Synthesis of Full-length, Virus Genomic DNA by Nuclei of Vaccinia-infected HeLa Cells

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SUMMARY

It is well established that vaccinia virus infection induces the synthesis of virus-specific DNA in cytoplasmic 'factories', which are sites of virus-specific transcription. The present study demonstrates that vaccinia virus-specific DNA is synthesized also in the nuclei of infected cells with a similar time course. Direct observation and radiolabelling confirm the integrity of isolated nuclei. Reconstitution experiments and inhibitor studies demonstrate that virus-induced DNA is synthesized de novo within nuclei and does not result from cytoplasmic contamination. Cell-specific DNA synthesis is inhibited completely after infection and nuclei of infected cells then synthesize DNA which co-sediments with virus genomic DNA in denaturing gradients. Restriction endonuclease cleavage and hybridization with a virus-specific probe indicate that this is full-length, virus genomic DNA. The biological implications of this are discussed.

INTRODUCTION

The replication of poxviruses has been described as an exclusively cytoplasmic event. Autoradiography of [3H]thymidine-labelled, vaccinia virus-infected cells has demonstrated that virus-induced DNA is synthesized in cytoplasmic inclusions (Cairns, 1960; Harford et al., 1966) analogous to the Guarnieri bodies detected by Feulgen staining (Goodpasture et al., 1932). Similarly, cytoplasmic inclusions containing both DNA and virus protein have been detected by acridine orange and immunofluorescent staining (Loh & Riggs, 1961). The association of newly replicated virus DNA with large, rapidly sedimenting aggregates and the transcription of virus-specific mRNA have been observed in cytoplasmic fractions of vaccinia virus-infected cells (Joklik & Becker, 1964; Becker & Joklik, 1964). Vaccinia virus DNA–membrane complexes have been isolated from the cytoplasm of infected cells (Dahl & Kates, 1970a) and shown to transcribe both early and late species of vaccinia virus mRNA using an endogenous RNA polymerase (Dahl & Kates, 1970b) and to contain virus-specific proteins associated with the DNA (Polisky & Kates, 1972; Sarov & Joklik, 1973). Electron microscopy confirms that such inclusions are the sites of in vivo assembly of progeny virus particles (Dales, 1963; Easterbrook & Rozee, 1971) and this process has been investigated as a model for membrane biogenesis (Dales & Mosbach, 1968). Thus, all classes of macromolecular synthesis expected for the eventual production of progeny vaccinia virions have been described as occurring in cytoplasmic 'factories'. Additionally, the formation of DNA-synthesizing, cytoplasmic aggregates has been demonstrated following vaccinia infection of L cells enucleated with cytochalasin B (Prescott et al., 1971).

However, a number of reports suggest also a nuclear role in the replication of poxviruses. Enucleate BSC-1 cells are unable to support the formation of mature progeny vaccinia virus (Pennington & Follett, 1974; Hruby et al., 1979), as are cells in which host DNA synthesis is inhibited by mitomycin C (Reich & Franklin, 1961). Additionally, it has been shown by autoradiography that input vaccina DNA is associated first with the cell nucleus and later with the cytoplasmic sites of virus DNA synthesis (Walen, 1971). More specifically, nuclei of
vaccinia virus-infected cells have been reported to incorporate $[^3 \text{H}]$thymidine into virus-specific DNA sequences with kinetics similar to those of incorporation into the cytoplasmic fractions (LaColla & Weissbach, 1975). Purified, isolated nuclei from such cells synthesize vaccinia DNA sequences in vitro, a process sensitive to vaccinia-specific antiserum (Bolden et al., 1975). Cell-specific DNA synthesis is inhibited after infection even with u.v.-irradiated vaccinia virus incapable of replication (Jungwirth & Launer, 1968).

The occurrence of poxvirus transcripts in infected cell nuclei has also been offered as evidence of a nuclear role in virus replication. Gafford & Randall (1976) demonstrated the presence of virus-specific RNA in nuclei of fowlpox virus-infected cells although this virus does not inhibit cell DNA synthesis as effectively as orthopoxviruses. More specifically, Bolden et al. (1979) have shown by reassociation kinetics that virus-specific RNA from nuclei of vaccinia virus-infected cells is restricted to a subset of the cytoplasmic virus-specific RNA sequences. Vaccinia virus replication is inhibited in normal cells pretreated with $\alpha$-amanitin but not in cell mutants resistant to this drug (Silver & Dales, 1982; G. McFadden, M. Silver & S. Dales, unpublished results), suggesting a role of the cell nuclear polymerase II in transcription of the virus genome.

