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Expression of the Vaccinia Virus A2.5L Redox Protein Is Required for Virion Morphogenesis

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In this article we report the initial biochemical, genetic, and electron microscopic analysis of a previously uncharacterized, 8.9-kDa, predicted thiol-redox protein. The name A2.5L was assigned to the corresponding vaccinia virus gene, which is conserved in all sequenced poxviruses. Multiple alignment analysis and secondary structure prediction indicated that the A2.5L gene product is an all-α-helical protein with a conserved Cxx(x)C motif in the N-terminal α-helix. The DNA replication requirement and kinetics of A2.5L protein accumulation in virus-infected cells were typical of a late gene product, in agreement with the predicted promoter sequence. The A2.5L protein was a monomer under reducing conditions, but was mostly associated with the vaccinia virus E10R redox protein as a heterodimer under nonreducing conditions. The A2.5L protein was detected in virus particles at various stages of assembly, suggesting that it is an integral component of intracellular virions. An inducer-dependent A2.5L null mutant was constructed: in the absence of inducer, infectious virus formation was abolished and electron microscopy revealed an assembly block with an accumulation of crescent membranes and immature virions. This stage of assembly block was similar to that occurring when the E10R and G4L redox proteins were repressed, which is compatible with the involvement of E10R, A2.5L, and G4L in the same redox pathway.

INTRODUCTION

Poxviruses comprise a diverse family of complex cytoplasmic DNA viruses, with genome sizes ranging from 140 to 380 kb, that infect a variety of animals, including mammals, birds, and insects (Moss, 2001). Despite their unique biological characteristics and specific modes of interaction with their hosts, members of different poxvirus genera have a similar reproduction cycle that is based on a conserved set of approximately 50 genes that are represented by orthologs in all sequenced poxvirus genomes. The genome of vaccinia virus, the prototype member of the orthopoxvirus genus, contains nearly 200 genes. Several of the conserved vaccinia virus genes remain completely uncharacterized but are inferred to have essential functions. One of these open reading frames (ORFs), predicted to encode an 8.9-kDa protein, was originally identified in the WR strain of vaccinia virus and was shown to be transcribed at late times after infection, although no protein was detected by translation of mRNA selected by DNA hybridization (Weinrich and Hruby, 1986). Subsequently, a global yeast two-hybrid analysis of vaccinia virus WR proteins revealed an interaction of the product of this ORF with the vaccinia virus E10R product (McCraith *et al.,* 2000). The gene of interest, however, had no name in the current nomenclature, which is based on the order of ORFs in the Copenhagen strain of vaccinia virus. This ORF escaped identification because of an apparent error in the published Copenhagen genome sequence (Goebel *et al.,* 1990), which is corrected here. Given the location of this ORF between the A2L and A3L genes, we assigned the name A2.5L to the gene and the corresponding protein. Here, we show that the A2.5L gene is conserved in all poxviruses and that the protein is essential for virus reproduction and, specifically, for virion assembly. Elsewhere, we demonstrate that the A2.5L protein forms part of a vaccinia virus-encoded redox pathway (Senkevich *et al.,* 2002).

RESULTS

A2.5L protein is conserved in all poxviruses

We noted that an ORF, which was predicted to encode an 8.9-kDa protein in the WR strain of vaccinia virus, was disrupted in the published Copenhagen strain sequence but intact in all other sequenced poxvirus genomes. This gene was resequenced using a PCR product generated from the Copenhagen strain DNA and an extra G was identified in position 48, restoring an ORF of the same size as that found in all other orthopoxviruses (GenBank

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FIG. 1. Multiple alignments of A2.5L orthologs. Invariant amino acid residues are shown by white letters against a black background and positions occupied by similar residues in all aligned sequences are shown against a gray background. The Cxx(x)C motif is indicated with asterisks. The secondary structure prediction is shown above the alignment; E indicates extended structure (β-strand) and H indicates the α-helix. The alignment includes three sequences of orthopoxviruses (top block), one representative sequence from each of the other genera of chordopoxviruses (middle block) and two sequences of the entomopoxviruses (bottom block). The proteins are denoted by the abbreviated virus name and gene identification number. Virus name abbreviations: VV_Cop, Copenhagen strain of vaccinia virus; VV_WR, WR strain of vaccinia virus; VAR, variola virus; YLDV, Yaba-like disease virus; SPV, swinepox virus; LSDV, lumpy skin disease virus; MYX, myxoma virus; MCV, molluscum contagiosum virus; FPV, fowlpox virus; MSV, *Melanoplus sanguinipes* entomopoxvirus; AMV, *Amsacta moorei* entomopoxvirus.

Accession No. AF516337). The corresponding region of our WR strain isolate was also sequenced (GenBank Accession No. AF516336), and we found several nucleotide substitutions leading to three amino acid replacements compared to the previously reported WR sequence (gi140133; Weinrich and Hruby, 1986). The new protein sequences are shown in Fig. 1. We assigned the name A2.5L to this gene and its protein product because of its location between the A2L and A3L ORFs of the Copenhagen strain of vaccinia virus.

