Electron Microscopic Studies on Assembly of Herpes Simplex Virus upon Removal of Hydroxyurea Block

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SUMMARY

The release of hydroxyurea-treated, herpes simplex virus-infected cells from the drug-induced block resulted in the prompt assembly of infectious virus. Electron microscope observations at sequential intervals following removal of the drug revealed considerable synchrony of replication. This synchrony permitted stages in the complex process of core assembly to be examined in detail. The data suggest that after partial or complete assembly the nucleoprotein enters the differentiated capsid to become enfolded.

INTRODUCTION

Hydroxyurea prevents the replication of herpes simplex virus (HSV) by inhibiting the synthesis of virus DNA. Although infectious virus is not made in the presence of the drug, virus proteins and glycoproteins are synthesized and intranuclear capsids are formed. The effects of hydroxyurea are reversible. Chemical studies reveal that upon reversal of the blockade DNA and protein synthesis resume and follow a pattern characteristic of untreated infected cultures with resulting assembly of infectious virus (Nii et al. 1968; Rosenkranz & Becker, 1973). Microscopic observations show that a considerable degree of synchrony of virus maturation follows release from hydroxyurea inhibition, thus permitting recognition of sequential stages in the apparent incorporation of the nucleoprotein into pre-formed capsids.

METHODS

Cell and culture media. Monolayer cultures of HeLa cells were grown in Eagle's minimal essential medium (MEM, Grand Island Biological Co. (GIBCO) Grand Island, N.Y.), supplemented with 10% foetal calf serum.

Virus. The Miyama strain of herpes simplex virus was used.

Treatment with hydroxyurea (HU). Monolayers were infected with HSV at a multiplicity of 2 in the presence of 10^{-2} M-HU. After 1 h adsorption at 37 °C the cultures were washed twice with BSS containing 10^{-3} M-HU. Fresh MEM media containing the same HU concentration was then added. After incubation for 18 h several cultures were harvested for electron microscopy. Others were washed three times with BSS to remove the drug and incubated with fresh media. Samples were withdrawn hourly for 12 h and processed for electron microscopy. As controls, uninfected cultures were treated with HU and released in the same manner.

Preparation of cells for electron microscopy. The cells were scraped, pelleted by centrifuging, and fixed in 1% glutaraldehyde. After washing, the samples were post-fixed in
osmium tetroxide and embedded in epoxy resin (Epon 812). Thin sections were stained with uranyl acetate and lead citrate.

_Treatment of sections with ethylene diaminetetraacetic acid (EDTA)._ Thin sections were stained with 1% uranyl acetate for 1 h, washed with distilled water and immersed in 0.2 m-EDTA at pH 7.0 for 30 min. After washing in distilled water the sections were stained with lead citrate for 5 min.

_Isolation of capsids._ HeLa cells, infected and treated for 14 h with 10^{-2} m-HU, were suspended in 1 ml of PBS buffer and disrupted by ultrasonic treatment for 10 min. After clearing by centrifuging at low speed the supernatant fluid was layered on a 20 to 50% sucrose gradient and centrifuged at 25,000 rev/min for 60 min at 5 °C using SW 25-1 rotor, and Beckman L-2 ultracentrifuge. The visible band was removed from the side of a tube with a needle, diluted with PBS to 40 ml and pelleted by centrifuging for 1 h at 18,000 rev/min in a Sorvall RC-2 centrifuge. The pellet was resuspended overnight in 1 ml PBS and centrifuged on a second sucrose gradient under the same conditions as before. The visible band was removed and negatively stained for electron microscopy with 0.5% ammonium molybdate.

_Plaque assay._ Monolayer cultures of HeLa cells were grown in 30 ml tissue culture flasks (Falcon Plastics, Oxnard, California) and infected with 0.1 ml portions of HSV dilutions. The infected cultures were placed on a rocker table for 1 h at 37 °C, washed three times with balanced salt solution (BSS, GIBCO), overlayed with 0.5% agarose in MEM maintenance medium and incubated for 72 h. The agarose was then removed by suction, and the cells fixed in 1% glutaraldehyde for 2 min. After washing with water the cells were stained with 1% gentian violet for 5 min.

**RESULTS**

_Production of infectious virus after release from HU block._

Production of infectious virus after release from HU inhibition was found to correspond to that of uninhibited cultures. The titres of infectious virus started to rise 5 h after removal of the drug and continued for 7 h. The same events can be followed for untreated cultures (Fig. 1).

_Hydroxyurea treated HSV-infected cultures._

Electron microscopic examination of HU-inhibited, HSV-infected cells showed capsids containing cores of low electron density (Fig. 2). By negative staining these capsids, which were readily purified on sucrose gradients, appeared to be intact and to contain an oval core (Fig. 3). Chemical analysis using radioisotopes revealed that the capsids and cores contained virus proteins and were devoid of DNA.

