertion itself. Therefore, fusing EGFP to the 3′ portion of HSV *UL49* completely abrogated HSV-1 VP22 residual biological activity in this context.

This study aimed to evaluate whether heterologous *UL49* could *cis* complement an MDV *UL49*-null phenotype. Our

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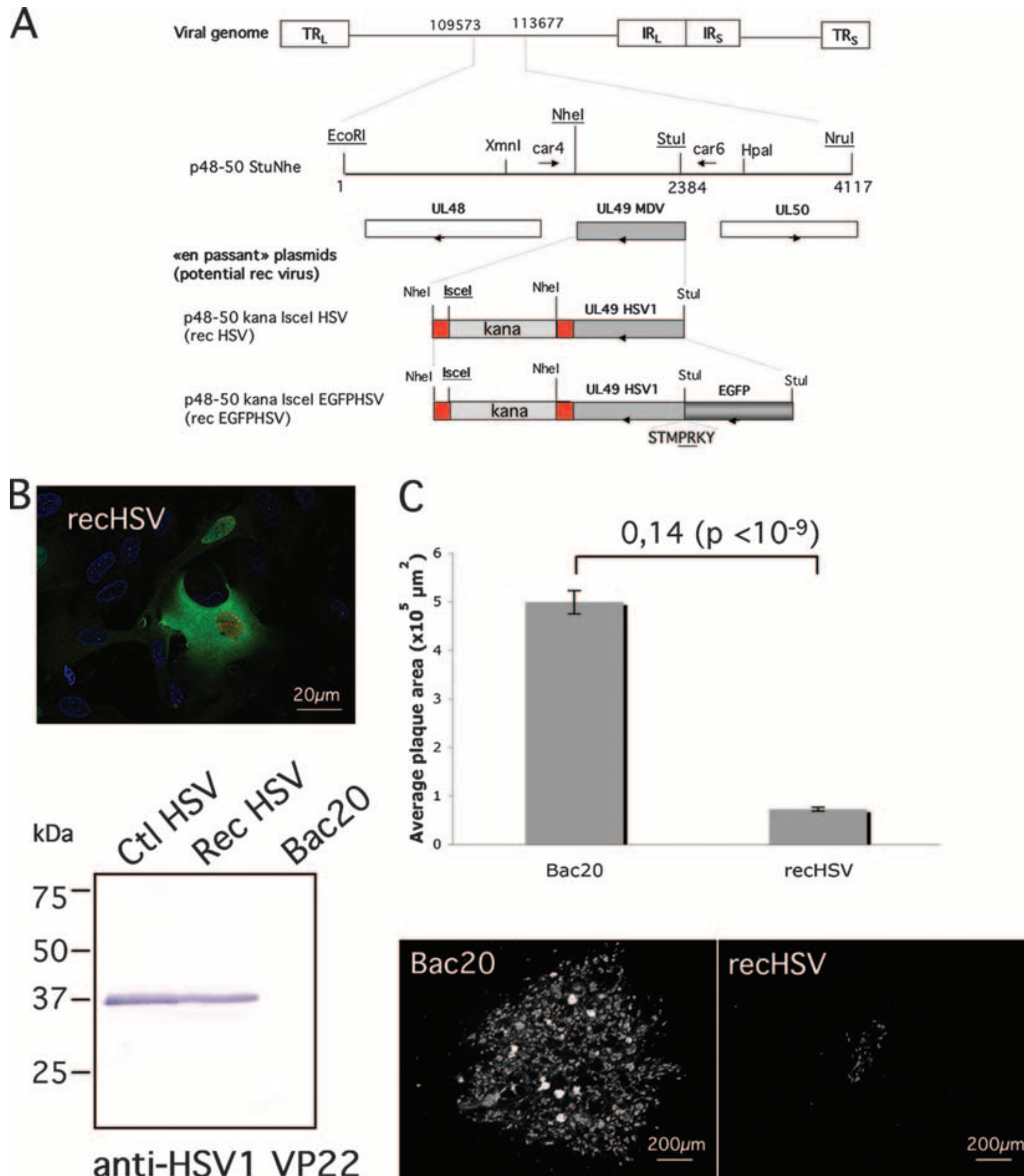