Standard searches of the nonredundant (nr) protein sequence database (NCBI, NIH, Bethesda) detected orthologs of A2.5L in all sequenced chordopoxviruses, but no statistically significant similarity to any other protein was detected. The apparent absence of orthologs of A2.5L in the two entomopoxviruses with sequenced genomes was unexpected, however, because yeast two-hybrid analysis of vaccinia virus proteins (McCraith *et al.,* 2000) revealed the interaction of A2.5L with E10R, which is highly conserved in all poxviruses and has an essential function in disulfide bond formation (Senkevich *et al.,* 2000b). Therefore, we considered the possibility that orthologs of A2.5L were, in fact, encoded in entomopoxvirus genomes but were not detected due to the small size of the protein and low sequence conservation. Since chordopoxvirus A2.5L sequences contain a conserved Cxx(x)C motif, which is implicated in the redox function of A2.5L (Senkevich *et al.,* 2002), we searched the sequences of small proteins (60–100 amino acids) encoded in the genome of *Melanoplus sanguinipes* entomopoxvirus (MSV) for this amino acid pattern. This search identified nine small proteins containing the Cxx(x)C pattern, which were further searched with a position-specific scoring matrix for chordopoxvirus A2.5L orthologs using the PSI-BLAST program. This search detected limited sequence similarity between A2.5L and the MSV168 protein. A search of the nr database with the MSV168 sequence detected a moderately conserved ortholog (AMV203) among the *Amsacta moorei* entomopoxvirus gene products (expectation value of 10^{-5} when the search was performed with the composition-based statistics turned off) but failed to detect significant similarity to any other proteins. Multiple sequence alignment of the chordopoxvirus A2.5L orthologs and their potential counterparts from entomopoxviruses showed, in addition to the conservation of the Cxx(x)C motif, the presence of another characteristic motif near the C-termini of these proteins, with two invariant, negatively charged residues, and several positions occupied by similar residues in all proteins (Fig. 1). Secondary structure predictions, which were performed separately for the multiple alignment of chordopoxvirus A2.5L orthologs and the pair-wise alignment of the potential orthologs from entomopoxviruses, showed similar patterns of predicted structural elements, namely six α-helices (Fig. 1). Therefore, despite the low sequence conservation in entomopoxviruses, we concluded that A2.5L belongs to the set of proteins conserved throughout the poxvirus family and is probably essential for virus reproduction.

Expression of the A2.5L gene during the vaccinia virus replication cycle

The start codon of the A2.5L gene forms part of a TAAATG sequence, which is typical of poxvirus late promoters (Davison and Moss, 1989; Senkevich *et al.,* 1997). To investigate the dynamics of A2.5L protein synthesis during the virus replication cycle, we constructed a recombinant vaccinia virus in which the 14-amino-acid V5 epitope tag coding sequence was linked to the 3′-terminus of the A2.5L gene, which accordingly remained under the control of the native A2.5L promoter (Fig. 2A). For this construction, another recombinant vaccinia virus, vE10R-HA (Senkevich *et al.,* 2000a), was used as the parental virus instead of the wild type. In vE10R-HA, the 9-amino-acid influenza hemagglutinin (HA) epitope tag coding sequence was attached to the 3′-terminus of the E10R gene. Thus, the final recombinant virus vE10R-HA/A2.5L-V5 contained E10R and A2.5L proteins with different tags, allowing the analysis of the interaction between these two proteins as predicted by a yeast two-hybrid system screen (McCraith *et al.,* 2000). Neither the growth nor the plaque size of this recombinant virus was impaired compared to that of the wild type (data not shown). The synthesis of the V5-tagged A2.5L protein of the expected size of ~10 kDa was demonstrated by reducing SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting with the anti-V5 antibody. The A2.5L protein was detected by 6 h postinfection and increased in amount thereafter as expected of a late protein (Fig. 2B). Also typical of a late protein, the synthesis of the A2.5L protein was not detected when viral DNA replication was inhibited with AraC (Fig. 2B). The kinetics of accumulation of A2.5L and E10R were similar, as shown by analysis of aliquots of the same samples in two parallel gels using the HA- and V5-tag-specific antibodies that recognized the tagged E10R and A2.5L proteins, respectively (Fig. 2B).

Additional samples, obtained 24 h postinfection as described above, were resolved by SDS–PAGE under nonreducing conditions, and proteins in adjacent lanes were analyzed with either the anti-HA or the anti-V5 antibody. A band with identical mobility (~23 kDa) was detected with each antibody, in addition to the bands corresponding to the monomers of E10R-HA and A2.5L-V5 (Fig. 2C). The molecular mass of this 23-kDa species corresponded to a heterodimer between E10R-HA and A2.5L-V5. As expected, the mobility of this protein was decreased slightly when the extract from cells infected with the parental vE10R-HA virus expressing untagged A2.5L protein was analyzed (Fig. 2C). In addition, the putative heterodimer was present in purified virions (Fig. 2C). Most of the A2.5L protein was detected as the 23-kDa species throughout the course of infection (data not shown), suggesting that the heterodimer forms soon after synthesis of the component polypeptides and is incorporated as such into virions. Further characterization of this disulfide-bonded heterodimer is presented elsewhere (Senkevich *et al.,* 2002).

