Interrelation between Viral and Cellular DNA Synthesis in Mouse Cells Infected with the Parvovirus Minute Virus of Mice

By N. HARDT, C. DINSART, S. SPADARI, G. PEDRALI-NOY AND J. ROMMELAERE

1 Laboratoire de Biophysique et Radiobiologie, Université libre de Bruxelles, rue des Chevaux, 67, B-1640 Rhode-St Genèse, Belgium and 2 Istituto di Genetica Biochimica ed Evoluzionistica, Consiglio Nazionale delle Ricerche, Via S. Epifanio, 14, I-27100 Pavia, Italy

(Accepted 25 May 1983)

SUMMARY

Mouse fibroblasts arrested in Go by isoleucine deprivation were inoculated with the autonomous parvovirus minute virus of mice (MVM). Infected cells were released from the Go block by transfer to complete medium and their progression to and through the S phase was monitored. The onset of viral and cellular DNA synthesis coincided, suggesting that cellular factor(s) required for MVM DNA replication became available as soon as cells entered the S phase. Cellular DNA synthesis was reduced to about 60% by MVM infection. However, this inhibition did not decrease significantly the overall rate of DNA replication in infected cells because it was compensated by concomitant viral DNA synthesis. MVM infection delayed the movement of the cells out of S phase by at least 5 h. At any time post-infection, more than 95% of both viral and cellular DNA synthesis was sensitive to inhibition by aphidicolin. Since this drug is highly specific for cellular DNA polymerase α, the data are consistent with a major role of this enzyme in the in vivo DNA replication of autonomous parvovirus. The assembly of 95% of virus progeny particles was concomitant with a late phase of viral DNA replication which accounted for 30% of the total viral DNA synthesized. The inhibition of this residual viral DNA replication by aphidicolin reduced dramatically the size of the burst of infectious particles; this observation concurs with other evidence to suggest that encapsidation is driven by a late replication event sensitive to this drug.

INTRODUCTION

Parvoviruses are among the smallest known animal viruses and contain a predominantly single-stranded, linear DNA genome (Tattersall & Ward, 1978). The replication of parvovirus DNA takes place in the cell nucleus and involves three steps: (i) the conversion of single-stranded DNA to double-stranded replicative forms, (ii) the replication of double-stranded DNA and (iii) the asymmetric production of progeny single-stranded DNA from replicative forms (Berns & Hauswirth, 1978). Parvoviruses of the non-defective subgroup are able to replicate autonomously provided that S phase-specific host cell function(s) is (are) available (Tattersall & Ward, 1978). The S phase-dependent step of the parvovirus life cycle is likely to be viral DNA replication, in particular the conversion of the input single-stranded genome to a duplex form (Wolter et al., 1980). The cellular ‘S factors’ essential for parvovirus replication have not been identified so far and the time of their appearance during the S phase is disputed (Parris & Bates, 1976; Wolter et al., 1980). Productive infection by the non-defective parvoviruses does not require a virion-associated DNA polymerase (Pritchard et al., 1978a) and is likely to rely on cellular DNA polymerases. Although both DNA polymerases α and γ have been shown to catalyse parvovirus DNA replication in vitro (Handa & Carter, 1979; Pritchard et al., 1981; Kollek & Goulian, 1981; Kollek et al., 1982; Faust & Rankin, 1982), the contribution of these enzymes to viral DNA synthesis in vivo is unclear. The involvement of DNA polymerase α is supported by indirect evidence showing a temporal correlation between this enzymic activity and...
and parvovirus DNA synthesis in infected cells (Pritchard et al., 1978b). Studies of the effect of parvovirus replication on the progression of cellular DNA replication have given contradictory results. Some authors have reported that parvovirus infection causes an early reduction in the rate of DNA synthesis (Salzman et al., 1972) whereas others have found no alteration in the progression of infected cells through S phase (Hampton, 1970; Parris & Bates, 1976).

