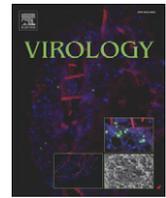




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## Vaccinia virus F16 protein, a predicted catalytically inactive member of the prokaryotic serine recombinase superfamily, is targeted to nucleoli

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### ABSTRACT

The F16L gene of vaccinia virus (VACV) is conserved in all chordopoxviruses except avipoxviruses. The crocodile poxvirus F16 protein ortholog has highly significant similarity to prokaryotic serine recombinases and contains all amino acids that comprise the catalytic site. In contrast, F16 orthologs encoded by other poxviruses show only marginally significant similarity to serine recombinases, lack essential amino acids of the active site and are most likely inactive derivatives of serine recombinases. Nevertheless, the conservation of F16L in non-avian poxviruses suggested an important function. However, a VACV mutant with the F16L gene knocked out replicated normally in dividing and quiescent cells. The F16 protein was synthesized early after infection and detected in virus cores. When expressed in infected or uninfected cells, F16 accumulated in nucleoli depending on the level of expression and confluency of cells. Evidence was obtained that F16 forms multimers, which might regulate concentration-dependent intracellular localization.

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### Introduction

The Poxviridae comprise a family of large, complex viruses with 130 to >300 kbp double-stranded DNA genomes that infect numerous vertebrate and invertebrate animals (Moss, 2007). Vaccinia virus (VACV) was used as the vaccine for smallpox eradication and is currently employed as a live recombinant vector for expression studies and development of vaccines against other pathogens and cancer (Moss, 1996). VACV, by far the most intensively studied poxvirus, replicates entirely in the cytoplasm of infected cells and encodes many of the proteins required for its growth as well as numerous proteins modulating virus–host interactions. The genome of VACV comprises a subset of genes that are common to all or most poxviruses and essential for replication in cell culture and another subset of genes that are specific for the orthopoxvirus genus, mostly nonessential in cell culture and involved in host interactions (Upton et al., 2003). Those genes that are conserved throughout the Poxviridae have been functionally characterized to varying degree of detail. However, many genes that are present in most but not all chordopoxviruses remain to be analyzed. One example is the uncharacterized VACV F16L gene, which has been retained in all chordopoxviruses except for avipoxviruses. Our interest in this gene, apart from its considerable evolutionary conservation, was stimulated by evidence that the F16 protein ortholog of Nile crocodile poxvirus (CRV) is homologous to site-specific serine recombinases of prokary-

otic transposons and bacteriophages (Afonso et al., 2006). Here we provide a detailed computational analysis of the F16 protein indicating that it is unlikely to have serine recombinase activity, and experimental data showing that the F16L gene is not required for virus growth in cell culture. Remarkably, the F16 protein specifically localized to the nucleoli of infected and uninfected cells.

### Results

*F16 belongs to the superfamily of prokaryotic site-specific Ser recombinases*

Our specific interest in the F16 protein of VACV was aroused by the report of Afonso and co-workers (Afonso et al., 2006) that the putative protein encoded by the 051 gene of CRV shares similarity with both bacterial transposon resolvases and homologs of VACV F16, which were detected in all chordopoxviruses except avipoxviruses. Starting with the VACV F16 sequence, an iterative database search using the PSI-BLAST program (Altschul et al., 1997) revealed a distant relationship to CRV051 and the lack of any detectable homolog in avipoxviruses. Whereas the CRV protein showed statistically highly significant similarity to numerous proteins of the Ser recombinase superfamily from bacterial and archaeal transposons and bacteriophages (Grindley et al., 2006; Olorunniji and Stark, 2010; Smith and Thorpe, 2002), the VACV F16 and the other poxvirus orthologs showed only marginally significant similarity (expect value of ~0.03 (Altschul et al., 1997)). Excluding CRV, the detection of homology of the F16 proteins with Ser recombinases required at least two PSI-BLAST iterations, accounting for the absence of such annotation in the

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literature. Nevertheless, the validity of this relationship was strongly supported by additional search results using the HHSearch method (Soding, 2005) that detects Ser recombinases as proteins with the greatest similarity to a Hidden Markov Model generated from the alignment of poxvirus F16 proteins with a 97% confidence.

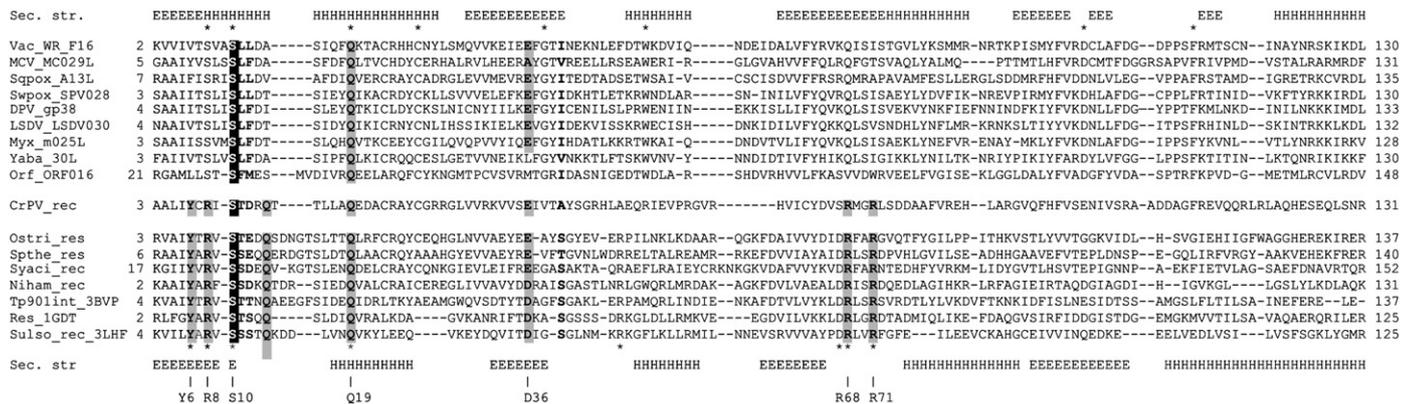
The region of sequence conservation between F16 proteins and Ser recombinases encompasses the entire catalytic domain of the latter that consists of approximately 140 amino acids (Fig. 1). All structural elements of the resolvase/recombinase domain appear to be conserved in F16 as indicated by the comparison of the predicted secondary structure of F16 and the crystal structure of resolvases of the Ser recombinase superfamily (Fig. 1). Strikingly, however, most of the individual conserved amino acid residues of the resolvases/recombinases have matches only in the CRV protein but not in the other poxvirus F16 sequences. Specifically, all 8 amino acid residues that comprise the catalytic site of the Ser recombinases, namely Tyr6, Arg8, Ser10, Gln14, Gln19, Asp36, Arg68, and Arg71 (using the amino acid numbering of Tn3 resolvase which is nearly identical to Res\_1GDT included in the alignment in Fig. 1) are conserved in the CRV protein (with the substitution of Glu for Asp36 that is found in many Ser recombinases), whereas only two, Ser10 and Gln19, are represented in the rest of the F16 proteins (Fig. 1). Although Ser10 is the main catalytic residue of Ser recombinases that is directly responsible for the nucleophilic attack on the phosphodiester bonds in the target DNA, the other residues in the catalytic site, in particular the three conserved Arg, are also essential for catalysis (Oloruniji and Stark, 2009, 2010). Given the concerted loss of 6 of the 8 catalytic residues, and in particular the three essential Arg residues, it can be confidently predicted that F16 proteins are inactivated derivatives of Ser recombinases that possess no enzymatic activity. Thus, whatever the function of these proteins in poxvirus reproduction might be, it does not involve site-specific recombination. Most of the Ser recombinases contain a DNA-binding helix-turn-helix domain to the C-terminus (or less commonly to the N-terminus) of the catalytic domain. The approximately 100 amino acid region following the