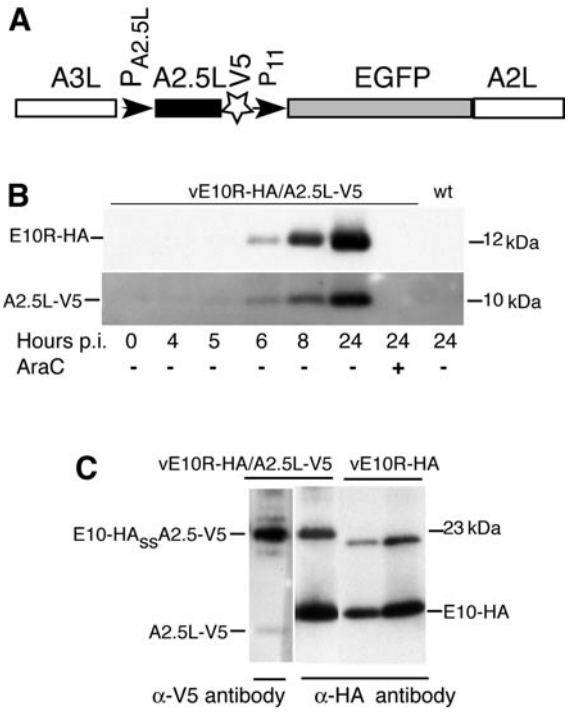
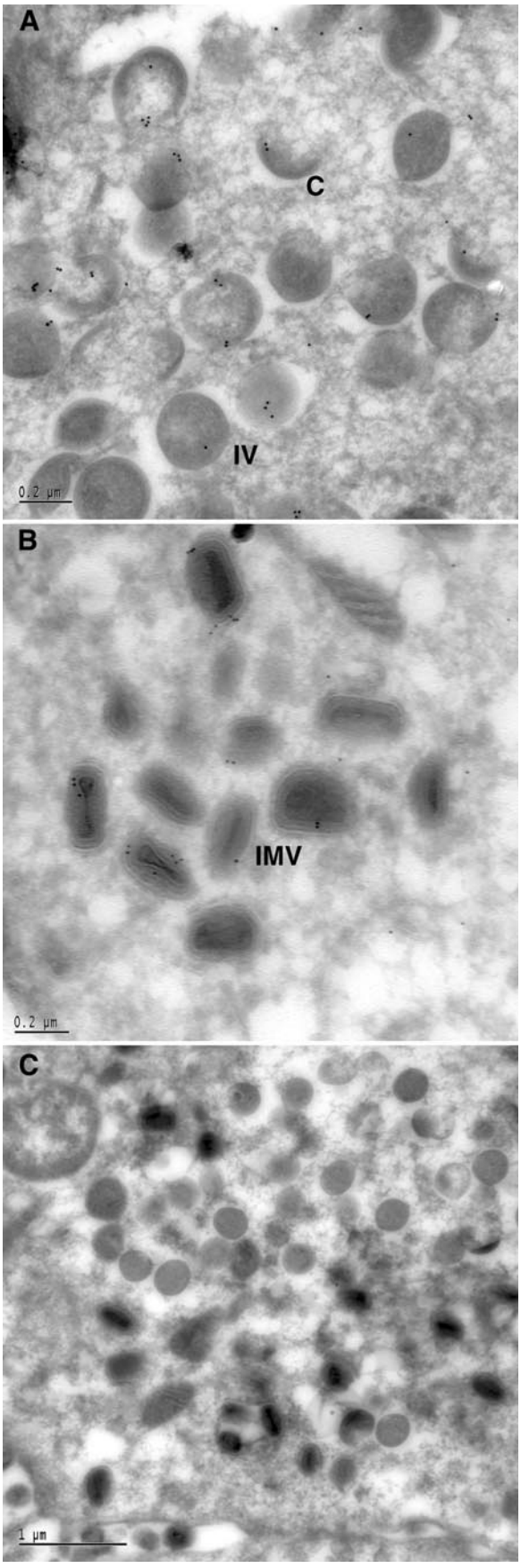


