Marek’s disease virus (MDV) homologues of herpes simplex virus type 1 UL49 (VP22) and UL48 (VP16) genes: high-level expression and characterization of MDV-1 VP22 and VP16

Fabien Dorange,1 Slimane El Mehdaoui,2 Chantal Pichon,3 Pierre Coursaget2 and Jean-François Vautherot1

1 Laboratoire de Virologie Moléculaire, Station de Pathologie Aviaire et de Parasitologie, Centre INRA de Tours, 37380 Nouzilly, France
2 Laboratoire de Virologie Moléculaire, INSERM EMIV-00-10, Faculté des Sciences Pharmaceutiques ‘Philippe Maupas’, 37200 Tours, France
3 Centre de Biophysique Moléculaire, Glycobiologie, CNRS UPR4301 et Université d’Orléans, rue Charles-Sadron, F-45071 Orleans Cedex 02, France

Introduction

Marek’s disease virus serotype 1 (MDV-1), the causative agent of T cell lymphoma in chickens, is classified as an alphaherpesvirus on the basis of its genome structure (Cebrian et al., 1982) and sequence homology with alphaherpesviruses (Buckmaster et al., 1988). Of the four distinct morphological components described for herpesvirus virions, i.e. core, capsid, tegument and envelope, the tegument is certainly the least well understood. In herpes simplex virus type 1 (HSV-1), the tegument is a macromolecular structure composed of at least 15 polypeptides, four of which are major structural components of the virion (VP1, VP13/14, VP16 and VP22, encoded by UL36, UL47, UL48 and UL49, respectively) (Spear & Roizman, 1972; Honess & Roizman, 1974). Although the tegument is described as an ‘amorphous region’, tegument proteins appear to form an organized layer outside the capsid, interacting with capsid proteins (Sodeik et al., 1997; Trus et al., 1999). These proteins are exposed to the intracellular environment early in virus infection and before the virus has reached the nucleus and initiated synthesis of the immediate-early (IE) proteins. Therefore, tegument proteins have been assigned a variety of functions, such as the shutting-off of host cell protein synthesis mediated by the tegument Vhs protein (Fenwick et al., 1978; Overton et al., 1994; Smibert et al., 1994) or the initiation of virus replication by VP16, a tegument phosphoprotein, which is a powerful transactivator of the five IE genes of HSV-1 (Campbell et al., 1984).

Information on the protein composition of MDV tegument and on the role of these proteins is not yet available. Sequence analyses have revealed striking similarities between MDV and HSV-1 regarding the genomic organization of the UL46 to UL49 genes. In MDV-1 and MDV-2, four collinear sequences were found to encode homologues of the HSV-1 UL46 to UL49 gene products (Izumiya et al., 1998; Yanagida et al., 1998). Genes UL49 and UL48 of Marek’s disease virus 1 (MDV-1) strain RB1B, encoding the respective homologues of herpes simplex virus type 1 (HSV-1) genes VP22 and VP16, were cloned into a baculovirus vector. Seven anti-VP22 MAbs and one anti-VP16 MAb were generated and used to identify the tegument proteins in cells infected lytically with MDV-1. The two genes are known to be transcribed as a single bicistronic transcript, and the detection of only one of the two proteins (VP22) in MSB-1 lymphoma and in chicken embryo skin cells infected with MDV-1 prompted the study of the transcription/translation of the UL49–48 sequence in an in vivo and in vitro expression system. VP16 was expressed in vitro at detectable levels, whereas it could only be detected at a lower level in a more controlled environment. It was demonstrated that VP22 is phosphorylated in insect cells and possesses the remarkable property of being imported into all cells in a monolayer. VP22 localized rapidly and efficiently to nuclei, like its HSV-1 counterpart. The DNA-binding property of VP22 is also reported and a part of the region responsible for this activity was identified between aa 16 and 37 in the N-terminal region of the protein.

Author for correspondence: Jean-François Vautherot.
Fax +33 2 47 42 77 74. e-mail jfvauthe@tours.inra.fr

0001-6946 © 2000 SGM
1993). Transcriptional studies of this region revealed the presence of mRNAs corresponding to UL46, UL47–46 and UL49–48, but no mRNA corresponding to UL48 alone was detected. In MDV, the bicistronic mRNA UL49–48 was found to direct the synthesis of VP22 and VP16 in vitro (Koptidesova et al., 1995).

