JOURNAL OF VIROLOGY, June 1977, p. 720-725

Vol. 22, No. 3
Printed in U.S.A.

Regulation of Herpesvirus Macromolecular Synthesis

VI. Synthesis and Modification of Viral Polypeptides in Enucleated Cells

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Received for publication 7 December 1976

Cells were enucleated with cytochalasin B after infection with herpes simplex virus 1. When protein synthesis was blocked by cycloheximide from the time of infection, mRNA for viral α-infected cell polypeptides (ICP) 4, 0, and 27 accumulated in the cytoplasm and was expressed after the removal of both drug and nucleus. A host protein, ICP 22, whose synthesis is stimulated in intact cells, was not made, and viral protein ICP 4, which is normally modified to a form that migrates more slowly in polyacrylamide gels, was not modified in the absence of the nucleus. After enucleation at 2 h postinfection, a number of viral β and γ proteins continued to be made, starting at 20 to 25% of the normal rates and declining with a half-time of about 2 h. The synthesis of ICP 4 declined more rapidly, suggesting that it is switched off in the cytoplasm.

The replication of human herpesvirus 1 (herpes simplex virus) involves a complex system of regulatory mechanisms controlling the production and posttranslational modification of proteins and their movements within the host cell. At least three groups of viral polypeptides have been identified: α polypeptides are made first and induce the later production of β proteins (6, 7). These in turn switch off α polypeptide synthesis and induce the production of γ polypeptides. The maximum rates of synthesis of α polypeptides occur 2 to 4 h after infection, and those of β polypeptides occur 5 to 7 h after infection. A number of α and β proteins are phosphorylated, and some are further modified after synthesis, acquiring a lower electrophoretic mobility in polyacrylamide gels. All α and some β proteins are translocated from the cytoplasm to the nucleus (L. Pereira, M. Wolff, M. L. Fenwick, and B. Roizman, Virology, in press).

To examine the function of the nucleus in the modification of viral proteins, in the decline of α and host protein synthesis and in the induction of β and γ viral polypeptide synthesis, we removed the nuclei from cells after infection by centrifugation in the presence of cytochalasin B (11) and examined the proteins made in the anucleate cytoplasts. Cytoplasts obtained from uninfected cells continue to make proteins at somewhat reduced rates for several hours, and viruses that do not require the nucleus, such as vaccinia, poliovirus, reovirus, vesicular stomatitis, and Semliki forest viruses, can multiply in them, yielding infectious progeny (3, 10).

MATERIALS AND METHODS

Cells and virus. Near-confluent monolayers of about 7×10^5 Vero (African green monkey kidney) cells were grown in plastic Leighton tubes (Nunc, Denmark). They were infected with 30 to 50 PFU per cell of the F strain of herpes simplex virus type 1 (2) in 0.5 ml of mixture 199 supplemented with 1% calf serum (medium 199-1). After 20 min at 20°C, the unadsorbed virus was removed and replaced with 1.5 ml of medium at 37°C (zero time).

Enucleation. Cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide (1 mg/ml) and diluted into medium 199-1 to yield a final concentration of 10 μg/ml. Leighton tubes containing 8 ml of this solution at 34°C were placed, flat surface toward the center, in a Sorvall SS34 fixed-angle rotor that had been prewarmed to 34°C. They were centrifuged at 10,000 rpm (12,000 $\times$ *g*) for 10 to 15 min without refrigeration. The pellet of nuclei, contaminated with whole cells, was resuspended and discarded. The surviving cytoplasts were covered with fresh medium without cytochalasin. Control experiments in which cells were labeled by overnight incubation with [^{3}H]thymidine and then enucleated showed that 1 to 2% of the acid-precipitable radioactivity remained in the monolayer of cytoplasts. An estimate of the amount of cytoplasmic protein retained in the cytoplast monolayer was made by scanning a positive transparency of the stained gel after electrophoresis and comparing the areas of a number of prominent peaks with those in whole cell controls. This indicated an approximately 60 to 75% survival of cytoplasmic material.

Labeling. The cells were labeled in medium containing L-[U-^{14}C]leucine, isoleucine, and valine (specific activity, 2.5 Ci/g) in place of the nonradioactive amino acids.

