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Viral and Host Deoxyribonucleic Acid Synthesis in Shope Fibroma Virus-infected Cells as Studied by Means of High-Resolution Autoradiography

RAOUL SCHERRER

Institut de Recherches sur le Cancer, Laboratoire de Microscopie Electronique, 94 Villejuif, France

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Incorporation of ^{3}H-thymidine by BSC-1 cells infected with Shope fibroma virus was studied by means of high-resolution electron microscopic radioautography. One-hour pulses with the radioactive precursor were given at various times after infection, during a one-step growth cycle of the virus. In the cytoplasm of infected cells, reacted grains occurred over foci of viroplasm; these foci are believed to represent the true sites of viral deoxyribonucleic acid (DNA) replication. Shope fibroma virus DNA synthesis began before 3 hr postinfection, reached a maximum at 8 to 9 hr, and then declined rapidly. It was demonstrated that the decline in ^{3}H-thymidine uptake is correlated with the onset of viral morphogenesis. In comparison with the noninfected culture, the nuclear labeling, which reflects host DNA metabolism, was slightly reduced by 4 hr postinfection. Inhibition became more marked as infection progressed, and host DNA synthesis was almost completely suppressed in late stages of viral development.

The Shope fibroma virus (SFV) has been classified as a member of the oncogenic poxviruses because it is capable of causing tumors in its natural host, the rabbit (25). Several in vitro studies of cell-SFV interactions have been attempted in order to determine in what way they may be different from or similar to those occurring with other poxviruses, particularly with the vaccinia virus subgroup whose infectious cycle has been extensively explored during the last 10 years. In general, cells infected with vaccinia virus show early cytopathic effect (CPE), leading sooner or later to cell lysis (1, 5). SFV, on the other hand, does not exhibit early CPE; rather, it induces diverse late responses, depending essentially upon viral and (or) cellular strains used (13, 26, 27). Among these late responses, pock induction (14, 15) has been most extensively studied, although the mechanism of pock formation still remains unclear. With respect to the viral maturation process, SFV shows all of the characteristic steps involved in the replication of the vaccinia subgroup (3, 4, 24); thus far, fundamental differences have not been found. In a recent biochemical study, Ewton and Hodes (6, 7) pointed out that SFV-mediated nucleic acid synthesis in HeLa cells resembles that occuring after infection with vaccinia virus, but it appears that the entire cycle of events is prolonged when compared with the vaccinia virus system. This seems to be true with respect to viral deoxyribonucleic acid (DNA) synthesis as well as cytoplasmic and nuclear ribonucleic acid (RNA) synthesis.

This study presents additional data concerning metabolic and ultrastructural modifications induced in vitro by SFV. Since it is known that poxvirus DNA replicates in the cytoplasm, advantage was taken of the high resolution radioautographic technique for studying viral and host DNA synthesis independently.

MATERIALS AND METHODS

Virus. The virus was originally isolated from a rabbit fibroma tumor. Crude virus preparations were obtained from complete monolayers of primary rabbit kidney cells inoculated with stock virus. After 4 or 5 days of incubation at 36 C, the cultures were frozen and thawed three times. Cell debris was centrifuged at 1,000 × *g* for 20 min and the supernatant fluid was centrifuged at 20,000 × *g* for 45 min. High-titer stocks were prepared from the pellet by suspension in nutrient medium containing 5% calf serum.

Cells and mode of infection. The continuous cell line (BSC-1) used for these experiments (21) was derived from a monkey kidney (cercopithecus). Cells were grown in Leighton tubes at 37 C in Eagle's medium containing fourfold concentrations of amino acids and vitamins and 5% calf serum. Cultures were infected when the cell sheet became confluent, generally 20 or 24 hr after seeding. An 0.2-ml sample of

virus suspension was added to each tube and was allowed to adsorb for 2 hr at 36 C. At regular intervals, the tubes were stirred gently to ensure redistribution of the virus. The virus was then removed, the cell monolayer was washed, the growth medium was replaced, and the cultures were reincubated at 36 C (19).

Incorporation of radioactive precursor. Tritiated thymidine (Radiochemical Center, Amersham, England; specific activity, 14 c/mmole), diluted to 100 μc/ml in medium containing 5% calf serum, was added to the cultures at different times after infection for pulses of 1 hr at 36 C. Control experiments were carried out simultaneously with the same pulses on noninfected cultures. The cells were subsequently fixed in situ by addition of the fixative into the tubes, after removing the radioactive medium. Cells were fixed with 1.6% glutaraldehyde for 20 min at 4 C, followed by 2% osmium tetroxyde for 30 min at room temperature. Each fixative was in Sörensen's phosphate buffer at *p*H 7.4.

In another experiment, the cells were pulse-labeled for 1 hr, as above, then washed and subsequently incubated with medium containing a 100-fold concentration of cold thymidine. Fixation was carried out after chases for 1, 2, or 3 hr.

High resolution radioautography. After dehydration in increasing concentrations of acetone, cells were embedded in situ with a 2- to 3-mm thick layer of Epon. Polymerization was carried out at 60 C.