recombinase homology domain of F16 does not show detectable similarity to any helix-turn-helix domains, although it is predicted to adopt a primarily alpha-helical fold (not shown). This unique domain might mediate functionally important protein-DNA and/or protein interactions.

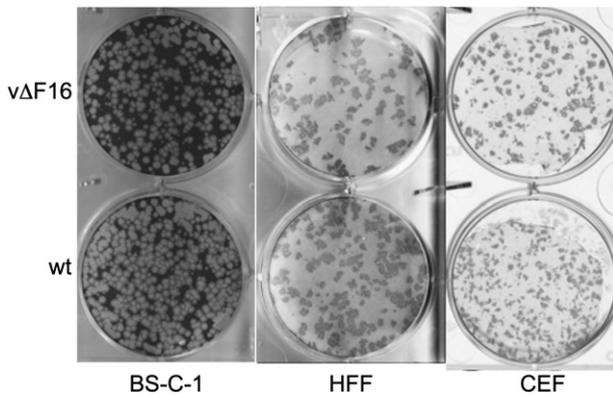
*F16 is non-essential for virus growth in cell culture*

The conservation of F16 among chordopoxviruses suggested that it had an important function. Yet the likelihood that the protein lacks Ser recombinase activity left us with no clue as to what that function might be. Our first question was whether F16 was essential for VACV replication. We succeeded in knocking out the F16L gene by replacing it with the enhanced green fluorescent protein (EGFP) gene under the control of the synthetic early/late VACV promoter (Chakrabarti et al., 1997). Green fluorescent plaques formed and the knockout virus vΔF16 was plaque purified, suggesting that the function of F16 is non-essential at least in BS-C-1 cells used for its isolation. Plaques produced by vΔF16 were compared with wild type (wt) virus on several conventionally used cell lines (BS-C-1, RK13, HeLa, and A543) as well as on two primary cell cultures, chick embryo fibroblasts and human foreskin fibroblasts (HFFs). No difference was detected in the plaque size or appearance of the mutant and the wt virus on any of the tested cells (Fig. 2 and data not shown). Furthermore, no differences in the yields of vΔF16 and wt virus were found in a single round of infection of BS-C-1, HeLa, and RK13 cells (Fig. 3A and data not shown).

Based on our recent studies with the VACV DNA ligase (Paran et al., 2009), we considered that the F16 protein might have a more critical role in resting cells. Replicate cultures of human foreskin fibroblasts cells were cultivated for 5 days in 0.2% serum to achieve quiescence, which was confirmed by a decrease in the amount of the cell cycle-dependent DNA ligase I transcript, and infected with a low multiplicity of each virus. Virus yields were determined over a 48 h period. However, no difference was found in the rate of formation or amount of wt and mutant viruses (Fig. 3B).



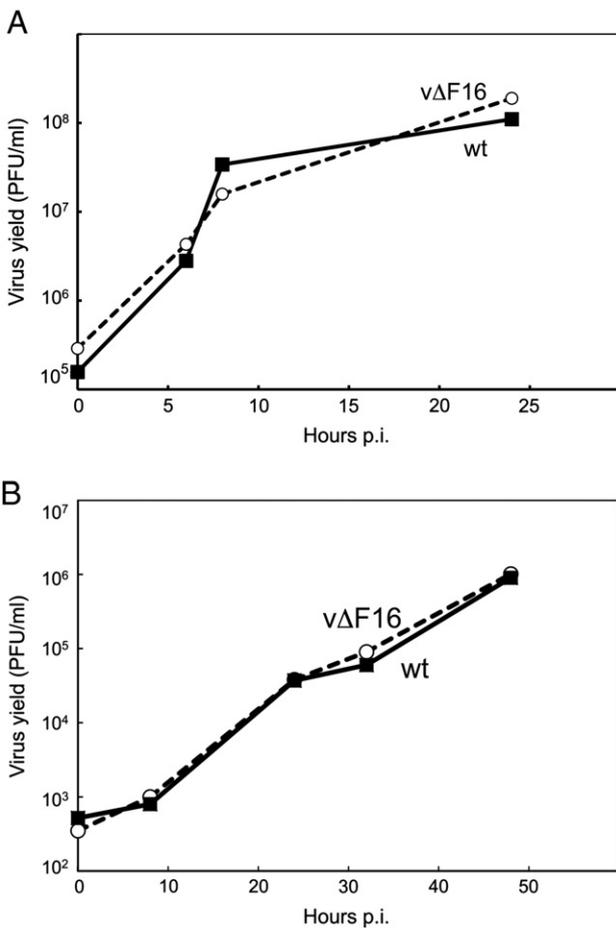
**Fig. 1.** Multiple alignment of chordopoxvirus F16 orthologs and selected serine recombinases. The viral protein selection consists of one representative of 9 of the 10 *Chordopoxvirus* genera that encode this protein (with the exception of avipoxviruses). The Ser recombinase selection consists of four sequences of putative resolvases and recombinases with the greatest similarity to F16 and three sequences for representative crystal structures. The secondary structure lines show the structural elements for F16 proteins that were predicted using the PSIPRED program (Buchan et al., 2010) and the structural elements extracted from the 1GDT structure; E indicates extended conformation (beta-strand) and H indicates alpha-helix. Asterisks above and beneath the alignment indicate invariant amino acid residues in the F16 sequences and in the selected Ser recombinase sequences, respectively. The catalytic residues of Ser recombinases and the matching residues in the poxvirus sequences are shaded gray. The catalytic serine is shown by white type on black background. The alignment was constructed using the MOFFT program (Katoh et al., 2005) and slightly manually adjusted to improve the alignment of secondary structure elements. The poxvirus sequences are denoted by an abbreviated virus name followed by the gene name; the Ser recombinase sequences are denoted by the abbreviated species name followed by abbreviated protein annotation; for the sequences with resolved crystal structures, the PDB Accession Numbers are indicated. For each protein, the number of the first and last amino acid residues in the aligned region is indicated before and after the sequence, respectively. The orthologs of F16 are 231 amino acids long in all orthopoxviruses and 209 to 230 amino acids in other genera. Abbreviations (GenBank accession number is given in parentheses): Vac\_WR, VACV, strain WR (YP\_232937.1); MCV, *Molluscum contagiosum* virus (NP\_043980.1); Sqpox, Squirrel poxvirus (ABD51455.1); DPV, Deerpox virus (YP\_002302379.1); LSDV, Lumpy Skin Disease Virus (NP\_150464.1); Myx, Rabbit myxoma virus (ACB28648.1); Yaba, Yaba monkey tumor virus (NP\_938286.1); Orf, Orf virus (NP\_957793.1); CrPV, Crocodilepox virus (YP\_784241.1); Ostri, *Oscillochloris trichoides* (ZP\_07686770.1); Spthe, *Sphaerobacter thermophilus* (YP\_003320473.1); Syaci, *Syntrophus aciditrophicus* (YP\_462099.1); Niham, *Nitrobacter hamburgensis* (YP\_579027.1); TP901-1, *Lactococcus* phage TP901-1; Res\_1GDP, Gamma-Delta Resolvase (from *E. coli* transposon); Sulso, *Sulfolobus solfataricus* (for the last three sequences, the PDB accession numbers are indicated instead of the GenBank accession numbers); res, resolvase; rec, recombinase.