We aimed to characterize MDV RB1B VP16 and VP22 because these proteins appeared to play a key role in the alphaherpesvirus life-cycle. In addition, differences in the transcriptional organization between MDV and HSV-1 have been reported. Both proteins were expressed in the baculovirus–insect cell system, which is known to produce recombinant proteins that are processed properly and targeted to their appropriate cellular locations. MAbS were raised against recombinant MDV-1 VP22 and VP16. VP22 was identified during virus infection or reactivation, whereas VP16 was not. This prompted us to study the transcription/translation of the UL49–48 sequence in different cell systems. VP22 was synthesized at high levels in all cell systems, whereas only low levels of VP16 could be detected in a limited number of expression systems.

We show that VP22 is a phosphorylated protein with a DNA-binding activity located at the N terminus of the protein. In addition, MDV-1 VP22 displays cell trafficking properties similar to those reported for its HSV-1 counterpart.

Methods

**Viruses and cells.** *Spodoptera frugiperda* (Sf9) and High Five cells (Invitrogen) were cultured at 27 °C in Grace’s insect medium (Gibco-BRL) supplemented with 10% foetal calf serum (FCS) (BIO MEDIA-France). Cells were infected with recombinant baculoviruses or the wild-type AcRP6-SC baculovirus (O’Reilly et al., 1992), at an m.o.i. of 1 p.f.u. per cell, for 72 h at 27 °C.

Chicken embryo skin cells (CESC) were obtained essentially as described by Silim et al. (1981) except that collagenase (0–2% in William’s E medium (WE)) was used instead of trypsin and that cell monolayers were established in gelatin-coated Petri dishes (0–2% gelatin in PBS for 15 min at 4 °C). The medium used for growing primary skin cells was WE supplemented with 2% FCS and 3% chicken serum. After infection, cells were maintained in WE containing 1% FCS and 1% chicken serum. COS-7 cells were cultured in DMEM (Gibco-BRL) supplemented with 10% FCS. RK13 cells were cultured in WE containing 10% FCS. LMH cells were cultured as described by Kawaguchi et al. (1987). The MSB-1 cell line was cultured in RPMI 1640 medium with 10% FCS. Cells were treated with 3 µM 5-aza- cytidine for 72 h to reactivate virus (Fynan et al., 1993).

**Infection of cells with MDV RB1B.** The first passage was performed by plating blood lymphocytes (PBLs), collected from an experimentally infected animal, on CESC. PBLs (2 × 10⁶) were plated on 1-day-old monolayers seeded in a 60 mm Petri dish. Cells were rinsed 1 h after the addition of PBLs and incubated at 41 °C for 1 week. Subsequent passages were performed by standard procedures (Sharma et al., 1969).

**Cell transfections.** Cell monolayers (COS-7, RK13 and CESC) were transfected with pcDNA3 vector containing the gene of interest by using lipofectAMINE (Gibco-BRL). CESC were infected by the MVA T7 strain of vaccinia virus (vTV7) at an m.o.i. of 3–5 (Sutter et al., 1995) before transfection with pET or pcDNA3 vectors.

**PCR and nucleotide sequence verification.** Fragments containing MDV-1 UL49, UL48 and UL49–48 were amplified by PCR. The template was MDV-1 strain RB1B genomic DNA extracted from a splenic lymphoma (Nucleobond AXG100; Macherey Nagel). Primers selected on the basis of the published sequence of UL49 and UL48 of the MDV-1 GA strain (Yanagida et al., 1993) were as follows: VP22F (GCGGCCCTCCATGGGATCCTGAAGGGC; SphI site underlined, Ncol site in bold), VP22R (GGAGCTCAGTGGTAATGGCTACACCTGCTAGC; SphI site underlined, PstI site in bold), VP16F (CCCCGGATCCATGGGAGCCAAATATGAGTTTTG; SmaI site underlined, BanHI site underlined, Ncol site in bold), VP16R (GGATCCATGGGATATCTCCTTGCA; BanHI site underlined, Ncol site in bold) and VP22n2 (GGATCCATGGAATTTAAGGGAGC; BanHI site underlined).

The UL49–48 sequence was obtained by using primers VP22F and VP16R. UL49N1 and UL49N2 encoding truncated N-terminal peptides VP22N1 (aa 1–15 deleted) or VP22N2 (aa 1–37 deleted), respectively, were obtained by using primers VP22R and VP22n1 or VP22n2 and pGEM-T UL49 as template.

PCR fragments were purified from agarose gels (Nucleospin Extract 2 in 1; Macherey Nagel) and cloned in the T-tailed vector pGEM-T (Promega). Sequences of inserts were determined by using universal primers — 21M13 and revM13 on a Perkin Elmer automatic sequencer.