Electrophoresis. Cells were lysed in 0.25 ml, and 75-μl samples were subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate as described elsewhere (4). Acrylamide was cross-linked with 4.0% of its weight of *N,N'*-diallyltartardiamide. Gels were stained with Coomassie brilliant blue, dried under vacuum, and left in contact with X-ray film for 1 to 2 weeks.

Densitometry of autoradiograms and computer-aided analysis of absorbance tracings. The autoradiograms were scanned with a soft-laser densitometer (BioMed Instruments, Inc., Chicago, Ill.) interfaced with a General Automation 16/45 digital computer. The analytical procedures were described elsewhere (5, 7).

RESULTS

Synthesis of α proteins. Viral mRNA specifying α polypeptides accumulates in cells infected in the presence of cycloheximide, an inhibitor of protein synthesis, and upon removal of the drug, the production of α polypeptides starts at an enhanced rate (6). To demonstrate that α polypeptides can be synthesized in cytoplasts, cultures of infected cells were either enucleated or treated with actinomycin D at the time of removal of cycloheximide (4 h postinfection) and then were immediately labeled with ^{14}C-labeled amino acids. The radioactive polypeptides extracted from the cells or cytoplasts were separated by electrophoresis in polyacrylamide gels. The first channel of the autoradiogram (Fig. 1) shows the infected cell polypeptides (ICP; 5) made in untreated cells 4 to 5 h after infection. The second channel shows the enhanced production of α ICP 4, 0, and 27 (and the reduced production of β ICP 6) upon reversal of the cycloheximide block. Production of ICP 22, probably a host protein (7), is also stimulated by this treatment. The third channel shows the polypeptides made after enucleation at the time of cycloheximide reversal and indicates that the mRNA's for ICP 4, 0, and 27 (as well as a small amount of mRNA for ICP 6) had already entered the cytoplasm before enucleation and were translated in cytoplasts. ICP 22, on the other hand, was less heavily labeled than in the cycloheximide-treated intact cell controls. ICP 4 made in the absence of the nucleus was more homogeneous than that produced in intact cells, which formed a broad band in the autoradiogram.

Modification of viral proteins. As mentioned in the Introduction, a number of viral polypeptides become modified after synthesis, resulting in a small change, usually a decrease, in electrophoretic mobility (Pereira et al., Virology, in press). The relatively sharp band of ICP 4 formed in cytoplasts (Fig. 1) suggested

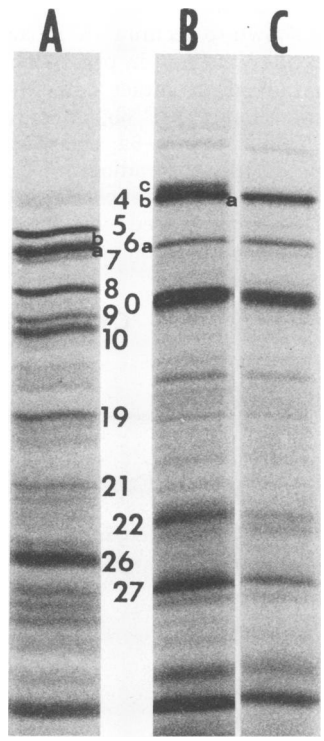

FIG. 1. *Autoradiograms of electrophoretically separated polypeptides from infected intact cells and enucleated cytoplasts. The intact cells and cytoplast cultures were labeled with ^{14}C-labeled amino acids (5 μCi/ml) at 4 to 5 h postinfection. Channel A: untreated infected cells. Channel B: infected cells were incubated in the presence of cycloheximide (50 μg/ml) from 0 to 4 h postinfection. The cycloheximide was then removed, and the cells were incubated in the presence of actinomycin D (1 μg/ml). Channel C: infected cells were incubated in the presence of cycloheximide (50 μg/ml) from 0 to 4 h postinfection. The cycloheximide was then removed, and the cells were enucleated. The polypeptides were subjected to electrophoresis in 8% polyacrylamide gels. The numbers refer to ICP; the letters a, b, and c refer to the primary translational product (a) and to the products of post-translational processing (b and c). In this figure, ICP 4b and c are prominent in autoradiograms of polypeptides extracted from actinomycin D-treated intact cells and absent from those of polypeptides extracted from cytoplasts. Similarly, ICP 6b is prominent in the autoradiograms of polypeptides extracted from untreated infected cells.*