These uncertainties concerning the interrelation between cellular and parvovirus DNA replication prompted us to investigate the time course of these processes in synchronized mouse cells following infection with the autonomous parvovirus minute virus of mice (MVM) (Ward & Tattersall, 1982). The data presented suggest that MVM DNA replication (i) depends on cellular function(s) expressed early in S phase, (ii) is accompanied by an inhibition of cellular DNA synthesis, and (iii) is inhibited by aphidicolin, a specific inhibitor of cellular DNA polymerase α (Ikegami et al., 1978; Pedrali-Noy & Spadari, 1979), to a similar extent as cellular DNA replication. Moreover, a late, aphidicolin-sensitive event in MVM DNA replication seems to drive the assembly of infectious progeny particles.

**METHODS**

**Cell culture and viral infection.** Mouse A9 cells (Littlefield, 1964) were accumulated in G₀ by isoleucine deprivation as described previously (Rommelaere et al., 1981). The arrested monolayer culture (4 × 10⁶ cells) was infected with the plaque-purified strain T of MVM (Tattersall, 1972) at 4 p.f.u./cell. Immediately after infection, cells were transferred to Eagle's minimal essential medium supplemented with 5% foetal calf serum.

**Autoradiography.** At intervals following MVM or mock infection, cells attached to glass slides were pulse-labelled with [³H]thymidine (TdR) (5 μCi/ml, 5 Ci/mmol) for 45 min and further incubated for 10 min in phosphate-buffered saline (PBS) containing 1 mg/ml of unlabelled TdR. Cultures were then rinsed, fixed and prepared for autoradiography, as described previously (Hardt et al., 1980).

**Virus titration.** At intervals post-infection, cells were collected and the virus was extracted by freeze-thawing (Tattersall et al., 1976). Extracts were assayed for infectivity by direct plaque assay (Tattersall, 1972). In some experiments, infected cells were exposed to 15 μM-aphidicolin from different times until 36 h post-infection, at which time the infectivity of cell-associated viral particles was measured.

**DNA analysis**

**Radioactive labelling.** At intervals after MVM or mock infection, cells were pulse-labelled with [³H]TdR (20 μCi/ml, 25 Ci/mmol) for 45 min. Cultures were then incubated for 7 min with PBS containing 1 mg/ml of unlabelled TdR. When present, aphidicolin was used at 15 μM during both the pulse and chase periods.

**Measurement of the overall rate of DNA synthesis.** A 0.1 ml amount of the cell suspension was collected on a Whatman GF/C filter. The filter was washed twice in 5% trichloroacetic acid (TCA) and once in ethanol. When dry, the filter was dipped in Econofluor scintillator (New England Nuclear) and the radioactivity was measured in a liquid scintillation spectrometer. It was verified that the rate of incorporation of [³H]TdR in both uninfected and MVM-infected cells was not changed by increasing the concentration of thymidine in the external medium from 0.8 μM (our standard radioactive labelling conditions) to 5 μM. Therefore, the incorporation of [³H]TdR is not likely to be affected by endogenous deoxyribonucleotide pools (Cleaver & Holford, 1965) and should provide a reliable measurement of DNA synthesis.

**Alkaline sucrose sedimentation analysis.** The cell suspension was exposed to 20 Gy of γ-radiation delivered from a Gamma cell 200 (195Co). A 4 ml gradient of 5 to 20% sucrose in 0.1 M-NaCl, 0.1 M-NaOH was formed on top of a 1 ml cushion of 50% sucrose. Cell suspensions were made 0.2 M in NaOH, incubated for 15 min at room temperature and layered (0.2 ml) on top of the gradients. These were centrifuged immediately at 4 °C, in the SW50.1 rotor in a Beckman model L ultracentrifuge at 48,000 rev/min for 4 h. After centrifugation, 24 to 26 fractions were collected and their radioactivity was measured by liquid scintillation spectrometry using Aquasol-2 fluid (New England Nuclear). DNA purified from MVM virions and labelled with [³H]TdR (Bourguignon et al., 1976) was used as a marker and centrifuged in parallel gradients.

