Unique Temperature-Sensitive Defect in Vaccinia Virus Morphogenesis Maps to a Single Nucleotide Substitution in the A30L Gene

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Marker rescue experiments demonstrated that the genetic lesion of a previously isolated vaccinia virus temperature-sensitive mutant which forms multilayered envelope structures with lucent interiors and foci of viroplasm with dense centers mapped to the A30L open reading frame. A single base change, resulting in a nonconservative Ser-to-Phe substitution at residue 17, was associated with degradation of the A30L protein at elevated temperatures.

Poxviruses are complex, enveloped DNA viruses that replicate entirely in the cytoplasm of infected cells (4). The morphogenesis of vaccinia virus, the prototype member of the poxvirus family, has been studied almost entirely by electron microscopy with the aid of conditional lethal mutants and specific antibodies. Dales and coworkers (1) characterized the defects in morphogenesis of a large collection of vaccinia virus temperature-sensitive (*ts*) mutants. The mutants were divided into 17 categories based on the stage of virion assembly that was reached at the restrictive temperature. Thus, category A mutants failed to form recognizable virus structures, category B mutants induced foci of viroplasm but no viral membranes, category C mutants elicited rudimentary viral membranes, and so on up to category Q, in which the virus particles appeared normal but lacked infectivity. Despite the potential value of this set of mutants, few were biochemically characterized or genetically mapped. We became interested in *ts6757*, an F-category mutant that was described as forming aberrant membrane structures with lucent centers and dense foci of viroplasm (1). Electron micrographs of cells infected with *ts6757* under nonpermissive conditions resembled those of a conditionally lethal mutant with an inducible A30L gene (6). The *ts6757* mutant was originally isolated from a stock of vaccinia virus IHD-W (a hemagglutinin-negative derivative of IHD-J) that had been treated with 1-methyl-3-nitro-1-nitrosoguanidine and then propagated in a derivative of mouse L₉₂₉ cells in the presence of bromodeoxyuridine (1). The *ts6757* mutant was characterized as forming small plaques at 33°C and very small plaques at 40°C in a mouse cell line.

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FIG. 1. Analysis of the synthesis and processing of viral proteins in cells infected with *ts6757* at permissive and nonpermissive temperatures. BS-C-1 cells were infected at a multiplicity of infection of 10 with vaccinia virus strain IHD-J at 37°C in the presence (+) or absence (–) of the drug rifampin (RIF) or with *ts6757* at 31 or 39°C. At 9 h after infection, replicate cultures were pulse-labeled for 30 min with [³⁵S] methionine. One set of cells was then harvested (Pulse), and another was washed and then incubated for 15 h in medium containing unlabeled methionine (Chase). The indicated drug and temperature conditions were maintained during the pulse and chase. Lysates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The masses and positions of marker proteins are shown on the left. The positions of precursor (P4a and P4b) and processed (4a and 4b) core proteins are indicated on the right.

FIG. 2. Marker rescue of *ts6757*. BS-C-1 cells were transfected with either the empty plasmid vector pGEM5Zf(+) or with the plasmid containing the A30L ORF and about 800 bp (left side) and 900 bp (right side) of flanking DNA from vaccinia virus WR (A30WR), IHD-J (A30IHDJ), or *ts6757* (A30TS6757). At 4 h after the transfection, the cells were infected with *ts6757* at a multiplicity of infection of 0.05 and incubated at 39°C for 3 days. The cells were harvested, and in each case the lysates were diluted 10-fold and two 0.5-ml samples were used to infect fresh BS-C-1 monolayers at 39°C. After 2 days, the monolayers were stained with crystal violet and photographed. The titers obtained after transfection of A30WR DNA and A30IHDJ DNA were 2.2×10^3 and 2.1×10^3 , respectively. In contrast, no plaques at 39°C were detected when either A30TS6757 DNA or pGEM5Zf(+) DNA was transfected.