The culture tubes were then broken, and the layer of Epon was detached from the glass (20). Uniform sections were obtained with the LKB Ultrotome and were transferred with a plastic ring to a glass slide previously covered with 1% collodion. The slides were then covered by dipping (10) with Gevaert NUC 307 emulsion (9; Anvers, Belgium; crystal size, 70 nm), allowed to dry, and stored at 4 C. Sections were exposed for 10 or 14 days and were developed in Kodak D-19 at 19 C for 5 min. After fixation with buffered hyposulfite (*p*H 6.0), the slides were washed and the collodion membrane was floated onto distilled water so that a 200-mesh grid could be placed under the sections. Slides were dried for 3 days at 37 C and the sections were subsequently stained with lead citrate for 10 min. The grids were then picked up and covered with a thin layer of carbon.

The background of the emulsion batches employed was always lower than 0.30 grains per 100 μ^2. The radioautograms were photographed at 80 kv with a Siemens Elmiskop I electron microscope, with an objective aperture of 30 μm.

Quantitation. The essential requirements for quantitative radioautography were carefully respected (8, 10). Only thin sections showing the same interference color (approximately 60 nm thick) were used. All sections were layered, exposed, and developed under similar conditions. All micrographs were taken at a nominal magnification of 3,000 ×. The concentration of the radioactivity over the nucleus was determined by counting the grains directly on the photographic plates with the aid of a dissecting microscope. The nuclear surface was measured by drawing its outline on paper. The resulting area was then cut out, and weights were converted into μ^2 by comparison with the weight of a known surface area of the same paper. With respect to progeny viral DNA synthesis, which takes place in the cytoplasm, ^{3}H-thymidine uptake occurred exclusively over foci of viroplasm which enlarge as infection progresses. Thus, for estimating viral DNA synthesis at a given time, only the radioactivity over foci of viral factories was evaluated. The rare nonspecific background radioactivity of the rest of the cytoplasm was discarded in the grain count. At each specified time, an average number of 30 cells, showing foci of viroplasm, were photographed with the electron microscope, silver grains were counted, and the average number of these grains per cell was calculated.

RESULTS

Light microscopy. SFV, like other pox viruses, multiplies in aggregates (viroplasm or factories) located in the cytoplasm. The affinity for acridine orange of the early viral inclusions renders them easily observable in the light microscope and permits a rapid analysis of the degree of infection inside a given cellular population. In our system, inclusions became apparent at 3 hr postinfection (PI) in a few cells (Fig. 1). Very often, several fac-

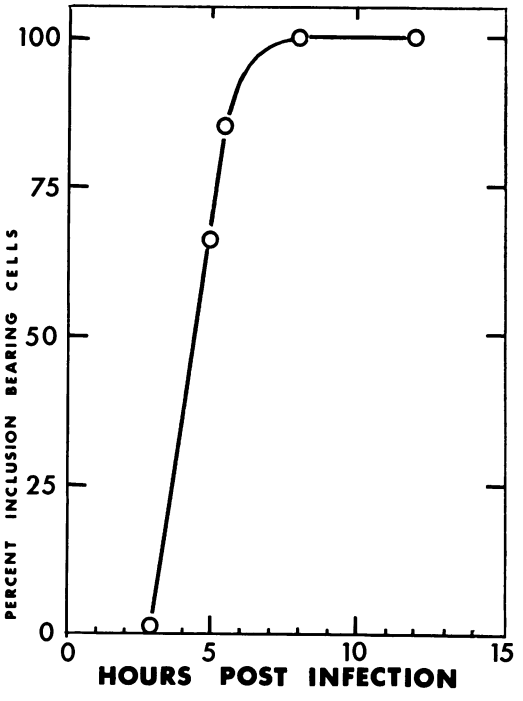

FIG. 1. *Appearance of inclusion-bearing cells as a function of time. Cover slip cultures in Leighton tubes were infected at time 0. At different times postinfection, they were fixed in Carnoy's fluid, stained with acridine orange, and subsequently examined in a fluorescence microscope.*

tories were seen in a single cell. At 5 and 8 hr PI, the percentage of cells containing inclusions was 75 and 100%, respectively. Since viral morphogenesis begins only after 10 hr, it is likely that all cells became infected during the adsorption time.

Viral DNA replication. Early factories could be labeled as soon as 3 hr PI. After a 1-hr pulse with tritiated thymidine, most of the reduced silver grains were located over dense cytoplasmic areas which are known to represent the sites of viral replication (24). Numerous sections showed three or four labeled foci in individual cells. In most instances, they occurred in the vicinity of the nuclear envelope (Fig. 2). At this stage of development, early factories consisted of dense, packed granules or short fibrils; this material was variably labeled at 4 hr, but foci without label were never found. The normal cell cytoplasm was often completely devoid of label. At later stages, a pronounced enlargement of viral factories occurred, concomitantly with fusion of all separate foci into a single large mass per cell (Fig. 3 and 4). At 6 hr and chiefly at 8 hr PI, the silver grains over these foci were sometimes so numerous that single grains could not easily be distinguished. At these times, foci still consisted of dense granular material, sometimes including clusters of ribosomes. Progeny virus could never be observed before the 10th hr. The sequence of steps in the elaboration of progeny virus particles at the ultrastructural level has been described in a previous publication (24). Foci of developing virus particles, such as the one illustrated in Fig. 5, were observed from 11 hr on. They contained immature spherical particles with complete membranes as well as clumps of viroplasm partially enclosed by incomplete membranes. Previously described electron-dense crystalline structures were also present (24). After a 1-hr pulse, such foci showed poor labeling as compared with that seen at 6 hr or 8 hr. In most instances, silver grains were predominantly located over the fibrogranular material of the viroplasm as in earlier stages, and the remainder was located over the crystalline inclusions. Occasionally, a few grains were associated with developing particles. At 16 hr PI, most foci examined after a 1-hr pulse contained no label. Intermediate stages between the spherical immature virus and the mature forms were observed at this time. Complete mature virions with a dumbbell-shaped core were also present.