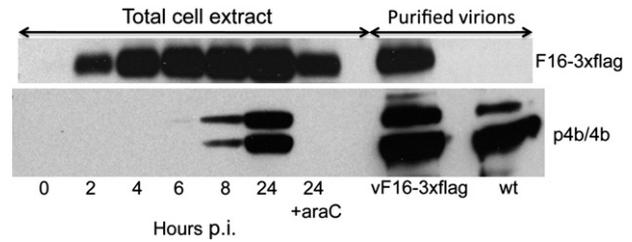
**Fig. 2.** Comparison of plaques produced by wt VACV and vΔF16. Monolayers of BS-C-1 African monkey kidney cells, primary HFF cells and primary chick embryo fibroblasts (CEF) were infected with wt VACV or vΔF16. After 48 h, the cells were stained with crystal violet (BS-C-1 cells) or antibody to VACV followed by a secondary antibody conjugated to horseradish peroxidase.

### F16 is an early protein and a component of the virion core

Inspection of the sequence immediately upstream of the F16 gene suggested the presence of an early promoter and the absence of a late promoter (Davison and Moss, 1989a, 1989b). Indeed, the F16 gene



**Fig. 3.** Kinetics of vΔF16 virus accumulation. (A) One step growth curve. BS-C-1 cells were infected at a multiplicity of 5 PFU per cell of wt VACV or vΔF16. At indicated hours post-infection (p.i.), the cells were harvested and the virus yields determined by plaque assay on BS-C-1 cells. (B) Replication and spread on resting cells. HFF cells were maintained for 5 days in medium containing 0.2% serum and then infected with 0.01 PFU of wt VACV or vΔF16. At indicated hours, the virus yields were determined as in panel A.



**Fig. 4.** Time course of F16 accumulation in infected cells. BS-C-1 cell monolayers were infected with vΔF16-3xflag and harvested at intervals post-infection (p.i.) in the absence of drug and at 24 h in the presence of araC. At each time point, aliquots were lysed with sodium dodecyl sulfate and reducing agent. In addition, virus particles were purified from Dounce homogenates by sedimentation through two successive sucrose cushions and one sucrose gradient. The proteins from the lysates and purified virions were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a membrane. The blot was probed with antibody to the flag epitope tag of F16 and to the late core protein 4b and its precursor p4b. Antibodies were detected by chemiluminescence.

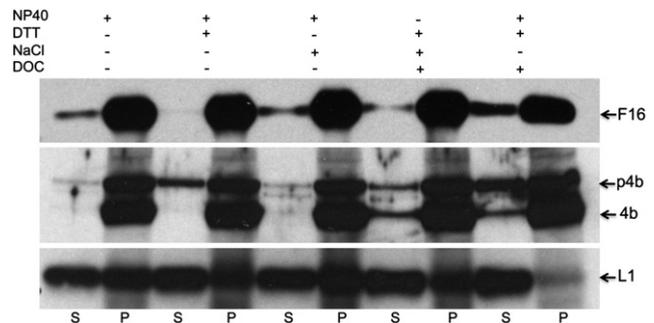
was expressed early in infection as shown by the accumulation of the F16 protein containing a 3xflag tag on the C-terminus (Fig. 4). F16-3xflag was detected by Western blotting at 2 h after infection, only slightly increased in amount between 4 and 24 h, and accumulated in the presence of cytosine arabinoside (araC), an inhibitor of DNA synthesis that prevents VACV intermediate and late gene expression. As a control, the blot was probed with antibody to the well-characterized late 4b core protein and its precursor p4b. These proteins were detected between 6 and 8 h after infection and were not seen in the presence of araC (Fig. 4).

F16-3xflag was detected in virions purified by sucrose gradient centrifugation following two sucrose cushions. As shown in Fig. 4, the ratio between F16 in virus particles and the 24 h cell extract was only slightly lower than the ratio for the abundant virion protein p4b/4b, suggesting that F16 is a bona fide virion protein.

VAC proteins are generally considered to be present in the core or membrane fraction of virus particles depending on their ability to be extracted from purified virions by non-ionic detergents in the presence or absence of reducing agent. We compared the extraction of F16-3xflag with well-characterized core (4b) and membrane (L1) proteins. F16-3xflag was mainly in the insoluble fraction, like 4b and unlike L1, during different treatments and was thus classified as a core protein (Fig. 5).