**Determination of viral DNA by DNA-DNA reassociation.** Samples of labelled cells were suspended in 0.02 M-Tris-HCl, 0.15 M-NaCl, 0.01 M-EDTA, pH 7.5 (2 × 10⁶ cells/ml) and lysed with SDS (0.6%) and self-digested Pronase (2 mg/ml) for 1 h at 37 °C. Extracted DNA was sheared to a length of 500 to 600 nucleotides by heating the lysates at 100 °C in the presence of 0.3 M-NaOH for 15 min. After cooling to 0 °C, samples were neutralized with KH₂PO₄ to a final pH 7.3 to 7.5 and dialysed overnight against 5 mM-Tris-HCl, 0.5 M-NaCl, 1 mM-EDTA, pH 7.7. The resultant DNA solutions were used for reassociation experiments as described by Pedrali-Noy & Weissbach (1977) except that an appropriate excess of both strands of non-radioactive viral DNA was added to each sample to drive the assembly of infectious progeny particles.
the reassociation reaction. This DNA was prepared by copying with Escherichia coli DNA polymerase I (Klenow fragment) self-primed single-stranded MVM DNA purified from viral particles, essentially as described by Bourguignon et al. (1976).

Chemicals. [Me-3H]TdR was from Amersham International. Aphidicolin was kindly supplied by Dr A. Todd, Imperial Chemical Industries, Macclesfield, Cheshire, U.K.

RESULTS

Pattern of overall DNA synthesis in MVM-infected cells

Mouse cells were accumulated in G₀ by incubation in isoleucine-depleted medium. Arrested cultures were transferred to complete medium and pulse-labelled with [³H]TdR at intervals. DNA synthesis was measured by determining the radioactivity incorporated into TCA-insoluble material and by cell autoradiography. Cultures released from the G₀ block resumed the cell cycle in a synchronous fashion. Fig. 1 (solid lines) illustrates the time course of cellular DNA synthesis. The traverse of S phase is indicated by a sharp and transient increase of both the fraction of labelled cells (Fig. 1 a) and the incorporated radioactivity (Fig. 1 b). Entry into S phase and maximum labelling occurred about 10 and 18 h following transfer to complete medium, respectively.

G₀-arrested cultures were infected with MVM at a multiplicity of 4 infectious units/cell, immediately prior to transfer to complete medium. MVM infection did not disturb significantly the entry of the cells into S phase (Fig. 1 a). In contrast, the labelling index increased for 5 h more in MVM- than in mock-infected cultures, indicating that the movement of MVM-inoculated cells out of the phase of DNA synthesis was delayed (Fig. 1 a). As illustrated by Fig. 1 (b), MVM infection caused little reduction in the maximum rate of DNA synthesis but slowed its decline, resulting in a 1.2-fold increase of the total amount of labelled DNA precursors incorporated within 35 h post-infection.

Time course of viral and cellular DNA replication

Incorporation of [³H]TdR into cellular and viral DNA was measured in cells pulse-labelled at intervals after G₀ release and MVM infection. The DNA isolated from infected cells was fractionated by sedimentation through alkaline sucrose gradients; it generated bimodal profiles (Fig. 2a). Molecules of high and low sedimentation rates co-migrated with DNA from uninfected cells and with purified viral DNA, respectively. The slow-sedimenting peak was used for the quantification of viral DNA (Fig. 2a). The fraction of virus-specific tritiated DNA was also determined by DNA-DNA reassociation in the presence of an excess of non-radioactive viral DNA (Table 1).