A 1-hr pulse was generally not sufficient to produce labeled progeny virus. On the other hand, longer exposure to tritiated thymidine produced too much label over the foci. Thus, chase experiments were performed, after a 1-hr pulse, in order to follow the incorporation of newly synthesized DNA into developing virions. Quantitative data could not be obtained by this kind of experiment, and it could only be ascertained that the longer the chase, the greater the number of labeled particles (Fig. 6 and 7).

Labeling rate. To determine the time course of viral DNA synthesis, radioautographs were treated quantitatively.

Up to 7 hr, there was a rapid stimulation of thymidine incorporation in the cytoplasm of infected cells (Fig. 8); maximal incorporation occurred at 8 to 9 hr PI, followed by a rapid decline of label. By 16 hr, the average labeling rate was only one-eighth of that seen at 9 hr. High-resolution radioautography thus demonstrated that the decline of ^{3}H-thymidine uptake is correlated with the occurrence of progeny immature particles. As expected, cytoplasmic incorporation in control cells was negative at all times specified.

Effect of SFV infection on host DNA synthesis. This investigation was carried out by quantitative analysis of ^{3}H-thymidine uptake into nuclei of infected as well as control cells. Figure 9 depicts the results obtained after a 1-hr pulse given at various times. Until 7 hr, most of the nuclei in noninfected cells showed a rather weak labeling as compared to later times. In most instances, silver grains were diffusely distributed over the nucleoplasm (Fig. 10), but occasionally they occurred preferentially over chromatin clumps disposed along the nuclear envelope or over the nucleolus-associated chromatin. From 7 hr on, nearly the entire cell population entered an S phase. As a consequence, cells taken at 12 and 16 hr showed intense nuclear labeling. At these times, the mean number of silver grains per nuclear surface was nearly six times greater that seen at 7 hr.

In contrast, infected cultures showed a poor labeling rate at all times examined (Fig. 11). A slightly reduced incorporation was already detectable at 4 hr PI. Until 9 hr, the synthetic events taking place in the cytoplasm were accompanied by a nearly constant, but low, incorporation rate of ^{3}H-thymidine into the nucleus.

This situation was modified as viral DNA synthesis began to decrease; at this point of viral development, nuclear uptake also decreased. At 12 hr, only a few grains were still scattered over the nucleoplasm, and, at 16 hr PI, most nuclei showed no label at all.

DISCUSSION

High-resolution radioautography was employed in this work in order to study viral DNA replication and to investigate the effect of SFV infection on host cell DNA synthesis. It is not within the scope of this study to discuss the value and limita-

