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Fate of Frog Virus 3 DNA Replicated in the Nucleus of Arginine-deprived CHO Cells

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SUMMARY

In a productive infection, frog virus 3 (FV 3) DNA was synthesized in both the cell nucleus and cytoplasm. The infection was aborted in arginine-starved Chinese hamster ovary cells and viral DNA replication was then restricted to the nuclear compartment. It has been demonstrated that the newly synthesized FV 3 DNA present in the nucleus is of genomic size. After the addition of arginine, this DNA is transferred into the cytoplasm and can be encapsidated. The formation of infectious particles occurred even if an inhibitor of DNA synthesis was added simultaneously with arginine. Although the synthesis of early FV 3 polypeptides and their intracellular distribution were comparable in the presence or in the absence of arginine, the production of late species was greatly reduced by arginine deprivation; one of these late proteins must be involved in the passage from the nuclear to the cytoplasmic phase of FV 3 DNA replication. This system made it possible to carry out an independent analysis of the nuclear events that comprise the first stage of FV 3 multiplication.

INTRODUCTION

A novel pattern of frog virus 3 (FV 3) replication has recently been proposed. DNA replicates in two stages: the first stage occurs in the nucleus where genome-size molecules are formed; the second step takes place in the cytoplasm where the replicating molecules are large concatamers (Goorha, 1982). This model also accounts for the earlier observations. Although the cytoplasm was described as the site of virus assembly, morphogenesis and DNA synthesis (Granoff et al., 1966; Bingen-Brendel et al., 1971; Kelly, 1975), some DNA was always replicated in the nucleus (McAuslan & Smith, 1968; Aubertin et al., 1970; Goorha et al., 1978) and the necessity for a functional nucleus was established (Goorha et al., 1977).

Based on the fact that most double-stranded DNA animal viruses have an arginine requirement for completion of their growth cycle (Russell & Becker, 1968; Prage et al., 1968; Spring et al., 1969; Obert et al., 1971; Archard & Williamson, 1971; Rouse & Schlesinger, 1972), previous experiments showed that in FV 3-infected arginine-starved BHK cells, the DNA was replicated and accumulated in the nucleus (Aubertin, 1975). Moreover, the functional competence of a viral genome during the course of arginine starvation was recently demonstrated in the case of adenovirus (Auborn & Rouse, 1982). We have made use of these results to investigate the fate of FV 3 DNA synthesized in the nucleus and to determine whether this DNA can be packaged into virions.

Some viral proteins are selectively concentrated within the nucleus (Elliott & Kelly, 1980; Martin et al., 1981), and are likely to participate in the replication. Since these proteins may play a role in the localization of the DNA in the various cell compartments, an analysis was made of their synthesis and phosphorylation under arginine deficiency.
METHODS

Cells and virus. Chinese hamster ovary (CHO) cells were grown in monolayers in alpha medium (Eurobio) supplemented with 10% calf serum. To deprive the cells of arginine, this medium was replaced 16 h before infection by alpha medium devoid of arginine and supplemented with only 0.5% dialysed calf serum.

FV 3 was produced and purified as described previously (Aubertin et al., 1973, 1980).

Infection conditions and labelling of proteins, phosphoproteins and DNA. Cells were infected at a multiplicity of 40 p.f.u. of FV 3. After an hour's adsorption, the unbound virus was eliminated and the cells were incubated at 29°C in alpha medium with or without arginine. The DNA was labelled at different times following infection, usually for 30 min, with 5 µCi [3H]thymidine (CEA, Saclay, France; sp. act. 25 Ci/mmol) per ml medium. Proteins were labelled for the same period of time (or as indicated in the legend) with [35S]methionine (Amersham International, sp. act. 900 Ci/mmol) at a concentration of 12 µCi/ml medium. Phosphorylation of the polypeptides was followed by the addition of 25 µCi [32P]orthophosphate (Amersham International) per ml phosphate-free medium.

Collection and fractionation of cells. After the labelling or chase period, the cells were washed with phosphate-buffered saline (PBS) and scraped up in this buffer. They were fractionated following the method described previously (Martin et al., 1981). When the samples were prepared for the determination of DNA polymerase activity, the PBS buffer used contained neither MgCl2 nor CaCl2.