The present investigation was prompted by an autoradiographic study showing a possible nuclear involvement during vaccinia virus replication in that a high proportion of cells in HeLa cell monolayers infected at low multiplicity with purified vaccinia virus and labelled with $[^3 \text{H}]$thymidine showed more than one discrete grain focus characteristic of virus infection. In infected cells showing such multiple foci, one was closely associated with the cell nucleus and distinguishable from the diffuse grain formation seen over the nuclei of a few cells in either infected or control cultures (see below). The suggestion that vaccinia virus-induced DNA species may be synthesized at different locations within the infected cell requires a cell fractionation procedure from which the products are closely defined. Many previous studies of the localization within the cell of events in the biochemistry of vaccinia virus replication have employed mechanical disruption (Dounce et al., 1955) of infected cells under conditions where nuclear damage is minimized (Joklik & Becker, 1964; Becker & Joklik, 1964; Dahl & Kates, 1970a, b; Polisky & Kates, 1972). Nuclei, removed by centrifugation, are contaminated by cytoplasmic debris and, presumably, some vaccinia virus DNA (Jungwirth & Launer, 1968). A number of treatments, including washing with citric acid (Dounce, 1943) or non-ionic detergents (Triton N-101; Berkowitz et al., 1969), have been used to free nuclei of cytoplasmic contamination. Additionally, a selective effect of the non-ionic detergent Nonidet P40 (NP40) on the cytoplasmic rather than the nuclear membrane of eukaryotic cells has been described (O'Brien, 1964) and this reagent has been used to obtain a crude, nuclear preparation from HeLa cells (Borun et al., 1967). A mixture of a similar, non-ionic detergent (Tween 40) and the ionic detergent sodium deoxycholate has been used to remove the cytoplasmic debris contaminating nuclei obtained by mechanical disruption of HeLa cells. This combination of detergents removes part of the nuclear membrane, resulting in intact nuclei bounded apparently by a single membrane only. Such nuclei are free of cytoplasmic contamination as judged by electron microscopy (Holtzman et al., 1966) or by the absence of mature ribosomal RNA (Penman, 1966). As vaccinia virus-infected HeLa cells vary in fragility throughout the virus growth cycle and, in this laboratory, mechanical homogenizers have proved unsatisfactory in the isolation of nuclei from such cells, aspects of the detergent methods described above were combined. This report describes the occurrence of distinguishable classes of virus-specific DNA in detergent-generated fractions of vaccinia virus-infected HeLa cells.

**METHODS**

_Virus, cell culture and infection of cultures_. The Lister strain of vaccinia virus was pock-purified by growth on the chorioallantoic membrane of fertile hens' eggs and the morphology of virus particles checked by electron microscopy. Infectivity titres were determined by plaque formation in monolayer cultures of Vero cells. Virus was propagated in, purified from and used to infect HeLa cell monolayer or suspension cultures at a multiplicity of approximately 5 p.f.u./cell as described previously (Joklik, 1962; Williamson & Archard, 1976). Stock cell cultures were propagated routinely in the absence of antibiotics: media used for experimental cultures contained 200 µg/ml kanamycin, 100 µg/ml streptomycin and 100 units/ml penicillin.
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In vivo labelling of cultures and virus. Cultures were labelled radioactively by incubation in media containing various concentrations of the precursors indicated. These included [6-3H]thymidine (5 Ci/mmole), deoxy- [6-3H]uridine (15 Ci/mmole) and [U-14C]uridine (487 mCi/mmol; Amersham International). Non-incorporated radioactivity was reduced by washing twice with Hank's balanced salt solution prior to recovery and fractionation of cells. Incorporation in pulse label experiments was stopped by washing twice with phosphate-buffered saline 'A' (PBSA) at 4 °C. In pulse-chase experiments, labelled cultures were washed twice at 37 °C and re-incubated with medium lacking the radiotracer but containing the unlabelled compound at a concentration at least 10-fold higher than that present in the labelling medium. The amounts of incorporated radioactivity in cell fractions were determined by trichloroacetic acid precipitation and liquid scintillation spectrometry essentially as described previously (Archard & Williamson, 1971). Samples labelled with both 3H and 14C were counted by an automatic external standard ratio method permitting independent determinations of the two isotopes.

Vaccinia virus labelled radioactively in the DNA was prepared by addition of [3H]thymidine (1-0 to 10 µCi per ml) to the maintenance medium following infection. Labelled virus particles were recovered from cytoplasmic fractions of infected cells by isopycnic centrifugation in preformed 20 to 50% potassium tartrate gradients essentially as described previously (Archard & Williamson, 1971).