that the modification of ICP 4 was prevented if the nucleus was removed. That this is so is seen more clearly in Fig. 2. In this experiment, the 1-h labeling period was followed by a 1-h incubation (chase) in the absence of radioactive amino acids. ICP 4 extracted from intact cells that had been labeled in this way formed two discrete bands, 4b and 4c, but cytoplasts con-

tained only ICP 4a. ICP 4b and 4c have previously been shown to accumulate in the nucleus (Pereira et al., Virology, in press). Three other changes occurred in intact cells during the chase. A new band, ICP 6b, appeared in the autoradiogram just above 6a, and this band was also visible in the autoradiogram of polypeptides extracted from the anucleate culture. Secondly, ICP 9a was replaced by a slower-migrating ICP 9b. Neither ICP 9a nor 9b was made in cytoplasts; presumably the appropriate mRNA had not yet entered the cytoplasm at the time of enucleation. Thirdly, ICP 10a was replaced by the slightly faster-migrating 10b, but this change does not appear to have occurred in cytoplasts.

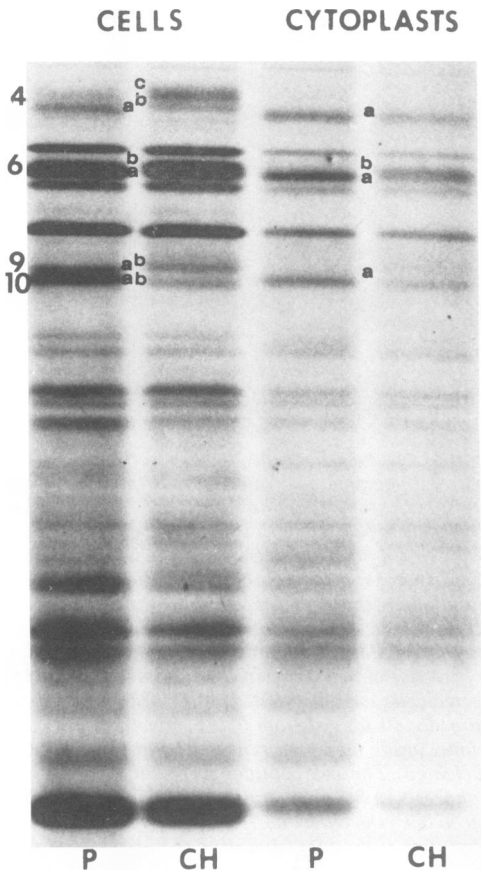

FIG. 2. *Modification of viral proteins after enucleation. Left two channels: autoradiograms of polypeptides extracted from whole infected cells that had been centrifuged without cytochalasin B at 2 h postinfection. Abbreviations: P, labeled from 2 to 3 h postinfection with 1 μCi/ml; CH, labeled 2 to 3 h postinfection with 1 μCi/ml and then chased for 1 h in nonradioactive medium. Right two channels: polypeptides extracted from cytoplasts enucleated at 2 h postinfection. Abbreviations: P, labeled from 2 to 3 h with 4 μCi/ml; CH, labeled from 2 to 3 h with 4 μCi/ml and then chased for 1 h. The two right channels contained approximately 60% of the protein in 1 and 2. The gel contained 9% acrylamide. Numbers indicate infected cell polypeptides that undergo modification during the chase.*

Pattern of protein synthesis. To study the effect of removal of the nucleus on the changing pattern of protein synthesis in infected cells, cytoplasts were labeled for 1 h at different times after enucleation, and identical fractions of the cell or cytoplast lysates were subjected to electrophoresis. The left half of the autoradiogram in Fig. 3 shows the pattern of polypeptide synthesis in intact cells. Other control expeirments (not illustrated) showed that the pattern of viral protein synthesis is not affected by merely exposing cells to cytochalasin B without centrifugation, nor is it affected by centrifugation without exposure to cytochalasin. In intact cells, the synthesis of ICP 4 and 27 declined after about 4 h; ICP 5 through 10 and 19 progressively increased, and ICP 16, 21, 26, and 36 appeared at later times. After enucleation at 2 h, however (right half of Fig. 3), ICP 4 to 8, 10, and 19 continued to be made at gradually declining rates over the next 5 h; ICP 9, 16, 21, 26, and 36 were not made.

