Replication of Animal Viruses in Differentiating Muscle Cells: Vaccinia and Herpes Simplex Virus Type 1

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SUMMARY

Cells cultured from the breast muscles of 11 to 12-day-old chick embryos were infected in the undifferentiated mitotic myoblast stage or in the terminally differentiated non-mitotic myotube stage with one of two DNA viruses, vaccinia and herpes simplex virus type 1 (HSV-1). DNA synthesis was measured and production of virus-specific DNA detected in cells infected as myoblasts or myotubes by isotope labelling, autoradiographic and buoyant density centrifugation techniques. Furthermore, fully fused myotubes resemble myoblasts in their ability to support productive infection by these DNA viruses although DNA replication and nuclear division have ceased in myotubes and only minimum levels of host-cell DNA polymerase activity are present.

We have examined the replication of animal viruses in avian muscle cells undergoing differentiation in vitro as an approach to understanding virus-host cell interactions. The intracellular processes that occur during differentiation of these cells have been studied extensively and it has been shown that terminal differentiation is accompanied by cell fusion into muscle fibres, by initiation of muscle specific protein synthesis and by the rapid decline of DNA polymerase activity concomitant with cessation of DNA replication (Lash et al. 1957; Stockdale & Holtzer, 1961; Okazaki & Holtzer, 1966; O'Neill & Strohman, 1969, 1970; Paterson & Strohman, 1972). Previously we examined the infection of muscle cells with influenza A viruses (O'Neill & Kendal, 1975; Cox et al. 1977) which have a requirement for a host-cell nuclear function in order to replicate. In the present study we have examined DNA synthesis and virus replication in differentiating muscle cells infected with vaccinia virus or herpes simplex virus type 1 (HSV-1). Vaccinia and HSV-1 were compared because virus DNA synthesis occurs in the cytoplasm and nucleus, respectively, for these viruses (Moss, 1974; Roizman & Furlong, 1974) and if different host cell components were involved in their replication, the growth of these two viruses might be affected in different ways by cellular differentiation. Our results demonstrate that fusion of muscle cells caused no apparent change in the ability of the cells to support the replication of either of these viruses, in contrast to the results obtained for polyoma and SV40 viruses which have been reported to infect myoblasts but not myotubes (Fogel & Defendi, 1967).

Muscle cell cultures were prepared as described previously (Cox et al. 1977). Chang liver cell-propagated stocks of HSV-1 strain VR 3 with infectivity titres of 5 to 7 × 10^7 plaque forming units (p.f.u.)/ml were provided by Dr S. Shore and human amnion (FL) cell-propagated stocks of the Wyeth vaccine strain of vaccinia virus with infectivity titres of 10^7 pock forming units per ml were provided by Dr J. Esposito. Growth of vaccinia was measured by the method of Downie & Dumbell (1947) and growth of HSV-1 was measured by the plaque titration method of Shore et al. (1976) or by determining the median tissue culture infectious dose (TCID_{50}) in WI-38 cells.

Muscle cells were infected at room temperature for 1 h with 10 to 20 p.f.u. or 5 to 10 pock

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Fig. 1. DNA synthesis in muscle cells infected with vaccinia or HSV-1 viruses. (a to d). Incorporation of $^3$H-thymidine into TCA-precipitable material. Results are the ratio of the cts/min incorporated in infected cells to the cts/min incorporated in mock-infected control cells. (a) HSV-1-infected myoblasts. (b) HSV-1-infected myotubes. (c) Vaccinia virus-infected myoblasts. (d) Vaccinia virus-infected myotubes. (e to f). Autoradiography of control or infected avian muscle cultures labelled with $^3$H-thymidine. Fixed preparations were covered with Kodak AR-10 stripping film and stored in a dry light-proof box for 2 weeks. After the film had been developed, preparations were stained with Nuclear fast red and examined under phase contrast optics. (e) Mock-infected myotubes labelled from 10 to 12 h p.i. (f) HSV-1-infected myotubes labelled from 10 to 12 h p.i. (g) Vaccinia infected myotubes labelled from 12 to 24 h p.i. (h) Mock-infected myotubes labelled from 12 to 24 h p.i.

forming units per cell nucleus for HSV-1 and vaccinia virus respectively. After the residual virus inoculum was removed and maintenance medium was added, cells were incubated at 35 °C. Initially cells were screened for the production of virus antigens by fluorescent antibody staining. Virus-specific antigens were detected in both the undifferentiated myoblasts and differentiated myotubes infected with either HSV-1 or vaccinia (not shown) indicating that virus messenger RNA was transcribed and translated. DNA synthesis was determined by adding methyl-$^3$H-thymidine (specific activity 58.7 Ci/mmol; New England Nuclear Corporation, Boston, Massachusetts) at a concentration of 1 μCi/ml to 60 mm Petri dishes of cells at the appropriate times post infection (p.i.). After incubation at 35 °C for 2 h, the cells were washed with ice-cold Hank’s balanced salt solution (HBSS) and cells from each dish were scraped into 1 ml of ice cold 5% trichloracetic acid (TCA). TCA precipitable material was collected on glass fibre filters (Whatman GF/C) and washed with 50 ml of 5% TCA and 20 ml of ether:ethanol (1:4). Filters were dried in a hot air oven and counted in toluene-based scintillation fluid. HSV-1 infection produced a rapid increase in the incorporation of $^3$H-thymidine into infected muscle cultures (Fig. 1a, b). By approx. 11 h p.i., incorporation had reached its maximum level of 300 to 350% of that in mock-infected
Fig. 1, (e) to (h). For legend see opposite page.
cells. Thereafter, thymidine incorporation decreased, and at 24 h p.i. incorporation in infected cells declined below the control level, reflecting the occurrence of cytopathic effect and a decline in cell numbers. Thymidine incorporation in vaccinia-infected myoblasts and myotubes increased slowly, reaching at least 150% of control level by 12 h p.i. and increasing to 400% by 24 h p.i. (Fig. 2c, d).