Quantification of DNA and protein syntheses. Protein synthesis and post-translational modification of proteins were measured as the acid-insoluble material incorporated into the subcellular fractions. Aliquots were spotted onto a Whatman 3MM paper square, immersed in 10% trichloroacetic acid (TCA), heated in 10% TCA in a boiling water-bath and washed according to the method of Mans & Novelli (1961). The paper squares were dried and the radioactivity associated was counted by liquid scintillation spectroscopy.

A similar procedure was followed for the determination of DNA synthesis, except that the TCA concentration was 5% and the heating step was omitted.

SDS-polyacrylamide gel electrophoresis. Denatured proteins were separated by electrophoresis using a discontinuous buffer system (Laemmli, 1970) under the conditions described by Martin et al. (1981). To analyse [35S]methionine-labelled proteins, the gels were treated and the fluorograms prepared as described by Bonner & Laskey (1974). The phosphoproteins were revealed by direct autoradiography using an intensifying screen (Cronex Lightning Plus, DuPont) (Laskey & Mills, 1977). Occasionally, to establish the ratio of [35S]methionine to [32P]orthophosphate incorporated into a given polypeptide, the autoradiogram was used as a guide to localize the molecule in the gel and the corresponding band excised from the dried gel. The pieces of gels were incubated in 500 µl H2O2 for 16 h at 37°C, scintillation fluid was added and the radioactivity was measured.

Sedimentation in neutral sucrose gradients. To analyse the virus particles produced, the cytoplasmic fraction was layered on a pre-formed linear (10 to 40%, w/w) sucrose gradient prepared in 0.01 M-Tris-HCl pH 8. Centrifugation was carried out for 1 h at 55000 g in a Beckman SW27 rotor. Fractions of 1 ml were collected, an aliquot of each was spotted onto a Whatman 3MM paper square, TCA-precipitated and the radioactivity was measured.

DNA analysis by sedimentation on alkaline sucrose gradients. The cell suspension, 106 cells in 1 ml buffer (0.15 M-NaCl, 1.5 mM-KH2PO4, 2.5 mM-KCl, 1 mM-Na2HPO4, 0.5 mM-EDTA, pH 7.4), was loaded on top of a 30 ml layer of 0.5 M-NaCl. The loaded gradients were kept overnight at 4°C and then centrifuged at 72000 g for 7 h. One ml fractions were collected and the radioactivity was determined after TCA precipitation, the acid-insoluble material being recovered on glass fibre filters (Whatman, GF/C).

DNA hybridization to filter-bound FV 3 DNA. FV 3 DNA was immobilized on diazobenzyloxymethyl (DBM)-paper using the commercially available paper supplied by Schleicher & Schuell. The activation step was performed as recommended by the manufacturer. Paper squares were incubated for 30 min in 30 ml 1 M-Tris–HCl to which 0.85 ml NaNO2 at a concentration of 10 mg/ml had been added, and they were then washed twice for 1 min in H2O and twice for 1 min with 20 mM-CH3COONa pH 4. Ten µg DNA, denatured by alkali treatment (15 min in 0.1 M-NaOH) and neutralized with 1.5 M-Na2HPO4, were spotted onto an activated paper square and allowed to dry at room temperature. The filters were washed four times for 10 min in 0.5 M-NaOH and with 0.1% SDS, 2 × SSPE (SSPE: 0.18 M-NaCl, 10 mM-Na2HPO4, 1 mM-EDTA, pH 7.7). Hybridizations were carried out in Eppendorf tubes in the following mixture: 50% deionized formamide, 1 × SSC (SSC: 0.15 M-NaCl, 0.015 M-trisodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.01 M-sodium phosphate pH 6.8, 10 µg sonicated calf thymus DNA, 2 µg poly(A) and the labelled DNA to be analysed, in a final volume of 300 µl.

Samples were incubated at 42°C for 48 h. The papers were washed three times for 10 min with 5 × SSC, similarly with 2 × SSC, 0.1% SDS and then likewise with 2 × SSC at 60°C. The filters were dried and the radioactivity was measured.