FIG. 2. Expression of the A2.5L-V5 and E10R-HA proteins in cells infected with vE10-HA/A2.5L-V5. (A) Construction of vE10-HA/vA2.5L-V5. The DNA used for the insertion of the V5 tag coding sequence at the 3′ end of the A2.5L gene is shown schematically. The enhanced green fluorescent protein (EGFP) gene was used as the reporter for the screening procedure. $P_{A2.5L}$ and P_{11} are the native A2.5L promoter and a strong late vaccinia virus promoter, respectively. This DNA was inserted into the vE10-HA virus by homologous recombination. (B) Time course of A2.5L-V5 and E10R-HA accumulation. Replicate cell cultures were infected with 5 PFU per cell of vE10R-HA/A2.5L-V5 or wild-type (wt) vaccinia virus as a control for 1 h; nonadsorbed virus was removed by washing the cell monolayers three times with medium, and incubation was continued for the indicated periods of time. Additional cells were incubated with AraC (40 μg/ml) for 2 h prior to the addition of the virus, and AraC was present continuously thereafter. Cells were collected at the indicated times postinfection (p.i.) and disrupted in SDS–PAGE loading buffer containing the reducing agent dithiothreitol. Proteins in total cell extracts were resolved by SDS–PAGE; E10R-HA and A2.5L-V5 were detected in parallel gels by Western blotting with anti-HA and anti-V5 antibody, respectively. The migration positions of standards, with indicated masses, analyzed on the same gel are shown on the right. (C) Detection of the E10R-A2.5L heterodimer ($E10\text{-}HA_{ss}A2.5\text{-}V5$). Cells infected as in the legend to (B) were collected at 24 h postinfection and disrupted in non-reducing SDS–PAGE loading buffer containing 20 M *N*-ethylmaleimide (Sigma). Duplicates of this sample were resolved in adjacent lanes of SDS–PAGE and analyzed by Western blotting with either anti-HA (first lane) or anti-V5 (second lane) antibody. Additional samples were prepared by the same procedure from cells infected with vE10R-HA for 24 h (third lane) or from vE10R-HA virions (fourth lane) purified as described previously (Senkevich *et al.,* 2000a).



A2.5L protein is a component of immature and mature virus particles

To determine the stage at which the A2.5L protein is incorporated into viral structures, ultrathin cryosections of cells infected with vE10R-HA/A2.5L-V5 were incubated with the anti-V5 antibody, followed by protein A conjugated to colloidal gold. Gold grains were associated with crescents, spherical immature virions, and brick-shaped intracellular mature virions (IMV) (Figs. 3A and 3B), as well as intracellular enveloped virions. The similar densities of the gold grains in immature and mature virions suggested that the association of the A2.5L protein with virus particles occurred at an early stage of morphogenesis. The specificity of protein A–gold labeling was demonstrated by the absence of significant background in cells infected with wild-type vaccinia virus (Fig. 3C).

Properties of an A2.5L inducible mutant

Because of the conservation of the A2.5L gene in all poxviruses, we suspected that its expression would be essential for virus replication in cell culture. A mutant with an inducible A2.5L gene (vA2.5Li) was constructed as previously described for a mutant with an inducible E10R gene (Senkevich *et al.,* 2000a). Briefly, this inducible mutant expressed the *Escherichia coli lac*-repressor, the bacteriophage T7 RNA polymerase regulated by the *lac*-operator and the A2.5L gene under the control of the T7 promoter, which was also regulated by the *lac*-operator (Fig. 4A). Thus, the *lac*-repressor inhibited two consecutive steps, the expression of the T7 polymerase and of the A2.5L gene, ensuring a high stringency of repression. Controlled expression of the inducible A2.5L gene was achieved by the addition of the desired concentration of isopropyl β-D-thiogalactoside (IPTG). The vA2.5L mutant virus did not form plaques in the absence of IPTG (Fig. 4B), and plaque number and size showed a positive correlation with the concentration of added IPTG, reaching the maximum at greater than 50 μM IPTG (data not shown). The plaques produced at optimal IPTG concentrations, however, were still slightly smaller than the plaques of the parental virus, vT7lacOI (Fig.4B).

To further assess the dependence of virus growth on the expression of the A2.5L gene, we determined the yield of cell-associated virus in the presence or absence of IPTG under one-step growth conditions (Fig. 4C). The replication of vA2.5Li was entirely dependent on the

FIG. 3. Immunoelectron microscopy of sections of cells infected with vE10R-HA/A2.5L-V5. Ultrathin cryosections of cells infected with vE10R-HA/A2.5L-V5 (A, B) or wild-type vaccinia virus (C) were incubated with monoclonal anti-V5 antibody and 10-nm gold particles conjugated to protein A. Typical fields containing predominantly IV (A) and IMV (B) are shown with a 0.2-μm marker. A larger field of the control wild-type virus is shown with a 1-μm marker. C, crescents; IV, immature virions; IMV, intracellular mature virions.

