Vaccinia virus replication requires active participation of the host cell transcriptional apparatus

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ABSTRACT The ability of vaccinia virus to replicate in BSC-40 monkey cells whose nuclei have been functionally inactivated was examined. Exposure of cell monolayers to ultraviolet radiation at doses that did not alter the cells’ capacity to support a subsequent infection by a cytoplasmic virus (vesicular stomatitis virus) caused a reduction to less than 10% in the observed yield of infectious progeny from vaccinia virus and herpes simplex virus (type I) infections. Similarly, replication of vaccinia virus was reduced to 5% by treatment of BCS-40 cells with α-amanitin (10 μg/ml), a potent inhibitor of nuclear mRNA synthesis. In both situations, ultraviolet irradiation and α-amanitin treatment, early and late vaccinia viral genes were expressed at high levels, but the newly synthesized virion components were not assembled into mature infectious particles. Taken together, these data suggest that the active involvement of the host cell nuclear transcriptional system is obligatory in the vaccinia virus replicative cycle.

Thought regarding a direct involvement of the host cell nucleus in poxvirus replication has undergone rather dramatic inversions within the last 10 years. Recent interest in this problem was sparked by LaColla and Weissbach (1), who found appreciable synthesis of vaccinia virus (VV) DNA in the nuclear fraction of infected HeLa cells after short pulses with [3H]-thymidine. Their paper also presents a thorough discussion of prior work relating to the question of nuclear participation in poxvirus replication. Evidence for a nuclear involvement in avian poxvirus replication came from morphological studies of nuclear inclusions (2), and recently virus-specific DNA, RNA, and protein have also been detected in the nucleus of fowlpox virus-infected cells (3, 4). Similar results were obtained with Yaba virus (5).

After the realization that VV possessed a virion transcription system, it was demonstrated that uncoating and viral DNA synthesis could occur in enucleated L cells (6). Further studies on the ability of VV to replicate in an enucleated monkey cell line confirmed these results but also demonstrated that enucleates failed to produce infectious progeny virus particles (7). The latter work suggested that some defect that was expressed as a failure to obtain mature infectious progeny occurred in enucleated cells. In these studies, however, the ability of the enucleates to synthesize normal amounts of viral DNA and late proteins was considerably below that observed in whole cells. This left open the possibility that failure to obtain mature virus might be due to a general cytopathic effect rather than to the absence of specific nuclear function(s). Recent studies in our laboratory (8) have confirmed and extended the findings of these previous workers in enucleates prepared from a number of different cell lines under conditions that resulted in little or no reduction in the amounts of virus-specific macromolecular syntheses. Thus it is unlikely that a failure to produce infectious progeny is related to a "winding-down" of the ability of enucleates to support virus-specific functions. Further studies of these enucleated cells by electron microscopy revealed a failure to produce normal mature or even immature viral particles. Therefore, it was apparent that, in the physical absence of the host cell nucleus, VV was unable to properly assemble viral constituents into mature progeny virus particles.

The experiments reported here have investigated the specific nature of the apparent nuclear involvement in the VV growth cycle in infected whole cells whose nuclei have been selectively inhibited. Results have been obtained that, surprisingly, suggest that the active expression of cell-coded function(s) is required for VV replication.

MATERIALS AND METHODS

Viruses and Cells. VV, WR strain, was obtained from the American Type Culture Collection and purified twice by selecting single plaques. Herpes simplex virus, type 1 (HSV-1), was provided by T. North. Purified vesicular stomatitis virus (VSV), Indiana strain, was donated by L. A. Ball.

BSC-40 monkey cells (M. Ensinger) were grown in Eagle’s minimal essential medium plus 10% heat-inactivated fetal calf serum. Mouse L cells (I. Tamm) were maintained in Eagle’s medium plus 5% heat-inactivated fetal calf serum.

Ultraviolet (UV) Irradiation. Subconfluent monolayers of BSC-40 cells were grown in 35 × 10 mm dishes (Falcon). Growth medium was removed and the cells were washed twice with 37°C phosphate-buffered saline. Monolayers were then exposed to a prewarmed 15-W germicidal lamp (General Electric no. G8T5, with emission primarily at 254 nm) at a distance of 30 cm [24 erg mm−2 sec−1 (1 erg = 10−7 J)] for the indicated periods of time. The UV treatment was terminated by replacing the covers on the dishes. Two milliliters of 37°C medium was added to each plate and the cells were equilibrated at 37°C for 30 min prior to further experimentation.

α-Amanitin. A sterile stock solution of α-amanitin (Sigma), 1 mg/ml in water, was used. The medium was removed from 35 × 10 mm dishes of BSC-40 cells and replaced with 1 ml per dish of α-amanitin-containing medium and placed in a 37°C incubator overnight. It was found that 18–24 hr of drug pretreatment was required to equilibrate the effective drug dosage. Also, α-amanitin was present throughout the course of viral infection to prevent changes in internal drug concentration.