Protein kinase activity. The enzyme activity was measured as described previously (Martin et al., 1981) and expressed as pmol of [32P], transferred during a 15 min reaction with a lysate prepared from 2.5 × 105 cells.
Determination of DNA polymerase activity. Endogenous DNA polymerase activity was assayed for 30 min at 30 °C in an incubation medium (final volume, 1 ml) containing 1 mM-ATP, 0-1 mM-dATP, 0-1 mM-dCTP, 0-1 mM-dGTP, 0-01 mM-dTTP, 5 μCi [α-32P]TTP (Amersham International; sp. act. 3000 Ci/mmol), 1 mM-dithiothreitol, 1 mM-EGTA, 40 mM-HEPES pH 7.8, 100 mM-KCl, 5 mM-MgCl₂, 0-5 mM-CaCl₂, and 30 μl of the subcellular fractions. The reaction was stopped by the addition of 20 mM-EDTA, 0-1% SDS.

DNA synthesis was determined by measuring the incorporation of labelled nucleotide into acid-insoluble material as described for DNA synthesized in vivo. The total DNA polymerase activity was determined with activated calf thymus DNA (50 μg) added to the reaction mixture described above.

Electron microscopic autoradiography. To localize the newly synthesized DNA, the cells labelled with [3H]-thymidine for 30 min (10 μCi/ml, sp. act. 25 Ci/mmol) were processed for electron microscopy as previously described (Martin et al., 1981) and the treatment of samples for autoradiography was carried out according to Larra & Droz (1970).

RESULTS

Influence of arginine starvation on the site and the kinetics of DNA replication

Non-confluent CHO cultures were pretreated overnight in alpha medium deficient in arginine, then infected with FV 3 and incubated either in alpha medium deprived of arginine or in complete medium. The newly synthesized DNA in infected cells was localized by pulse labelling with [3H]-thymidine for 30 min for different periods of time following infection and the distribution in subcellular fractions, prepared as described in Methods, was determined. The cytoplasm was separated into two fractions: a cytoplasmic fraction obtained after homogenization of the cells in hypotonic buffer and centrifugation to eliminate the nuclei and associated material, and a 'NP40 fraction' resulting from the washing of the crude nuclear pellet with the non-ionic detergent Nonidet P40. Under normal growth conditions, this NP40 fraction is enriched in viroplasms containing viral DNA and proteins, whereas mainly DNase-resistant material is recovered in the cytoplasmic fractions (Martin et al., 1981). The rate of DNA synthesis in the nuclear fraction increased with time and levelled off in both cases at about 7 h, although arginine deficiency reduced that rate by a factor of two (Fig. 1 a). However, whereas after 4 h the amount of newly synthesized DNA increased in the NP40 and cytoplasmic fractions when cells were incubated in a normal medium, in the absence of arginine the quantity of acid-insoluble 3H-labelled material recovered in these fractions remained very low and represented less than 20% of the total incorporation (Fig. 1 a).

The endogenous DNA polymerase activity (measured without the addition of template) associated with the subcellular fractions after 6 h of infection was found to be higher in the nuclei isolated from arginine-starved cells than from cells maintained in arginine-containing medium (Fig. 1 b). The opposite situation was observed in the NP40 and cytoplasmic fractions, the activities being very low for arginine-deprived cells. This was to be expected, as the amount of viral DNA present in the cytoplasm is very small. The total DNA polymerase activity measured after the addition of exogenous DNA was lower in arginine-starved cells (Fig. 1 c), reflecting the decreased rate of protein synthesis, in the nuclear and NP40 fractions it was four- and threefold lower respectively. However, it is interesting to note that in the cytoplasm the amounts of free DNA polymerase were almost identical whether the cells received arginine or not. Thus, if DNA replication does not take place in the cytoplasm of arginine-deprived cells, it can not be related to the absence of DNA polymerase in this cell compartment.