Although Fig. 1 (b) shows that the overall rate of DNA synthesis in infected cells closely approximated that of uninfected cells, Fig. 2 (b) clearly shows that cellular DNA replication was markedly depressed after MVM infection. However, this inhibition, presumably due to some event(s) in MVM replication, was compensated for by a concomitant increase in viral DNA synthesis. The extent of the inhibition of cellular DNA synthesis occurring within 35 h after infection varied from 20 to 60% in independent experiments. Correspondingly, viral DNA replication accounted for a fraction, which ranged from 25 to 65% of total incorporated radioactivity. The reason for this fluctuation in the level of viral DNA synthesis is not known. The time courses of viral and cellular DNA synthesis in infected cultures overlapped to a great extent (Fig. 2b; Table 1). The onset of replication of both types of DNA could not be dissociated and took place around 10 h after infection and release from G₀. Infected cells achieved the highest rate of viral DNA replication about 5 h after residual cellular DNA synthesis started to decline.

Effect of aphidicolin on viral DNA replication and encapsidation

Viral replication was tested for its sensitivity to aphidicolin. The drug was used at a concentration, 15 μM, that inhibits over 95% of cellular DNA replication (Pedrali-Noy & Spadari, 1980) with no effect on RNA and protein synthesis (Pedrali-Noy et al., 1982). Consistently, aphidicolin reduced [³H]TdR incorporation into cellular DNA of both mock- and MVM-
infected A9 cells to 3 or 5% of its normal level at any time after release from G₀ (Fig. 1b and 2b). Moreover, viral DNA in MVM-infected cells was as sensitive to aphidicolin as cellular DNA replication, irrespective of time post-infection (Fig. 2b). Aphidicolin did not alter the fraction of the culture involved in DNA synthesis but rather the level of incorporation per replicating cell (data not shown). The influence of the inhibition of DNA synthesis on the formation of

![Graphs and diagrams showing DNA synthesis in synchronized A9 cells infected with MVM.](image_url)
**MVM DNA synthesis**

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>Total DNA†</th>
<th>Viral DNA‡</th>
<th>Cellular DNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2050</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>19</td>
<td>27800</td>
<td>3367</td>
<td>24433</td>
</tr>
<tr>
<td>26</td>
<td>28679</td>
<td>6564</td>
<td>22115</td>
</tr>
<tr>
<td>32</td>
<td>6219</td>
<td>1293</td>
<td>4926</td>
</tr>
<tr>
<td>45</td>
<td>5720</td>
<td>795</td>
<td>4925</td>
</tr>
</tbody>
</table>

* Cultures were pulse-labelled with [3H]TdR and lysed at intervals following release from G₀ block and infection. Radioactivity incorporated into TCA-insoluble material was measured.
† Total DNA extracted from infected cells.
‡ Virus-specific DNA, as determined by DNA-DNA reassociation. The reassociation reaction was completed within 8 min in the presence of 22 μg/ml of non-radioactive viral DNA. Values were corrected for the extent of reassociation of cellular DNA under those conditions (approximately 5%).
§ Total DNA synthesis minus viral DNA synthesis.
ND, Not done.

Fig. 3. Effect of aphidicolin on progeny virus formation in MVM-infected A9 cells. At intervals after MVM infection, A9 cells were either collected (closed symbols, continuous line) or further incubated in the presence of 15 μM-aphidicolin until harvest at 36 h post-infection (same open symbols, interrupted lines). Collected cells were disrupted and the infectivity of associated viral particles was measured. The dotted line is the cumulative curve of viral DNA synthesis calculated from Fig. 2(b) and considered as 1.0 at 36 h post-infection.