We acquired *ts6757*, which had been deposited by S. Dales in the American Type Culture Collection, and confirmed the stringency of the mutant by plaque assay using BS-C-1 monkey cells at 39°C. Because the phenotype of the mutant had not been biochemically characterized, we analyzed viral protein synthesis at 31 and 39°C. Vaccinia virus gene expression is

FIG. 3. Stability of A30L protein of *ts6757* under permissive and nonpermissive conditions. BS-C-1 cells were infected with vaccinia virus WR, IHD-J, or *ts6757* at a multiplicity of infection of 10 and incubated at 31, 37, and 39°C. After 24 h, the cells were harvested and whole-cell lysates were analyzed by electrophoresis on a 10 to 20% polyacrylamide gel in SDS-Tricine buffer. The resolved proteins were transferred to nitrocellulose and analyzed by Western blotting using an antiserum directed to the 11 C-terminal amino acids of the A30L protein (upper panel). The blot was then stripped and reprobed with antibody to the G4L protein (lower panel). The masses and positions of the marker proteins are shown on the left. The positions of the A30L and G4L proteins are shown on the right. Lane U, uninfected cells at 37°C.

FIG. 4. Visualization of immature envelope structures formed by *ts6757* and vA30Li under nonpermissive conditions. BS-C-1 cells were infected with 10 PFU of either vA30Li in the absence of an inducer at 37°C (A) or *ts6757* at 39°C (B). After 20 h, the cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were then prepared by osmication, dehydration, and embedding in Epon resin. Thin sections were obtained, placed on grids, and stained with uranyl acetate and Reynold's lead citrate. Images were acquired using a Philips CM100 electron microscope. Arrows point to examples of multilayered membranes.

FIG. 5. Effect of temperature on the morphology of globular viroplasm. BS-C-1 cells were infected with vA30Li in the absence of an inducer at 37°C (A) or 39°C (B) or with *ts6757* at 37°C (C) or 39°C (D) and processed for transmission electron microscopy as described in the legend to Fig. 4.

temporally regulated, and viral DNA replication is required for synthesis of the intermediate- and late-stage proteins. Thus, an assessment of the stage at which virus growth is inhibited can be made by labeling infected cells at various times with radioactive amino acids and resolving the proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography. It was evident from such an analysis of *ts6757* that the switch from early to late gene expression occurred normally. An autoradiogram displaying similar late protein band patterns from cells infected with the wild-type virus or the *ts* mutant at 31 and 39°C is shown in Fig. 1.

The morphogenesis of vaccinia virus involves the proteolytic cleavage of certain core proteins (2, 3, 5). Typically, mutants blocked at an early step of morphogenesis exhibit a defect in protein processing. Pulse-chase experiments indicated that cleavage of the major core proteins P4a and P4b was inhibited at the nonpermissive temperature (Fig. 1), which is consistent with such a morphogenesis block. This defect was similar to that which occurred when assembly was blocked with the drug rifampin (Fig. 1).

Because the characteristics of *ts6757* were similar to those of vA30Li, we tried to rescue the mutant by transfection of DNA containing the wild-type A30L gene. Successful marker rescue was expected to produce recombinants capable of replicating and forming plaques at the nonpermissive temperature of 39°C. The A30L open reading frame (ORF) and flanking sequences were amplified by PCR of DNA from vaccinia virus strain WR, IHD-J, or *ts6757* and inserted into a pGEM plasmid to form pGEMA30WR, pGEMA30IHDJ, or pGEMA30TS, respectively. Numerous plaques ($>2 \times 10^3$ per ml) that formed at 39°C were observed using lysates from cells infected with *ts6757* and transfected with the A30L gene from WR or IHD-J (Fig. 2). In contrast, no plaques (<10 per ml) were observed when BS-C-1 monolayers were inoculated with lysates from *ts6757*-infected cells transfected either with the A30L gene derived from the *ts6757* mutant or with the vector alone (Fig. 2). These results indicated that a mutation within the A30L ORF or flanking sequence was responsible for the *ts* phenotype of *ts6757*. To identify the mutation, the entire A30L ORF and the flanking sequences in the plasmid vectors used for transfection were sequenced. Except for a few silent mutations, only one difference in the DNA of WR, IHD-J, or *ts6757* was found: a C-to-T substitution resulting in a Ser-to-Phe change in amino acid 17 of the A30L ORF product of *ts6757*. Based on these results, we concluded that the single nonconservative amino acid substitution was responsible for the *ts* phenotype. The identity of the WR and IHD-J sequences was not surprising, as both were derived from the New York City Board of Health strain of vaccinia virus.