**Construction of UL49, UL49N1, UL49N2, UL48 and UL49–48 sequences under the control of the T7, polyhedrin and CMV promoters.** After verification of the sequences, the five fragments were cloned in pET22B+ expression vector and in the pFastBac 1 shuttle vector.

For this purpose, UL49, UL49N1, UL49N2, UL48 and UL49–48 were released from pGEM-T vectors and cloned in PET and pFastBac 1 vectors by using the restriction enzymes ad hoc. UL48 and UL49–48 were released from pGEM-T and pFastBac 1 and cloned in pcDNA3.

**In vitro translation by using rabbit reticulocyte lysate.** The UL49, UL48 and UL49–48 genes, cloned into pET or pcDNA3 vectors, were transcribed and translated by using the Tnt T coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine (Amersham) according to the manufacturer’s instructions.

**Production of recombinant virus.** Recombinant baculoviruses were generated by using the Bac-to-Bac baculovirus expression system (Gibco-BRL).

For each recombinant baculovirus, the presence of a foreign gene was verified by PCR amplification with primers VP22F, VP22R, VP16F and VP16R. For this purpose, the supernatant of infected cells was centrifuged at 4,300 rpm for 1 h to pellet the virus. Virus pellets were resuspended with 100 µl lysis buffer (10 mM Tris–HCl pH 7.4, 3 mM MgCl₂, 0.5% Triton X-100, 0.05% Tween 20, 1 mg/ml proteinase K) and viral DNA was purified by phenol–chloroform–isoamyl alcohol extraction.

**MAb production.** MAbs to baculovirus-expressed VP16 and VP22 were raised by using conventional procedures with minor modifications (Shulman et al., 1978). In the initial fusion experiment, the antigen consisted of crude extracts of Sf9 cells that had been infected by either Bac UL49 (VP22) or Bac UL48 (VP16). For a second fusion, BALB/c mice were immunized with a nuclear extract from Bac UL49-infected Sf9 cells. The immunization regime and the fusion protocol were as described previously (Thouvenin et al., 1997). Screening for hybridomas secreting antibodies against VP22 or VP16 was performed by indirect immunofluorescence on baculovirus-infected Sf9 or High Five cell monolayers.
The subsequent steps, cloning of hybridomas and ascites fluid production, were performed as described previously (Vautherot et al., 1992). Antibody isotyping by ELISA was carried out by using a mouse MAb isotyping kit (ISO-2; Sigma).

Indirect immunofluorescence (IF). Infected cell monolayers were fixed and permeabilized in ethanol–acetone (75:25) at −20 °C for 30 min. After blocking in PBS (0·137 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄) with 0·05% Tween 20 (PBST), ascites fluids diluted 1:1000 or hybridoma supernatant diluted 1:4 in PBST were added to fixed cells and incubated at 37 °C for 1 h. Subsequent steps were carried out as described previously (Vautherot & Laporte, 1983). Monolayers were inspected with a BH2 Olympus microscope equipped with a fluorescence illuminator or an MRC1024 confocal microscope (Bio-Rad). Images were obtained with a Kalman acquisition device and processed with the Adobe Photoshop software.

Immunoprecipitation of radiolabelled proteins. Six-well plates containing High Five cells infected for 24 h were incubated in the presence of 100 µCi [³²S]methionine or 60 µCi [³⁵S]orthophosphate per well. Twenty-four h later, infected cells monolayers were washed with PBS and harvested in 500 µl hypertonic buffer (400 mM NaCl, 0·1 mM EDTA, 0·5 mM DTT, 10 mM HEPES–NaOH pH 7·9). Samples were centrifuged at 1800 r.p.m. (IEC, rotor 3229) for 5 min, resuspended and frozen at −80 °C until use. Two hundred µl labelled supernatant was diluted in 200 µl RIPA (500 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl pH 8) before addition of 1 µl ascites fluid. After 1 h at 37 °C, 1 µl rabbit anti-mouse IgG was added and the mixture was incubated for 30 min at 37 °C. Subsequent steps were performed as described previously (Vautherot et al., 1992) until the separation of proteins by PAGE.

SDS–PAGE and Western blotting. SDS–PAGE and Western blotting were carried out essentially as described previously (Vautherot et al., 1992). For Western blotting, proteins separated by PAGE were transferred to nitrocellulose membranes by transverse electrophoresis in 10 mM CAPS–NaOH buffer pH 11 (Sigma). Membranes were blocked with 20 mM Tris–HCl pH 7·4, 150 mM NaCl and 3% non-fat dried milk for 16 h at 4 °C. Nitrocellulose membranes were then incubated for 1 h at 37 °C with ascites fluid diluted 1:100 in IE buffer (150 mM NaCl, 20 mM Tris–HCl pH 7·4, 0·05% Tween 20 and 3% non-fat dried milk). Bound antibodies were detected by enhanced chemiluminescence (ECL; Amersharm-Pharmacia) with peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:50000 in IE buffer. Between steps, membranes were washed three times in IE buffer containing 1% non-fat dried milk.