Autoradiography. HeLa monolayers, grown on 1 cm glass coverslips, were mock-infected or infected with vaccinia virus suspended in Eagle's medium at a multiplicity of 0-2 p.f.u./cell. Cultures labelled from 2-5 to 3-5 h post-infection by the addition of 1-0 µCi/ml [3H]thymidine were fixed with two changes of 2-5% (v/v) glutaraldehyde in 0-1 M-sodium cacodylate buffer pH 7-2 for a total of 1 h at room temperature. After washing overnight in similar buffer, the fixed cultures were autoradiographed at 4 °C for 5 days using Kodak AR10 stripping film. After development and fixation of the autoradiographs, the cells were examined through the emulsion using Erlich's haematoxylin/eosin and examined by light microscopy.

Cell fractionation. Monolayer cultures were fixed and the cells recovered using 0-02% EDTA in PBSA. Cells from suspension cultures were recovered by centrifugation at 200 g for 2 min. After washing with 0-25 M-sucrose in 0-01 M-phosphate buffer pH 7-3, aliquots of 2 × 10^6 to 5 × 10^6 cells were resuspended in 5 ml volumes of 0-25% NP40 (Shell U.K. Ltd) in buffered sucrose solution. These suspensions were allowed to stand at room temperature for 2 min, agitated using a vortex mixer and the resulting crude nuclei sedimented by centrifugation at 200 g for 2 min. The supernatants were retained as the cytoplasmic fraction and the nuclear pellets were resuspended thoroughly in 4-5 ml volumes of phosphate-buffered sucrose solution using a vortex mixer and the resulting crude nuclei sedimented by centrifugation at 200 g for 2 min. The supernatants were retained as the cytoplasmic fraction and the nuclear pellets were regarded as purified nuclei.

Electron microscopy. Pellets of untreated cells or nuclei sedimented after detergent treatment were fixed in situ for 1 h using two changes of 2-5% (v/v) glutaraldehyde in 0-1 M-sodium cacodylate buffer pH 7-2, at room temperature. The pellets were washed in cacodylate buffer and post-fixed for 1 h in 1% osmium tetroxide and re-washed. The fixed material was dehydrated through a graded series of ethanol solutions, infiltrated and flat-embedded in resin (Spurr, 1969). Sections were cut using an LKB Ultratome and examined with an AEI EM6B electron microscope.

Rate zonal sedimentation in denaturing gradients. Radioactively labelled DNA from isolated cell nuclei or virus particles was analysed by rate sedimentation in 5 to 21% isokinetic alkaline sucrose gradients. Gradients were made in polypropylene tubes (boiled previously for 1 h in 0-001 M-NaOH, 0-1 M-EDTA) from sucrose solutions prepared in 0-7 M-NaCl, 0-005 M-EDTA, 0-3 M-NaOH (gradient vol. 11-5 ml) using a constant-volume mixing device essentially as described by Noll (1967). Volumes of 0-25 ml of 1 M-NaOH were floated on the gradients followed by 0-25 ml aliquots containing untritiated vaccinia virus or 1 × 10^5 to 2 × 10^5 nuclei and 0-02 M-EDTA. These were allowed to lyse in situ for 30 min at room temperature and the gradients centrifuged at 21 °C and 200 000 g for 90 min. Fractionation was by upward displacement and incorporated radioactivity was determined by liquid scintillation spectrometry in a Triton:toluene:water system (3:6:1) after neutralization of fractions.

Preparation of DNA and restriction endonuclease cleavage. DNA was extracted from purified virus or nuclei of control or vaccinia virus-infected cells as described previously (Mackett & Archard, 1979). Restriction fragment generaes generated by cleavage with endonuclease Hind III or XhoI were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (Southern, 1975) under conditions described previously (Archard & Mackett, 1979).

In vitro labelling of DNA and DNA–DNA hybridization. DNA was labelled by nick translation (Rigby et al., 1977). Reaction mixtures (100 µl contained 1 µg DNA, 2 to 10 µM-deoxyribonucleoside triphosphates including 2 to 5 µCi [α-32P]deoxycytidine triphosphate or [α-32P]deoxythymidine triphosphate (sp. act. approx. 350 Ci/mmol; Amersham International), 50 mM-Tris–HCl pH 7-4, 5 mM-MgCl2, 1-4 mM-2 mercaptoethanol, 5 µg bovine serum albumin and 2 units DNA polymerase I (Boehringer); DNase was omitted. After incubation at 14 °C for 2 h, the reactions were stopped by addition of proteinase K to 200 µg/ml and incubation at 37 °C for 10 min. Reaction mixtures were deproteinized by phenol extraction and the DNA re-isolated by exclusion chromatography on Sephadex G-50 (Pharmacia) followed by ethanol precipitation.
Fig. 1. Autoradiography of [3H]thymidine-labelled HeLa cell cultures. Cells were mock-infected (a) or infected with vaccinia virus at a multiplicity of 0-2 p.f.u./cell (b) and labelled from 2-5 to 3-5 h post-infection.