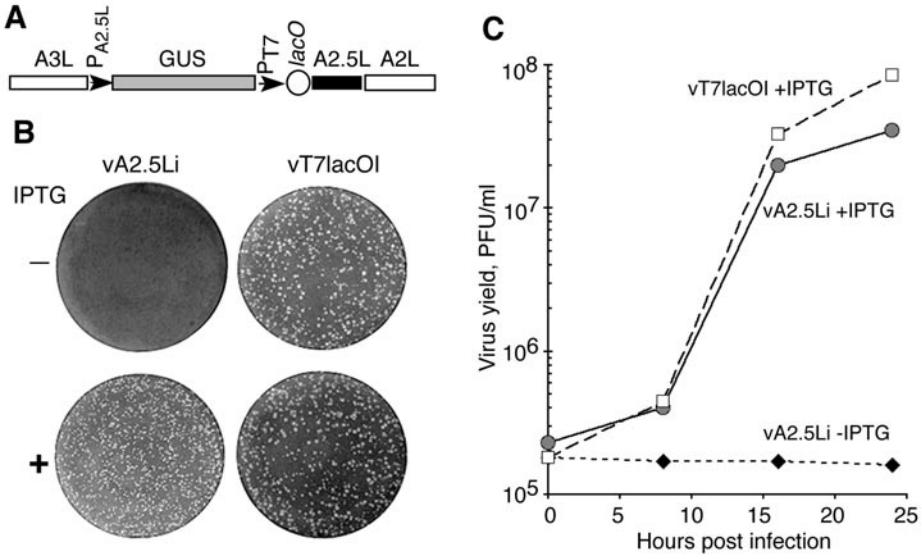


FIG. 4. Effect of IPTG on replication of vA2.5Li. (A) vA2.5Li. The DNA used for the insertion of the T7 promoter (PT7) and *lac*-operator (lacO) sequences upstream of the A2.5L gene is shown schematically. The β-glucuronidase (GUS) gene was used as the reporter. This construction was inserted into the vT7lacOI virus via homologous recombination. (B) BS-C-1 cell monolayers were infected with vA2.5Li or vT7lacOI in the presence or absence of 100 μM IPTG as indicated. Cells were stained with crystal violet at 48 h after infection. (C) The effect of IPTG on the replication of A2.5Li under one-step growth conditions. BS-C-1 cell monolayers were infected with 5 PFU of vA2.5Li or vT7lacOI per cell in the presence or absence of 100 μM IPTG. Cells were harvested at the indicated times after infection and the virus titers were determined by plaque assay in the presence of 100 μM IPTG.

addition of IPTG, as no increase in the amount of vA2.5Li was detected in the absence of IPTG during a 24-h period (Fig. 4C). In contrast, in the presence of 100 μM IPTG, the amount of vA2.5Li increased more than 100-fold during this time. The kinetics of replication of the vA2.5Li in the presence of IPTG was similar to that of the parental vT7lacOI, except that the total accumulation of vA2.5Li was slightly reduced (Fig. 4C). The yield of the vA2.5Li increased dramatically when the concentration of IPTG was raised from 0 to 75 μM; a further rise in the IPTG concentration up to 1 mM had no effect on the virus yield (not shown).

Trans-complementation of vA2.5Li and the functional requirement of the CxxxC motif

To confirm that the defect in vA2.5Li was caused solely by the repression of the A2.5L gene and not by effects on neighboring genes, we sought to demonstrate complementation by *trans* expression of A2.5L from a plasmid. To this end, cells infected with vA2.5Li in the absence of IPTG were transfected with a plasmid containing the V5-tagged A2.5L gene under the control of the vaccinia virus strong late P11 promoter. The plasmid-expressed A2.5L rescued the mutant, with the virus yield increasing more than 10-fold (Fig. 5). Under these complementation conditions, the yield of the virus was several times lower than that in the presence of inducer (Fig.5), probably reflecting the relative inefficiency of transfection.

The A2.5L protein contains only two cysteines (Cys17 and Cys21), which are conserved in all poxviruses. A2.5L

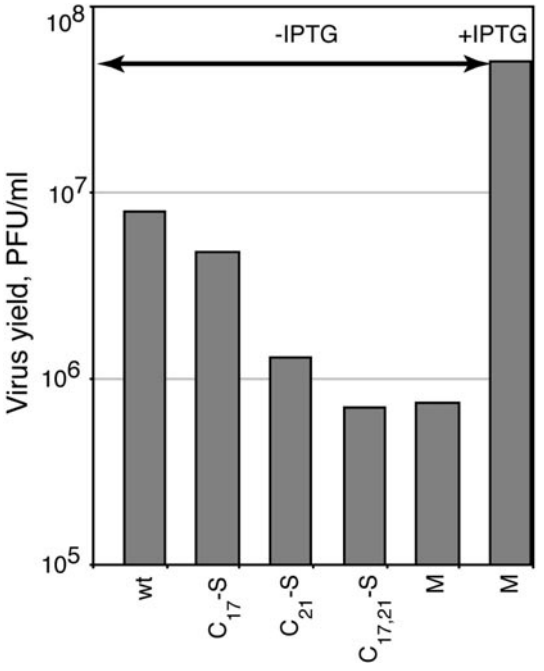


FIG. 5. Complementation of vA2.5Li with plasmids expressing wild-type or mutated A2.5L-V5. BS-C-1 cell monolayers were infected with vA2.5Li in the presence or absence of 100 μM IPTG. After 2 h, the cells were transfected with plasmids containing wild-type or mutated (cysteine(s) replaced with serine is indicated for each mutant) A2.5L-V5 gene under control of the strong late promoter (P11) or mock-transfected (M). Cells were harvested after 18 h and the virus titers were determined by plaque assay in the presence of 100 μM IPTG.