The relative rates of synthesis of ICP 4, 5, 6, and 7 were calculated from scans of the autoradiographic images shown in Fig. 3 as described in the legend to Fig. 4. In cytoplasts, there was a general decline, with a half-life of about 1.5 h for ICP 4 (the same as in intact cells from 4 to 5 h onwards) and about 2 h for ICP 5, 6, and 7. That the rate of synthesis of ICP 4 declined more rapidly than those of ICP 5, 6, and 7 in cytoplasts as well as in intact cells can be deduced from Fig. 4, where for each time interval the rate of synthesis of each polypeptide was related, as described in the legend to that figure, to the sum of the four rates and normalized with respect to the rates at the earliest time.

The relative rates of synthesis of the six host polypeptides marked with dots in Fig. 3 are shown in Fig. 5. In this instance, the rates were calculated from the ratio of the integrated absorbance of the autoradiographic image of a band at each time interval to that at the first labeling interval. The curves for all six polypeptides were similar, and average values are shown. Host protein synthesis declined in infected cells from 4 to 5 h, with a half-life of about 2 h, and in infected cytoplasts from the time of enucleation, with a half-life of about 3 h. In uninfected cytoplasts in another experiment, also shown in Fig. 5, the rates of synthesis of the same host proteins declined, with a half-life of about 5 h.