The localization of newly synthesized DNA was further supported by electron microscopic autoradiographs. For this, cells infected and incubated in the appropriate medium were labelled with [3H]-thymidine, 30 min before processing for electron microscopy as indicated in Methods. Typical distributions of the grains in the cells at 5 h post-infection are presented in Fig. 2. In the majority of the infected cells maintained in arginine-free medium, grains were found predominantly in the nucleus (Fig. 2 a), whilst for cells incubated in normal medium, they were concentrated in cytoplasmic viroplasms (Fig. 2 b).

The DNA, which is synthesized and remains concentrated in the nucleus under arginine starvation, can reach the cytoplasm when optimal growth conditions are restored and the medium is supplemented with arginine. Indeed, in cells pulse-labelled with [3H]-thymidine for
Fig. 1. Influence of arginine starvation on the intracellular distribution of DNA and DNA polymerase in FV 3-infected cells. Arginine-starved CHO cells were infected with 40 p.f.u./cell, then (a) incubated in alpha medium with (○) or without (●) arginine. □ Control culture. They were labelled for 30 min with [3H]thymidine at the time indicated after infection, collected, fractionated and DNA synthesis determined as described in Methods. The DNA polymerase activity was measured in subcellular fractions (N, nuclear; NP40, Nonidet P40; Cyt, cytoplasmic) prepared after 6 h infection, either without (b) or with (c) the addition of exogenous DNA. The composition of the reaction mixture is given in Methods and the incubation was for 20 min at 30 °C. Cells infected and maintained in complete medium (□) or in arginine-minus medium (■), and arginine-starved control cells (●) are shown.

30 min at 5 h post-infection and re-incubated after elimination of the radioactive precursor in arginine-containing medium for 2 h, only 20% of the labelled DNA was associated with the nuclei; this value is close to the one obtained for the DNA synthesized in the presence of arginine (Table 1). When cells were kept in arginine-depleted medium, 75% of the DNA remained in the nuclear fraction after a 2 h chase, whereas immediately after labelling 77% was recovered in this fraction.

Compartmentalization of the newly synthesized DNA in the nucleus and its passage into the cytoplasm is corroborated by the numbers of grains on electron microscopic autoradiographs. As the cytoplasm is not totally free of grains, cells were identified as having DNA predominantly in the nucleus when more than 20 grains were found there and less than five in the cytoplasm. This was the case for over 50% of the arginine-deprived cells when examined immediately after a 30 min [3H]thymidine pulse (Table 1). Following a 2 h chase, in the presence of arginine, only 30% of the cells showed a nuclear concentration. The movement of the newly synthesized DNA from the nucleus to the cytoplasm is thus controlled by an arginine-dependent factor. Hybridization experiments of newly synthesized DNA with immobilized viral DNA showed that 63% and 84% of the DNA produced, respectively, in arginine-starved cells and cells kept in complete medium at 5 h post-infection, was of viral origin.
Fig. 2. Electron microscopic autoradiographs of DNA in FV 3-infected CHO cells, showing the intracellular localization of newly synthesized DNA, labelled with [3H]thymidine from 4-5 to 5 h post-infection, in cells incubated in arginine-depleted medium (a) or in complete growth medium (b). Bar markers represent 1 μm.
Table 1. Transfer to the cytoplasm of DNA replicated in the nuclei of FV 3-infected arginine-deprived cells

<table>
<thead>
<tr>
<th>Incubation medium*</th>
<th>Time after infection (h)</th>
<th>Total cells (T)</th>
<th>Nuclear fraction (N)</th>
<th>% N/T</th>
<th>Total number of cells examined (T)</th>
<th>Predominant nuclear concentration (N)</th>
<th>% N/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Arg</td>
<td>5</td>
<td>19241</td>
<td>14875</td>
<td>77</td>
<td>165</td>
<td>86</td>
<td>52</td>
</tr>
<tr>
<td>+ Arg</td>
<td>5</td>
<td>33520</td>
<td>20190</td>
<td>60</td>
<td>169</td>
<td>43</td>
<td>22</td>
</tr>
<tr>
<td>± Arg</td>
<td>7</td>
<td>7420</td>
<td>5587</td>
<td>75</td>
<td>83</td>
<td>26</td>
<td>31</td>
</tr>
</tbody>
</table>

* Cells incubated in arginine-plus (+) or -minus (−) medium were labelled, from 4.5 to 5 h after infection, with [3H]thymidine. The distribution of the DNA was analysed immediately or at 7 h after a chase period in the same medium or in medium supplemented with arginine, at 5 h (+Arg).