Virus Growth. Medium was removed from 35 × 10 mm dishes of BSC-40 cells and the monolayers were washed twice with 37°C Eagle’s minimal essential medium (without serum). Next, virus inocula (0.2 ml), diluted to the desired multiplicity with phosphate-buffered saline plus 1 mM MgCl2, were added

Abbreviations: VV, vaccinia virus; VSV, vesicular stomatitis virus; HSV-1, herpes simplex virus (type I); UV, ultraviolet.

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and the plates were incubated at 25°C with occasional rocking. After 30 min the inocula were removed, monolayers were washed twice with phosphate-buffered saline to remove unadsorbed virus, and 1 ml of medium per dish was added. Infected cells were incubated at 37°C for 24 hr. Dishes were then subjected to 2 cycles of freeze-thawing, duplicates were pooled, and the crude lysates were titered as described (8), except that HSV-1 and VSV samples were not trypsinized, to determine total plaque-forming units produced. VV and HSV-1 were titered on BSC-40 cell monolayers; VSV was assayed on L cell monolayers.

Incorporation of radioactive precursors and analysis of VV-specific macromolecules synthesized in infected cells were carried out exactly as reported (8).

Radioisotopes and Materials. [methyl-3H]Thymidine (55.6 Ci/mmol; 1 Ci = 3.7×10¹⁰ becquerels) and [35S]methionine (647 Ci/mmol) were obtained from New England Nuclear. All tissue culture media and sera were from GIBCO. Pancreatic deoxyribonuclease I (DNase) (2839 units/mg) was purchased from Worthington.

RESULTS

Absence of VV Growth in UV-Irradiated BSC-40 Cells. To functionally inactivate the nuclei within intact BSC-40 cells, monolayers were exposed to low levels of UV irradiation. The primary result of such treatment is to introduce pyrimidine dimers in regions of nucleic acids that contain adjacent pyrimidines (9-11). Both DNA replication and RNA transcription are blocked beyond such dimers (12). Under the experimental conditions used here, DNA and RNA syntheses were reduced to 10-20%, as measured by incorporation of radioactive precursors, and these nucleic acid biosynthetic capabilities were not regained during the course of the experiments. Protein synthesis, and presumably the structural integrity of most small molecules, was relatively unaffected. Fig. 1 shows the results of exposing BSC-40 cells to increasing amounts of UV irradiation and then infecting with VV, VSV, or HSV-1. The growth of VSV (a cytoplasmic virus) was not inhibited, thereby confirming that the cytoplasm of the UV-irradiated BSC-40 cells was still capable of supporting viral macromolecular synthesis. In contrast, replication of HSV-1 (a nuclear virus) was reduced to less than 10%, as has been reported (13). Surprisingly, VV exhibited virtually the same sensitivity to UV pretreatment of the cells as HSV-1 did, thus classifying it, by this criterion, as a nuclear virus. However, the exact nature of the nuclear requirement was unclear in that UV irradiation inhibited a number of host cell processes. Therefore a more selective manner of inhibiting nuclear gene expression was sought.

α-Amanitin Inhibition of VV Growth. In animal cells, α-amanitin at low doses (0.1-10 μg/ml) has proven to be a relatively specific inhibitor of RNA polymerase II—i.e., messenger RNA synthesis—and has little effect on RNA polymerases I and III (14). By comparison, VV DNA-dependent RNA polymerase, whether assayed in the core or in soluble form, is resistant to this drug at very high concentrations (250 μg/ml) (15). Therefore, carrying out viral infections in the presence of α-amanitin should test whether de novo host messenger RNA synthesis is required for virus growth.

Because α-amanitin is usually taken up slowly by animal cells, it was of importance to ascertain the effects of this drug on BSC-40 cells. After 24-hr pretreatment with α-amanitin at 10 μg/ml, the cells retained their normal morphology, excluded trypan blue, took up neutral red (a vital dye), and did not differ in cell number as compared to the controls. While DNA and protein synthesis were virtually unaffected, incorporation of [3H]uridine into trichloroacetic acid-insoluble material was reduced to 10% of control levels, in agreement with previous studies (16).

The effect of concentrations of α-amanitin ranging from 0 to 10 μg/ml of viral replication in BSC-40 cells was examined (Fig. 2). Production of infectious progeny virus by VSV was completely insensitive to these doses of α-amanitin, while growth of VV was reduced to as little as 5%. These results can be compared to previous studies demonstrating that adenovirus replication is also inhibited by α-amanitin (16). To rule out any side effects of α-amanitin on the VV particle itself, a virus suspension was incubated with drug at 10 μg/ml for 4 hr at 4°C, with no resultant loss in infectivity. Thus it would appear that activity of host cell RNA polymerase II is necessary for VV infection.