Labelled DNA was denatured in 0-1 M-NaOH at 100 °C for 5 min, neutralized with HCl in the presence of 100 mm-Tris-HCl pH 7-4 and 2 x 10^5 to 10 x 10^5 Cerenkov ct/min were hybridized for 60 h to representative nitrocellulose membrane filter strips bearing transferred, restricted vaccinia virus or HeLa cell DNA. Pretreatment of filters, conditions of hybridization and subsequent washing were similar to those described by Jeffreys & Flavell (1977). Strips were autoradiographed for 3 to 14 days at -70 °C using presensitized Fuji Rx X-ray film and Hannimex Mach II intensifying screens.

RESULTS

Autoradiography

A proportion of cells in uninfected, control cultures showed a diffuse pattern of grain formation over the entire area of the nucleus, indicative of cell-specific DNA synthesis (Fig. 1 a). Typically, 166 of 582 cells (29%) in uninfected cultures showed such diffuse, nuclear labelling. This grain pattern was also observed over the nuclei of some cells in cultures infected with vaccinia virus at low multiplicity. In contrast, a proportion of cells in infected cultures showed only an intense localized focus of grain formation, not confined to the nucleus (Fig. 1 b) and presumptive evidence of cytoplasmic, virus DNA synthesis (Cairns, 1960). After infection at an estimated multiplicity of 0-2, 92 of 582 cells (16%) showed such localized, cytoplasmic labelling indicating an actual e.o.p. of 0-16. However, additional grain formations were observed associated with the nuclei of a proportion of such infected cells. Of 239 infected cells 114 (48%) showed a second focal grain formation in this position while 46 of 226 (20%) showed diffuse, nuclear labelling. Counted independently, 100 of 152 infected cells (66%) showed grain formations of either type, located over the nucleus and in addition to focal cytoplasmic labelling. Infected cells having both types of grain formation over the nucleus were rare. The proportion of doubly infected cells expected at this low multiplicity was only 6% and the virus suspension appeared mono-disperse on examination by electron microscopy. These results suggest that a
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Fig. 2. Occurrence of incorporated radioactivity in the nuclear wash fraction of [3H]thymidine-labelled HeLa cells after treatment with increasing concentrations of mixed detergents (SDC, sodium deoxycholate).

Fig. 3. Effect of initial NP40 concentration on the subsequent occurrence of incorporated radioactivity in cytoplasmic (●) or nuclear wash (○) fractions of control (a) or vaccinia virus-infected (b) HeLa cells labelled with [3H]thymidine.

The proportion of infected cells, too high to be explained by multiple infection, incorporates thymidine into nuclear-associated sites with an autoradiographic appearance similar to cytoplasmic sites of virus DNA replication and that cell nuclear DNA synthesis is reduced.

Development of the detergent fractionation procedure

HeLa cell monolayers labelled in the DNA by incubation for 15 min in medium containing 1·0 μCi/ml [3H]thymidine were disrupted using 0·25% NP40 in buffered isotonic sucrose solution. Aliquots of the resulting crude nuclei were washed with various concentrations of the mixed detergents and the proportion of incorporated radioactivity present in the nuclear wash fractions was determined (Fig. 2). This remained relatively constant up to 0·1% NP40, 0·05% sodium deoxycholate, above which concentrations greatly increased amounts of label were recovered in the nuclear wash indicating that nuclear lysis had occurred. On this basis, final concentrations of 0·067% NP40, 0·033% sodium deoxycholate were used in subsequent fractionations. In further experiments, vaccinia virus-infected or mock-infected HeLa cell monolayers were labelled as above and aliquots disrupted using various concentrations of NP40. The resulting crude nuclei were washed using the mixed detergent conditions described and the proportions of incorporated radioactivity present in cytoplasmic or nuclear wash fractions determined (Fig. 3). The results were similar qualitatively for infected or mock-infected cultures. Radioactivity recoverable in the cytoplasmic fraction increased slightly with increasing NP40 concentrations but that recoverable in the nuclear wash increased markedly, suggesting that the initial detergent treatment affects the subsequent efficacy of the mixed detergent wash. These effects were optimal in the presence of 0·25% NP40 which itself did not
Fig. 4. Electron micrographs of HeLa cells before fractionation (a), after NP40 treatment (b) and after washing with mixed detergents (c). The nuclear membrane is indicated (NM) and the bar marker represents 1 μm.
Table 1. *Re-isolation of nuclei from infected or control cells after mixing with cytoplasmic fractions of *[^3]H*thymidine-labelled vaccinia virus-infected HeLa cells*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity recovered (d/min)</th>
<th>% of Total</th>
<th>Radioactivity recovered (d/min)</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>278445</td>
<td>92.1</td>
<td>327129</td>
<td>92.2</td>
</tr>
<tr>
<td>Nuclear wash</td>
<td>22404</td>
<td>7.4</td>
<td>24494</td>
<td>6.9</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1565</td>
<td>0.5</td>
<td>3024</td>
<td>0.9</td>
</tr>
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result in nuclear lysis. In several experiments, the radioactivity incorporated in the combined cytoplasmic and nuclear wash fractions of uninfected cells varied between 10% and 20% of the total. These values are compatible with the data of Volpe & Eremenko (1973) which show that thymidine incorporated into HeLa cell mitochondria DNA during the S and G2 phases may constitute 25% of the total.