Infectious progeny particles was studied in MVM-infected cultures exposed to aphidicolin from different times to harvest time at 36 h after infection. It is apparent from Fig. 3 that little virus production occurred in the presence of aphidicolin. In the absence of the drug, most of the viral progeny was assembled during the interval from 24 to 36 h post-infection. The production of the burst was concomitant with a late phase of viral DNA replication accounting for only 30% of the total viral DNA synthesized from the time of infection. The inhibition of this residual DNA synthesis by aphidicolin (Fig. 2b) was associated with a dramatic reduction of the burst size (Fig. 3). Thus, although a major fraction of total viral DNA had been synthesized by 24 h, little progeny single-stranded DNA was available for encapsidation unless an aphidicolin-sensitive event(s) occurred during the subsequent 12 h.
At any time after infection, aphidicolin reduced viral DNA synthesis by more than 95% (Fig. 2b). Parvovirus DNA replication involves the conversion of input single-strands into duplex parental replicative forms (RF). The multistep amplification of parental RF DNA gives rise to a large pool of RF molecules and eventually to progeny single-stranded DNA (Berns & Hauswirth, 1978). Parvovirus replication accounts for up to 75% of uninfected cell DNA synthesis within a 35 h period which comprises the crossing of S phase (Fig. 2b). Most of the incorporation of $^{3}$H]dThd into viral DNA is associated with the viral DNA amplification phase; the conversion of the few 5000 nucleotides-long input genomes contributes minimally to total viral DNA synthesis. Consequently, the results presented indicate that aphidicolin prevents most of the amplification of parvovirus DNA, but they provide no information on the drug sensitivity of parental RF formation.

Aphidicolin is known to inhibit cellular nuclear DNA replication (Geuskens et al., 1981). The inhibition of viral DNA synthesis by this drug is unlikely to result merely from the inhibition of cellular DNA replication since viral DNA synthesis does not require concomitant cellular DNA replication (Parris & Bates, 1976). A more likely interpretation is that an aphidicolin-sensitive effector participates in a step of MVM DNA replication which is limiting for the amplification of viral DNA throughout the virus life cycle.

The dependence of parvovirus DNA replication on host cell enzymes (Rhode, 1978) and the specificity of aphidicolin for cellular DNA polymerase α strongly suggest that the latter enzyme is involved in viral DNA synthesis. It cannot be ruled out, however, that the drug affects other as yet undiscovered cellular or viral factors involved in parvovirus DNA synthesis. The role of DNA polymerase α in DNA replication of autonomous parovirus is further supported by the temporal correlation between this enzymic activity and viral DNA synthesis in vitro (Prichard et al., 1978) and by the ability of partially purified preparations of this enzyme to catalyse steps of viral DNA synthesis in vitro (Prichard et al., 1981; Kollek et al., 1982; Faust & Rankin, 1982; Hübischer et al., 1982; M. Günther, personal communication).

The inhibition of MVM DNA synthesis was greater than 95% and not significantly different from that of cellular DNA synthesis (Fig. 2b). Such a sensitivity to aphidicolin would be consistent with the fact that amplification of the DNA of autonomous paroviruses relies essentially or even exclusively on DNA polymerase α, as does the replication of cellular chromosomal DNA. However, the DNA amplification of autonomous paroviruses is a multistep process involving the replication of RF molecules and the asymmetric production of single-stranded DNA of viral polarity (Berns & Hauswirth, 1978). The temporal sequence and the enzymology of these steps are still unclear. The permanent need for DNA polymerase α therefore does not rule out additional polymerase requirements for the completion of viral DNA synthesis. Such a possibility is raised by recent studies showing that DNA polymerase γ participates in parvovirus DNA replication in vitro (Kollek et al., 1982; M. Günther, personal communication).