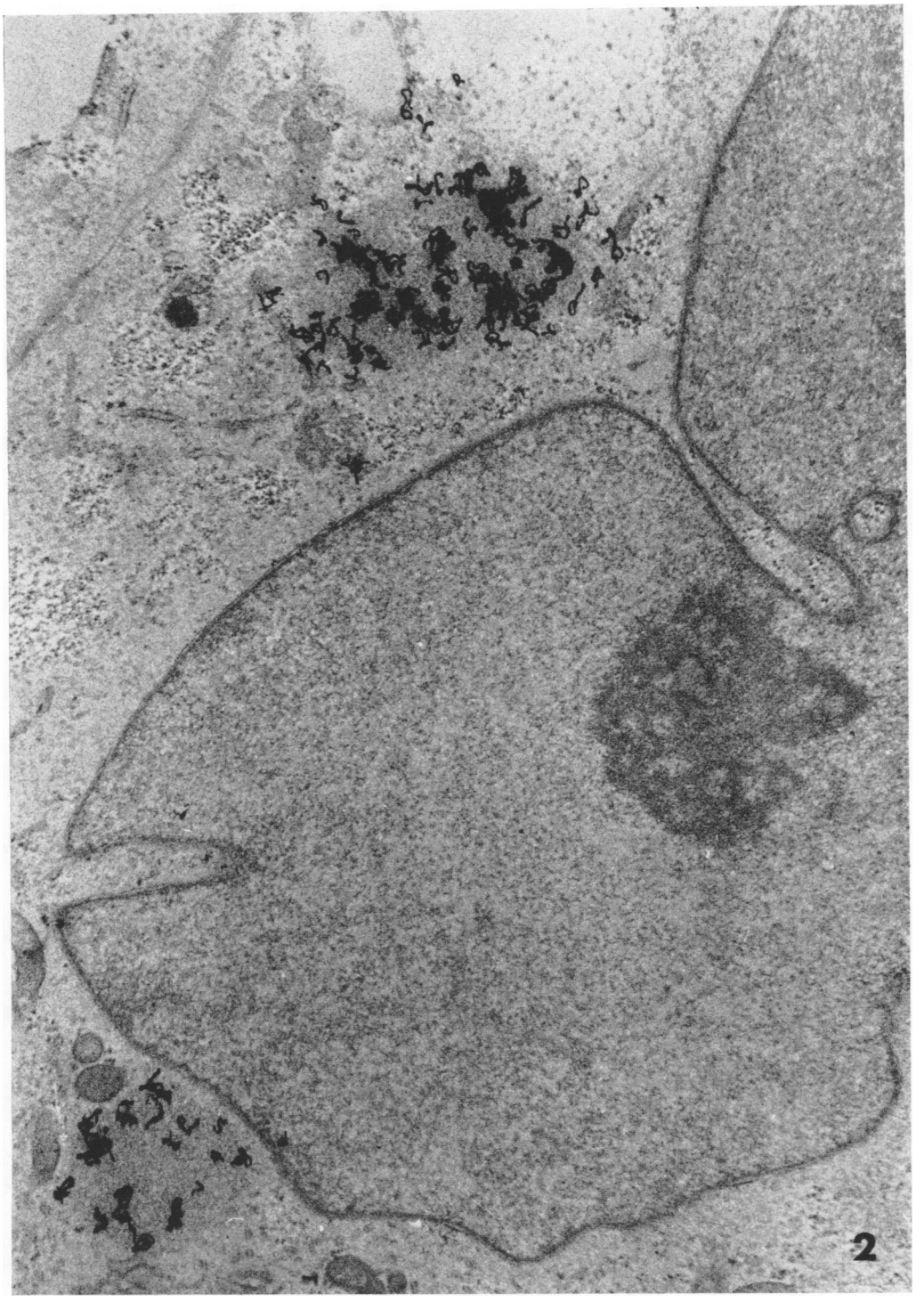

FIG. 2. *Electron microscopic autoradiography of a BSC cell, 3.5 hr after infection with SFV, showing two labeled foci of viroplasm. One-hour pulse with tritiated thymidine.* × *13,000.*