The products of the fractionation were examined by phase-contrast microscopy or electron microscopy. Phase-contrast microscopy demonstrated that NP40 treatment disrupted all cells and resulted in crude nuclei obviously contaminated by cytoplasmic debris. Additional washing with mixed detergents resulted in nuclei with no visible cytoplasmic contamination (not shown). Electron microscopy of sectioned, unfractated cells clearly demonstrated the cytoplasmic membrane and the bilaminar nuclear membrane. Crude nuclei derived by NP40 treatment showed adherent cytoplasmic material and the double nature of the nuclear membrane remained obvious, but nuclei washed subsequently with mixed detergents were free of cytoplasmic contamination and appeared to be bounded by a single membrane only (Fig. 4). The nuclear wash fraction thus consists of that part of the cytoplasm which was originally perinuclear together with some membranous material derived from the outer layer of the nuclear envelope.

The possibility of adsorption of cytoplasmic, virus DNA to isolated nuclei was examined in mixing experiments. Crude nuclei, obtained by NP40 treatment of uninfected or virus-infected cells at 3 h post-infection, were mixed with aliquots of the cytoplasmic fraction obtained by NP40 treatment of infected cells radiolabelled for 15 min at 3 h after infection in medium containing 1-0 μCi/ml[^3]H*thymidine. The nuclei were re-isolated, washed with mixed detergents and the amounts of incorporated radioactivity recovered in the cytoplasmic, nuclear wash and nuclear fractions were determined (Table 1). The results obtained using nuclei isolated from either infected or control cells were similar. About 7% of the labelled material added as cytoplasmic fraction from infected cells was recovered with the initial, nuclear pellets. Subsequent washing with mixed detergents removed the bulk of this and less than 1% of the radioactivity added initially remained associated with the purified nuclei. These experiments demonstrate also that under the conditions adopted for routine fractionation, cytoplasmic structures containing virus DNA do not co-sediment with nuclei.

Incorporated thymidine in fractions of virus-induced or uninfected cells

Infected or mock-infected HeLa cell monolayers were pulse-labelled at various times after infection by incubation for 10 min in medium containing 1-0 μCi/ml[^3]H*thymidine. The cells were recovered, fractionated and the amounts of incorporated radioactivity present in the fractions determined as described. The data shown in Fig. 5 are representative of many experiments. The rate of incorporation of thymidine into the cytoplasmic fraction of infected cells increased rapidly soon after infection, reached a maximum at 2-5 h and then declined (Fig. 5a), while incorporation into this fraction of mock-infected controls remained low and constant. The maximum rate of thymidine incorporation found in the cytoplasm of infected cells was about 20-fold greater than that in the cytoplasm of the control cells at the same time. These results are similar to those described previously for vaccinia virus-infected cells fractionated by mechanical disruption (Joklik & Becker, 1964). A similar pattern of virus-induced incorporation
was observed in the nuclear wash fractions (b) although the maximum rate of thymidine incorporation found in these fractions of infected cells was generally greater than that in the cytoplasmic fraction of the same cells, indicating that much vaccinia virus-induced DNA synthesis is perinuclear. The rate of thymidine incorporation in the nuclei of vaccinia virus-infected cells was stimulated soon after infection although the overall rate of DNA synthesis declined earlier and more slowly than that in cytoplasmic or nuclear wash fractions (c).
Thymidine incorporation into nuclei of infected cells reflects the net rate of DNA synthesis and, as HeLa cell nuclear DNA synthesis is inhibited specifically and completely by 3 h after infection with vaccinia virus (Jungwirth & Launer, 1968; and Fig. 8 below), an initial stimulation followed by a declining net rate of thymidine incorporation represents continuing virus-induced DNA synthesis. The rate of incorporation in nuclei of control cells increased erratically in these non-synchronized cultures, due presumably to manipulations involved in mock infection.