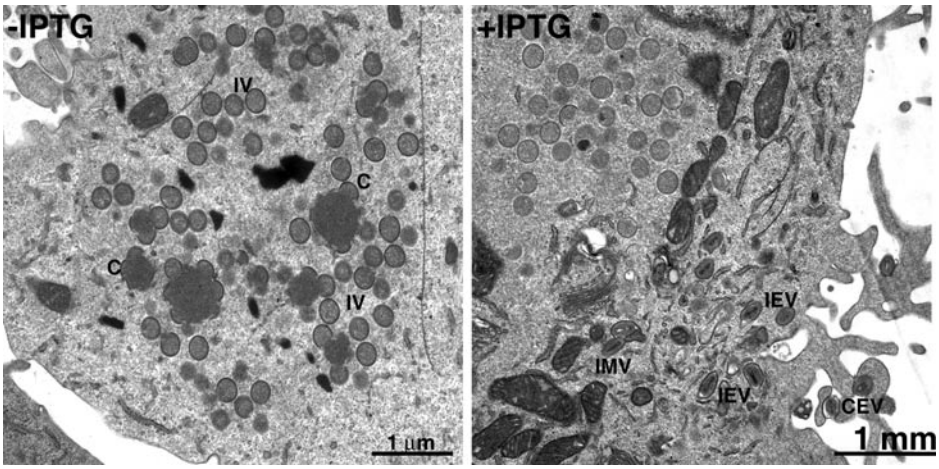


FIG. 6. Electron microscopy of vA2.5Li-infected cells in the presence or absence of IPTG. BS-C-1 cell monolayers were infected for 24 h with vA2.5Li in the presence or absence of 100 μM IPTG, fixed, and embedded in EPON, and ultrathin sections were prepared. C, crescents; IV, immature virions; IMV, intracellular mature virions; IEV, intracellular enveloped virions; CEV, cell-associated enveloped virions. Bars, magnification.

orthologs form a CxxxC pattern in orthopoxviruses and a CxxC pattern in other poxviruses (Fig. 1). We proposed elsewhere that this Cxx(x)C motif functions as a redox active site (Senkevich *et al.,* 1997) and subsequently demonstrated that A2.5L is involved in a vaccinia virus-specific disulfide bond-formation pathway (Senkevich *et al.,* 2002). At least one of these two cysteines should be involved in the formation of the E10R–A2.5L disulfide-bonded heterodimer (Fig. 2C). We assessed the ability of plasmids in which the Cys17, Cys21, or both cysteines of A2.5L were replaced by serines to complement the defect in vA2.5Li in the absence of IPTG (Fig. 5). The replacement of both cysteines of A2.5L completely abolished the ability of the plasmid to rescue virus replication; the replacement of Cys 21 had nearly as severe an effect, whereas the replacement of Cys17 had little effect (Fig. 5). The mutated and wild-type A2.5L proteins were all expressed at approximately the same level (data not shown), indicating that the complementation defects did not result from different levels of expression or from the instability of the mutated proteins.

The A2.5L protein is essential for the formation of mature intracellular virus particles

The above experiments indicated that A2.5L protein is synthesized at late times, is associated with IMV, and is required for virus replication. The effect of A2.5L repression on virion morphogenesis was investigated using transmission electron microscopy. Cells infected with vA2.5Li in the presence of IPTG contained the expected range of virus structures, indistinguishable from those produced by the parental vT7lacOI or wild-type virus (Fig. 6). Such infected cells contained clusters of mature virions and intracellular enveloped virions, as well as immature virions (IV) and some precursor crescent membranes. In the absence of IPTG, however, mature virions were rare and typical fields of vA2.5Li-infected cells contained crescents adjacent to large masses of granular material and numerous IVs (Fig. 6).

DISCUSSION

This study provides the initial biochemical, genetic, and electron microscopic analysis of the previously uncharacterized product of a vaccinia virus ORF encoding a protein with predicted molecular mass of 8.9 kDa. We assigned the name A2.5L to the gene and its product since the former is located between the previously recognized A2L and A3L genes. The A2.5L gene was not identified in the complete genome sequence of the Copenhagen strain of vaccinia virus because of an omitted residue leading to a frame shift in the coding sequence. After the correct sequence was restored, standard database searches showed that the A2.5L gene is present in all available genomes of chordopoxviruses. Detecting the orthologs of A2.5L in entomopoxviruses turned out to be a nontrivial task because of the small size of the protein and relatively low sequence conservation. However, once candidates were identified using a specially designed sequence analysis protocol, the conservation of the Cxx(x)C motif, another C-terminal motif, and the predicted secondary structure strongly suggested that entomopoxviruses indeed encode A2.5L orthologs. Thus, we concluded that the A2.5L gene belongs to the set of conserved poxvirus genes and should have an essential role in the poxvirus life cycle.