Preparation of nuclear extracts. Infected or mock-infected cells were dislodged by scraping and washed twice in ice-cold PBS. Pellets were incubated for 10 min at 4 °C in PBS containing 1% NP-40 and centrifuged for 10 min at 1400 g. The resulting pellet was resuspended in 400 mM NaCl, 0·1 mM EDTA, 10 mM HEPES–NaOH pH 7·9 containing 1% Triton X-100 and centrifuged at 15000 g for 1 min. The final pellet (nuclear extract) was resuspended in Laemmli sample buffer (Laemmli, 1970) and heated at 95 °C for 1 min.

Cell-import assay of VP22. VP22 and its deletion mutants were extracted from recombinant baculovirus-infected insect cells by freeze–thawing in the hypertonic buffer described above. Extracts were diluted 1:5 in culture medium and added to semi-confluent 1-day-old cell monolayers. VP22 import was visualized at different times by IIF with anti-VP22 MAbs.

DNA-binding assay. Nuclear extracts were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 30 min at room temperature with 0·2% BSA that had been boiled for 5 min in 0·1 M potassium phosphate, 30 mM HEPES–NaOH (pH 7·4). Renaturation of bound proteins was carried out overnight at 4 °C in 50 mM Tris–HCl (pH 7·4), 1 mM EDTA, 200 mM NaCl, 10% glycerol, 0·1% NP-40. DNA binding was assayed for 30 min at room temperature in 5 mM MgCl₂, 50 mM NaCl, 30 mM HEPES–NaOH pH 7·4 by using an 85 bp digoxigenin–dUTP-labelled PCR product obtained from plasmid pBR322. Blots were washed in 50 mM NaCl, 0·05% Tween 20, 15 mM Tris–HCl pH 8, incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche) and revealed by using NBT/BCIP according to the manufacturer’s instructions.

Results

Nucleotide sequence of the UL49 and UL48 gene of MDV-1 strain RB1B

The nucleotide sequences of the UL49, UL48 and UL49–48 PCR products exhibited more than 99% identity to MDV-1 strain GA (Yanagida et al., 1993). The RB1B UL49 gene was 750 nt long and presented only one nucleotide change relative to MDV-1 GA, at position 244 (A→G). The consequence of this is a replacement of a threonine by an alanine residue. The UL48 gene (1284 nt) and the intergenic region (107 nt) exhibited 100% identity to those of MDV-1 GA.

The UL49–48 fragment used throughout this study had two mutations, one at position 846 (C→T) in the intergenic region and one at position 1449 (T→C) in the UL48 gene.

Two point mutations, presumably introduced by Taq polymerase during amplification of the UL49 gene, were cloned and expressed since they yielded two C-terminally truncated VP22 proteins: VP22S and VP22MS (Fig. 1a). In VP22S (168 aa), the last 14 residues represent a missense sequence, generated by a one nucleotide deletion at position 465 (UL49M1). VP22MS (187 aa) is encoded by UL49–48M2, in which a C→T mutation at position 562 introduced a stop codon.

N-terminal deletion mutants VP22N1 and VP22N2 (Fig. 1a) lacking the potential nuclear localization signal (NLS) sequence(s) (Izumiya et al., 1998) were obtained by PCR amplification as described above. UL49, UL48, UL49–48 and their mutants UL49M1, UL49–48M2, UL49N1 and UL49N2 were cloned in the baculovirus system.

Expression and characterization of VP22 and VP16 in insect cells

Virus titres of the different recombinant baculoviruses were determined by plaque assay and ranged from 5 × 10⁹ to 1 × 10⁷ p.f.u./ml. Before testing for protein expression, the presence of the inserted gene was confirmed by PCR on each recombinant baculovirus.

