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Superinfection Exclusion of Vaccinia Virus in Virus-Infected Cell Cultures

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Vaccinia virus-infected BSC 40 cells do not permit the replication of superinfecting vaccinia virus. The extent of superinfecting virus propagation depends on the time of superinfection; there is 90% exclusion by 4 hr after the initial infection, and more than 99% by 6 hr. When superinfection is attempted at 6 hr after infection, the superinfecting virus is incapable of carrying out DNA replication or early gene transcription, demonstrating that an early event in the virus life cycle is inhibited. The rate of adsorption of the superinfecting virus is unaltered which shows that exclusion is affected at a point between adsorption and early gene transcription. In order to exclude superinfection, the primary infecting virus does not require replication of its DNA or expression of its late genes but it must express one or more early genes.

INTRODUCTION

Vaccinia virus, a member of the Poxvirus family, performs its replication cycle in the cytoplasm of infected cells. In order to follow this unusual life cycle, vaccinia encodes many of the proteins required for virus DNA replication and viral gene expression. In addition, the virus encapsidates several of the enzymes employed in early virus transcription and mRNA processing. The vaccinia virus infection, therefore, provides a unique opportunity to study gene expression, DNA replication, and DNA recombination in a genetically well-defined system in an eukaryotic environment.

In several laboratories, a combined genetic and biochemical approach is being followed in order to unravel the steps in the vaccinia life cycle. One genetic technique that is often informative in other viral systems is the analysis of mutant virus propagation following superinfection of mutant viral-infected cell cultures with a related virus variant. However, the possibility that superinfection of vaccinia virus-infected cell cultures is inefficient was raised first by Joklik (1964) during his initial studies on the rabbit poxvirus uncoating pathway. Later, Moss et al. (1971) described the results of an experiment that demonstrated the exclusion of superinfecting rifampicin-resistant vaccinia virus from cells infected with wild-type vaccinia. It is not clear from these few experiments whether superinfection exclusion is a general property of poxvirus-infected cells. In addition, if exclusion occurs, it is not known which step in the superinfecting virus’s life cycle is inhibited or which steps in the initial virus infection are required.

In this report, we establish that vaccinia virus is excluded from superinfecting vaccinia-infected cells within 4 hr after the onset of the initial virus infection. Furthermore, we show that replication of the superinfecting virus is inhibited at a point between the virus adsorption step and early virus gene transcription. Finally, we demonstrate that early gene expression but not viral DNA replication or late gene expression is required from the infecting virus to effect exclusion of the superinfecting virus.

MATERIALS AND METHODS

Cells and viruses

The African Green Monkey cell line BSC 40 was employed throughout these experiments (Condit and Motyzka, 1981). Wild-type vaccinia WR and ts mutants C17, C24, E69, and C42 have been described previously (Condit and Motyzka, 1981; Condit et al., 1983; Ensinger, 1982; Ensinger and Rovinsky, 1983; Seto et al., 1987). VSC8 and VSC9, vaccinia virus that possess the Escherichia coli β-galactosidase gene downstream from the vaccinia promoters P11 and PTK, respectively, were constructed as described (Chakrabarti et al., 1985) from the plasmids pSC8 and pSC9, generously provided by Dr. Bernard Moss. Virus were titrated at 31°, 37°, or 40°, as described (Condit and Motyzka, 1981). For large-scale preparations, virus were propagated in forty 150-mm dishes and isolated from cell extracts by sedimentation through 36% sucrose followed by velocity sedimentation through a 25 to 40% sucrose density gradient (Joklik, 1982). 3H-labeled virus were prepared by replication in medium containing 50 μCi [3H]thymidine per 150-mm dish and were purified from forty 150-mm dishes as described above. The purified
Superinfection exclusion assay