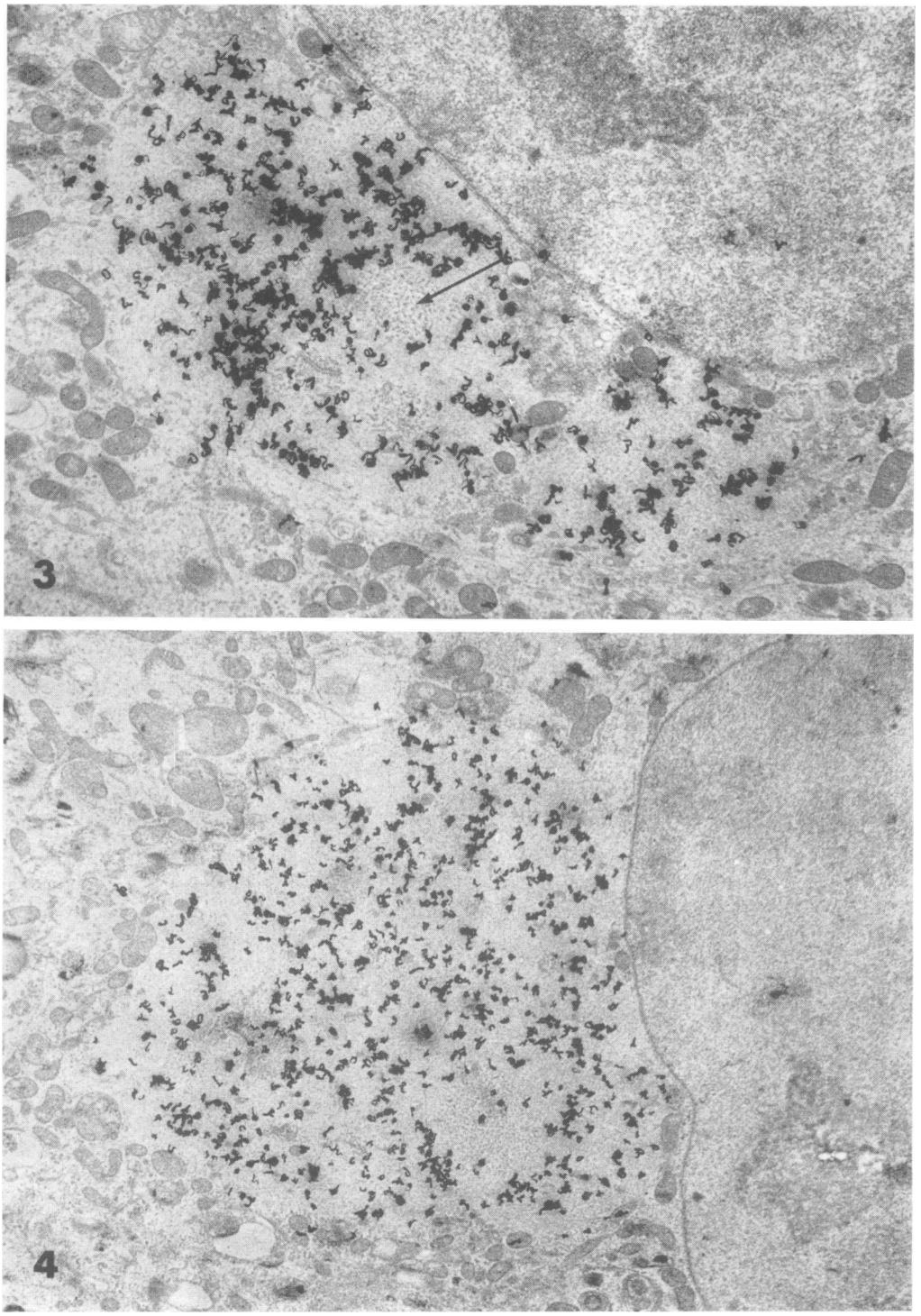

FIG. 3. *BSC cell 6 hr after infection with SFV. One-hour pulse with tritiated thymidine. Intense labeling of two foci of viroplasm which are fusing. Cluster of ribosomes* (→). × *9,000.*

FIG. 4. *BSC cell 8 hr after infection with SFV. One-hour pulse with tritiated thymidine. An enlarged focus of viroplasm shows intense labeling. Progeny virus particles are still absent at this point of development.* × *5,000.*

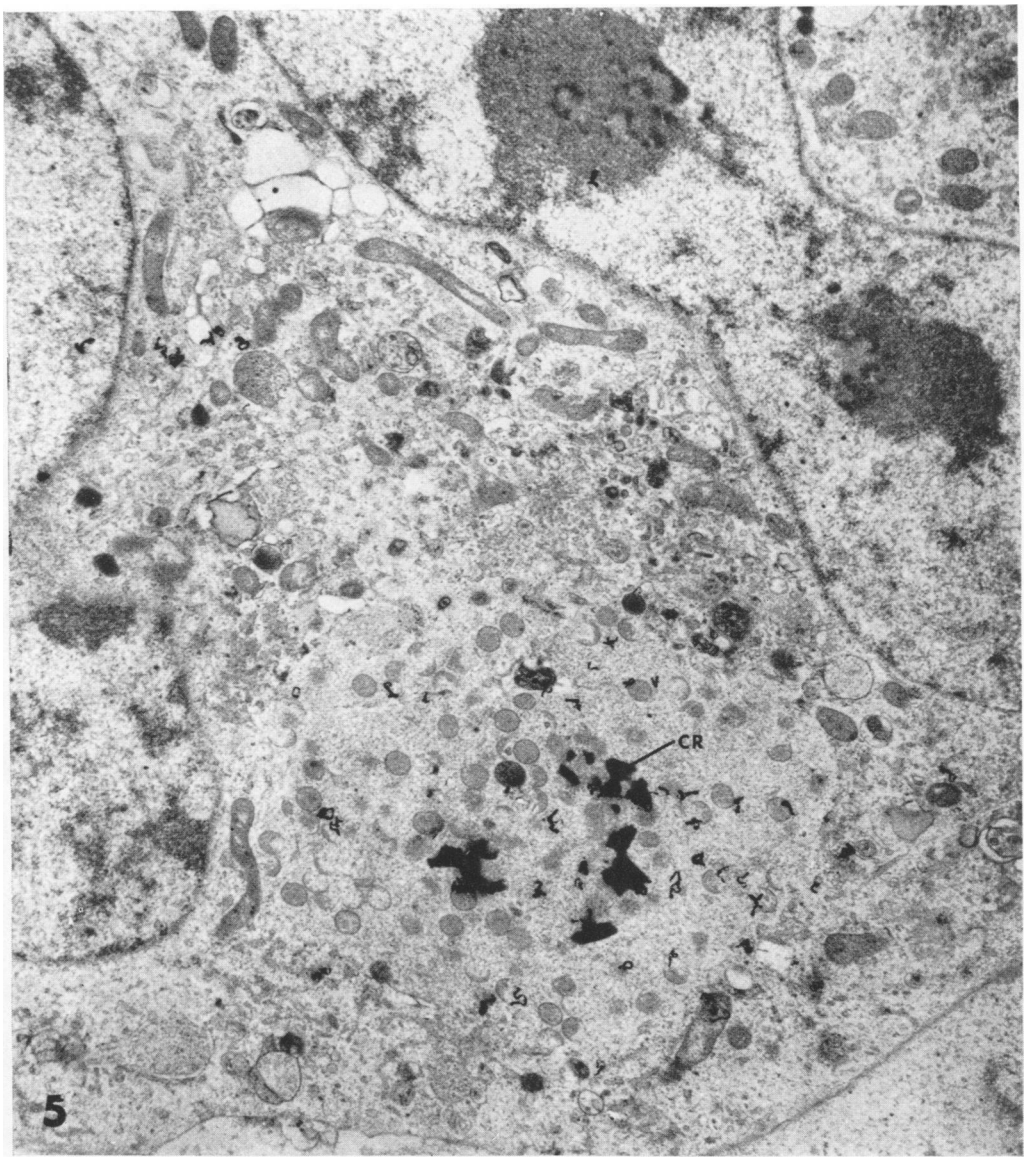

FIG. 5. *Example of a BSC cell 12 hr after infection with SFV. One-hour pulse with tritiated thymidine. A large cytoplasmic factory shows incomplete forms of progeny virus and dense crystalline structures (CR). Poor labeling as compared to Fig. 4.* × *9,000.*

tions of this technique as a tool for quantitative analysis. This problem has been examined in detail by several authors (2, 10).