**Size of DNA strands**

To determine whether full-length copies of the viral genome were produced in the nucleus or not, the sedimentation characteristics of DNA synthesized under arginine deficiency were compared to DNA replicated under normal conditions. For this, two infected cultures maintained in arginine-depleted or complete alpha medium were labelled with [3H]thymidine for 30 min at 5 h post-infection and the cells collected immediately afterwards. Zonal sedimentations were performed in alkaline sucrose gradients as described in Methods. To avoid any breakdown of the DNA which can occur during the preparation of the nuclear fraction and which has been observed in the first experiments (data not presented), the whole cells were layered on top of the gradients. Although the majority of the labelled DNA migrated at the same position as genomic DNA, a broad spectrum of the strand sizes was noticed, with a small fraction of the molecules being longer than the genome (Fig. 3a). However, the distribution of the DNA in the gradients was identical, irrespective of the infection conditions. To follow an eventual conversion of this DNA, cells infected and labelled as above were re-incubated for 20 h in the presence of an inhibitor of DNA synthesis, one culture being at that time supplemented with arginine. Fig. 3(b) shows a comparison of the DNA strand sizes. In all cases, a more homogeneous distribution of the DNA in the gradient could be observed; this distribution is analogous to that of the virion DNA. This was also true for the cultures maintained under arginine starvation, but the total amount of DNA recovered was significantly lower than that present in cells where protein synthesis resumed after the addition of arginine. Thus, considering that more than 80% of the DNA analysed in arginine-depleted cells was located in the nucleus, it is clear that genome-size molecules are present in this cell compartment.

**Functional FV 3 DNA is synthesized in the absence of arginine**

Virus assembly did not occur under arginine starvation. However, after restoration of normal medium, the growth cycle could be completed in the absence of additional DNA synthesis.

Two batches of arginine-starved CHO cultures were infected with FV 3 (m.o.i. 5 p.f.u/cell) and incubated either in normal or in arginine-depleted medium. After 8 h, in some cultures DNA synthesis was stopped by the addition of arabinosylcytosine (Ara-C) and, in the case of arginine-starved cells, the medium was supplemented with arginine. The production of infectious virus 24 h or 48 h later was measured by titration of the cell lysates on BHK cell monolayers (Table 2). Indeed, whilst in the cultures deprived of arginine there was no increase in the virus titre from 8 h to 48 h post-infection, the addition of arginine allowed the formation of virus particles and this took place in the absence of any new DNA synthesis. The amount of infectious
FV 3 DNA in arginine-deprived cells

Fig. 3. Alkaline sucrose gradient analysis of pulse-labelled DNA synthesized in FV 3-infected CHO cells. The infected cultures maintained in complete alpha medium (●) or in arginine-minus medium (○) were labelled for 4.5 to 5 h post-infection with \[^{3}H\]thymidine and analysed immediately (a). After the labelling period, some cultures were re-incubated in the presence of Ara-C (10^{-3} M) either in the same medium (normal medium, ●; arginine-minus medium, ○) or after being supplemented with arginine (○); they were analysed 20 h later (b). The vertical arrows indicate the position of virus particle DNA analysed in parallel. Infection and sedimentation conditions were as indicated in Methods. Sedimentation is from left to right.

Table 2. Production of infectious FV 3 particles affected by arginine starvation

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Infectious particles (p.f.u./ml) at time post-infection (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+ Arginine; at 8 h p.i.*</td>
<td>3 x 10^5</td>
</tr>
<tr>
<td>+ Ara-C (10^{-3} M)</td>
<td>2.2 x 10^6</td>
</tr>
<tr>
<td>− Arginine; at 8 h p.i.</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>+ Arg + Ara-C (10^{-3} M)</td>
<td>1.4 x 10^6</td>
</tr>
</tbody>
</table>

* Post-infection.

particles produced under these conditions after 48 h was almost identical to that obtained in cultures incubated in normal medium which received Ara-C at 8 h.