**Fate of incorporated thymidine in fractions of virus-infected and uninfected cells**

Virus-induced DNA synthesis appears to be associated with particular fractions of infected cells. In further experiments, infected or mock-infected suspension cultures were pulse-labelled for 5 min in the presence of 1.0 μCi/ml [3H]thymidine at 3 h, when the rate of vaccinia virus-induced DNA synthesis is high and cell nuclear DNA synthesis is inhibited completely (see below). Cultures were washed and re-incubated in medium containing a 103-fold molar excess of unlabelled thymidine and fractionated as described at times up to 12 h after infection, by which time a large proportion of the final yield of progeny virus is isolatable from similar infected cultures (Archard & Williamson, 1971). In control cultures, most of the thymidine incorporated during the pulse was found associated with nuclei and this situation was maintained throughout the experiment (Fig. 6a). In infected cultures, however, most of the incorporated radioactivity found immediately after pulsing was associated with the nuclear wash and with the cytoplasmic fraction. The amount of incorporated thymidine present in the nuclear fraction increased with time after pulsing, apparently at the expense of that in the nuclear wash whilst incorporated thymidine in the cytoplasmic fraction remained relatively constant (Fig. 6b). These results indicate that vaccinia virus-induced DNA is not synthesized initially in association with the nuclear membrane and transferred subsequently to the cytoplasm but that some incorporated thymidine detected initially in the nuclear wash fraction may appear later in the nuclei. However, the rate of transfer of radiolabelled DNA from the nuclear wash fraction to nuclei is not sufficient to account for the amount of virus-induced DNA found in isolated nuclei immediately after pulse-labelling.

**Effects of inhibitors on incorporated thymidine found in fractions of infected cells**

Virus-induced DNA synthesis, detected subsequently in different fractions of infected cells, was distinguished by the effects of various inhibitors. Aliquots of infected HeLa cells in suspension were pulse-labelled by incubation for 15 min at 3 h post-infection in medium containing 1.0 μCi/ml deoxy[6-3H]uridine and various concentrations of fluorodeoxyuridine (FUdR). After terminating the pulse and washing, cells were fractionated by detergent treatment and the amounts of incorporated radioactivity associated with the fractions determined (Fig. 7). Incorporation of radioactivity detected subsequently in the nuclear wash fraction or in nuclei was inhibited markedly, being reduced in the presence of 10⁻⁶ M-FUdR to 1% or 2% respectively of that found in non-inhibited controls. In contrast, incorporation of radioactivity detected subsequently in the cytoplasmic fraction was relatively resistant to inhibition and, in the presence of 10⁻⁶ M-FUdR, was 23% of that found in non-inhibited controls (mean data from four experiments).

Analogous experiments using cytosine arabinoside gave results similar qualitatively to those obtained using FUdR. In the presence of 10⁻⁵ M-cytosine arabinoside, incorporation in the nuclear wash fraction and the nuclei was reduced to 9% and 11% respectively, compared with the non-inhibited controls but incorporated radioactivity in the cytoplasm was 46% of that in controls (mean data from two experiments).

These results demonstrate that virus-induced DNA synthesis in the nuclei and nuclear wash fractions of infected cells occurs under different conditions from that in the cytoplasm.