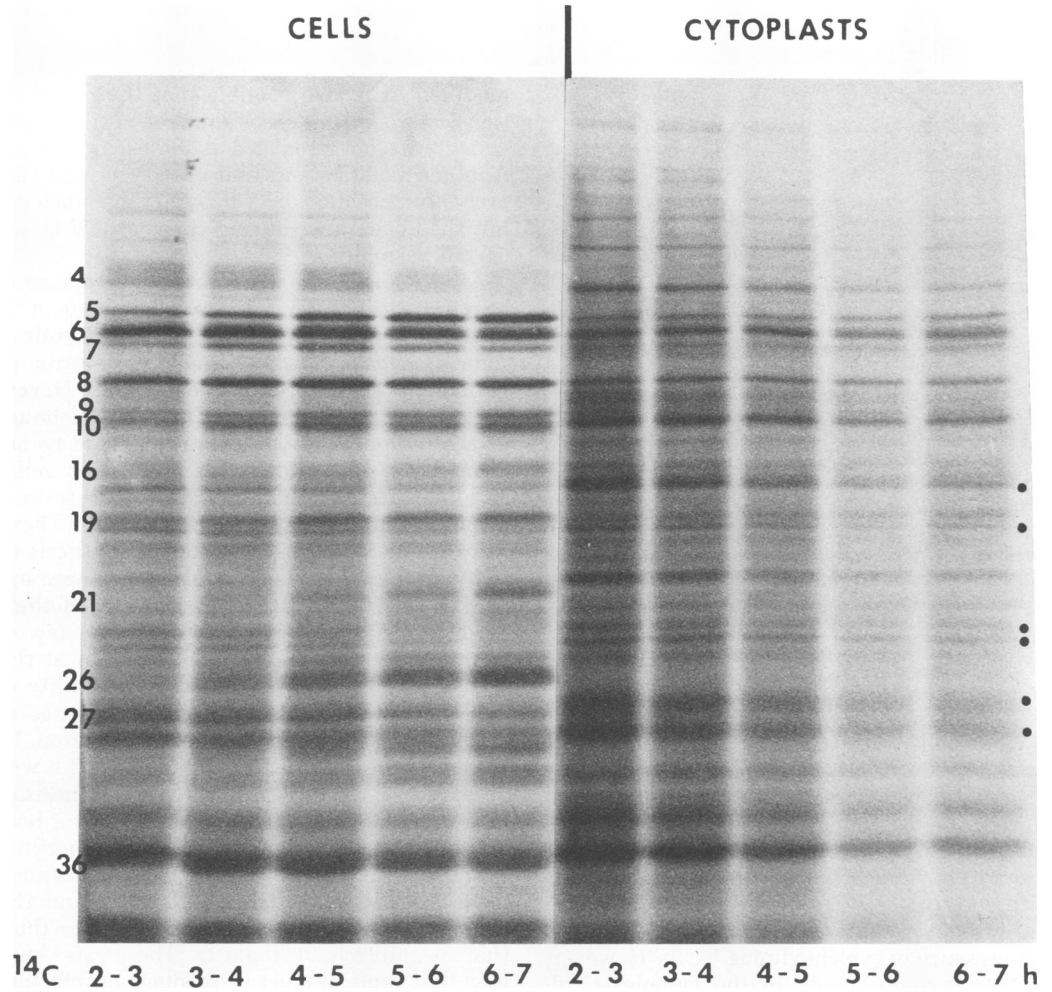

FIG. 3. *Effect of enucleation of viral protein synthesis. Autoradiogram of polypeptides labeled during the time intervals indicated (hour postinfection). Left panel: infected cells exposed to cytochalasin B at 2 h under enucleation conditions, but not centrifuged, and labeled with 1 μCi/ml. Right panel: cells enucleated at 2 h and labeled with 4 μCi/ml. Anucleate samples contained approximately 60% of the cytoplasmic protein in whole cell lysates. Gel contained 9% acrylamide. The numbers refer to infected cell polypeptides; the processed forms (a, b, c, etc.) are not indicated. The dots on the right refer to host polypeptides.*

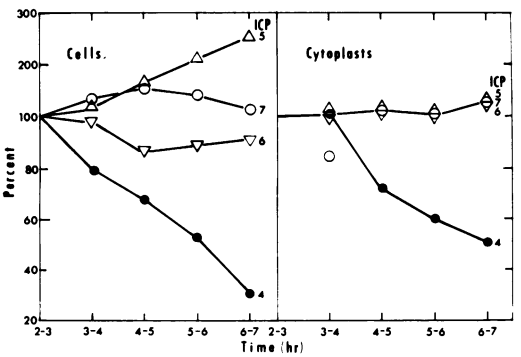

FIG. 4. *Synthesis of ICP, 4, 5, 6, and 7 in intact cells and cytoplasts. The percent normalized rates shown were calculated from scans of autoradiograms shown in Fig. 3. For example, for ICP 4, the percent normalized rate at time t was calculated from:*

$$\frac{A_t 4}{A_0 4} \times \frac{A_0\,(4, 5, 6, \text{and } 7)}{A_t\,(4, 5, 6, \text{and } 7)} \times 100$$

where A_t and A_0 are the integrated absorbances of the autoradiographic images of individual bands or group of bands at time t and at the time of the first labeling interval (2 to 3 h, respectively). Note that for the polypeptide whose synthesis is declining, the half-life can be determined from measurements of relative rates of synthesis. The relative rate of synthesis of ICP 4 at time t relative to that at time 0 is given by

$$\frac{A_t 4}{A_0 4} \times \frac{A_0\,(4, 5, 6, \text{and } 7)}{A_t\,(4, 5, 6, \text{and } 7)}.$$

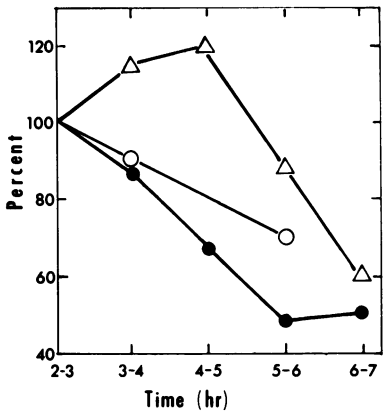

FIG. 5. *Changes in the rates of synthesis of host proteins in infected cells (△) and in infected (●) and uninfected (○) cytoplasts. Absorbances of the six absorbance bands marked with dots in Fig. 3 were each expressed as a percentage of the absorbance of that band in the first sample, and average values were plotted. The data for the same host proteins in uninfected cytoplasts (○) were taken from a different experiment.*

DISCUSSION

When cells were treated with cycloheximide for the first 4 h of infection and then both the drug and nuclei were removed, α ICP 4, 0, and 27 were made, indicating that their mRNA's had entered the cytoplasm, in accord with the observation that RNA capable of hybridizing with certain specific regions of the viral DNA was present in the cytoplasm of cells infected in the presence of cycloheximide (8, 9). However, ICP 22 was not made in the cytoplasts, although Fig. 3 shows that it was made in nonenucleated cells if actinomycin was added at the time of cycloheximide reversal. Therefore, the experiment suggests that, although mRNA for ICP 22 accumulated in the presence of cycloheximide, it did not pass into the cytoplasm until the drug was removed. ICP 22 is suspected of being a host protein whose production is stimulated after infection in the presence of cycloheximide and also in uninfected cells in the presence of the arginine analogue, canavanine (7).