To ascertain that the infectious particles formed resulted from the encapsidation of the DNA synthesized in arginine-depleted cells and not of parental DNA, \[^{3}H\]thymidine was added to arginine-starved cultures from 7 to 8 h post-infection; the labelled medium was then replaced by the normal medium containing Ara-C and 24 h later the cytoplasmic fraction was analysed by sedimentation on a sucrose gradient (under the conditions habitually used for virus purification). When the radioactivity was measured and the infectious particles present in the gradient fractions were titrated, a close correlation was found between the distribution of \(^{3}H\)-labelled DNA and infectivity in the gradient (Fig. 4). Thus, it is clear that \[^{3}H\]thymidine incorporated into DNA under arginine starvation is encapsidated into mature particles after re-incubation of the cultures in complete medium.

Protein synthesis under conditions of arginine depletion

The synthesis of FV 3 polypeptides is sequentially ordered and regulated (Willis et al., 1977; Cordier, 1979; Elliott & Kelly, 1980; Martin et al., 1982). To establish whether a lack of arginine
Fig. 4. Analysis of progeny particles formed by encapsidation of DNA synthesized under arginine starvation. Arginine-depleted cells, infected for 7.5 h with FV 3 (m.o.i. 5 p.f.u./cell) were labelled with \([^{3}H]\)thymidine for 30 min. The radioactive precursor was removed and the cells re-incubated in the presence of arginine and Ara-C (10^{-3} M) for 20 h. A cytoplasmic fraction was prepared and analysed by sedimentation on a neutral sucrose gradient as described in Methods. The radioactivity was measured after TCA precipitation of aliquots of the gradient fractions (○). Distribution of infectious virus particles was followed by titration of the fractions on BHK cell monolayers and quantification of p.f.u. (●). Sedimentation was from right to left.

would significantly affect the production of some polypeptide species or merely result in a reduced rate of synthesis for all of them, the proteins synthesized at different times after infection and present in the three subcellular fractions were analysed. In the time interval of 2 to 8 h post-infection, some modifications could be noted. After 2 h the amount of \([^{35}S]\)methionine incorporated in cells incubated in the absence of arginine was half that of cells incubated in the presence of arginine; by 8 h the difference was more pronounced as the rate of synthesis continued to decrease slightly in cells deprived of arginine while it doubled in normal cultures. At the beginning of infection, 65, 20 and 15% of the proteins were recovered respectively in the cytoplasmic, NP40 and nuclear fractions (data not presented).

A similar distribution was found later in cells lacking arginine, whereas in its presence the quantity of protein in the NP40 fraction increased with the time of infection, reaching 40% of the total by 8 h after infection. The percentage in the nuclear fraction remained close to 15. In the absence of cytoplasmic DNA there was no viroplasm formation and the amount of viral proteins in the NP40 fraction was therefore low. Electrophoretic analysis of the polypeptide species synthesized shows clearly that the early groups (immediate early and delayed early) were produced in the absence of arginine (Fig. 5). However, there were only traces of late polypeptides such as 48K and 15K, while polypeptides 70K, 63K or 12K were hardly detectable. The intracellular distribution was similar to that found under normal conditions, polypeptides 90K, 54K, 42K and 31K being concentrated in the nucleus and polypeptide 16K in the cytoplasmic fraction.

At 5 h, in the presence of arginine there is already a late pattern. The nuclear fraction contained the polypeptides 90K, 70K, 63K, 31K, 12K at concentrations higher than in the cytoplasm while 58K, 56K, 16K were almost exclusively found in the cytoplasmic and NP40 fractions. After 8 h of infection, the total protein synthesis decreased in arginine-starved cells: one early polypeptide, 31K, was more affected than the others.