Dishes (60 mm) containing fresh confluent monolayers of BSC 40 cells were infected with an initial virus at an m.o.i. of 10. After 30 min at the appropriate temperature, the unadsorbed virus inoculum was removed and the monolayer was washed with prewarmed PBSAM (170 mM NaCl, 3.35 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 10 mM MgCl₂, and 0.01% bovine serum albumin), and 4 ml of prewarmed medium was returned to the dish. This is defined as time zero. At a specified time, superinfection was initiated by removing the medium and adding a virus inoculum at an m.o.i. of 10. After 30 min, the unadsorbed superinfecting virus was removed, the monolayer washed, and the medium returned. Virus propagation was permitted to proceed for a total of 48 hr at 37° or 40°, or 72 hr at 31°. The infected cell monolayers were scraped and the virus were titered after the virus were released from the infected cells by two cycles of freeze/thawing followed by sonication. VSC8 or VSC9 plaques were identified by overlaying with agar containing 0.05% neutral red and 150 μg/ml of X-gal as described by Chakrabarti et al. (1985). In experiments involving ts mutant virus, the initial infection and the superinfection were carried out at either the permissive or nonpermissive temperature. The virus yield was titered at the permissive temperature to permit quantitation of both the ts mutant and the VSC8, and at the nonpermissive temperature to identify the clear plaque recombinant virus.

Quantitation of superinfecting virus DNA replication and early gene transcription

In order to quantify superinfecting virus DNA replication and early viral gene transcription, a series of 100-mm dish cultures of BSC40 cells were infected with wild-type virus as described above. After 6 hr, the cultures were superinfected with VSC9, which has the E. coli β-galactosidase gene under the control of the early vaccinia thymidine kinase promoter. At times after superinfection, RNA and DNA were prepared from the infected cell cultures (Pacha and Condit, 1985; Esposito et al., 1981). The level of superinfecting virus DNA replication was determined by Southern transfer analysis (Southern, 1975). Total cell DNA was cleaved with BamHI, which excises the β-galactosidase gene as a 3-kb fragment. DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a nick-translated β-galactosidase DNA fragment. Early gene transcription was measured by

![Fig. 2. Analysis of superinfecting virus DNA replication. Monolayer cultures of BSC 40 cells were infected with wild-type virus at an m.o.i. of 10. Six hours postinfection, the cultures were superinfected with VSC9 at an m.o.i. of 10. At times after superinfection, cells were scraped and DNA was isolated. Total DNA was cleaved with BamHI to release a 3 kb Escherichia coli β-galactosidase DNA fragment. A Southern transfer was carried out and probed with 32P-β-galactosidase DNA. WT + VSC9, DNA samples from superinfected cell cultures; VSC9, DNA samples isolated from VSC9-infected cells; 0 to 6, hr postinfection; C, Cial; B, BamHI; and HindIII are molecular weight markers.](image-url)
Northern transfer analysis (Thomas, 1983) in which the same β-galactosidase DNA probe was employed.

Adsorption assay

Cell cultures in 35-mm dishes were infected with wild-type virus at an m.o.i. of 10. At times after initiating the infection, cultures were superinfected with 3H-labeled wild-type virus at an m.o.i. of 10. At times after initiating the superinfection, the inoculum was removed and the monolayer was washed with 2 ml of PBS (170 mM NaCl, 3.35 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The cells were scraped in PRS and added to an equal volume of 0°C, 20% trichloroacetic acid. After 10 min on ice, the cell debris was collected by suction onto nitrocellulose filter disks and dried and the radioactivity determined.

Ultraviolet inactivation

Purified vaccinia was irradiated by a modification of the procedures previously described (Moss, 1968; Bablanian et al., 1981). Sucrose density gradient purified wild-type virus was diluted to 10⁶ PFU/ml in PBSAM. One milliliter of the virus solution, containing a small magnetic stirring bar, was placed in a 35-mm dish in an ice tray on a magnetic stirring motor and gently stirred. One or two germicidal lamps, GE G15T8, were placed at set distances from the dish of virus and illuminated for a set length of time. A calibration was carried out using a Blak-Ray J-225 shortwave ultraviolet intensity light meter. The following conditions were employed: 80 erg/mm², 1 bulb, at 30 cm, 5 sec; 350 erg/mm², 1 bulb, at 20 cm, 5 sec; 780 erg/mm², 2 bulbs, 20 cm, 5 sec; 1750 erg/mm², 2 bulbs, 10 cm, 5 sec; 17,100 erg/mm², 2 bulbs, 5 cm, 30 sec. The virus titer was determined at 37°C.