_Stages in capsid maturation after release from HU block._

Upon removal of HU from infected cultures there ensued a lag period of 3 h after which the capsids rapidly increased in number. At this time nearly all the capsids contained electron dense punctate bodies (Fig. 4). As early as the third hour electron dense strands could be traced from the nucleoplasm to the interior of the capsid (Fig. 4 and Fig. 5, arrows). The similarity in appearance of the capsids suggested that a considerable degree of synchrony was initially achieved by release of the block. Although synchrony appeared to diminish with time, the forms described invariably predominated at the intervals cited. Capsids containing cores of low density and resembling those seen in HU-treated cells were only occasionally encountered (see lower left of Fig. 4, arrow) suggesting that some capsids assembled during the block persist. The punctate cores continued to be numerous in
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Fig. 1. Synthesis of infectious HSV. ▲—▲, HeLa cells infected with HSV and treated with 10^{-2} M-HU at time 0; ○—○, HU-treated cells infected with virus for 18 h and released from block at time 0; ■—■ control, cultures infected at time 0 and not treated with HU.

the 4 h sample, but by the fifth hour, a striking single or double bar-shaped core appeared (Fig. 6a, b). It is likely that many of the capsids contain a double bar and that the appearance of a bar often reflects an artefact consequent upon the level and angle of sectioning. Capsids containing bars were numerous and predominated by the sixth hour. At 7 to 8 h both capsids containing bar-shaped cores and capsids with the dense oval cores characteristic of those found in mature virus particles were observed. Eight to nine hours after release of HU block, capsids containing the latter cores predominated (Fig. 7). At this time a considerable number of mature, enveloped virus particles were observed in both cytoplasmic vacuoles and extracellular spaces. Fig. 8 summarizes the sequential stages of development which were encountered. The first two micrographs show fibres which appear to be in the process of passing from the nucleoplasm into the interior of the capsids. Such filaments were repeatedly encountered. At first the filaments were irregular and poorly defined while those seen at later stages were more sharply demarcated (Fig. 4, 5, 7 and 8a, b, h).

Efforts to isolate capsids by sucrose gradient purification during the process of DNA incorporation at 3, 5 and 7 h following removal of the block were unsuccessful, the vast majority appearing broken or empty by negative staining.

**EDTA treatment of thin sections**

To determine whether the filaments apparently entering the capsids from the nucleoplasm contained DNA, advantage was taken of the observation by Furlong, Swift & Roizman (1972) that EDTA preferentially solubilizes the uranyl bound to DNA in thin sections.
Fig. 2. Capsids with cores of low electron density in the nucleus of a HU-treated HeLa cell 18 h after infection.

Fig. 3. Negatively stained capsids purified from HSV-infected HU-treated cultures. In the absence of DNA synthesis the cores in this and the preceding figure are presumably composed of protein.

Fig. 4. Capsids with punctate and filamentous-shaped core structures in the nucleus of infected cells 3 h after release from HU inhibition. Synchrony is reflected by the fact that the cores appear to be at similar stages of differentiation.

Accordingly, thin sections of HSV-infected cultures at 3, 5, 7 and 9 h after drug release were treated with EDTA after uranyl acetate staining. The results of these experiments revealed that: (1) Chromatin of the nuclei was unstained and the nuclei seemed empty when compared with non-EDTA-treated cells. (2) 75% of the enveloped virus particles lost their electron dense core and appeared devoid of internal structure. The fact that some cores appeared not to be altered by EDTA is consistent with the hypothesis previously advanced (Miyamoto & Morgan, 1971) that there may be two distinct types of particles. (3) In addition, the punctate bodies and bars characteristic of early stages in assembly disappeared.
Fig. 5. 3 h post release. The cores containing filaments are viewed at higher magnification. When properly oriented within the section there is a strong suggestion of symmetry in the arrangement of the filaments.
Fig. 6 (a) and (b). Capsids with bars and spherical cores of differing size and density 5 h after release of the block.
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(4) Lastly, the filaments in process of entering the capsids from the nucleoplasm were no longer seen (Fig. 9). These findings suggest that the punctate bodies, bars and filaments are composed of, or at least contain, DNA.

DISCUSSION

Studies of the effect of HU on the development of several viruses have revealed that although synthesis of both virus and host DNA is blocked, some, if not all, of the virus proteins are formed (Levy et al., 1968; Nii et al., 1968; Breese & DeBoer, 1969; Morishima, Komano & Onodera, 1971). The effect of the drug is reversible (Nii et al., 1968; Nordemskjold & Krakoff, 1968; Jasty & Chang, 1970; Zweig, Rosenkranz & Morgan, 1972; Rosenkranz & Becker, 1973), and after release, replication of infectious virus occurs. It is important to emphasize that the virus development as judged by DNA and protein synthesis is similar to that observed in untreated cells (Zweig et al., 1972; Rosenkranz & Becker, 1973).
Fig. 8. Capsids arranged in the presumed sequence of development. Fine filaments intrude from the nucleoplasm and assume an ordered and probably symmetrical arrangement within the capsid. One or two bars form, which increase in size and density. The double bars merge and finally the oval, dense and tightly wound core characteristic of the mature virus particle appears.