It has been reported (1, 7; Pereira et al., Virology, in press) that ICP 4 is modified after synthesis to the slower electrophoretically migrating forms, 4b and 4c. All three forms are phosphorylated (Pereira et al., Virology, in press). Whereas the original form, 4a, is found in the cytoplasm, 4b and 4c are predominantly nuclear (Pereira et al., Virology, in press), suggesting that the transition of 4a to 4b rapidly follows, or is rapidly followed by, transport from cytoplasm to nucleus. The experiments reported here favor the former alternative, since in the absence of the nucleus ICP 4a is not modified. It is also possible that the modification of 4a is mediated by another viral protein which was not produced in enucleated cells. Another modification, that of ICP 10, was also probably inhibited by removing the nucleus, but the appearance during chase of ICP 6b occurred normally.

After enucleation, the rate of cytoplasmic host and viral protein synthesis fell to about 20 to 25% of the value in intact cells as a result of some damage or loss sustained during centrifugation in the presence of cytochalasin B. Nevertheless, synthesis continued for several hours at a rate appreciably higher than could be accounted for by the few remaining intact cells, and the patterns of synthesis differed in several respects from those found in intact cells. There were no increases in the rates of synthesis of individual viral proteins, and no new ones appeared after enucleation, as expected if their production were controlled by the entry of mRNA into the cytoplasm rather than at the translational level. The functional stability of mRNA's can be estimated from the rates of decay of protein synthesis after enucleation. In infected cytoplasts, a half-life of about 2 h was observed for the declining rates of synthesis of ICP 5, 6, and 7, compared to about 5 h for host proteins in uninfected cytoplasts. In infected cytoplasts, the synthesis of ICP 4 declined faster than that of ICP 5, 6, and 7, and the synthesis of host proteins declined faster than that in uninfected cytoplasts. These rates suggest that some product or products of infection that have reached the cytoplasm by 2 h, or are made in the cytoplasm after enucleation, inhibit the synthesis of ICP 4 and host proteins. The observation (6) that after removal of a cycloheximide block ICP 4 declines more slowly in the presence of actinomycin than without it also suggests a cytoplasmic switch-off mechanism.

ACKNOWLEDGMENTS

M. F. is on leave of absence from Sir William Dunn School of Pathology, University of Oxford. The studies were aided by grants from the American Cancer Society (VC 103L) and from the National Cancer Institute (CA 08494 and CA 19264).

LITERATURE CITED

1. **Courtney, R. J., and K. L. Powell.** 1975. Immunological and biochemical characterization of polypeptides induced by herpes simplex virus types 1 and 2, p. 63-73. *In* G. de The, M. A. Epstein, and H. zur Hausen, Oncogenesis and herpesviruses. II. IARC, Lyon.
2. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. 3:357-364.

3. **Follett, E. A. C., C. R. Pringle, and T. H. Pennington.** 1975. Virus development in enucleate cells. J. Gen. Virol. 26:183-196.
4. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. 14:640-651.
5. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in the infected cell. J. Virol. 12:1347-1365.
6. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14:8-19.
7. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. U.S.A. 72:1276-1280.
8. **Jones, P. C., G. S. Hayward, and B. Roizman.** 1977. Anatomy of herpes simplex virus DNA. VI. α RNA is homologous to noncontiguous sites in both the L and S components of viral DNA. J. Virol. 21:268-276.
9. **Kozak, M., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis: nuclear retention of non-translated viral RNA sequences. Proc. Natl. Acad. of Sci. U.S.A. 71:4322-4326.
10. **Pennington, T. H., and E. A. C. Follett.** 1974. Vaccinia virus replication in enucleate BSC-1 cells: particle production and synthesis of viral DNA and proteins. J. Virol. 13:488-493.
11. **Prescott, D., D. Myerson, and J. Wallace.** 1972. Enucleation of mammalian cells with cytochalasin B. Exp. Cell Res. 71:480-485.