FIG. 3. Construction and cell-to-cell spreading of recombinant MDV containing the HSV-1 *UL49* gene. (A) Schematic representation of the two en passant plasmids constructed and used for the first step of recombination in *E. coli*. (B) Expression of HSV-1 VP22 in recHSV-infected CESC analyzed by fluorescence and immunoblotting. CTLHSV corresponds to CESC transfected with an HSV-1 VP22 expression vector. (C) Plaque size comparisons and plaque pictures viewed on the red channel.

study brings the first evidence for a structural/functional conservation among four VP22 proteins from different avian and human genuses. Although VP22 functional homologies within highly homologous mardiviruses were not surprising, the increased plaque formation observed after the introduction of HVT VP22 points at the potential emergence of more pathogenic MDV strains. These findings are reminiscent of lethal mutations affecting the gB gene in pseudorabies virus that were fully *trans* complemented with BoHV-1 gB, which shares 63% amino acid identity with pseudorabies gB (10). In the present study, the functional complementation of the MDV *UL49*-null phenotype provided by HSV VP22 was more striking because the two proteins are very divergent. This suggests that protein-protein interactions required for MDV cell-to-cell spreading and involving VP22 may then be partially preserved among VP22 orthologs. A recent study supporting this hypothesis has shown functional proteins interactions between *UL34*/*UL31* heterologous pairs within the *Betaherpesvirus* subfamily (14). Lastly, the present study confirms that MDV can be a useful and valuable model for studying various aspects of VP22 function, as well as identifying alphaherpesvirus proteins involved in cell-to-cell spreading.

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HSV-1 VP22). We also thank P. Castelnau and M. Sitbon for their comments on the manuscript.

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SUPPLEMENTAL DATA.

Table 1. Primers used in this study.

Primers	Sequence (5'-3')	Description
StuIEGFP5	aAGGCCTatggtgagcaagggcgaggagc	For amplification in 5' of fusion **EGFP** gene and cloning in StuI site (in upper case)
3REGFPStuI	ccAGGCCTcttgtacagctcgtccatgcc	For amplification in 3' of **EGFP** sequence and cloning in StuI site (upper case)
HPRS49_5FScaI	ggAGTACTcattgggagattcggatagacg	For amplification in 5' of **HPRS24 GaHV3 UL49** gene with underlined mutated ATG and cloning in ScaI site (upper case) of pEGFP-C3
HPRS49_3R	ggttcgaactattcactctcgctactatag	For amplification in 3' of **HPRS24 GaHV3 UL49** gene and cloning in pEGFP-C3
HPRS49_3RNheI	ggGCTAGCctattcactctcgctactatag	For amplification in 3' of **HPRS24 GaHV3 UL49** gene and cloning in NheI site (upper case) of p48-50 StuNhe
HVT49_5FBglII	ggAGATCTttgggagacagcgaagggcg	For amplification in 5' of **HVT UL49** gene with underlined mutated ATG and cloning in BglII site (upper case) of pEGFP-C1
HVT49_3RXbaI	ggtctagactactcactatcgctggttcgt	For amplification in 3' of **HVT UL49** gene and cloning in pEGFP-C1
HVT49_3RNheI	ggGCTAGCctactcactatcgctggtt	For amplification in 3' of **HVT UL49** gene and cloning in NheI site (upper case) of p48-50 StuNhe
5FUL49HSV1StuI	aaAGGCCTatgacctctcgccgctccgt	For amplification in 5' of **HSV-1 UL49** gene and cloning in StuI site (upper case) of p48-50 StuNhe
3RUL49HSV1NheI	ggGCTAGCtcactcgacgggccgtctgg	For amplification in 3' of **HSV-1 UL49** gene and cloning in NheI site (upper case) of p48-50 StuNhe
NheHSV5Fkana	cg**aggcct**gataactaagatataatattaaacagtaagatatgaacaagtgcTAGGGATAACAGGGTAATCGATTT	For "en passant mutagenesis and construction of the p48-50 UL49 kana IsceI plasmids. Sequence of the last 44bp in 3' of HSV1 UL49 is underlined (corresponding to the red boxes in FIG. 3) , NheI sites used for the construction of "en passant" plasmids are in bold, complementary sequences to I-SceI site in NheHSV5Fkan and to kanamycin gene are in upper case
Nhe3Rkana	aat**aggcct**CATATGCTAGCCAGTGTTACAACCAATTAACC	
car4	ggatgtctataaaagacgac	Previously described primers (3). For PCR amplification from pUL48-50 plasmids or for sequencing. Positions indicated in figures 1 and 2
car6	tgtttaaagaggagtggtaa	
seqUL41_3R	CTCTGGAAGCGTGTCAAACG	For amplification of **MDV UL41** gene and sequencing
seqUL41_5F	ACAGAACTGTAGTACTTGTG	
Seq_in_UL41_R	CCGTGTAGTTGGAAACATC	For sequencing **MDV UL41** gene
Seq_in_UL41_F	GGCGACTACATGCACGATTG	