In nuclear extracts from Bac UL48-infected cells, a distinct band was detected corresponding to a protein of 49 kDa, the predicted molecular mass of VP16 (Fig. 1b, lane 7). VP22 and the truncated forms of VP22 were also detected in nuclear extracts from cells infected with the corresponding recombinant baculoviruses (Fig. 1b).
VP22S, VP22MS, VP22N1, VP22N2 and the full-length VP22 migrated more slowly than predicted, exhibiting molecular masses of 24, 25, 28, 26 and 30 kDa when the predicted molecular masses were 19, 21, 26, 23-5 and 27 kDa, respectively. These differences between predicted and observed molecular mass might be due to post-translational modifications of the VP22 protein (see below). VP22 was also detected in nuclear extracts of cells co-infected by Bac UL49 and Bac UL48 (Fig. 1b, lane 8) as well as in Bac UL49–48-infected cells (Fig. 1b, lane 6). VP16 was detected in a nuclear extract from co-infected cells (Fig. 1b, lane 8) but not in a nuclear extract from Bac UL49–48-infected cells. A faster-migrating product was detected in nuclear extracts of cells infected by Bac UL49 and Bac UL49–48 (VP22) (Fig. 1b, lanes 3, 6 and 11).

MAbs to recombinant VP22 and VP16 were selected from two fusions. IIF experiments showed that MAb I13 (IgG1) bound specifically to Sf9 or High Five cells infected by Bac
MDV UL49 and UL48 gene products

UL48. MAb I13 reacted with recombinant VP16 in Western blotting, defining a denaturation-insensitive epitope (data not shown). MAb D18 (IgG1) exhibited a very strong reactivity to S9 or High Five cells infected with Bac UL49 or Bac UL49–48 (data not shown), but failed to react with denatured VP22 and was unable to recognize VP22S. A second fusion experiment yielded a panel of six anti-VP22 MAbs (B12, B17, D1, G4, G22 and L13). MAb G22 (IgG1) was the only one that recognized the shortest C terminus-deletion mutant of VP22, VP22S. Despite a rather limited difference in length between VP22S and VP22MS (33 aa), all MAbs except G4 (IgG1) recognized VP22MS, suggesting that this 33 aa stretch is of major importance for epitope conservation. MAb L13 (IgG2a) recognized VP22 and VP22MS in Western blot experiments and was used to monitor the synthesis of viral VP22 (see below).

Post-translational modifications of VP22 proteins

VP16 and VP22 have been reported to be phosphorylated proteins in HSV-1. We therefore examined whether these proteins were phosphorylated in MDV-1 by using the baculovirus expression system. Two radiolabelled bands corresponding to VP22 and VP22MS, exhibiting molecular masses of 33 and 29 kDa for VP22 (Fig. 2; VP22) and 22 and 23 kDa for VP22MS (Fig. 2; VP22MS), were visualized after both 32P and 35S labelling. By contrast, VP16 did not appear to be phosphorylated in High Five cells (Fig. 2; VP16).

Detection of VP22 and VP16 in MDV-infected cells

In order to assess whether VP16 and VP22 were synthesized during MDV infection, IIF assays were performed on CESC infected by MDV-1 strain RB1B. MDV RB1B replication in CESC induced a cytopathic effect characteristic of MDV, i.e. appearance of plaques of rounded cells. VP22 was detected at high levels in cells of these plaques as well as in surrounding cells by MAbs D18/1 and L13 (Fig. 3 a–c) and MAbs B17, D1 and B12 (data not shown). VP22 had a nuclear localization, apparently diffused in multinucleated myotubes (Fig. 3 a) and to cells adjacent to the infected plaque (Fig. 3 b, c). By contrast, MAbs G22 and G4 detected VP22 in infected cells in the centre of the virus plaques (Fig. 3 d). Further study of the apparent difference between the staining patterns obtained with different MAbs will be conducted to assess whether the reactivity of MAB G22 and G4 was indeed restricted to VP22 in infected cells. In immunoblotting experiments, viral VP22 was detected by MAB L13 in whole-infected-cell extracts (Fig. 4 b, lane 1) and in the nuclear fraction when subcellular fractions were tested (Fig. 4 b, lane 4). VP22 was present in MDV-infected CESC as early as 4 h post-infection and the amount of protein increased steadily during the course of infection (data not shown).

No significant staining of infected cells was obtained with anti-VP16 MAb I13 used under the same conditions. Further testing of MDV-infected cell extracts yielded no detection of the VP16 protein in Western blotting experiments. As we could not detect VP16 in MDV-infected cells, we initiated a study on the transcription/translation of the UL49–48 sequence.