**Sedimentation characteristics of nuclear DNA**

DNA species present in nuclei isolated from vaccinia virus-infected or mock-infected cells were analysed by rate zonal sedimentation in alkaline sucrose gradients and compared with
DNA present in particles isolatable from the cytoplasmic fractions of similar cells at a late stage of infection. HeLa cell monolayers were prelabelled in the DNA by incubation for 36 h in medium containing 0.1 μCi/ml [14C]thymidine. After washing and re-incubation for 3 h to reduce unincorporated radioactivity, aliquots were infected with vaccinia virus or mock-infected, and pulse-labelled for 10 min at 3 h post-infection by incubation in medium containing 1.0 μCi/ml [3H]thymidine. Nuclei were isolated by detergent fractionation and lysed on alkaline sucrose gradients as described. Additional HeLa cell cultures were infected or mock-infected and incubated for 18 h in the presence of [3H]thymidine. The cytoplasmic fractions of these cultures were layered on preformed potassium tartrate gradients, centrifuged as described and acid-precipitable radioactivity in aliquots of gradient fractions was measured to locate particles containing DNA (data not shown). Peak fractions were dialysed to reduce salt concentration and aliquots lysed on alkaline sucrose gradients as before. Denaturing gradients containing nuclear or cytoplasmic DNA from infected or mock-infected cells were centrifuged
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3\textsuperscript{H}thymidine before mock infection sedimented as a broad peak to a position in the gradients indicative of high molecular weight. Infection with vaccinia virus slightly reduced the sedimentation rate of such prelabelled cell DNA, although not to the extent described by Parkhurst et al. (1973) (Fig. 8a). Nuclei of control cells incorporated \([3\text{H}]\)thymidine into similar DNA during the pulse at 3 h after mock infection, while those of vaccinia virus-infected cells incorporated \([3\text{H}]\)thymidine into a slower sedimenting class of DNA resulting from \textit{de novo} synthesis after infection (Fig. 8b). Analogous patterns of labelling were observed when DNA of nuclei isolated from vaccinia virus- or mock-infected cells and digested with 0.1% Sarkosyl, 0.1% preincubated Pronase, 0.1 M-EDTA in saline sodium citrate at 37 °C overnight was sedimented in 5 to 21% sucrose gradients in 1.0 M-NaCl, 0.005 M-EDTA, 0.05 M-Tris-HCl pH 7.8 (data not shown). DNA recovered in progeny virus particles from the cytoplasmic fraction of vaccinia virus-infected cells at 18 h post-infection co-sedimented in denaturing gradients with virus-induced nuclear DNA pulse-labelled at 3 h after infection. In contrast, DNA from cytoplasmic particles of control cells and presumed to be mitochondrial DNA remained near the top of the gradients (Fig. 8c). The relative amount of \([3\text{H}]\)thymidine incorporated into nuclei of vaccinia virus-infected cells during the pulse at 3 h was similar to that into nuclei of mock-infected cells under the same conditions (infected : mock-infected = 1.08 when normalized against incorporated \([14\text{C}]\)thymidine prelabel) but was found exclusively in a new species of virus-induced DNA co-sedimenting with virion DNA. These data demonstrate that vaccinia virus infection results in \textit{de novo} nuclear synthesis of virion-length DNA not linked covalently to cell DNA and that cell nuclear DNA synthesis is completely inhibited by this time.

Specificity of virus-induced DNA in fractions of infected cells

The sequence specificity and organization of virus-induced DNA detected in nuclei isolated from vaccinia virus-infected HeLa cells was tested by hybridization with radiolabelled vaccinia virus genomic DNA. DNA extracted from purified virus or from nuclei of control or vaccinia virus-infected cells prepared at 4 h or 16 h after infection was digested with restriction endonuclease \textit{HindIII} or \textit{XhoI} and the fragments immobilized on nitrocellulose membrane filters after separation by agarose gel electrophoresis. \(^{32}\text{P}\)-labelled, total vaccinia virus DNA hybridized specifically to restriction fragments of nuclear DNA from infected cells which co-migrated in electrophoresis with all fragments present in control digests of vaccinia virus genomic DNA, but failed to hybridize to restricted DNA from nuclei of uninfected cells (Fig. 9 is illustrative of many experiments). No virus-specific sequences were detected in fragments of altered mobility and the known terminal fragments resulting from \textit{HindIII} cleavage (B and G; Wittek et al., 1977) or \textit{XhoI} cleavage (F, 2-molar; Mackett & Archard, 1979) are represented. These data demonstrate that vaccinia virus-specific DNA sequences exist in the nuclei of infected cells as intact genomes and that integration of full-length molecules into cell DNA does not occur to any great extent.

DISCUSSION

The data reported raise two major questions of interpretation: one concerns the validity of the observations and another their biological significance in virus replication.

The first requires that purified nuclei isolated from infected cells are not contaminated by virus-specific DNA synthesized in the cytoplasm of those cells and that novel DNA species detected in nuclei arise \textit{in situ} from \textit{de novo} virus-induced synthesis. The cell fractionation procedure is based on established principles. The conditions of detergent treatment do not result in nuclear lysis as judged by release of incorporated \([3\text{H}]\)thymidine. Neither light nor electron microscopy reveals contamination of nuclei by cytoplasmic debris: on the contrary, electron microscopy suggests that purified nuclei are leached of chromatin and so the extent of nuclear DNA synthesis, quantified by radiolabelling, is underestimated. Mixing experiments demonstrate that adventitious virus-specific DNA from the cytoplasm of infected cells does not adsorb to or co-sediment with nuclei purified from similar cells.