### F16-3xflag is targeted to the nucleoli of infected and uninfected cells

Most VACV virion proteins are expressed after viral DNA replication and are present predominantly in cytoplasmic factories, which are the sites of viral DNA, RNA and protein synthesis as well as

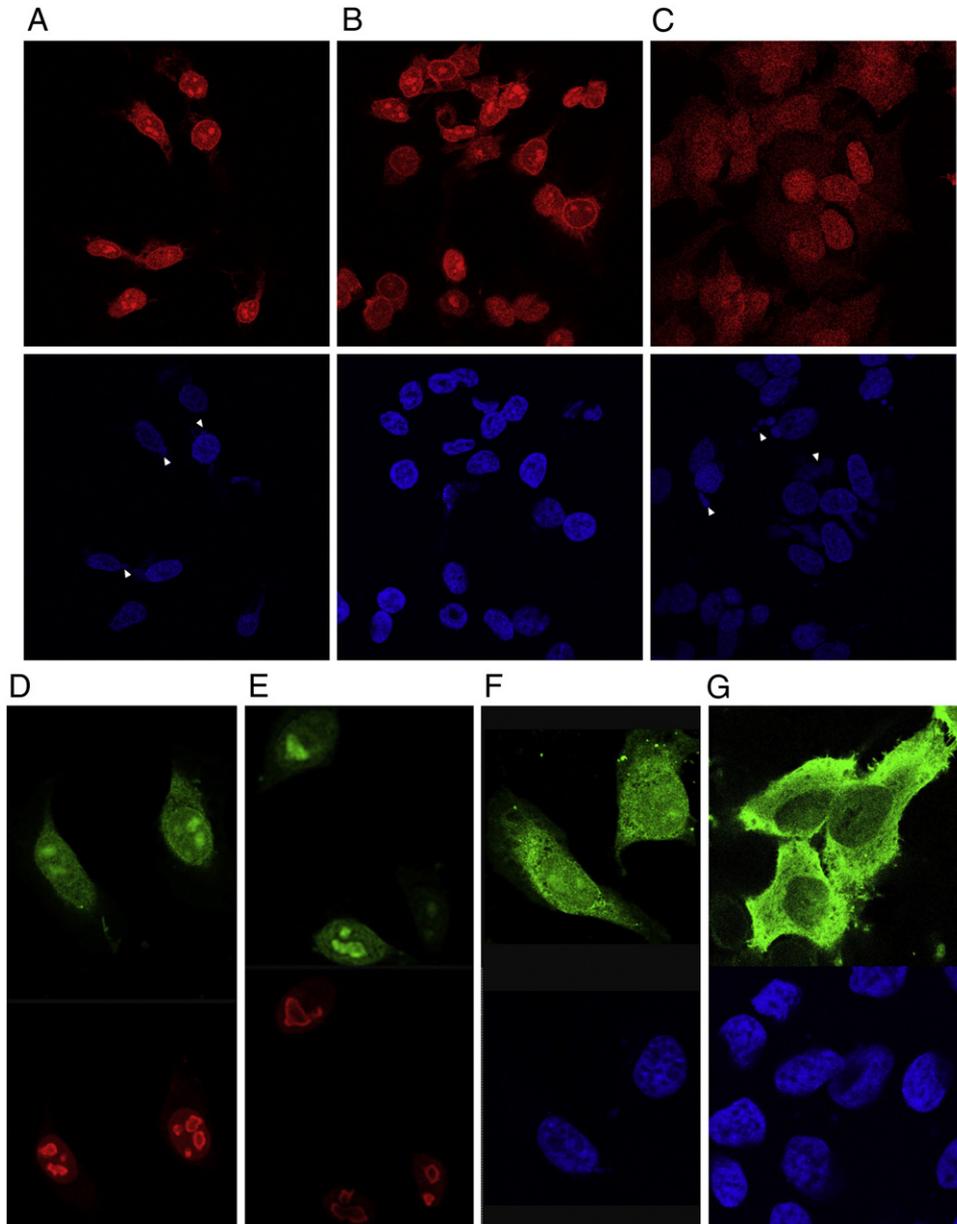


**Fig. 5.** Fractionation of proteins from purified vF16-3xflag virions. Virus particles were purified as described in the legend to Fig. 4 and treated with NP40 detergent with or without dithiothreitol (DTT), NaCl, and deoxycholate (DOC) as indicated by plus and minus signs. The suspensions were centrifuged and the supernatant (S) and pellet (P) fractions were analyzed by Western blotting with antibodies to the flag epitope of F16, the 4b core protein and its precursor p4b, and to the L1 membrane protein.

virion assembly (Katsafanas and Moss, 2007). F16, however, was expressed starting early in infection, before viral DNA synthesis and factory formation. Unexpectedly, in non-confluent cells before 8 h of infection F16 was detected in the nuclei, where it was concentrated in the nucleoli and to some extent on the nuclear membrane in addition to the cytoplasm (Fig. 6A). F16 was detected in virus factory regions, presumably accounting for its incorporation into virions, but was not concentrated there (Figs. 6A and C). Localization in the nucleoli also occurred when cells were infected in the presence of the DNA replication inhibitor araC (Fig. 6B). In confluent cells and at later times after infection, F16 was distributed throughout the cell but there was still some nucleoplasmic staining (Fig. 6C and data not shown). A

similar inverse correlation between localization in nucleoli and density of cell monolayer has been observed for the nucleoprotein NP of influenza virus (Bui et al., 2002).

Furthermore, when a human codon optimized copy of the F16 gene under the control of the cytomegalovirus (CMV) immediate early promoter was expressed at relatively low levels in non-confluent, uninfected cells, nucleoli targeting also occurred (Figs. 6D and E). Two other VAC proteins, I3 and B1, containing a C-terminal 3xflag tag and expressed from a transfected plasmid under the control of the CMV promoter were analyzed in parallel with F16-3xflag, and no nucleolar or nuclear membrane staining was detected (not shown). The association of F16 with nucleoli was confirmed by



**Fig. 6.** Intracellular localization of F16-3xflag in infected and uninfected cells. (A–C) HeLa cells infected with vF16-3xflag for 7 h. A, infected non-confluent HeLa cells; B, same in the presence of araC; C, infection as in panel A but cells were confluent. In panels A, B and C, F16 was stained with anti-flag monoclonal mouse antibody followed by a secondary antibody conjugated with AlexaFluor 647 and appears red; DNA stained with DAPI appears blue. White arrowheads point to cytoplasmic viral DNA factories. (D–G) Uninfected HeLa cells transfected with plasmid encoding F16-3xflag. D and E, relatively low level expression in non-confluent cells; F and G, high level of expression in non-confluent and confluent cells, respectively. In D, E, F and G, F16 was stained with an anti-flag polyclonal rabbit antibody followed by a secondary antibody conjugated with AlexaFluor 488 and appears green; in D, nucleolin was stained with an anti-nucleolin polyclonal rabbit antibody followed by a secondary antibody conjugated with AlexaFluor 647 and appears red; in E, nucleophosmin was stained with an anti-nucleophosmin monoclonal mouse antibody followed by a secondary antibody conjugated with AlexaFluor 647 and appears red; in F and G, DAPI staining appears blue.