RESULTS

Vaccinia virus superinfection is excluded from virus-infected cells

In order to determine whether vaccinia virus-infected cell cultures can support the replication of superinfecting vaccinia virus, a series of monolayer cultures were infected with wild-type virus and at various times after the primary infection, the cultures were superinfected with vaccinia virus VSC8. After incubation for 48 hr, the virus preparations were titered in the presence of neutral red and X-gal to permit the visualization of clear wild-type plaques and blue VSC8 plaques, respectively. The data in Fig. 1 show that by 4 hr after the primary infection, the ability of the superinfecting virus VSC8 to replicate is dramatically reduced, and by 6 hr, VSC8 replication is decreased to less than 1% of the initial level. Although there are minor variations in the time course of exclusion (compare Fig. 1 and Fig. 5), this observation holds true whether the infection is carried out at 31°C, 37°C, or 40°C. Quantitation of the clear plaques formed by the wild-type infecting virus shows that the superinfecting virus did not alter plaque formation by the initial infecting virus.

Replication of the superinfecting virus is inhibited at a step between virus adsorption and early gene transcription

In order to test whether the superinfecting virus DNA is capable of replication, a corioe of cell monolayers was infected with wild-type virus, and at 6 hr after the primary infection, superinfection was carried out with VSC9. At times after initiating the superinfection, the
infected cells were collected and total DNA was isolated. DNA was cleaved with BamHI, which releases the β-galactosidase gene as a 3-kb fragment, and a Southern transfer analysis was carried out using a 32P-labeled β-galactosidase DNA fragment as a probe. In Fig. 2 it can be seen that there is no increase in the amount of VSC9 DNA in the superinfected cell cultures, while in the control VSC9 virus-infected cells, VSC9 DNA begins to accumulate by 2 hr and reaches high levels by 4 hr after infection.

A Northern transfer analysis was carried out in order to determine if early gene transcription from superinfecting virus can be observed. In Fig. 3 it is apparent that a stable 3-kb β-galactosidase mRNA appears by 4 hr after infection and is replaced by a heterogeneous smear of late mRNA at 6 hr, consistent with the expression of an early virus gene. In the superinfecting virus cultures, however, there is no evidence for synthesis of the early β-galactosidase mRNA demonstrating that early gene transcription from the superinfecting virus genome is greatly reduced.

Since the exclusion event must occur at a very early step in the virus infection, the rate of virus adsorption to infected cell cultures was measured. In Fig. 4 it can be seen that although there is up to a 40% decrease in the initial adsorption of the superinfecting virus, the rate of virus attachment to the infected cell monolayers is virtually unaffected by the time at which superinfection is initiated. At 60 min of incubation, three to four virus particles remain bound per cell. As a result of these experiments, we conclude that the superinfecting virus is excluded at a point in the virus life cycle between the adsorption of the superinfecting virus to

![Graph](image)

**Fig. 4.** Measurement of the rate of adsorption of superinfecting virus to the infected cell monolayers. Cell monolayers were infected with wild-type virus at an m.o.i. of 10. At different times after infection, the medium was removed and the cells were overlayed with 3H-labeled virus at an m.o.i. of 10. At various times after addition of the superinfecting virus, the inoculum was removed and the monolayer was washed. The cells were collected and added to 0%, 20% trichloroacetic acid, and the precipitate radioactivity was measured. 0 hr, 2 hr, 4 hr, 6 hr represent time after the initial infection that the adsorption assay was initiated. Each point is the average of three determinations.
Time of Superinfection

**Fig. 6.** Effect of uv irradiation on the ability of a virus to exclude superinfection. Wild-type virus was diluted to 10^9 PFU/ml in PBSAM and treated with ultraviolet light at the indicated energy. The irradiated virus was then used as the initial infecting agent in a standard superinfection assay. The results of each set of infections are presented as a ratio of PFU of VSC8 determined at each time point of superinfection to PFU of VSC8 at time zero of superinfection. The uv dose employed, ergs/mm², is indicated on each line.

Virus early gene expression is required to exclude superinfection

To test if either DNA replication or late viral gene expression was required from the primary infecting virus in order to exclude superinfection, we analyzed the ability of a DNA^- ts virus to mediate exclusion at the permissive and nonpermissive temperatures. A series of cell monolayers was infected with the ts mutation C17, in gene D5R (Condit and Motyczka, 1981; Niles et al., 1986; Seto et al., 1987; Roseman and Hruby, 1987; Evans and Traktman, 1987), at either 31° C or 40° C, and a standard superinfection assay was carried out. In Fig. 5 it can be seen that exclusion is rapid at either 31° C or 40° C whether wild-type virus or C17 was employed as the primary infecting virus, demonstrating that virus DNA replication is not required to effect exclusion. Since late gene expression is dependent on viral DNA replication (Oda and Joklik, 1967), we can also conclude that late transcription is not required. This observation is found when another DNA^- virus, i.e., C24 or E69(gene D5R), or C42 (DNA polymerase) is used as the infecting virus (data not shown).