Transcription/translation of UL49–48

In vitro expression system. Studies of in vitro transcription and translation of the UL49–48 sequence revealed the synthesis of...
Fig. 3. Immunofluorescence analysis of the expression of VP22 and VP16. CESC infected with MDV were fixed and stained with MAb D18 (a, b), MAb L13 (c) or MAb G22 (d). Panels (b)–(d) were prepared with a confocal microscope. COS-7 cells, transfected by pcDNA3-UL49–48, were fixed and immunostained with MAb D18 (e) or I13 (f).
two proteins with molecular masses of 33 and 49 kDa (Fig. 5a, lane 5). Two UL49–48 sequences originating from other PCRs performed on MDV-1 RB1B were cloned under the control of the T7 promoter. These sequences, carrying different mutations either in the UL48 gene or in the intergenic region, encoded the same products (33 and 49 kDa) (Fig. 5a, lanes 6 and 7). The 33 kDa protein corresponded to the VP22 protein encoded by the UL49 gene alone (Fig. 5a, lane 2) and the 49 kDa protein corresponded to the VP16 protein encoded by the UL48 gene alone (Fig. 5a, lane 1). The 33 kDa protein was immunoprecipitated by MAb D18 (Fig. 5b) and the 49 kDa protein was immunoprecipitated by MAb I13 (Fig. 5c), confirming that the translated products corresponded to VP22 and VP16. Translation of the UL49–48M2 mRNA yielded the VP16 protein (49 kDa) and the VP22MS protein (Fig. 5a, lane 4), which again were immunoprecipitated by their respective MAbs (Fig. 5b, c, lanes 4).

Transcription/translation of UL48, UL49–48 and UL49–48M2 sequences yielded the VP16 protein (49 kDa) and two other bands of lower molecular mass (48 and 43 kDa). These faster-migrating forms could be attributed to internal initiations that are in-frame with the first AUG of the UL48 mRNA.

Other internal initiations could easily be detected with the transcription/translation of UL49–48 sequences cloned in-frame with the bacterial peptide leader gene, where two major bands with molecular masses of 33 (VP22–bacterial peptide leader fusion protein) and 30 kDa (VP22) were detected (Fig. 5a, b, lanes 5 and 7).

Data obtained from the in vitro expression system confirmed that the VP16 protein could be expressed from a bicistronic mRNA (Koptidesova et al., 1995). However, since it has been reported that reticulocyte ribosomes can initiate inappropriately at each AUG codon (Joubert et al., 2000; Kozak, 1986), the production of the VP16 protein from transcription/translation of the UL49–48 sequence was monitored in cells.

**In vivo expression systems.** VP16 could be detected by IIF only in CESC transfected with pcDNA3-UL48 and at a lower level in cells transfected with pcDNA3-UL49–48 or pET UL48 after vvT7 infection. Surprisingly, transfection of pcDNA3-UL48 in the absence of vvT7 infection yielded no detectable VP16 in CESC. VP16 could not be detected after transfection of any of the pcDNA3-based vectors in RK13 cells, whereas it was detected at low levels in COS-7 cells transfected with either pcDNA3-UL48 or pcDNA3-UL49–48 (Fig. 3f). By contrast, VP22 was detected at high levels in all expression systems. In Bac UL49–48-infected insect cells, only VP22 was detected by IIF, Coomassie blue protein staining or Western blotting; VP16 was not detected (see above).

**Intercellular transport of VP22**

In MDV-1-infected primary CESC, VP22 appeared to be excreted from infected cells in virus plaques to the nuclei of surrounding cells (Fig. 3a). As one of the most striking features of HSV-1 VP22 is its remarkable ability to migrate from cell to cell and to be imported and relocated to the nucleus, we studied the import properties of MDV VP22.

Several mammalian and avian cell lines were tested for their ability to import the recombinant full-length VP22 or its N- and C-terminal mutants extracted from baculovirus-infected insect cells (Fig. 6a). A time-course experiment on the import of VP22 in RK13 cells showed that VP22 was present mainly in the cytoplasm 5 min after initial contact with the cells; the protein was detected in the cytoplasm and nucleus after a 10 min incubation and, after 30 min, VP22 was localized predominantly in cell nuclei, showing an enrichment around the nuclear rim (Fig. 6b). Recombinant VP22 was able to enter and to concentrate within nuclei of many other different cell lines of avian or mammalian origin, e.g. LMH (Fig. 6c), COS-7, Sp2/O and avian lymphoid cell lines.

Interestingly, N- and C-terminally truncated proteins (VP22N1, VP22N2 and VP22MS) retained their ability to be imported into LMH cells (Fig. 6c), suggesting that the central part of VP22 (aa 38–188) is involved in the intercellular trafficking.