Because the background level of thymidine incorporation into fused cells is minimal, it is possible to visualize directly the site of incorporation in virus-infected myotubes by means of autoradiography (Stockdale & O’Neill, 1972). For this purpose fused muscle cultures were infected 72 h after plating when maximum cell fusion had occurred and cross-striations were visible. Infected and mock-infected cultures were labelled with 1 μCi/ml of "H-thymidine from 10 to 12 or from 12 to 24 h p.i. and autoradiography was carried out as described by Rogers (1967). The nuclei of control cells remained essentially free of radiographic grains (Fig. 1e) whereas muscle cells infected with HSV-1 contain nuclei that incorporated "H-thymidine during a 2 h labelling period from 10 to 12 h p.i. (Fig. 1f). This autoradiographic observation confirms the finding that herpes virus induces thymidine incorporation in myotubes and demonstrates that newly synthesized DNA accumulates in the nucleus after a 2 h labelling period. However, in vaccinia-infected cells labelled during the same time period, specific sites of thymidine incorporation were observed in the cytoplasm of only a few infected cells (not shown). When infected cells were labelled from 12 to 24 h p.i. much more thymidine incorporation was observed in infected (Fig. 1g) than

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**Fig. 2.** Distribution of "H-thymidine in nucleic acids from infected muscle cells after equilibrium sedimentation in CsCl density gradients. (a) HSV-1-infected myotubes labelled from 3 to 12 h p.i. (b) HSV-1-infected myotubes labelled from 12 to 24 h p.i. shown with the profile of "P-labelled HSV-1 DNA (●–●). (c) HSV-1-infected myoblast labelled from 3 to 12 h p.i. (d) HSV-1-infected myoblasts labelled from 12 to 24 h p.i.
in control cells (Fig. 2h) and newly synthesized DNA accumulated at numerous cytoplasmic sites in the infected cells.

HSV-1 DNA has a buoyant density of approx. 1.73 g/ml in CsCl (Kieff et al. 1971) and can be separated by equilibrium density centrifugation from avian cellular DNA which has a density of approx. 1.702 g/ml (Schildkraut et al. 1962). Therefore, to determine if the DNA accumulating in the nuclei of HSV-1 infected muscle cells was of host or virus origin, DNA from infected cultures was centrifuged to equilibrium in CsCl density gradients using procedures similar to those described by Kaplan (1969). HSV-1 DNA labelled with $^{32}$P was obtained by purifying HSV-1 nucleocapsids (Martin et al. 1971) from virus grown in the presence of 50 µCi/ml of $^{32}$P-orthophosphate from 3 to 48 h p.i. Nucleocapsids were disrupted with 1% Sarcosyl (NL 97) and mixed with the HSV infected cell lysates before centrifugation to serve as a DNA marker. Cell monolayers labelled with 1 µCi/ml of $^3$H-thymidine were scraped from Petri dishes and washed three times in ice-cold 2 × SSC (0.3 M NaCl and 0.03 M Na citrate, pH 7.2). Washed cells were suspended in 2 × SSC and lysed with SDS (3% final concentration). Lysed cellular material was mixed thoroughly with 4.5 ml of caesium chloride solution (21.8 g CsCl added to 15 ml 2 × SSC) and centrifuged in a Beckman SW 50.1 rotor for 48 h at 35,000 rev/min. Fractions were collected from the bottom and 10 µl samples of each fraction were taken for refractive index determination and 25 µl samples were taken for scintillation counting with PCS solubilizer (Amersham-Searle, Arlington Heights, Illinois). DNA from HSV-1 infected myotubes comprised approx. 60% virus DNA, as judged by the separation of virus and avian cell DNA on CsCl density gradients, whether labelled from 3 to 12 h p.i. or from 12 to 24 h (Fig. 2a, b). However, DNA from HSV-1 infected myoblasts labelled 3 to 12 h p.i. (Fig. 2c) comprised approx. 80% cellular and only 20% virion DNA. The proportion of DNA with the buoyant density of virus DNA increased to about 60% when DNA was labelled from 12 to 24 h p.i. (Fig. 2d). These observations are consistent with the fact that host DNA synthesis occurs actively in myoblasts before fusion, but declines in the myoblast population during the time corresponding to the first 12 h of infection.

The synthesis of virus antigen and DNA observed in both infected myoblasts and myotubes was associated with virus replication; since, in cultures of myoblasts and myotubes, about 2 log$_{10}$ of infectious HSV-1 and vaccinia were produced by about 24 and 48 h p.i., respectively.

Thus the DNA synthetic activity stimulated in fully differentiated muscle cells after infection with vaccinia or HSV-1 is presumed to largely represent replication of virus genomes. In support of this conclusion are the observations that the newly synthesized DNA accumulating in vaccinia-infected myotubes was located predominantly in the cytoplasm whereas in HSV-1 infected myotubes, accumulation occurred in the nucleus. Furthermore, in HSV-1-infected myotubes the majority of newly synthesized DNA had a buoyant density similar to that of HSV-1 DNA.

Cellular DNA synthesis and DNA polymerase activity are barely detectable in myotubes. Since this occurs without the use of chemical inhibitors or cell mutants the myotube cell system may prove to be of considerable value in answering questions concerning the relationship of virus and host cell DNA polymerases, such as whether properties of host DNA polymerases in eukaryotic cells are altered after virus infection, and whether replication of pox and herpes virus DNAs depends entirely on proteins specified by the virus genomes.
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