The experimental data supported a vital role for the A2.5L gene; its repression severely inhibited virus reproduction and plaque formation. Electron microscopy indicated that the block in virus reproduction occurred after the formation of crescents and spherical immature particles, preventing later stages of morphogenesis. A further analysis showed that A2.5L, together with the two

other vaccinia virus thiol-redox proteins E10R and G4L, participates in the formation of the disulfide bonds in certain virion membrane proteins, including L1R (Senkevich *et al.,* 2002). Notably, the same stage of virus morphogenesis was interrupted when any of these four genes (A2.5L, E10R, G4L, and L1R) were repressed (Ravanello and Hruby, 1994; Senkevich *et al.,* 2002; White *et al.,* 2000; and the present work). Our finding that the A2.5L protein is required at the same stage of virus morphogenesis as E10R and G4L is consistent with their roles in a common disulfide bond pathway rather than independent roles as structural proteins. Moreover, the functional impairment of E10R and G4L that occurred upon cysteine to serine mutations in their CxxC motifs indicated that the redox activity of these proteins is crucial for virus reproduction (Senkevich *et al.,* 2000a; White *et al.,* 2002). The inability of mutated A2.5L, with both cysteines changed to serines, to complement virus growth and the lesser effect of the Cys17 replacement were consistent with the effects of such mutations on L1R disulfide bond formation (Senkevich *et al.,* 2002). The E10R and G4L proteins belong to the widespread ERV1/ALR and thioredoxin superfamilies, respectively, which interact in at least one known cellular disulfide bond-formation pathway (Sevier *et al.,* 2001). In contrast, A2.5L appears to be a unique poxvirus protein with no known homologues to suggest its evolutionary origin. Further computational and experimental analyses of proteins involved in disulfide bond formation will show whether distant homologues or nonhomologous functional counterparts of A2.5L are present in other systems.

MATERIALS AND METHODS

Cells and viruses

Standard procedures for preparation and maintenance of cell cultures, propagation, titration, and purification of vaccinia virus were described previously (Earl *et al.,* 2001). BS-C-1 cells (ATTC CCL6) were used for all experiments. All recombinant vaccinia viruses were derived from the WR strain (ATTCVr119). VT7lacOI (Alexander *et al.,* 1992), the parental virus for generating the IPTG-inducible mutant, contains an IPTG-inducible copy of the bacteriophage T7 RNA polymerase gene and the *E. coli lac*-repressor gene inserted into the nonessential thymidine kinase locus. VA2.5Li is a vaccinia virus recombinant with an inducible A2.5L gene (see below). VE10R-HA is a recombinant vaccinia virus containing the E10R gene with a C-terminal HA tag; its construction was described in detail previously (Senkevich *et al.,* 2000). vE10R-HA/A2.5L-V5 is a derivative of vE10R-HA containing the V5 tag on the C-terminus of A2.5L gene (see below).

Construction of vE10R-HA/A2.5L-V5 and vA2.5Li

vE10R-HA/A2.5L-V5 was made from vE10R-HA by attaching the sequence GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG encoding the 14-amino-acid-long V5 epitope (GKPIPNPLLGLDST) to the 3′-terminus of the A2.5L gene. The procedure was the same as that for the construction of vE10R-HA (Senkevich *et al.,* 2000a), the only difference being that enhanced green fluorescent protein (EGFP) was used as a screening marker instead of the *E. coli* β-glucuronidase gene. In brief, recombinant PCR was used to join individual PCR products (Expand High Fidelity PCR System, Roche Molecular Biochemicals) and assemble a DNA segment containing the A2.5L gene with the V5 epitope tag sequence, followed by the EGFP gene under control of the vaccinia strong late P11 promoter, and flanking sequences of ~500 bp at both ends to allow homologous recombination into the vaccinia virus genome (Fig. 3B). This final PCR product was used directly for homologous recombination with vE10R-HA. The green fluorescent plaques were visualized under an inverted fluorescent microscope (Leica) with a standard FITC (fluorescein) filter set and picked at 48 h after infection.

vA2.5Li, the recombinant vaccinia virus with the IPTG-inducible A2.5L gene, was made from vT7lacOI using the procedure described previously for the construction of vE10Ri (Senkevich *et al.,* 2000a). The final product (Fig. 2B) was assembled using recombinant PCR and inserted into vT7lacOI by homologous recombination. In addition to the *lac*-repressor and the IPTG-inducible T7 polymerase of the parental vT7lacOI, vA2.5Li contains the A2.5L gene under the control of the *lac*-operator-regulated T7 promoter.

Expression vectors

The A2.5L gene was amplified by PCR (Expand High Fidelity PCR System) using DNA from the WR strain as a template and cloned in the pGEM-T easy vector (Promega). The strong late P11 promoter sequence [GAATTTCATTTTGTTTTTTTCTATGCTATAAATG (the start codon is underlined)] was included at the 5′ end of the forward PCR primer and the V5 epitope coding sequence was included at the 3′ end of the reverse PCR primer. Cysteine to serine mutations of the A2.5L gene were constructed by recombinant PCR using primers containing the corresponding nucleotide substitutions. All constructs were verified by sequencing.

Transfection of vaccinia virus-infected cells with expression plasmids

BS-C-1 cells in a 24-well plate were infected with five plaque-forming units of virus per cell and 2 h later were transfected with 0.25 μg of plasmid that had been preincubated with 2 μl of Lipofectamine 2000 (Invitrogen)

according to the manufacturer's protocol. Where indicated, IPTG was added to a final concentration of 100 μM at 2 h after transfection.