---

Fig. 4. VP22 expression in MDV-infected CESC. (a) Protein profiles of cellular and subcellular extracts. Lanes: 1–2, whole-cell extracts from infected (lane 1) and non-infected (2) cells; 3–4, cytoplasmic extract (3) and nuclear fraction (4) of MDV-infected CESC (position of VP22 is indicated). (b) Western blot analysis of VP22 expression by using anti-VP22 MAb L13. Extracts are the same as those used in (a).
Fig. 5. In vitro transcription/translation of UL49–48 sequences. Proteins were labelled with [35S]methionine and separated by 10% PAGE (a). T7 products were immunoprecipitated with either MAb D18 (b) or MAb I13 (c). Lanes: 1–7, transcription/translation of UL48 (lane 1), UL49 (2), UL49 plus UL48 (3), UL49–48M2 (4), UL49–48 (5) and two other UL49–48 sequences (6 and 7); M, molecular mass markers.

DNA-binding property of VP22

In DNA-binding assays, two major bands were identified in Bac UL49 nuclear extracts, having molecular masses of 33 and 29 kDa, corresponding to VP22 and its faster-migrating form (Fig. 7, lane 3). Moreover, VP22S, VP22MS and VP22N1 showed similar binding properties (Fig. 7, lanes 1, 2 and 7) whereas VP22N2 did not bind DNA, suggesting strongly that the main DNA-binding site is located in a region ranging from aa residues 16 to 37.

Discussion

As VP16 and VP22 have been shown to play major roles during the replication of the prototype alphaherpesvirus, HSV-1 (Elliott et al., 1995), we aimed to characterize the MDV-1 homologues of these two tegument proteins.

VP22 was detected in CESC infected with MDV and in MDV tumour cell line MSB-1 by using anti-VP22 MAbs (data not shown). MAbs G22 and G4 differed from the other MAbs as they led to low fluorescence signals in an infected monolayer. Morrison et al. (1998) reported that a MAb to HSV-1 VP22, P43, recognized the unphosphorylated form of VP22, giving low intensity staining when reacted with infected cells. Whether G22 and G4 MAbs have low affinity for VP22 or recognize an epitope masked by the association of VP22 with a nuclear protein or with DNA or by post-translational modification remains to be established.

We report the absence of detection of VP16 during MDV infection in CESC or MDV reactivation in MSB-1. Several hypotheses were considered regarding this lack of detection, when HSV-1 VP16 could readily be detected throughout the infection (Morrison et al., 1998). Lack of reactivity of MAb I13 to VP16 expressed in mammalian or avian cell systems is unlikely, as MAb I13 recognized VP16 expressed from pcDNA3-UL48 both in CESC (after vvT7 infection) and in COS-7 cells. However, we cannot rule out the possibility that VP16 might be phosphorylated by a virus kinase, inducing a modification of the epitope defined by MAb I13.

The translatability of VP16 from the UL49–48 transcript, reported to encode VP16 in MDV-1 and herpesvirus of
turkeys (Koptidesova et al., 1995; Kopacek et al., 1997), was studied in several expression systems. We confirmed that VP16 was synthesized in reticulocyte lysates (Koptidesova et al., 1995) and showed that this protein can be expressed from the bicistronic mRNA in a more controlled environment. Indeed, VP16 was detected in COS-7 cells and CESC (infected by vvT7) transfected by pcDNA3-UL49–48. Other bicistronic eukaryotic mRNAs having a structure similar to UL49–48, i.e. two non-overlapping ORFs separated by an intercistronic sequence, are known to encode two functional proteins (Bouhidel et al., 1994; Wang et al., 1987). However, VP16 was expressed at very low levels in COS-7 cells transfected with either pcDNA3-UL48 or pcDNA3-UL49–48 and could not be detected in RK13 cells or CESC (not infected with vvT7) transfected with the same plasmid. Amplification of pcDNA3 vectors that have the SV40 ori by the presence of T antigen in COS-7 cells (Bennett et al., 1989) could account for the detection of VP16 in COS-7 cells only. Our initial results in CESC show that the T7 promoter/vvT7 system appears to be more efficient for VP16 expression than expression by pCMV.

Fig. 6. Import of VP22. (a) Protein profiles (Coomassie blue staining) (upper panel) and Western blot (lower panel) of high-salt extracts made from Bac UL49- (lane 1) and Bac UL48- (2) infected cells. VP22 was detected in the Western blot by using MAb L13. Extracts were added to the medium covering cells on coverslips at a final dilution of 1:5. (b) Time-resolved import of VP22 in RK13 cells. RK13 cells were fixed after 5, 10 or 30 min incubation and immunostained with MAb D18. (c) VP22 uptake in LMH cells. LMH cells were incubated with either VP22 or the N-terminal mutants VP22N1 and VP22N2 or with the C-terminal deletion mutant VP22S. MAbs D18 or L13 were used to detect VP22, VP22N1 or VP22N2. The level of background staining is given by the VP22S panel, as MAb L13 does not react with VP22S. Cells were examined with a ×40 objective.
alone. Further work will be directed towards the study of the transcriptional patterns of the UL48 gene and the UL49–48 sequence.