This supports the assumption that the structures shown are the normal intermediates in virus replication.

As previously mentioned, electron microscopic study of virus differentiation after removal of HU reveals considerable synchrony in the appearance of developmental forms. At 3 h most of the infected cells contain capsids enclosing thin filaments or punctate bodies. Presumably the punctate bodies represent filaments in cross section. The filaments tend to assume parallel orientation and in cross section often exhibit symmetrical arrangement. Similar structures have been seen in several electron microscopic studies of herpes virus
Fig. 9. Thin sections of HSV-infected cells treated with 0.2 M-EDTA. (a) 12 h after release of HU block. Most electron dense cores have been removed by treatment. (b) 9 h after release of HU block. Following treatment, neither cores nor filaments are observed in developing capsids.

development (Nii, Morgan & Rose, 1968; McCombs et al. 1971; Nazerian et al. 1971; Nii, 1971) but never in the numbers we observed 3 to 5 h after release of the block. Irregular filaments in the nucleoplasm frequently terminate at the capsid or appear to be in passage directly into the core. Presumably such filaments contain, or are composed of, nucleoprotein since they are susceptible to treatment with EDTA, an agent that was shown selectively to remove the uranyl stain from DNA (Furlong et al. 1972). At 5 h many capsids contain bar-shaped, electron dense cores. By the eighth hour after reversal of the HU block, the filaments become more sharply defined and they can be seen more clearly in passage from the nucleoplasm directly through the capsid into the core (striking examples of the phenomenon are also evident in Fig. 10 of the paper by Nii (1971) and in Fig. 8 and 9 of the paper by Nii et al. 1969). By the tenth hour there are relatively few bar forms, and the core has begun to assume the dense, oval appearance characteristic of the mature virus.
particle. By the twelfth hour filaments extending from the nucleoplasm to the core are no longer encountered and capsids are in process of envelopment and transport to the cytoplasm.

Concerning the process by which DNA passes from the nuclear matrix into the capsids it should be noted that the appearance of the capsid in thin section does not reflect the state of its assembly. For example, infected cells depleted of arginine contain numerous capsids which appear to be structurally intact. However, they can be shown to be pervious since ferritin-conjugated antibodies penetrate and cluster on the core (C. Morgan, unpublished observations). Moreover, release of the core after entry of the virus to initiate infection seems to occur through a capsid which, in thin sections at least, appears to be structurally intact (Miyamoto & Morgan, 1971). The probability that at early stages of differentiation the capsid is only partially assembled is supported by failure, despite repeated attempts, to isolate capsids containing filaments or bars in sucrose gradients at 3, 5 and 7 h after release of the block. Presumably these early forms of the capsid are incompletely assembled and thus more fragile and liable to disruption than those encountered later in the course of development.

Of particular interest in this connexion are recent studies (Simon, 1972; King, Lenk & Botstein, 1973; Laemmli & Favre, 1973) concerning the relation between virus DNA maturation and capsid assembly in T4 and P22 bacteriophage systems. It has been shown that during the replication of these bacteriophages, long DNA strands (concatemers) are formed, which are precursors for the shorter mature virus DNA (Dove, 1966; Salzman & Weissbach, 1967; Frankel, 1968). The long DNA strands are believed to be cut to the mature DNA length after entering the capsids. The phage capsids pass through a series of morphogenic steps before they accept the DNA. Cleavage or removal of protein from the early head structures (proheads) apparently prepares the capsid for entry of the DNA (Luftig, Wood & Okinaka, 1971; King et al. 1973; Laemmli & Favre, 1973). In the case of P22 phage a so-called scaffolding protein, which appears as part of the early head structure, is believed to be responsible for folding the incoming DNA molecule. This protein is not present in the mature virus particles (King et al. 1973). In the case of HSV it has been shown that the nuclear capsids contain at least one protein (P22A) not found in the capsids isolated from the mature virus particle (Gibson & Roizman, 1972). Whether this protein is analogous to that encountered in studies of P22 phage remains to be determined.

The foregoing observations together with the sequence of micrographs we have obtained lead us to the following hypothesis. The virus nucleoprotein is synthesized in the nuclear matrix and passes into the capsids, which are pervious at early stages of development. The folding process is undoubtedly a complex and ordered sequence, which is reflected by the structures herein described. Although symmetry is often encountered in the spatial arrangement of the early filaments seen in cross-section the manner in which they are coiled is not yet clear. The filaments become tightly wound into dense bar shaped bodies, which ultimately coalesce into the characteristic oval dense core of the mature virus particle. Envelopment of the capsid with the passage to the cytoplasm completes the process of assembly. The isolation of intermediate structures containing only a fraction of the genome would support the observations in this report. The use of HSV ts mutants now available may assist in the study of this problem.

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