In an attempt to determine whether early virus gene expression is required for exclusion, the primary infecting virus was treated with various doses of ultraviolet light in order to achieve different levels of inactivation of viral gene expression. The ability of the uv-inactivated virus to exclude superinfection was then determined. In Fig. 6 it can be seen that both the time course of exclusion and the degree of exclusion are dramatically altered by uv treatment of the initial infecting virus.

In order to assess the effect of the uv treatment on infecting virus gene expression, the rate of both viral and host protein synthesis was monitored by pulse-labeling (Fig. 7). In the absence of uv treatment, the typical pattern of early and late virus protein synthesis, as well as the shut off of host protein synthesis, was observed (Salzman and Sebring, 1967; Moss and Salzman, 1968). For virus treated at 80 erg/mm², the appearance of late proteins was delayed. At 200 erg/mm² there was a substantial decrease in late virus protein synthesis and failure to shut off early protein synthesis. This is consistent with a loss in the ability to replicate viral DNA. At a higher level of irradiation, late virus protein synthesis was lost and early protein synthesis was severely reduced. At even the highest level of uv treatment, however, host protein synthesis continues to be shut off, although both the rate of shut off and the extent of shut off are reduced about twofold (Moss, 1968; Bablanian et al., 1981).

The effect of uv treatment on virus protein synthesis was more precisely quantified by measuring the rate of synthesis of an early protein, D12L, and a late protein, D8L, by immunoprecipitation from pulse-labeled virus-infected cell cultures. It can be seen in Fig. 8 that the expression of the late gene D8L was severely reduced when virus were treated at 200 erg/mm². A decreased level of gene D12L expression can be observed at 200 erg/mm² which is lost at doses greater than 350 erg/mm².

The results of these experiments are summarized in Fig. 9. Ultraviolet irradiation decreases the virus titer up to 6 orders of magnitude. The ability of the irradiated virus to express the early gene D12L is severely diminished at a level above 200 erg/mm². Over the same range of uv treatment, the primary infecting virus loses its ability to exclude superinfection. From a comparison of the uv dose dependence of the expression of gene D12L and the loss of the ability to exclude superinfection, we observe that the rate of early gene expression is directly correlated with the ability of the infecting virus to exclude superinfection, and we conclude that
the expression of one or more early genes is required for rapid and complete exclusion of the superinfecting virus.

**DISCUSSION**

A vaccinia virus-infected cell culture is capable of supporting the replication of superinfecting vaccinia virus for 1 to 2 hr after infection. At times greater than 2 hr, replication of the superinfecting virus is greatly reduced. By 4 hr postinfection, virus propagation decreases to less than 10% of the initial value and by 6 hr, virus replication is excluded greater than 99%. Although there is some variability in the kinetics of exclusion between experiments, wild-type and several ts mutant vaccinia virus are capable of excluding superinfecting virus.
the ability of the infecting virus to exclude superinfection and its rate of early gene expression. Selected portions of the results presented in Figs. 6 and 8 are compared. The titer of uv-treated virus at each dose of uv light, X. The rate of synthesis of D12L protein at 2 hr postinfection for virus treated with different doses of uv light. The synthesis of the D12L protein in the autoradiograph in Fig. 8 was quantified by densitometric analysis and presented as a percentile where the synthesis of the D12L protein in the untreated virus-infected cell is 100%. O. A measure of the replication of the superinfecting virus at 4 hr postinfection in cells preinfected with uv-treated virus. The ratio vscc8 vs c8, in Fig. 6 at 4 hr postinfection is converted to a percentile by multiplication by 100 and is plotted at each energy of uv treatment of the primary infecting virus.