Figure 1

```
1                                          50
     MDV        ~~~~~~~~~~ ~~~~~MG SE R.. KSER   . L..GY ..  AYDDVSIPA
     GAHV3      ~~~~~~~~~~ ~~~~~MG SD R.. KSSR   . TMRTS DN  AHIS.S RA
     HVT        ~~~~~~~~~~ ~~~~~MG SE G.. KYER   P VYHSHQDG TGGTDGT ..
     VZV        ~~~~~~~~~~ ~~~MASS GD RLC SNAV   KTT.... SY  G..QYR .A
     HSV1       MTSRRSVKSG PREVPRDEYE DLYYTPSSGM A .PDSP DT  RRGALQ RS
     CONSENSUS  ---------- -------D-- ---R----RR -S-----P-- S------T--

                51                                                100
     MDV         . PSTRTQR NLNQ DL... .......... .......... .... KH...
     GAHV3       . DSS...K NESP RI... .......... .......... .... .....
     HVT         . KSTRSLQ SPPR DYLHA SRVTSNRHAR SPPRAELPRS TRRQ AHHAE
     VZV         . SVVVGPP DDSD SLGYI TTVGADSPSP VYADLYFEHK NTTPRVHQPN
     HSV1        Q GEVRFVQ YDES YALYG GSSSEDDEHP EVPRTRRPVS GAVL GPGPA
     CONSENSUS  R-R------- ----D----- ---------- ---------- ----S-----

                101                                               150
     MDV        ........GP FTD......H .PTQKHKSAK .AVSE..... DVSS.T.T.R
     GAHV3      .........P .PS......H .SLQRRRSVK .IERK..... DSSSET.Q.R
     HVT        SSPPEERPGP .SD......H RSLQRRKSVK ....E..... VEPANT.S.K
     VZV        DSSGSEDDFE DIDEVVAAFR EARLRHELVE DAVYENPLSV EKPSRSFT.K
     HSV1       RAPPPPAGSG GAGRTPTTAP RAPRTQRVAS KAPAAPAAET TRGRKSAQPE
     CONSENSUS  ---------- ---------- ---------- ---------- ----------

                151                                               200
     MDV        G.GFTNK... ...P  K  V   VQSN . A      SSAS  T RSN VA
     GAHV3      GESLSSK... ...V  K  A   IEKG . A    T ASAT  T RSN LV
     HVT        SSSIPLG... ...Q  R  V   VQKN . M   S  TSRT  H KSN VA
     VZV        NAAVKPK.LE DSPK  P  A G IASGRPIS      KTAT  S CGP PS
     HSV1       SAALPDAPAS TAPT SKTPA QGLA..RKLH      PNPDA P TPRVAG
     CONSENSUS  ---------- ----RA-PG- RA----K-F- FSTAP----S -W---T--FN

               201                                               250
     MDV        Q M  G  AT   QY  YQG  LA  RQDP     E  DAF S R    K  IQ
     GAHV3      E     G  AA   QY  YRG  LS  RRNA     A  EEF A R    K  IQ
     HVT        QH    C  AA   RY  FRG  LA  NKEP    DEQ EDF V R    K  VR
     VZV        K     E  RR   AMQ QKA  EAA NSNP   N A  DRL T G    R  VH
     HSV1       K     A  GR L AM  RMA  VQ  DMSR    DED NEL G ITT R  VC
     CONSENSUS  -RVFC-AV-- VA--HA---A --LW----PR TN-EL---L- -AVI-IT--E

                251                                               300
     MDV         P  MGE .  TCARKLLE S G SQGNENVK  KSE T.TKS ERTR G EIE
     GAHV3       A  LDE .  ACTRKLSE S G SPDMGNPK  ....... S QYGK D D.E
     HVT         PY LEE .  SCTQRFME T G GSADNKPK  ... S.G S ERDVES EGS
     VZV         L  IQA .. .......N A D GGG....A  VSK GHN K TGDLQG MG.
     HSV1        K  LQR N  LVNPDVVQDV DAATATRG.R  AAS PTE P RAPA SAS.R
     CONSENSUS  G-NL---A-E --------E- -L-------- S---R---R- ----R-G---

                301                                       342
     MDV        IKS .DPGSH RTHNP. TPA .TSRRHHSSA RGYRS DSE~ ~~
     GAHV3      .ST VDKRDR RSKTPG APT .TSRRHYSSS RGNYS ESE~ ~~
     HVT        FNS....GAR RPIAIA L.V .SSAQSFADS PGERT DSE~ ~~
     VZV        .NE MYAQVR KPKS.. TDT QTTGRITNRS RARSA RTDT RK
     HSV1       PRR VE~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~
     CONSENSUS  ---P------ ------R--- ---------- -----S---- --
```