The kinetics of incorporation of radiolabelled nucleosides in the presence of inhibitors of
Fig. 9. Hybridization of \(^{32}\)P-labelled vaccinia virus genomic DNA to restriction fragments of DNA extracted from nuclei of control cells (1) or from purified vaccinia virus (2) or from nuclei isolated at 4 h (3) or 15 h (4) post-infection from cells infected with vaccinia virus. DNA was cleaved with endonuclease \(HindIII\) (H) or \(Xhol\) (X), fragments separated by agarose gel electrophoresis and immobilized on nitrocellulose filters.

DNA synthesis distinguish in each case between nuclear and cytoplasmic DNA in infected cells. FUdR or cytosine arabinoside, both of which preferentially inhibit virus-induced nuclear DNA synthesis in this system, are nucleoside analogues and so are expected to accumulate within nuclei although they have different mechanisms of inhibition. Ethidium bromide, which in contrast preferentially inhibits virus-induced cytoplasmic DNA synthesis in this system, was shown previously to inhibit selectively the synthesis of a class of vaccinia virus-specific DNA which sedimented faster than virus genomic DNA in denaturing gradients (Archard, 1979). This intercalating agent is preferentially bound by circular DNA (Radloff et al., 1967) and completely inhibits the production of infectious progeny vaccinia virus at 20 \(\mu\)g/ml. This concentration has no inhibitory effect on nuclear-associated DNA synthesis in vaccinia virus-infected cells but results in a marked inhibition of virus-induced cytoplasmic DNA synthesis (data not shown). It may be related that Moyer & Graves (1981) have shown that a proportion of replicating virus-specific DNA in rabbitpox virus-infected cells exists in a multimeric form. Whatever the mechanisms, the ability of these inhibitors to distinguish between virus-induced DNA synthesis detected subsequently in different fractions of infected cells confirms that such syntheses took place originally in discrete regions of the cell and apparent virus-induced nuclear DNA synthesis does not result from contamination with cytoplasmic DNA.

Pulse–chase experiments demonstrate that vaccinia virus-induced DNA detected in cytoplasmic fractions does not originate in the nuclei of infected cells. However, a proportion of
Vaccinia virus DNA in infected cell nuclei

the DNA labelled initially in the nuclear wash fraction appears eventually in the nuclei although the rate of such transfer is insufficient to account for the amount of virus-induced DNA present in nuclei prepared immediately after labelling at a time when cell nuclear DNA synthesis is inhibited. It is clear that the bulk of such DNA is synthesized in situ.

Sedimentation analysis of DNA from purified nuclei demonstrates that vaccinia virus infection results in total inhibition of cell nuclear DNA synthesis and a switch to de novo synthesis of a novel species which co-sediments with virus genomic DNA. Restriction endonuclease cleavage and hybridization with a virus genomic probe demonstrates that such nuclei contain DNA which is homologous to all vaccinia-specific sequences, organized as intact virus genomes and readily detectable by 4 h post-infection. It is remarkable that nuclei in which cell-specific DNA synthesis is inhibited by a component of the infecting virus particle (Pogo & Dales, 1974) remain capable not only of synthesis of virus-specific sequences but also of the processing events which generate unit length virus genomes with covalent terminal crosslinks.

The biological role of vaccinia virus-specific nuclear DNA remains obscure. Whilst it is clear that a large proportion of total virus DNA is synthesized in association with the cell nucleus, there is no evidence that this is eventually relocated in the cytoplasm as demonstrated with frog virus-3 (Goorha et al., 1978). However, it is established that vaccinia virus-specific DNA synthesis is highly redundant and that the bulk is not incorporated into progeny virus particles but remains susceptible to experimental degradation by DNase (Joklik & Becker, 1964; L. Archard, unpublished observations). The presence and replication of vaccinia-specific DNA within the cell nucleus might be regarded as fortuitous rather than obligatory as transfection experiments demonstrate that mammalian cells in culture readily take up adventitious DNA into the nuclei. However, vigorous investigations have failed to demonstrate the production of mature progeny virus in enucleated cells despite the synthesis of substantial amounts of virus-specific DNA and the controlled expression of early and late virus proteins (Pennington & Follett, 1974; Hruby et al., 1979). The possibility remains that the limited set of transcripts synthesized from virus-specific DNA in the nuclei of vaccinia virus-infected cells (Bolden et al., 1979) by the α-amanitin-sensitive cell RNA polymerase II (Silver & Dales, 1982) includes sequences essential for subsequent virus maturation. Host polymerase II function is required also in the replication of frog virus-3 (Goorha, 1981). It may be that differential hybridization using cDNA complementary to mRNA synthesized in vaccinia virus-infected cells in the presence of α-amanitin would identify species of virus mRNA transcribed in non-inhibited or α-amanitin-resistant cells and essential for the completion of virus replication.

REFERENCES


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