Very little new virus assembly occurred in the presence of aphidicolin (Fig. 3). Although a major fraction of viral DNA had been synthesized by 24 h after infection, cells did not contain, at this stage, a large pool of progeny viral strands which could be encapsidated in the absence of an aphidicolin-sensitive event. The specificity of this drug points to the dependence of MVM production on DNA synthesis. The inhibition by aphidicolin of residual cellular DNA replication is unlikely to account for the reduced formation of infectious particles since the assembly and processing of autonomous paroviruses do not require the host cell to be in S phase (Parris & Bates, 1976; Richards et al., 1978). It follows that the burst of virus encapsidation appears to require concomitant synthesis of viral DNA. A similar conclusion was drawn from the effect of hydroxyurea on MVM production by rat cells (Richards et al., 1977, 1978). Little, if any, free single-stranded DNA was found in infected cells (Tattersall & Ward, 1976), suggesting that the production of progeny strands is tightly coupled to their packaging. The ultimate step in the formation of viral single strands is thought to be their displacement from duplex forms by asymmetric replication (Berns & Hauswirth, 1978). It was proposed that this strand displacement synthesis may be driven by encapsidation (Tattersall & Ward, 1976; Richards et al., 1978) thereby accounting for the dependence of the assembly of new particles on continuing viral DNA synthesis.
The burst of MVM packaging was associated with a late phase of viral DNA replication accounting for only 30% of the total viral DNA synthesized after infection (Fig. 3). Since virion morphogenesis lags behind the amplification of the pool of RF DNA (Ward & Tattersall, 1982), this late phase is likely to be mainly devoted to the asymmetric strand displacement synthesis of single-stranded progeny DNA. The aphidicolin sensitivity of both virion assembly and late viral DNA replication strongly suggests that DNA polymerase α participates in vivo in the displacement synthesis. This conclusion is consistent with the aphidicolin sensitivity of this reaction in parvovirus DNA replication in vitro (Pritchard et al., 1981; Kollek et al., 1982).

A9 cells were accumulated in G0, inoculated with MVM and released from growth inhibition. MVM uptake was presumably completed when cells entered into S phase since this process is independent of the cell cycle and is carried out within a few hours after infection (Linser et al., 1979; J. Rommelaere, unpublished results). Therefore, initiation of viral DNA replication was mainly limited by the dependence of the conversion of the parental genome into duplex RF, on cellular factors expressed specifically during the S phase ('S factors') (Tattersall, 1972). The onset of viral DNA synthesis was found to coincide with the burst of cellular DNA replication (Fig. 2b). Consistently, the first viral double-stranded DNA was detected in early S phase (Wolter et al., 1980). In contrast, other authors reported that viral DNA synthesis was delayed until the end of the S phase (Parris & Bates, 1976). Taken together, these data suggest that cellular S factor(s) might be available throughout the S phase and that the relative timing of cellular and viral DNA synthesis may vary depending on the system and cell synchronization method used.

Viral DNA synthesis was accompanied by an inhibition of cellular DNA replication detectable as soon as 10 h after infection (Fig. 2b). Viral DNA synthesis might compete with cellular DNA replication since it appears to make use, in particular, of the cellular DNA polymerase α (see above) and it accounts for as much as 75% of thymidine incorporation during a normal S phase (Fig. 2b; Parris & Bates, 1976). The decrease in RNA and protein syntheses (Parris & Bates, 1976) and the degeneration of nuclear elements (Richards et al., 1977) which occur at the time of viral DNA synthesis and virion assembly, respectively, might also participate in the reduction of cellular DNA replication. The inhibition of cellular DNA synthesis was compensated by concomitant viral DNA replication. Therefore, MVM infection caused only a slight decrease of the rate of overall thymidine incorporation, which was restricted to early S (Fig. 1b). Similarly, cells inoculated with the bovine (Parris & Bates, 1976) and hamster osteolytic (Hampton, 1970) parvoviruses proceed through S phase without a significant reduction in the rate of total DNA synthesis.

Taken together, the data presented and discussed point to the intimate relationship between DNA replication of autonomous parvoviruses and their host cells with regard to their nuclear location, cell cycle dependency and enzymology. In this respect, parvoviruses appear to provide an attractive model for studying the molecular mechanism of DNA replication in eukaryotic cells.

We wish to thank Professor M. Errera for his support and Drs J. Tal and J. J. Cornelis for helpful discussions. This work was supported by an agreement between the Belgian Government and the Université libre de Bruxelles 'Actions de Recherche Concertées', the European Communities [contract BIO-359-B(G) and ENV/335/B], the Ministère de la Santé Publique (contract G/826/1981), the Fonds National de la Recherche Médicale (contract 3.451.4.80) and partly by the 'Programma finalizzato Controllo delle Malattie da Infezione'. J. Rommelaere is Chercheur Qualifié du Fonds National Belge de la Recherche Scientifique.

REFERENCES


1998


(Received 7 March 1983)