MDV VP22 as well as VP22MS appeared as two phosphorylated forms when expressed in insect cells, suggesting that the phosphorylation site(s) is located within the first 188 aa of VP22. Elliott et al. (1999) have reported two serine phosphorylation sites within HSV-1 VP22 and assigned the N-terminal site (\(SSS\)) as a substrate of casein kinase II (CKII) and the C-terminal site (\(SAS\)) as a substrate of an unknown kinase. Further experiments will be carried out on the identification of putative phosphorylation sites within VP22 and particularly on VP22N2, which lacks the consensus CKII serine phosphorylation site located at residue 19 within MDV-1 VP22. ADP-ribosylation could also account for the observed increase of the apparent molecular mass of recombinant VP22, as reported for HSV-1 (Blaho et al., 1994). An ADP-ribosylation consensus sequence, \((R/P)RA(P/S)R\), has been determined for viral proteins (Blaho et al., 1994) and a sequence with high similarity to this consensus sequence exists on MDV-1 VP22, extending from K to P (KPRAKP).

Our results on the import of VP22 as a function of time showed that VP22 was identified first in the cytoplasm of cells and progressively reached and concentrated in the nuclei.
Indeed, recombinant MDV-1 VP22 is imported from the culture medium to the nucleus of different cell types including lymphoid cell lines of avian or murine origin, as described for HSV-1 VP22 (Elliott & O’Hare, 1997). Different fixation protocols were tested but the use of paraformaldehyde (PFA) led to a low detection of VP22 in MDV-infected cells and in recipient cells (data not shown), as if PFA quenched the fluorescence of MDV VP22, as reported for HSV-1 VP22 (Brewis et al., 2000).

N- and C-terminally truncated VP22 proteins were imported in LMH cells, suggesting that the core region of the protein (aa 38–188) is important for VP22 import. Alignment of MDV and HSV-1 VP22 proteins using DIALIGN 2.1 (Morgenstern, 1999) revealed a region of high similarity between the two proteins that extends from residue 93 to 173 of MDV VP22. This 80 residue sequence, also outlined when VP22 of MDV, HSV-1, varicella-zoster virus and bovine herpesvirus-1 (BHV-1) are aligned (Fig. 8), may be involved in intercellular trafficking of the VP22 homologues. Nuclear import involves several interactions and mechanisms, such as attachment of the protein to the cytoplasmic membrane, entry into cells, targeting to the nucleus and nuclear concentration. Staining of MDV VP22 in myotubes present in infected CESC monolayers suggests that VP22 could interact with the cytoskeletal network (actin microfilaments and microtubules), as its HSV-1 counterpart does, and might utilize these networks for its intracellular transport and nuclear accumulation (Elliott & O’Hare, 1998). Izumiya et al. (1998) have reported a potential NLS in the N-terminal part of the protein [RRK-(S/E)RRRS]. However, the N-terminally truncated proteins VP22N1 and VP22N2 still retained the ability to be imported into nuclei, suggesting either that the NLS is located elsewhere in the protein or that this small protein (30 kDa) can diffuse through the nuclear membrane and be retained by binding to nuclear components. Recombinant MDV VP22 exhibited powerful DNA-binding activity, as reported for HSV-1 VP22 (Knopf & Kaerner, 1980), which was assigned to aa 16–37. Interestingly, VP22N2 (aa 1–37 deleted) still retained the ability to be imported into nuclei, but appeared to be washed away after a 3 h chase, suggesting that the DNA-binding region is required for the retention of VP22 in the nucleus.

Whether VP22 is a tegument component remains to be established and the role of VP22 in MDV-1 infection is not known. Liang et al. (1995) have reported that a mutant of BHV-1 carrying a deletion of the UL49-homologous gene was able to replicate in MDBK cells, although it showed an impaired replication efficiency. Construction of MDV mutants with the UL49 gene deleted will be of major help in understanding the role of VP22 in MDV infection.

The laboratories of Pierre Coursaget and Jean-François Vautherot are members of the IFR Biologie Comparée des Transposons et Virus. The technical assistance of A. Francineau for all cell-culture experiments is gratefully acknowledged. We also wish to thank K. Osterrieder (BFAV-Riems, Germany), P. Midoux (CBM-CNRS-Orleans, France) and D. Rasschaert (INRA-Tours, France) for their suggestions and comments.

References


Received 27 January 2000; Accepted 20 May 2000