colocalization with nucleolin/C23 (Fig. 6D) and nucleophosmin/B23 (Fig. 6E). Different anti-flag antibodies were used in the latter double-staining experiments, namely an anti-flag mouse monoclonal antibody with an anti-nucleolin rabbit polyclonal antibody and a rabbit polyclonal anti-flag with mouse monoclonal anti-nucleophosmin antibody. Thus, two independent anti-flag antibodies detected the same staining pattern for F16, corroborating the specificity. As in infected cells, the localization of F16 depended to a great extent on the level of expression in an individual cell. In transfected cells with high F16 expression level, either the entire cell or only the cytoplasm was stained with flag antibody (Figs. 6F and G). The same pattern of F16 accumulation was detected in HeLa cells, BS-C-1 and RK13 cells infected with vF16-3xflag or transiently transfected with a plasmid expressing F16-3xflag from the CMV promoter (not shown).

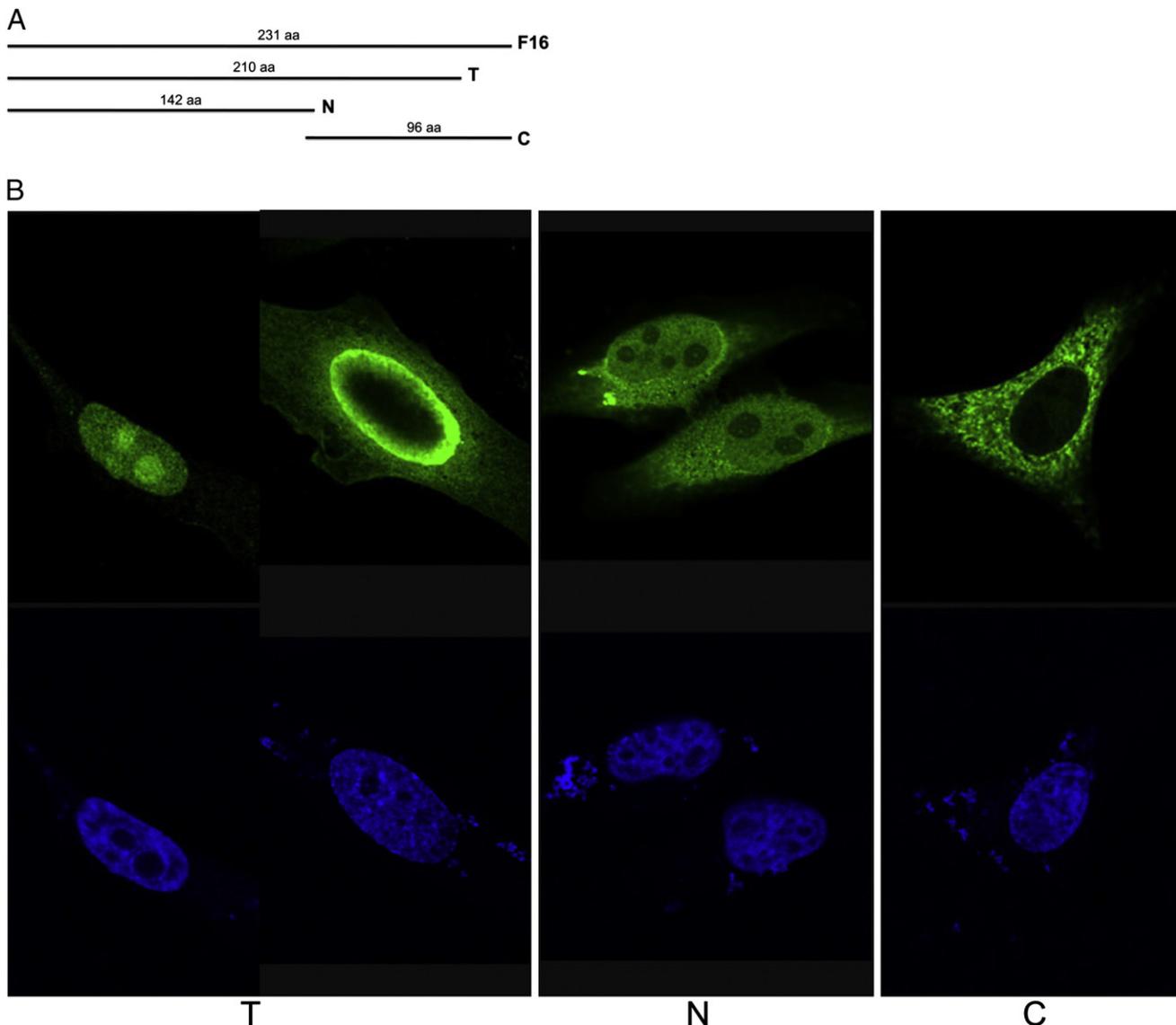
#### Invariant Ser8 and 11 are not required for targeting F16 to nucleoli

All poxvirus orthologs of F16 share the invariant Ser11 that aligns with the catalytic Ser10 of the Ser recombinases (Fig. 1). The striking conservation of this Ser in all poxvirus orthologs in spite of the absence