Determination of protein kinase activity, a late function (Silberstein & August, 1976),
Fig. 5. Influence of arginine starvation on the polypeptide species synthesized in FV 3-infected cells. Fluorogram of a 12.5% polyacrylamide gel slab containing electrophoretically separated pulse-labelled ([35S]methionine, 30 min) protein in infected, arginine-starved CHO cells (40 p.f.u./cell). Cells were re-incubated after adsorption in the following: complete alpha medium (+ A), alpha medium devoid of arginine (−A); arginine-depleted medium until 5 h post-infection and then supplemented with arginine (+ + A). The numbers give the time (h post-infection) of cell collection. Fractionation of the cells was as indicated in Methods; the cytoplasmic fractions (Cyt) from 1-12 × 10⁵ cells, NP40 and nuclear (N) fractions from 2-25 × 10⁵ cells were analysed. The fluorograms presented were obtained after an exposure of 1.5 days for the cytoplasmic fractions and 5 days for the NP40 and nuclear fractions. The left column shows the structural polypeptides of FV 3, labelled with [35S]methionine, used as mol. wt. markers. The numbers in the margin indicate the polypeptide mol. wt. (× 10⁻³).

confirmed the strong reduction in the synthesis of group III polypeptides. Indeed, the respective activities of lysates prepared with cells infected for 8 h and incubated in the presence or in the absence of arginine were 0.75 and 0.15.

When the starved cultures were supplemented with arginine at 5 h and the proteins analysed 1 h afterwards, late species such as polypeptide 70K as well as polypeptides of mol. wt. higher than 90K could already be found in the nucleus. At 8 h, the profiles and the distribution were almost identical to those observed in cells maintained for the entire period in complete medium.

**Phosphorylation of the polypeptides**

The transfer of phosphate onto polypeptide chains may be a means of controlling their biological activities, particularly in modifying their affinity for DNA (Wilcox et al., 1980).

Thus, as the synthesis of protein kinase was greatly depressed by arginine starvation, and considering that this enzyme may be involved in the phosphorylation of the early polypeptide species, the ratio of [35S]methionine to [32P]orthophosphate incorporation into polypeptides was measured between 1 h and 8 h after infection (Table 3). The data indicated that phosphorylation was only slightly lower for polypeptides synthesized in the absence of arginine no matter what their localization. To check whether individual polypeptides had been differentially affected, we analysed polypeptides 90K and 31K, two species found concentrated essentially in the nucleus and known to be phosphate acceptors (Martin et al., 1981). The ratio of [35S]methionine to [32P]orthophosphate incorporated into the species present in the various cell compartments was determined as described in Methods (Table 3). The consequence of arginine starvation was more pronounced for the polypeptide 31K present in the cytoplasm whereas in the nucleus only small differences were observed.
Table 3. *Relative phosphorylation of polypeptides in the different cell compartments*

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>Culture medium</th>
<th>Ratio [35S]methionine/[32P]orthophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ arginine</td>
<td>Cytoplasmic fraction</td>
</tr>
<tr>
<td>Total proteins</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td>90K</td>
<td>+</td>
<td>20.2</td>
</tr>
<tr>
<td>31K</td>
<td>+</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>25.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>32.5</td>
</tr>
</tbody>
</table>

It should be emphasized that the degree of phosphorylation of a given polypeptide can be three- to fivefold higher in the nucleus than in the cytoplasmic fraction. This is true for polypeptides of apparent mol. wt. 90K and 31K, synthesized in cells maintained in complete or in arginine-depleted medium.

**DISCUSSION**

Arginine starvation was used as a means of restricting FV 3 DNA replication to the nucleus. Indeed, we have shown that approximately 80% of the newly synthesized DNA in infected, arginine-deprived cells was found in the nucleus and remained there except when the medium was supplemented with arginine. Under normal conditions, when the rate of DNA synthesis is at its highest (7 to 9 h post-infection), only 40% of the newly replicated molecules are recovered with the nuclear fraction.

Thus, according to the pattern proposed by Goorha (1982), stage 1 of DNA replication occurs in the absence of arginine and the passage from stage 1 to stage 2 is greatly impaired but probably not totally blocked.

Although it cannot be excluded that a small fraction of the DNA recovered in the cytoplasmic fraction may stem in part from a breakdown of some nuclei, electron microscopic autoradiography enabled grains to be found also in the cytoplasm.