Fig. 9. Correlation between the ability of the infecting virus to exclude superinfection and its rate of early gene expression. Selected portions of the results presented in Figs. 6 and 8 are compared. The titer of uv-treated virus at each dose of uv light, X. The rate of synthesis of D12L protein at 2 hr postinfection for virus treated with different doses of uv light. The synthesis of the D12L protein in the autoradiograph in Fig. 8 was quantified by densitometric analysis and presented as a percentile where the synthesis of the D12L protein in the untreated virus-infected cell is 100%. O. A measure of the replication of the superinfecting virus at 4 hr postinfection in cells preinfected with uv-treated virus. The ratio vscc8 vs c8, in Fig. 6 at 4 hr postinfection is converted to a percentile by multiplication by 100 and is plotted at each energy of uv treatment of the primary infecting virus.

The site at which the inhibition of the superinfecting virus replication occurs was determined at 6 hr postinfection. Southern transfer analysis demonstrates that the superinfecting virus does not undergo DNA replication, and Northern blot analysis shows that early genes are not transcribed. However, the rate of superinfecting virus adsorption to the infected cell monolayer is unaltered at times of superinfection up to 6 hr postinfection. We conclude that the replication of the superinfecting virus is inhibited at a point between virus adsorption and early gene transcription.

The genetic requirements for superinfection exclusion were determined. Through the use of temperature-sensitive mutations in genes which exhibit a DNA− phenotype as the primary infecting virus, it was possible to demonstrate that DNA replication and late gene expression are not required. The need for early gene expression was tested by quantifying superinfection exclusion mediated by virus which had been uv inactivated to different extents. Ultraviolet inactivation alters the kinetics of exclusion such that the greater the intensity of uv treatment of the infecting virus, the less replication of the superinfecting virus is inhibited. Through a quantitation of the affect of uv treatment on host and virus protein synthesis it was possible to correlate the degree of inactivation of virus early gene expression with the ability to exclude superinfection, demonstrating that early virus gene expression is required to affect exclusion of superinfection.

There are at least three models which may explain superinfection exclusion. In the first model, after the initial infection, an early protein accumulates which is directly responsible for preventing the penetration, uncoating, or transcription of the superinfecting virus. This model is consistent with the need for early gene expression from the infecting virus and the observed kinetics of exclusion. In the second model, the expression of early genes from the initial infecting virus activates a host anti-viral activity which prevents either uptake or early gene expression from the superinfecting virus. Since replication of the initial infecting virus proceeds unabated, we must assume that the replication of the initial virus is beyond the control of this putative anti-viral agent. The final model recognizes that vaccinia has evolved to initiate replication in an uninfected cell and that the physiology of the infected cell may be substantially altered by the early events which occur during the initial infection. As a result of this initial infection, a superinfecting virus encounters a cellular environment which may be unfavorable. In this model, exclusion is an indirect consequence of early gene expression from the initial infecting virus.

The superinfection exclusion phenomenon raises several important points. First, genetic experiments requiring superinfection are not possible with vaccinia virus if superinfection is carried out later than 2 hr postinfection. Second, superinfection exclusion can have an affect on virus plaque assays. For example, in plaque assays carried out at a high particle to PFU ratio, i.e., low dilutions of ts mutants plated at the nonpermissive temperature, exclusion will cause revertant virus to form small plaques, because although the mutant virus cannot replicate at the nonpermissive temperature, it will infect a cell and prevent superinfection by the revertant virus. Third, it is worth considering superinfection exclusion as an additional property of the vaccinia virus infected cell, and realizing that a full understanding of the molecular basis of exclusion will provide a greater appreciation of the vaccinia virus life cycle. Fourth, since superinfection exclusion requires early gene expression, one might be able to take advantage of this observation to select for conditional lethal muta-
tions of vaccinia virus altered in functions required for early gene expression.

In other virus systems, interference with superinfecting virus replication is well documented. Influenza virus synthesizes a neuraminidase which destroys the cell-surface receptor required for virus infection (Palese et al., 1974). Corona virus express an esterase which may carry out a similar function (Vlasak et al., 1989). In the case of herpes virus, the cell-surface expression of glycoprotein D inhibits superinfection by preventing virus penetration but not adsorption (Johnson and Spear, 1989). It is often argued that these exclusion mechanisms prevent reinfection by progeny virus and result in their release into the infected organism. Perhaps the exclusion phenomenon described for vaccinia virus serves a similar function.

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