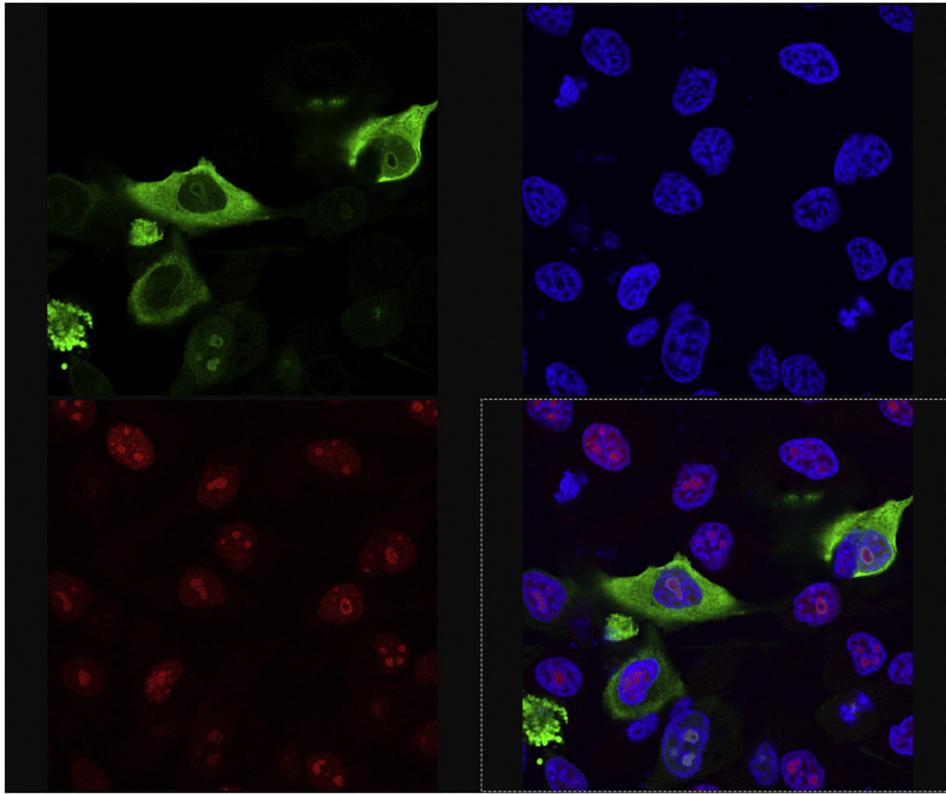
of other catalytic residues could suggest an important function, e.g., phosphorylation required for nucleolar targeting. Similarly, Ser8 is conserved in all poxvirus F16 orthologs except for CRV. To assess the potential importance of Ser11 and Ser8 for the targeting of F16 to the nucleoli, two mutants were constructed in which Ser11 or Ser8 was replaced by Ala. However, no difference in nucleoli targeting was observed after transfection of the corresponding plasmids into cells (not shown). Thus, these conserved serine residues are not required for the targeting of F16 to the nucleoli.

#### Contributions of different portions of the F16 protein to subcellular localization

The F16 protein does not contain known nuclear and/or nucleolar targeting signals (Emmott and Hiscox, 2009). In order to determine which part of the protein might be responsible for targeting to nuclei and nucleoli, we investigated the localization of a truncated mutant of F16 as well as the N-terminal and C-terminal domains of F16 expressed separately. In the truncated mutant of F16 (T), the 21 C-terminal amino acids were removed (Fig. 7A). This region includes a



**Fig. 7.** Localization of flag-tagged F16 deletion mutants in transfected HeLa cells. (A) Schematic representation of deletions in F16. T, truncation mutant; N, N-terminal domain; C, C-terminal domain. (B) Localization of deletion mutants. F16 was stained with an anti-flag polyclonal rabbit antibody followed by a secondary antibody conjugated with AlexaFluor 488 and appears green; DAPI staining appears blue.



**Fig. 8.** RNA synthesis in HeLa cells transfected with F16-3xflag. Cells were transfected for 48 h and incubated with the uridine derivative EU for the last 3 h. F16 was stained with an anti-flag polyclonal rabbit antibody followed by a secondary antibody conjugated with AlexaFluor 488 and appears green; DAPI staining appears blue; RNA containing 5-ethynyl uridine (EU) was stained with AlexaFluor 647 azide and appears red. A merge is shown in the lower right box.

predicted alpha-helix that is conserved in the chordopoxvirus F16 homologs (not shown) and potentially could be involved in protein–protein interactions. The T mutant was still targeted to the nucleoli, but showed much less cytoplasmic staining than the full-length protein (Fig. 7B). In addition, when highly expressed, the T mutant exhibited a characteristic halo that lined the inner side of the nuclear membrane (Fig. 7B). The N-terminal domain, which is homologous to Ser recombinases, and the unique C-terminal domain of F16 were not targeted to the nucleoli under any conditions. The N-terminal domain was distributed throughout the cell but was specifically excluded from the nucleoli, whereas the C-terminal domain was localized exclusively to the cytoplasm (Fig. 7B). These results suggest that for the nucleolar targeting of F16 interaction between structural elements located in the N-terminal and C-terminal domains might be required.

#### Expression of F16 did not abrogate r RNA synthesis

As nucleoli are the sites of rRNA synthesis, we investigated whether expression and nucleolar localization of F16 reduced r RNA synthesis. Cells were transfected with F16-3xflag expressed from the CMV promoter, incubated for 48 h during the last 3 h of which the uridine analog 5-ethynyluridine (EU) was present (Jao and Salic, 2008). Following fixation, F16-3xflag was detected by antibody and the labeled RNA was discerned by “click” chemistry, a copper-catalyzed covalent reaction between azide and alkyne. A comparison of F16 transfected and non-transfected cells in the same field did not suggest decreased rRNA synthesis (Fig. 8).

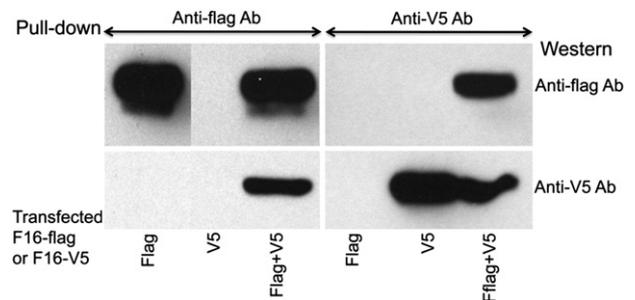
#### F16 forms multimers

The absence of nuclear/nucleolar targeting signals suggested to us that interaction of F16 with a cellular protein might modulate

its intracellular localization. Attempts to show an interaction of F16 with cellular nucleolin or nucleophosmin by coimmunoprecipitation, however, were unsuccessful (TGS, unpublished). However, we did find that F16 forms multimers by cotransfecting cells with plasmids expressing F16-3xflag and F16-V5. Both tagged versions of F16 coimmunoprecipitated with each other following incubation with anti-flag or anti-V5 antibody as shown by Western blotting (Fig. 9). These results indicate that F16 exists as dimers or higher order multimers.