Three conclusions can be drawn from the results presented here. (i) DNA replicated in the nucleus of arginine-depleted cells is of genome size; (ii) the DNA synthesized under arginine starvation is functional and, upon restoration of the normal growth conditions, can be encapsidated to form infectious particles; (iii) DNA synthesis is restricted to the nucleus, most likely because the expression of late genes is extremely limited.

The conclusion that genome-size molecules are present in the nucleus agrees with the recent work of Goorha (1982). The observation of subgenomic size molecules reported earlier was made after the isolation of the nuclei and a partial degradation may have occurred during cell fractionation. The relatively heterogeneous distribution of the DNA in denaturing gradients even after a chase is probably related to the structure of the genome, which contains single-stranded breaks in one DNA strand (Kelly & Avery, 1974).

The recovery of more than 50% of the DNA labelled during arginine starvation in particles after an incubation in complete medium definitely indicates that nuclear DNA has been encapsidated. Although DNA replicated in the nucleus is of unit length, it does not preclude the hypothesis of an involvement of concatemeric molecules in DNA packaging. In fact, larger precursor molecules can still be formed in the cytoplasm by recombination of unit size molecules, an event which is likely to occur quite frequently between molecules presenting a circular permutation and terminal redundancies (Goorha & Murti, 1982).

As to why FV 3 replication is impaired in arginine-starved cells, the data presented point to a defect in the expression of the late genes. Early polypeptides are produced and they are distributed in the cell compartments as in the normal infection, but at 8 h post-infection, the synthesis of polypeptide 31K is depressed. The degree of phosphorylation in the nucleus is only slightly lower.

Some of these early polypeptides present in the nucleus must be involved in the replication of viral DNA. In cells infected with herpes simplex virus and deprived of arginine, viral DNA-
binding proteins involved in DNA replication could also be identified (Becker et al., 1980). In parenthesis, it may be recalled that the phosphorylation of the two FV 3 polypeptides analysed, 90K and 31K, was strikingly different in the various subcellular fractions under either restrictive or optimal conditions. The properties of these molecules may therefore be modified depending on their location in the cells. Numerous species of herpes simplex virus polypeptides are phosphorylated but the extent of phosphorylation of the proteins accumulating in the nucleus is not significantly different from that of their cytoplasmic counterparts (Wilcox et al., 1980). However, in this case, the nucleus is the site of early events as well as of particle assembly.

It is clear that in FV 3-infected, arginine-starved cells, only traces of late polypeptide species are synthesized, if at all. This is certainly not related to the difficulty of ensuring an efficient polymerization of the amino acids in cells with a reduced pool of arginine, as the relative abundance of early versus late species indicates that the block is specific to late polypeptides. When added to normal medium, canavanine, an arginine analogue, did not interfere with the synthesis of late polypeptides; therefore, it has been suggested that the presence of arginine at a functional site of the molecules involved in the expression of late genes is unlikely (Elliott & Kelly, 1980). However, addition of canavanine to arginine-depleted medium had a more dramatic effect: it modified the regulation of the early species and did not allow the production of late polypeptides (Cordier, 1979; Martin et al., 1981).

Whether or not the transcription of the late genes occurs remains to be established. In this respect, it is interesting to make the comparison with vaccinia virus multiplication in arginine-depleted cells. Although vaccinia DNA replication takes place, late mRNAs are not synthesized (Obert et al., 1971) but transcription can resume upon the addition of ornithine or polyamines. However, the presence of arginine is necessary for the production of infectious particles (Obert et al., 1980).

The use of arginine deprivation has made it possible to differentiate the nuclear step of FV 3 replication from the later stage occurring in the cytoplasm. The recent description by Goorha (1982), of a ts mutant, defective in this second stage, has provided the genetic evidence for a two-stage replication. When data are available, a comparison of the genes expressed at the non-permissive temperature in cells infected with the mutant with that of a wild-type under arginine starvation may help to determine whether different events control the transition of FV 3 DNA replication from the nucleus to the cytoplasm.

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REFERENCES


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