In this investigation, reproductible quantitative data was obtained under controlled experimental conditions. The most important factor which made this possible was the synchronization of viral replication. Such a synchronization was obtained by inoculating a homogenous population of cells with a sufficiently large number of viruses so that all cells were infected within the time of adsorption.

As could be expected, exogenous thymidine was partially utilized in infected cells for the synthesis of a cytoplasmic viral DNA. The strict localization of label over foci of viroplasm suggests that these foci represent the true sites of viral DNA replication, rather than accumulation sites for DNA synthesized elsewhere. A similar conclusion was formulated by Harford et al. (12) and by

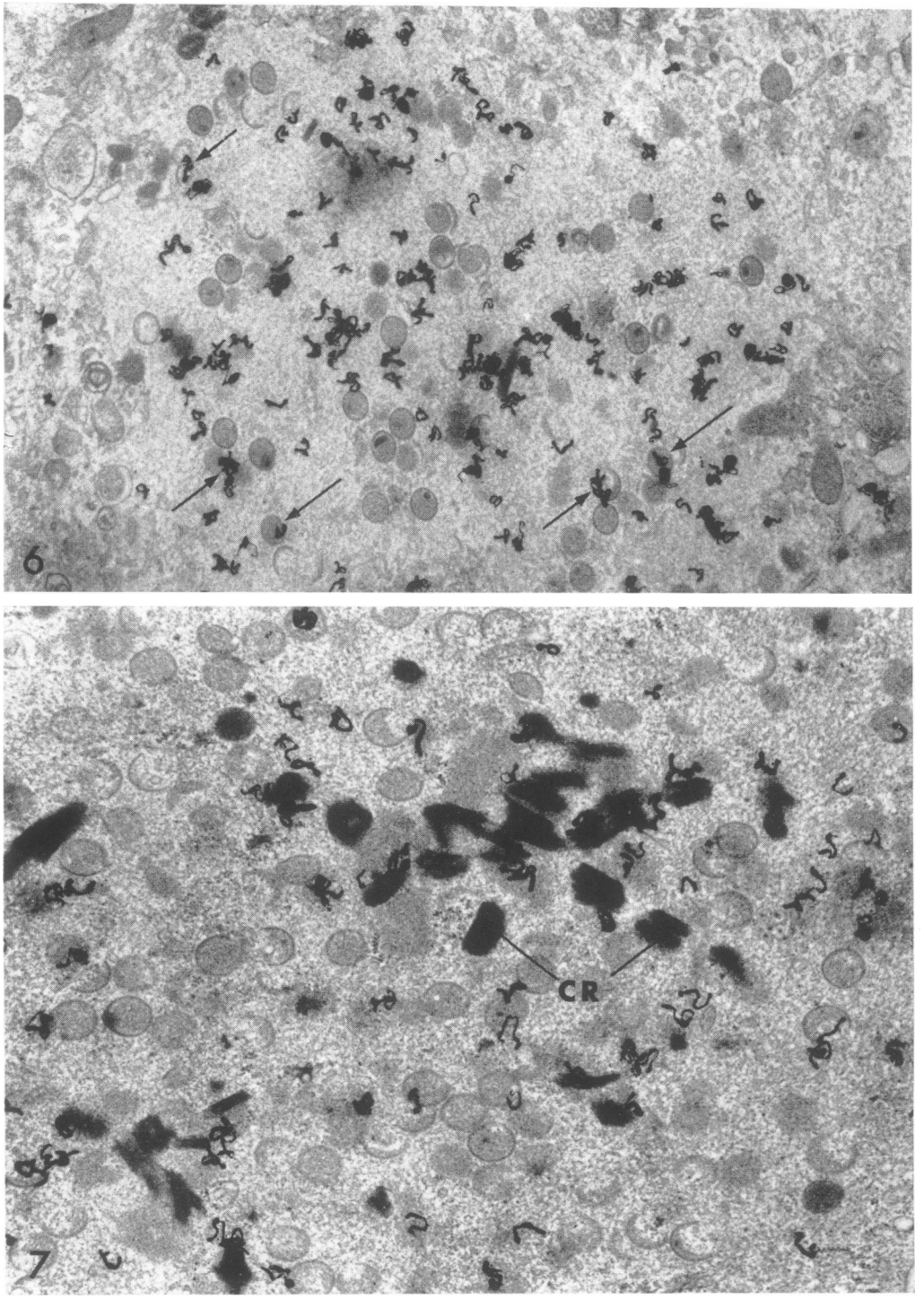

FIG. 6. *Factory site in a BSC cell infected with SFV. One-hour exposure to tritiated thymidine (from 11 to 12 hr PI) followed by a chase for 3 hr. Numerous silver grains are associated with developing virus particles (→).* × *12,000.*

FIG. 7. *Same experiment as shown in Fig. 6. A few silver grains are associated with immature virus particles and with dense crystalline structures (CR).* × *18,000.*