#### Discussion

The homology between F16 and Ser recombinases has interesting evolutionary implications. The CRV homolog of F16 is the first likely Ser recombinase to have been detected in any eukaryote or eukaryotic



**Fig. 9.** Formation of F16 multimers. HeLa cells were cotransfected with plasmids expressing F16-3xflag and F16-V5 tag or with F16-3xflag and a control plasmid or F16-V5 and a control plasmid. Cytoplasmic extracts were immunoprecipitated on agarose beads with anti-flag or anti-V5 antibodies. Agarose-bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-flag or anti-V5 antibodies.

virus (Afonso et al., 2006). Thus, the most parsimonious evolutionary scenario involves acquisition of a Ser recombinase gene by the ancestor of chordopoxviruses from a transposon or a bacteriophage. This scenario is reminiscent of other probable bacterial or bacteriophage contributions to the evolution of poxviruses, in particular, the Holiday junction resolvase A22 (Garcia et al., 2000) and the predicted deubiquitinase G6 (Senkevich et al., 2008) as well as the primase-helicase D5 and the DNA-packaging ATPase A32 that are shared with other nucleocytoplasmic large DNA viruses (Koonin and Yutin, 2010). The subsequent evolution of chordopoxviruses apparently involved the abrogation of the recombinase activity of F16, as evidenced by the substitution of key amino acids in the active site region. This loss of activity occurred after the divergence of CRV [the deepest chordopoxvirus branch (Afonso et al., 2006; Koonin and Yutin, 2010)] from the common ancestor with the other chordopoxviruses but prior to the radiation of the latter, and the complete loss of this gene in the avipoxvirus branch. The loss of recombinase activity was apparently accompanied by an overall acceleration of evolution and apparently a change of function.

Assuming the latter scenario, what is the acquired new function of the poxvirus F16 homologs? Given its conservation and early expression shown here, a role in viral DNA replication or recombination seemed one possibility. However, knockout of the F16L gene had no measurable effect on virus growth in any of the many tested cell cultures including resting primary HFFs. Another gene with similar biological features is the G6R gene that is conserved in all poxviruses and encodes a putative deubiquitinating enzyme (Senkevich et al., 2008). Knockout of the G6R gene has no effect on virus growth in all tested cell cultures and leads only to a slight attenuation in intranasal mouse infection. Conservation of these genes in most poxvirus genera suggests that their functions are not directly involved in the virus growth cycle but may be involved in generic aspects of virus–host interaction as opposed to interactions between viruses and their specific hosts.

The targeting of the F16 protein to the nucleoli, whether expressed by VACV or by transfection in uninfected cells, was unexpected. However, the myxoma virus ankyrin repeat protein M148R, which also lacks a defined nucleolar localization motif, has been reported to localize in the nucleolus (Blanie et al., 2009). Deletion of M148R does not perturb replication *in vitro* but reduces virulence in a rabbit model. Another VACV protein, E3L, has been detected in the nuclei of infected cells but its nuclear function (if any) has not been characterized (Yuwen et al., 1993). Although F16 had a 3xflag tag, the latter is unlikely to cause nuclear or nucleolar targeting as the VACV proteins I3 and B1, containing a C-terminal 3xflag tag and expressed from a transfected plasmid under the control of the CMV promoter, were analyzed in parallel with F16–3xflag and no nucleolar or nuclear membrane staining was detected (TGS, unpublished). Moreover, the 3xflag has been used in numerous studies of subcellular localization of proteins, and no specific effect on nuclear and/or nucleolar targeting has been reported. In particular, the 3xflag tag was used in a genome wide screen of herpesviruses for protein subcellular localization, with no specific effects on protein targeting (Salsman et al., 2008).

The amino acid sequence of F16 does not contain known nuclear or nucleoli targeting signals (Emmott and Hiscox, 2009) and our studies indicated that both the N- and C-terminal regions of F16 are required for localization to nucleoli. A likely possibility is that F16 targets the nucleus and nucleoli by interacting with a cellular protein. Thus far, we have been unable to detect an interaction of F16 with cellular nucleolin or nucleophosmin by coimmunopurification (TGS, unpublished) but interactions with other cellular proteins remain a possibility. We did find that F16 interacts with itself to form homodimers or higher order multimers. If there is competition between F16 binding to itself and to a cell protein, this could explain the inverse correlation of nucleolar targeting with the level of

expression and confluency of cells. Conditions in which the concentration of F16 is increased (high expression) or the cell protein is decreased (confluency), might favor multimer formation and cytoplasmic localization.

Targeting of viral proteins to nucleoli is a frequent theme in the study of virus–host interactions. For example, a genome-wide screen of three distinct herpesviruses has shown that at least 12 herpesvirus-encoded proteins specifically localize to the nucleolus (Salsman et al., 2008). Targeting of viral proteins to nucleoli has been described not only for DNA viruses such as herpesviruses, adenoviruses, and papovaviruses, and RNA viruses, such as retroviruses and influenza, that possess a defined nuclear stage of reproduction, but also for proteins of RNA viruses without any nuclear stage such as coronaviruses, arteriviruses and filoviruses as well as several groups of RNA viruses of plants (Hiscox, 2007; Hiscox et al., 2010; Taliansky et al., 2010). Concomitantly, it has become evident that nucleoli are not only the factories of ribosome biosynthesis, as previously thought, but multifunctional and dynamic structures that contain hundreds of cellular proteins and are involved in a variety of signaling pathways, including cell cycle control, regulation of cell division, apoptosis, antiviral defense, cancer, and more (Boisvert et al., 2007; Sirri et al., 2008). For many viruses, trafficking of viral proteins through nucleoli is an important step in their growth cycle, and accordingly, the viral proteins directed to nucleolus are essential for the viral replication in cell culture. For example, herpesvirus and human immunodeficiency virus proteins that are targeted to the nucleolus have been implicated in viral mRNA processing (Hiscox et al., 2010). Several proteins of adenoviruses and herpesviruses cause redistribution of nucleolar components, reorganization of the nucleolus and interference with nucleolar function, including disruption of rRNA synthesis, processing and trafficking (Hiscox et al., 2010).

VACV affects various host cell processes, in particular translation is shut down at least partly through decapping of host mRNAs (Parrish et al., 2007) and by localization of the translation apparatus within viral factories (Katsafanas and Moss, 2007). Host transcription is impaired by mechanisms that have not been well characterized (Puckett and Moss, 1983). Jefferts and Holowczak (Jefferts and Holowczak, 1971) reported decreased nucleolar RNA synthesis in VACV-infected L cells and detected peptide from <sup>14</sup>C-labeled virions in nucleoli. In our studies, the F16 protein was specifically targeted to the nucleoli of transfected HeLa cells but it had no obvious effect on rRNA synthesis as determined by EU incorporation and click chemistry. In a preliminary experiment, we did not see greater EU labeling of HeLa cell nucleoli in cells infected with the F16 deletion mutant compared to wt virus (TGS, unpublished). Thus, F16 may affect another, perhaps signaling functions of the nucleoli. Further studies of this phenomenon could reveal an additional layer of manipulation of host cell functions by poxviruses.