62 **Figure 1 legend:** Polypeptide sequences of MDV VP22 and four alphaherpesviruses
63 orthologs were aligned, using PileUp followed by Pretty in the GCG package
64 (Accelerys, USA). Consensus residues are indicated when at least four out of five
65 sequences have the same residue at that position. Only differences to the consensus
66 are written. The grey box represents the conserved central domain.
67
68

68 **MATERIALS AND METHODS SUPPLEMENT**

69

70 **Primers.** The primers used in this study are listed in Table 1 of this section.

71

72 **Generation of recombinant MDV viruses expressing heterologous VP22 fused**
73 **or not to EGFP in N-term.** All recombinant MDV viruses were derived from the
74 genome of the attenuated strain 584Ap80C, cloned as a bacterial artificial
75 chromosome (Bac20).

76 ***1. By homologous recombination in CESC.*** To investigate the functional
77 homologies between the mardiviruses VP22, we generated two recombinant viruses
78 with the UL49 derived respectively from GaHV3 and HVT fused in 3' of the EGFP
79 sequence.

80 *1.1. Construction of the EGFPUL49 GaHV3 and HVT genes*. The GaHV3 UL49 gene
81 was amplified by PCR from HPRS24 viral DNA (provided by Dr Venugopal Nair) with
82 primers HPRS49_5FscaI and HPRS49_3R, resulting in a 744 bp-fragment bearing a
83 5' ScaI site and a TTG codon in place of the original ATG from GaHV3 UL49. In the
84 same way, the HVT UL49 gene was amplified by PCR from vaccinal HVT viral DNA
85 with primers HVT49_5FBglII and HVT49_3RXbaI, resulting in a 868 bp-fragment with
86 a 5' BglII site and a ATG to TTG codon substitution. These PCR fragments were
87 cloned into pGEM-Te (Promega, Madison, WI), then subcloned into pEGFP-C3 and
88 pEGFP-C1, respectively by using the ScaI and PstI or the BglII and SalI sites,
89 resulting in pEGFPUL49 GaHV3 and pEGFPUL49 HVT plasmids. In order to

90 introduce a unique StuI site at the 5' end and a unique NheI at the 3' end of the two
91 EGFPUL49 genes, each EGFPUL49 fusion gene was amplified by PCR from its
92 respective pEGFPUL49 plasmid by using StuIEGFP5 and HPRS49_3RNheI for
93 GaHV3 or HVT49_3RNheI for HVT and cloned into pGEM-Te, resulting in pGEMTe-
94 EGFPGaHV3 and pGEMTe-EGFPHVT. The EGFPUL49 GaHV3 gene encoded a
95 482-aa protein with 239 aa corresponding to EGFP, 2 aa corresponding to a spacer,
96 and 241 aa corresponding to VP22. The EGFPUL49 HVT gene encoded a 527-aa
97 protein with 239 aa corresponding to EGFP, 5 aa corresponding to a spacer, and 283
98 aa corresponding to VP22.
99 *1.2. Construction of shuttle plasmids p48-50 EGFPUL49*. The p48-50 Stu plasmid
100 was described earlier (3). A unique NheI site was introduced immediately upstream
101 of the UL49 stop codon resulting in the p48-50 StuNhe plasmid. The UL49 gene was
102 then replaced with either the EGFPUL49 GaHV3 or EGFPUL49 HVT fusion genes.
103 To this end, the StuI-NheI fragments from the pGEMTe-EGFPGaHV3 and pGEMTe-
104 EGFPHVT (1457bp or 1592bp respectively), were ligated to the StuI-NheI 6364-bp
105 fragment of the p48-50 StuNhe, yielding the shuttle plasmids, p48-50 EGFP49GaHV3
106 and p48-50 EGFP49HVT, respectively. The final construct was verified by DNA
107 sequencing (MWG Biotech Sequencing service, Ebersberg, Germany).
108 *1.3. Generation of the recombinant MDV expressing the EGFPUL49 mardivirus*
109 *orthologs.* The procedure was described earlier (3). Briefly, 2μg of the appropriate
110 shuttle plasmid and 3 μg of Bac20ΔUL49 BACmid DNA were co-transfected into
111 CESC. Recombinant viruses were detected after one passage. The recEGFPGaHV3