The phenotype of a *ts* mutant can result from misfolding of the affected protein at the nonpermissive temperature, leading either to instability or to malfunction. The effect of the Ser-to-Phe substitution on the stability of the A30L protein was determined. BS-C-1 cells were infected with *ts6757*, WR, or IHD-J and incubated for 24 h at 31, 37, or 39°C. Lysates were analyzed by electrophoresis on a 10 to 20% polyacrylamide-SDS-Tricine gel and by Western blotting using an A30L anti-peptide polyclonal antibody (6). At the permissive temperature of 31°C, the intensities of the bands corresponding to the A30L protein in cells infected with WR, IHD-J, or *ts6757* were sim-

ilar (Fig. 3). In contrast, at the nonpermissive temperatures of 37 and 39°C, only a faint band was observed in cells infected with *ts6757* compared to those in cells infected with the wild-type WR and IHD-J viruses (Fig. 3). The specificity of this effect was demonstrated by stripping and reprobing the blot with antibody to the viral late cytoplasmic protein encoded by the G4L gene. The stability of the G4L protein was unaffected by temperature (Fig. 3). These results indicated that the Ser-to-Phe change rendered the A30L protein unstable at nonpermissive temperatures.

If the phenotype of *ts6757* was caused by the instability of the A30L protein, then electron microscopic images at the nonpermissive temperature should be virtually identical to those of vA30Li in the absence of an inducer, since in both cases the defect is due to the deficiency of the A30L protein. Indeed, nonpermissive infections with either mutant were reported to result in the accumulation of envelope structures with lucent interiors, some of which had a multilayered or onionskin appearance (1, 6). However, infections with *ts6757* caused the formation of large globular cytoplasmic masses containing a dense core contiguous with a distinctive lacelike outer layer (1), whereas the corresponding masses in cells infected with vA30Li were uniformly dense (6). This discrepancy could be attributed to (i) different parental virus strains (IHD-J versus WR), (ii) different host cells (RK-13 versus BS-C-1), (iii) different incubation temperatures (39 versus 37°C), or (iv) different fixation and staining procedures used in the two laboratories. To discriminate between these possibilities, we compared the phenotypes of the two mutant viruses under identical conditions. We confirmed the accumulation of envelope structures with lucent centers, some of which had multilayered membranes, in cells infected with *ts6757* or vA30Li under nonpermissive conditions (Fig. 4). We also confirmed the appearances of the globular masses. Except for holes containing normal cytoplasm, they were uniformly dense out to their margins in cells infected with vA30Li at 37°C in the absence of the inducer (Fig. 5A), whereas they had dense centers and lacelike outer regions in cells infected with *ts6757* at 39°C (Fig. 5D). However, when cells were infected with vA30Li at 39°C in the absence of the inducer, the outer layer of the masses also had a lacelike appearance (Fig. 5B). Furthermore, when cells were infected with *ts6757* at 37°C, which is still nonpermissive, the cytoplasmic masses were uniformly dense (Fig. 5C). Remarkably, the distinctive morphology was entirely due to a 2°C difference in temperature. Among the *ts* mutants analyzed by Dales and coworkers (1), the lacelike pattern around globular dense structures was unique to *ts6757*, implying that it is specifically related to the loss of the A30L protein. One possible explanation is that a putative A30L-binding protein becomes heat sensitive when it is not complexed with its partner.

In conclusion, despite different genetic backgrounds and types of conditional lethal mutations, the nonpermissive phenotypes of vA30Li and *ts6757* are virtually identical. This is undoubtedly due to the fact that both viruses behave as if they have null mutations: the A30L gene of vA30Li is repressed, whereas the A30L protein of *ts6757* is unstable.

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