## Materials and methods

### Cells and viruses

Standard procedures for preparation and maintenance of HeLa and BS-C-1 cells originally obtained from the American Type Culture Collection (Manassas, VA) and propagation, titration and purification of VACV were used (Earl et al., 1998a, 1998b). Primary HFFs were provided by A. McBride (National Institute of Allergy and Infectious diseases, Bethesda, MD), primary chick embryo fibroblasts were prepared from 10-day-old embryos and used in the first passage. On monolayers of BS-C-1 cells, VACV produced clear plaques that were visible after staining with crystal violet; on other cell monolayers, where plaques were less easily discerned, infected cell foci were detected by immunostaining with anti-VACV antibody, followed by protein A conjugated to horseradish peroxidase (Carroll and Moss, 1997).

## Recombinant viruses

Recombinant viruses were derived from the WR strain (ATCC VR-1354). v $\Delta$ F16 and vF16-3xflag were constructed by inserting a universal DNA fragment containing the EGFP gene preceded by VACV early/late synthetic promoter CTAAAATTGAAATTTATTTTTTTTTTTTGAATA-TAA via homologous recombination into the desired place in the VACV genome. The universal fragment (E/L-GFP) was surrounded on both sides by 500 nt flanks to allow homologous recombination into the specific site. All DNA pieces were assembled by overlapping PCR and transfected into BS-C-1 cells infected with 1–2 PFU/cell of VACV. After 24 h, lysates were prepared, new BS-C-1 cell monolayers were infected and green fluorescent plaques of recombinant viruses were isolated and clonally purified. In v $\Delta$ F16, the coding sequence of the F16 gene excluding 19 nucleotides from the 3' end was replaced by the E/L-GFP cassette. Replacement of F16 by GFP was confirmed using PCR primers that flanked F16; the size of the product was that predicted. In vF16-3xflag the sequence encoding the 3xflag GACTCAAAGACCAT-GACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACCAAGTGA was linked to the last codon of F16, followed by the E/L-GFP cassette and then by the last 7 nucleotides of the F16 ORF including its stop codon. The 3'-end nucleotides of F16 ORF were kept to prevent disruption of the downstream F15L gene promoter.

## Kinetics of virus replication

Duplicate wells containing cells in 24 well plates were infected with virus for 1 h, washed 3 times with medium, incubated for different times at 37 °C and harvested. Infected cells were lysed by 3 freeze-thaw cycles, sonicated, and virus titers were determined by plaque assay on BS-C-1 cells in semi-liquid 0.5% methylcellulose medium.

## Western blotting

Total cell extracts in SDS-PAGE loading buffer were sonicated to reduce viscosity and proteins were analyzed on NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Membrane was incubated with antibodies in 5% nonfat milk in phosphate buffered saline (PBS)-0.1% Tween-20 for different times at room temperature or 4 °C, then secondary horseradish peroxidase-conjugated antibodies, and the blot was developed with SuperSignal West Pico, Dura or Femto Chemiluminescent Substrates (Pierce, Rockford, IL) depending on hemiluminescence intensity.

## Expression of F16-3xflag and its derivatives in mammalian cells

A copy of F16-3xflag gene optimized for expression in human cells was synthesized by GeneArt (Regensburg, Germany) and cloned into pcDNA3.1(+) vector (Invitrogen), which contains the CMV immediate early promoter. Mutations and deletions were constructed by overlapping PCR and cloned into the same vector. HeLa cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer and analyzed after 48 h. As controls, plasmids containing two additional VAC proteins, I3 and B1, containing a C-terminal 3xflag tag under the control of the CMV promoter were constructed.

## Antibodies

All commercially available primary antibodies were from Sigma (St. Louis, MO). For confocal microscopy, anti-flag M2 mouse monoclonal, anti-flag rabbit polyclonal, anti-nucleolin rabbit polyclonal and anti-B23 mouse monoclonal antibodies were used followed by AlexaFluor 488 or 647 goat anti-mouse or goat anti-rabbit antibodies (Invitrogen). For Western blotting horseradish peroxidase conjugated M2 mouse monoclonal antibody and polyclonal rabbit

antibodies against VACV proteins L1 (R180), provided by G. Cohen and R. Eisenberg (University of Pennsylvania) and p4b/4b (R. Doms and B. Moss, unpublished) were used followed by secondary horseradish peroxidase conjugated antibodies.

## Immunoprecipitations

BS-C-1 cells, in 12-well dishes, were cotransfected with plasmids expressing F16-3xflag and F16-V5 or cotransfected with each plasmid and a vector plasmid. After 48 h of incubation at 37 °C, cells were disrupted in PBS-0.5% NP40. Cytoplasmic extracts were preincubated with IgG beads for 1 h at +4 °C and one-half of each extract was loaded on agarose beads with covalently bound anti-flag antibody (Sigma) or anti-V5 antibody (Sigma) and incubated overnight at +4 °C with constant rotation. Agarose beads were washed 4 times with PBS-0.5% NP40, proteins were eluted in SDS-polyacrylamide gel loading buffer and resolved in a SDS 10% polyacrylamide gel, transferred to a nitrocellulose membrane and incubated with horse radish peroxidase-conjugated anti-flag antibodies (Sigma), then the membrane was stripped and incubated with horse radish-conjugated anti-V5 antibody (Invitrogen).

## RNA synthesis

For detection of cellular RNA by confocal microscopy, the Click-it Kit RNA Imaging kit with Alexa Fluor 594 azide (Invitrogen) was used. HeLa cells were transfected and incubated with uridine derivative EU for the last 3 h of a 48 h incubation and then fixed and stained with Alexa Fluor 594 azide according to the manufacturer's protocol.

## Confocal microscopy

HeLa cells on coverslips were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, incubated with primary antibodies in PBS-5% fetal calf serum for 1–18 h at room temperature or 4 °C, stained with secondary Alexa Fluor antibodies for 1 h followed by DAPI staining for 10 min and mounted on slides with ProLong Gold antifade reagent (Invitrogen). A Leica SP2 inverted four-channel microscope was used for imaging.

## Protein sequence analysis

The search of the non-redundant protein sequence database (NCBI, NIH, Bethesda) was performed using the PSI-BLAST program with the conditional compositional score matrix adjustment (Altschul et al., 1997, 2005). Additional search of protein sequence databases was performed using the profile Hidden Markov Model approach implemented in the HHsearch program (Soding, 2005). Protein secondary structure was predicted using multiple alignments as implemented in the PSIPRED program (McGuffin et al., 2000).

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