112 and the recEGFPHVT viruses used in this study never exceeded 5 and 4 passages in
113 culture, respectively.
114 ***2. By homologous recombination in E. coli, EL250 strain.*** To investigate the
115 functional homologies between the MDV VP22 and the orthologous HSV-1 protein,
116 two recombinant bacmids were constructed with "en passant" mutagenesis, as
117 previously described (17).
118 *2.1. Construction of the UL49 HSV-1 intermediate plasmids*. The HSV1 UL49 gene
119 was amplified by PCR from pGE109 (provided by Dr Gillian Elliott) with primers
120 5FUL49HSV1stu and 3RUL49HSV1NheI, resulting in a 922bp-fragment which
121 incorporated StuI and NheI sites at the 5' and 3' ends, respectively, of UL49. This
122 PCR fragment was cloned into pGEM-Te generating the pGEM-Stu49HSVNhe. Then
123 the Stu-NheI fragments, 914bp from pGEM-Stu49HSVNhe and 6364bp from p48-50
124 StuNhe were ligated resulting in p48-50 HSV.
125 The EGFP sequence was PRC-amplified from plasmid pEGFP-C1 with primers
126 StuIEGFP5 and 3REGFPStuI and cloned in pGEM-Te generating pGEM-stuEGFPstu
127 plasmid. Then the 723bp StuI-StuI EGFP fragment from pGEM-stuEGFPstu was
128 ligated with the p48-50 HSV opened with StuI resulting in p48-50 EGFPHSV. The
129 EGFPUL49 HSV gene encoded a 542 aa protein with 239 aa corresponding to
130 EGFP, 2 aa corresponding to a spacer and 301 aa corresponding to VP22.
131 *2.2. Construction of the two "en passant" plasmids p48-50 kana IsceI*. The "IsceI
132 *aphAI*" cassette was amplified by PCR from pEPkan-S with NheHSV5Fkan and
133 Nhe3Rkana primers. The 1058bp- PCR fragment was cloned into pGEM-Te, resulting

134 in pGEM-NheKanaNhe. The 1039bp-Nhe I fragment from this plasmid was next
135 introduced into p48-50 HSV or into p48-50 EGFPHSV, both opened with NheI
136 resulting in "p48-50 kana IsceI HSV" or "p48-50 kana IsceI EGFPHSV", respectively.
137 *2.3. Construction of two bacmids with ''en passant'' mutagenesis.* For each first
138 recombination, we transformed electro-competent EL250 bacteria containing the
139 bac20 bacmid with one digestion fragment of each "en passant" plasmid. The
140 digestion fragments were either the 2911bp-XmnI/HpaI from p48-50 kana ISceI HSV
141 or the 3634bp-XmnI/HpaI from p48-50 kana IsceI EGFPHSV. After the second
142 recombination, the C^RK^S clones were selected. The UL49 region of the two resulting
143 recombinant bacmids, named recEGFPHSV and recHSV, were sequenced in its
144 entirety on both strands (MWG-biotech) between XmnI/HpaI sites.
145 *2.4. Generation of the recombinant MDV expressing the HSV-1 UL49 gene*. Each
146 purified bacmid (3μg) was transfected into 50% confluent CESC grown in 35-mm
147 dishes by the calcium phosphate precipitation method. The recHSV virus was
148 detected after one passage. The virus used in this study never exceeded 4 passages
149 in culture.