![Graph](image1.png)

**FIG. 1.** Effect of increasing doses of UV irradiation on the ability of BSC-40 cells to support subsequent viral infections. Duplicate dishes of BSC-40 monolayers (1.75×10⁶ cells per dish) were exposed to UV irradiation for the indicated times and then infected at a multiplicity of 10 plaque-forming units per cell with VSV (•). HSV-1 (○), or VV (□). After 24 hr, progeny virus was harvested and titered. In the absence of UV treatment the control titers were as follows: VSV, 8×10⁷; HSV-1, 4×10⁷; VV, 3×10⁸ (titers equal total progeny plaque-forming units per dish).

![Graph](image2.png)

**FIG. 2.** Effect of α-amanitin on viral replication in BSC-40 cells. Pairs of BSC-40 monolayers (9×10⁶ cells per dish) were exposed to the indicated concentrations of α-amanitin for 24 hr, then infected with VSV (•) or VV (□) at a multiplicity of 10 plaque-forming units per cell. After 24 hr, progeny virus was harvested and titered. With no drug present the control titers were as follows: VSV, 3×10⁷; VV, 2.3×10⁸ (titers equal total progeny plaque-forming units per dish).
VV Replicative Steps in UV-Irradiated and α-Amanitin-Treated BSC-40 Cells. Although neither UV-irradiated nor α-amatin-treated BSC-40 cells were capable of producing infectious progeny virus from VV infections (Figs. 1 and 2), it was not clear at which stage viral development was arrested. To this end, various parameters of viral gene expression were examined in VV-infected BSC-40 cells that had been subjected to 240 ergs mm$^{-2}$ of UV irradiation or α-amatin at 5 μg/ml, conditions sufficient to cause a reduction in virus yield to 10–15%. The results of these experiments, conducted as previously detailed, are displayed in Table 1 along with data previously obtained in our laboratory concerning growth of VV in enucleated cells (8). The results obtained in the three very different cases were remarkably similar. Both early and late VV genes were transcribed, and the resultant transcripts were processed and translated into apparently functional polypeptides at high levels (>70% of control, Fig. 3). Primary and secondary uncoating processes also occurred normally. Although viral DNA was synthesized at a high rate (>80% of control) and with the correct temporal sequence (Fig. 4), this newly synthesized DNA was not packaged into a DNase-in-

### Table 1. VV replicative cycle in infected BSC-40 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptake/Uncoating</th>
<th>Early gene expression</th>
<th>Uncoating II/DNA synthesis</th>
<th>Late gene expression</th>
<th>Protein modification</th>
<th>Virus assembly</th>
<th>Infectious progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Enucleated</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UV-irradiated</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.
* Conversion of P4a and P4b to 4a and 4b.
† Assayed by measuring conversion of newly synthesized, radioactively labeled viral DNA from a DNase-sensitive to a DNase-resistant form (17).

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**DISCUSSION**

Previous studies of VV-infected enucleated mammalian cells (7, 8) indicated that, in the physical absence of the host cell nucleus, VV was unable to properly package viral constituents into mature viral particles and suggested that a host nuclear
function(s) was required. The experiments reported have confirmed and extended this notion, and have shed light on the molecular nature of this host cell–virus interaction. The inability of VV to grow in UV-irradiated cells whose nuclei fail to synthesize appreciable amounts of DNA or RNA indicates that the mere physical environment of the nuclear body or the presence of its constituent macromolecules, other than nucleic acids, is not sufficient. Because it is unlikely that the doses of UV light employed here would cause direct inactivation of enzymes present within the nucleus, this suggests that interaction of a nuclear enzyme with the viral genome was not the observed requirement. Alternatively, it is possible that the required activity turns over extremely rapidly or else UV irradiation might abolish some necessary transport step. α-Amanitin inhibition of VV replication, in the absence of any demonstrable effects on viral functions, indicates that activity of host cell RNA polymerase II—i.e., mRNA synthesis—is necessary. When this result is considered in conjunction with the UV data, it appears that transcription of host nuclear genetic information and expression of the resultant function(s) are involved in VV replication.

Whatever the specific nature of the above function(s), its absence is consistently expressed as a defect in viral assembly. Whether this is due to a direct requirement at this replicative step or reflects a lesion earlier in infection is not yet clear. In either case, it would seem of importance to continue research into the molecular identity of the required host function(s) and their mode of interaction with the viral target sites. Also, it will be interesting to ascertain whether these functions reflect normal short-lived host cell gene products or represent induction of previously quiescent genetic information.

Another double-stranded DNA virus once thought to replicate in the cytoplasm (frog virus 3) has recently been demonstrated to undergo a nuclear phase that involves the initiation of DNA replication, which then continues in the cytoplasm, and the synthesis of viral RNA (18). At present, it may be said that no eukaryotic DNA virus has been demonstrated to be truly “cytoplasmic” in the sense of having achieved total independence from the nucleus. The elucidation of the precise nuclear requirement(s) for VV replication may shed a new light on virus–host cell interactions and may lead to the identification of some novel nuclear function.

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