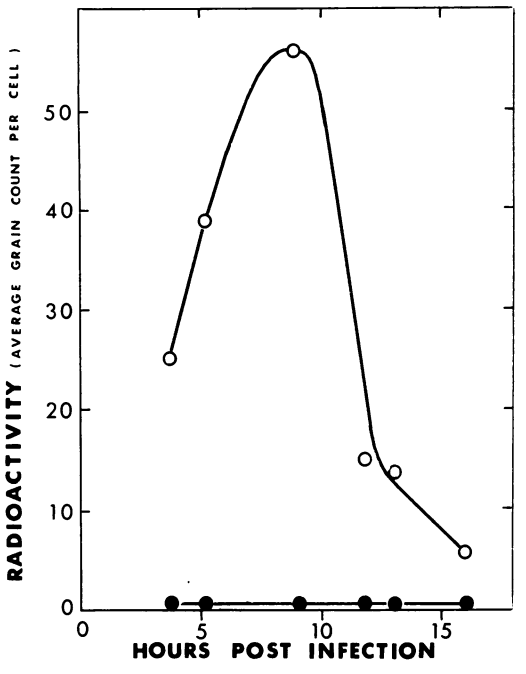

FIG. 8. *Cytoplasmic incorporation of tritiated thymidine. Infected (○) and noninfected (●) cultures were exposed to tritiated thymidine for 1 hr and were fixed at the specified times. A total number of 200 cells, showing foci of viroplasm, were photographed with the electron microscope, cytoplasmic silver grains were counted, and the average number of these grains per cell was calculated. The cytoplasm of uninfected cells was examined in the same manner.*

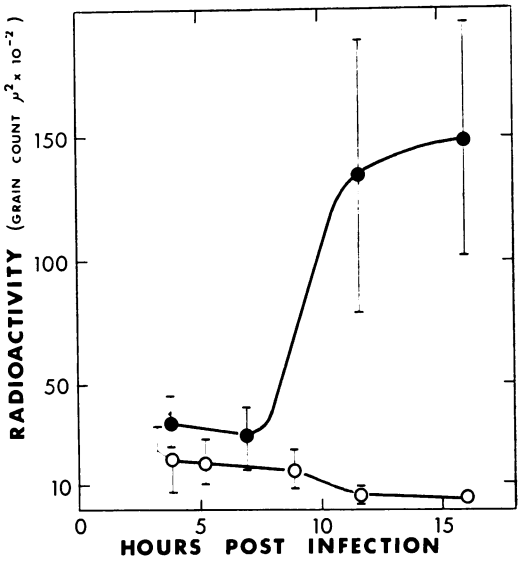

FIG. 9. *Nuclear uptake of tritiated thymidine. Infected (○) and noninfected (●) cells were exposed to tritiated thymidine for 1 hr and were fixed at the specified times. A total of 400 nuclei were photographed with the electron microscope.*

Joklik and Becker (16), who studied the uptake of ^{3}H-thymidine in cells infected with vaccinia virus. Furthermore, our results are in full agreement with previous light microscope radioautographic data (17); these data indicated that, in FL cells infected with SFV and subsequently exposed to ^{3}H-thymidine, all of the so called "B" type inclusions were labeled, whereas other areas of cytoplasm were entirely free from silver grains.

Present data indicate that progeny viral DNA synthesis starts before 3 hr PI. As the infection progresses, ^{3}H-thymidine uptake undergoes marked stimulation. Quantitative data clearly show that the maximal rate of incorporation occurs at 8 to 9 hr PI, followed by a rapid decline. By 15 hr, progeny viral DNA synthesis is nearly negligible.

This time course of viral DNA synthesis is in full agreement with earlier data presented by Ewton and Hodes (7), who studied, by biochemical means, the incorporation of exogenous thymidine in the cytoplasmic fraction of HeLa cells infected with SFV. In addition, we established that the events which lead to the decline of DNA synthesis are correlated with the onset of viral morphogenesis. Coating of progeny viral genome by membranous structures never occurs before 10 to 11 hr PI. Comparable to the vaccinia virus system (23), this may indicate that the proteins which are incorporated into mature virions and which probably also participate in membrane formation are made late in the infectious cycle. In the vaccinia virus system, late proteins are made at 6 hr PI and thereafter, whereas viral DNA replication is almost complete by 6 hr PI. (16, 22). Our findings suggest a similar set of events, but with a greater lag as compared with the vaccinia system.

However, morphogenesis of SFV, like that of vaccinia virus (12), is not associated with a complete cessation of viral DNA synthesis. Viral factories containing many immature as well as mature virions were still capable of incorporating small amounts of exogenous thymidine, indicating that DNA replication was not completely arrested.

With respect to host DNA modifications induced by SFV, very little biochemical evidence is available at present. This problem is of particular interest if one considers that contradictory reports have appeared in recent years concerning the proliferative potentialities of fibroma-infected cells (13, 15, 26). However, it is likely that the cellular response varies with the viral or cell strains used as well as with the experimental conditions employed (26). From their light radioautographic