Western blot analysis

Infected or infected and transfected cells were collected by centrifugation and solubilized in nonreducing SDS-loading buffer (Invitrogen) containing 20 mM *N*-ethylmaleimide (NEM; Sigma) or NuPage sample reducing agent (Invitrogen). All lysates were sonicated and boiled. Proteins were resolved by SDS–PAGE in 10–20% Tricine gels (Invitrogen). After PAGE, proteins were transferred to a nitrocellulose membrane, incubated with peroxidase-conjugated anti-HA monoclonal rat high-affinity antibody (3F10, Roche Molecular Biochemicals) or mouse monoclonal anti-V5 antibody (Invitrogen), and detected with a chemiluminescence detection kit (Pierce).

Electron microscopy

Electron microscopy was performed as described previously (Wolffe *et al.,* 1996), except that BS-C-1 cells were used in all procedures. For immunoelectron microscopy, mouse monoclonal anti-V5 antibody (Invitrogen) was used at a 1:250 dilution, followed by the addition of colloidal gold-conjugated protein A.

Protein sequence analysis

The nonredundant protein sequence database (NIH) was searched using the BLASTP program, and iterative searches were performed using the PSI-BLAST program (Altschul *et al.,* 1997). Position-specific scoring matrices were generated using the –C option and run using the –R option of PSI-BLAST (Schaffer *et al.,* 1999). Multiple sequence alignments were constructed using the CLUSTAL-X program (Thompson *et al.,* 1997) and adjusted on the basis of PSI-BLAST search results. Protein secondary structure was predicted using the PHD program, with multiple sequence alignments submitted as queries (Rost *et al.,* 1994).

REFERENCES

Alexander, W. A., Moss, B., and Fuerst, T. R. (1992). Regulated expression of foreign genes in vaccinia virus under the control of bacteriophage T7 RNA polymerase and the *Escherichia coli lac* repressor. *J. Virol.* **66,** 2934–2942.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.

Davison, A. J., and Moss, B. (1989). The structure of vaccinia virus late promoters. *J. Mol. Biol.* **210,** 771–784.

Earl, P. L., Moss, B., Wyatt, L. S., and Carroll, M. W. (2001). Generation of recombinant vaccinia viruses. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds.), Vol. 2, pp. 16.17.1–16.17.16. Greene Pub. Assoc./Wiley–Interscience, New York.

McCraith, S., Holtzman, T., Moss, B., and Fields, S. (2000). Genome-wide analysis of vaccinia virus protein–protein interactions. *Proc. Natl. Acad. Sci. USA* **97,** 4879–4884.

Moss, B. (2001). Poxviridae: The viruses and their replication. *In* "Fields Virology" (D. M. Knipe and P. M. Howley, Eds.), Vol. 2, 4th ed., pp. 2849–2883. Lippincott–Williams & Wilkins, Philadelphia.

Ravanello, M. P., and Hruby, D. E. (1994). Conditional lethal expression of the vaccinia virus L1R myrstylated protein reveals a role in virus assembly. *J. Virol.* **68,** 6401–6410.

Rost, B., Sander, C., and Schneider, R. (1994). PHD—An automatic mail server for protein secondary structure prediction. *Comput. Appl. Biosci.* **10,** 53–60.

Schaffer, A. A., Wolf, Y. I., Ponting, C. P., Koonin, E. V., Aravind, L., and Altschul, S. F. (1999). IMPALA: Matching a protein sequence against a collection of PSI-BLAST-constructed position-specific score matrices. *Bioinformatics* **15,** 1000–1011.

Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. (1997). The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. *Virology* **233,** 19–42.

Senkevich, T. G., Weisberg, A., and Moss, B. (2000a). Vaccinia virus E10R protein is associated with the membranes of intracellular mature virions and has a role in morphogenesis. *Virology* **278,** 244–252.

Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2000b). A viral member of the ERV1/ALR protein family participates in a cytoplasmic pathway of disulfide bond formation. *Proc. Natl. Acad. Sci. USA* **97,** 12068–12073.

Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2002). Complete pathway for protein disulfide bond formation encoded by poxviruses. *Proc. Natl. Acad. Sci. USA* **99,** 6667–6672.

Sevier, C. S., Cuozzo, J. W., Vala, A., Aslund, F., and Kaiser, C. A. (2001). A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation. *Nat. Cell Biol.* **3,** 874–882.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25,** 4876–4882.

Weinrich, S. L., and Hruby, D. E. (1986). A tandemly-oriented late gene cluster within the vaccinia virus genome. *Nucleic Acids Res.* **14,** 3003–3016.

White, C. L., Senkevich, T. G., and Moss, B. (2002). Vaccinia virus G4L glutaredoxin is an essential intermediate of a cytoplasmic disulfide bond pathway required for virion assembly. *J. Virol.* **76,** 467–472.

White, C. L., Weisberg, A. S., and Moss, B. (2000). A glutaredoxin, encoded by the G4L gene of vaccinia virus, is essential for virion morphogenesis. *J. Virol.* **74,** 9175–9183.