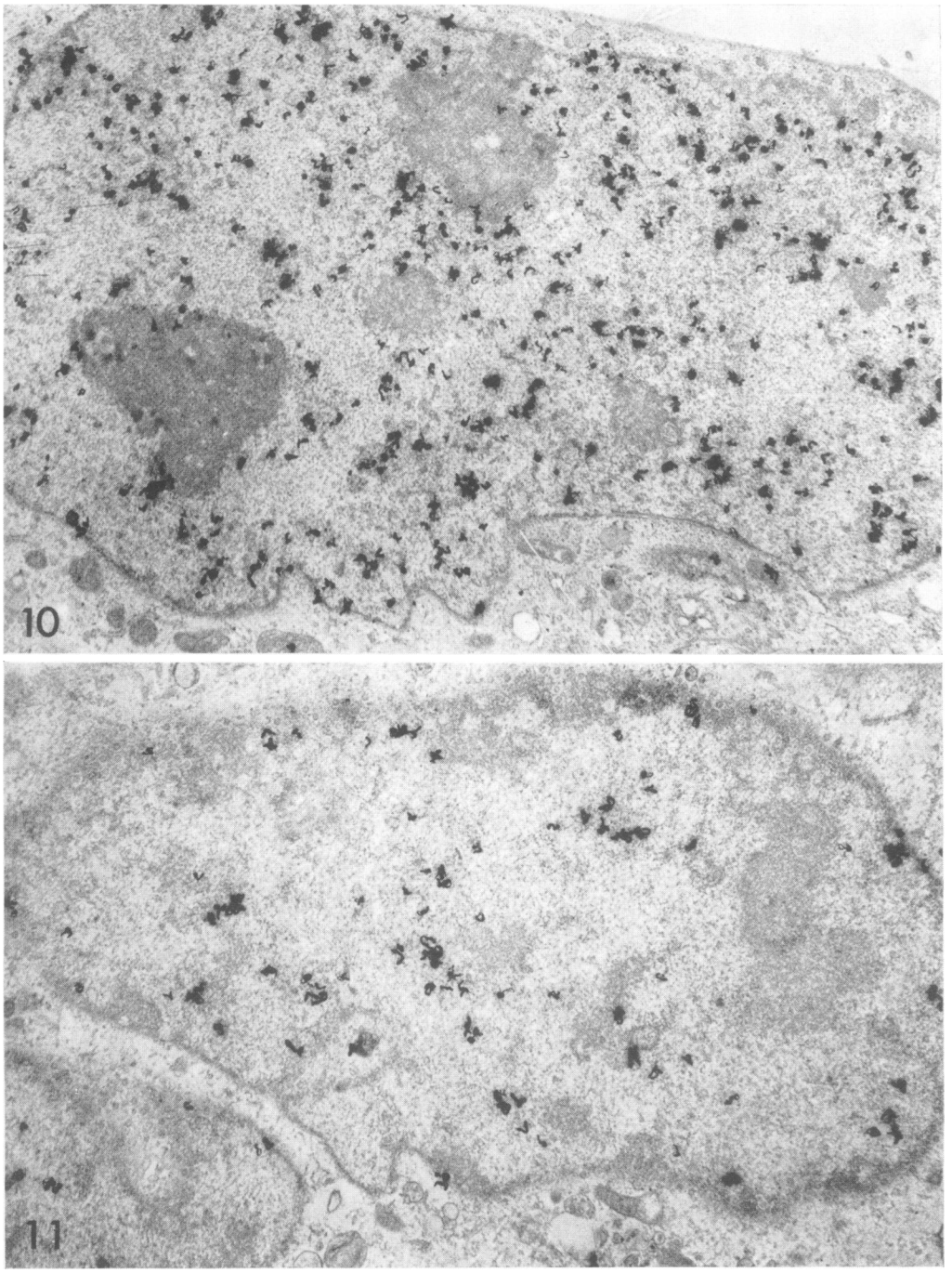

FIG. 10. *Electron microscopic autoradiography of a noninfected BSC cell. One-hour pulse with tritiated thymidine (control, 9 hr). Silver grains are primarily located over the nucleoplasm and the marginal chromatin.* × *6,750.*

FIG. 11. *Electron microscopic autoradiography of a BSC cell 9 hr after infection with SFV. One-hour pulse with tritiated thymidine. The nucleus shows a weak labeling as compared to the control in Fig. 10.* × *8,000.*

study, Kato et al. (17, 18) pointed out that the incorporation of ^{3}H-thymidine into nuclei of inclusion-bearing cells was almost completely suppressed.

Our methods permitted a more precise analysis with respect to host DNA metabolism. In our system (*see* Fig. 9), host DNA synthesis was reduced, on the average, by 50% 4 hr PI as compared with the noninfected cultures, in which the majority of cells were not in a true *S* phase at that time. It is very likely that the degree of inhibition varies according to the physiological state of the host cell at the time of infection. Inhibition becomes more marked as infection progresses and host DNA synthesis is almost completely suppressed in late stages of viral development. It seems very unlikely that this suppression of host DNA synthesis would be modified after 16 hr PI. In fact, a degenerative process always occurs at later times, leading to the complete destruction of the cultures.

With respect to the mechanism responsible for the inhibition of host DNA synthesis, no data are available at present concerning SFV infection, but this problem has been more fully explored in the vaccinia virus system. In this system, it has been found that input viral protein may be responsible for the early drastic inhibition of host DNA synthesis which occurs soon after infection (11, 16). We cannot, however, infer that the same mechanism exists in the fibroma virus system. It is well known (1, 5) that vaccinia virus exhibits an early toxic effect, whereas fibroma virus does not. Thus, the relationship of viral protein to host DNA synthesis may be another aspect of the host-viral relationship which differentiates the two viruses.

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