Cell Division Does Not Affect Sendai Virus Genome Replication in Persistently Infected BHK Cells

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(Accepted 9 July 1984)

SUMMARY

The extent of Sendai virus genome replication in persistently infected BHK cells actively growing or at confluence was followed by estimation of the [3H]uridine incorporated into intracellular nucleocapsid RNA. First, we showed that, in the presence of actinomycin D, actively growing persistently infected cells were taking up threefold more [3H]uridine than resting cells. This higher uptake exhibited by growing cells was observed neither in persistently infected cells in the absence of actinomycin D, nor in acutely infected cells in the presence of actinomycin D. Assuming that the cellular pool of unlabelled uridine stays constant, we used a correction factor for this difference in [3H]uridine uptake and estimated [3H]uridine incorporation in nucleocapsid RNA, normalizing the data either to the amount of cell or of viral template. Results showed that the viral genome replication, expressed either way, was not significantly influenced by cell growth conditions.

Paramyxoviruses and rhabdoviruses are well known to be capable of establishing persistent infections in their host cells in culture (Walker, 1968). While virus infections at high multiplicities develop a cytopathic effect leading to cell death, in persistent infections virus replication must be restricted in such a way that the cellular machinery is not disturbed by the infection. Viral factors, such as homologous defective interfering (DI) particles or mutant viruses, are known to exert such a restriction (in an as yet unknown way) during the establishment and probably also later during maintenance of viral persistence (Holland et al., 1980; Youngner & Preble, 1980). The question remains whether the host cell can participate in modulating the extent of viral replication. For instance, it is known that, upon acute infection of lymphocytes with measles virus, a member of the Paramyxoviridae, the stimulation of cells with phytohaemagglutinin (PHA) greatly enhances the extent of viral production by 10000-fold (Joseph et al., 1975). Similarly, the production of virus particles by a culture, called CAR₆, persistently infected by the rhabdovirus, vesicular stomatitis virus (VSV) was shown to depend on the growth conditions of the cells (Holland et al., 1978). Virus production ceased when CAR₆ cells were maintained in conditions minimal for cell division, i.e. no trypsinization but replacing medium to maintain cell viability and remove dead cells. It resumed, however, when the cells were stimulated to grow. These two examples suggested that virus replication was restricted in resting cells and, therefore, that the cell growth conditions could modulate the extent of viral replication. We decided to test this hypothesis using a BHK cell line (BHK-Pi) persistently infected with Sendai virus, another member of the Paramyxoviridae family, 60 weeks after establishment and measured the extent of viral genome replication in different cell growth conditions.

BHK-Pi was established by co-infecting BHK cells with a high multiplicity of standard (St) Sendai virus mixed with DI particles (Roux & Holland, 1979). In the persistently infected culture, close to 100% of the cells are infected at any time and, as in normal acute infection, all the viral proteins are synthesized in the same relative molar ratios (Roux & Waldvogel, 1981, 1982). Viral genome replication takes place continuously with a constant excess of DI over St.
Fig. 1. Uptake of [3H]uridine by BHK-Pi cells in the presence of AMD. Samples of (b) confluent (A259/PD > 4) or (a) actively growing (A259/PD < 2) BHK-Pi cells were labelled with 10 μCi/ml [3H]uridine in the presence of 2 μg/ml AMD for various periods of time. At the end of the labelling periods, the medium was discarded, the cells were washed twice with cold phosphate buffer and collected in 1 ml 10% TCA. After 30 min on ice, samples were centrifuged at 12000 r.p.m. for 15 min and the supernatants were separated and used to estimate the TCA-soluble c.p.m. The pellets were washed once with 10% TCA, once with ether, resuspended in 0.8 M-NaOH and incubated overnight at 37 °C. Aliquots of the resuspended pellets were used to estimate (i) the TCA-insoluble c.p.m. by direct liquid scintillation counting and (ii) the protein concentration of the cell material (Lowry et al., 1951). Every time point was analysed in duplicate and the mean of the two values is shown: •—•, total; ○—○, TCA-soluble; ●—●, TCA-insoluble.

Fig. 2. Uptake of [3H]uridine by BHK-Pi cells in the absence of AMD. As in the legend to Fig. 1, except that AMD was omitted.

Fig. 3. Uptake of [3H]uridine by St virus-infected BHK cells in the presence of AMD. As in the legend to Fig. 1, except that BHK-21 cells were acutely infected with St Sendai virus and labelled 18 to 24 h post-infection in the presence of AMD.

For the estimation of [3H]uridine incorporation into NC-RNA to be valid, one has first to ensure that the specific activity of the 3H-labelled NC-RNA does not vary with the cell growth
Table 1. Total uptake of $[^{3}H]$uridine in the presence of AMD*

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-St</td>
<td>2.55</td>
<td>3.6</td>
<td>6.85</td>
</tr>
<tr>
<td>BHK-Pi</td>
<td>2.04</td>
<td>1.99</td>
<td>5.75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratio: growing/confluent</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.32)</td>
<td>(2.70)</td>
<td>(2.39)</td>
<td>(1.25)</td>
</tr>
</tbody>
</table>

* Standard virus (BHK-St) and persistently (BHK-Pi) infected BHK cells, actively growing or at confluence (for definition, see text) were labelled with $[^{3}H]$uridine and the total uptake by the cells was estimated after 6 h as described in the legend to Fig. 1. Results obtained in three separate experiments are shown.

† m (mean of ratios from three experiments): BHK-Pi, 3.53; BHK-St, 1.34.

conditions. As the specific activity of the $^{3}$H-RNA depends on the specific activity of the $[^{3}H]$uridine pool, and as this latter depends on the cellular uptake of $[^{3}H]$uridine, it became essential to follow the uptake of $[^{3}H]$uridine in actively growing or confluent cells during the labelling period used in our experiments. Fig. 1 shows that the uptake of $[^{3}H]$uridine by BHK-Pi estimated in the presence of AMD varied with the cell growth conditions. Relative to the cell mass, the amount of $[^{3}H]$uridine incorporated in the growing cells, found mainly as TCA-soluble uridine because of the presence of the AMD, exceeded that found in confluent cells. Table 1 shows that the values of $[^{3}H]$uridine uptake by the growing cells repeatedly exceeded those measured in resting cells by a factor of 3 to 5. As shown in Fig. 2, this was not observed when growing BHK-Pi cells were labelled in the absence of AMD (here most of the radioactivity was found in TCA-insoluble counts since ribosomal RNA synthesis was no longer inhibited). Moreover, it was not observed in BHK cells acutely infected with St virus, even in the presence of AMD (Fig. 3 and Table 1). Therefore, the particular sensitivity to AMD in $[^{3}H]$uridine uptake only applied to BHK-Pi. Whether this peculiarity was triggered by persistent infection remains an open question. It is however important for the correct interpretation of our data. If one assumes that the pool of unlabelled intracellular uridine stays constant under different cell growth conditions then the results imply that the specific activity of the uridine pool can change and therefore that the efficiency of viral genome replication has to be corrected for the difference in $[^{3}H]$uridine uptake. Therefore, a dividing factor of 3.53, calculated as the mean of the differences in $[^{3}H]$uridine uptake between growing and confluent cells observed in the three experiments presented in Table 1 [(2.7 + 2.39 + 5.5)/3], was applied to all the values of $[^{3}H]$uridine incorporated in growing cells.

Incorporation of $[^{3}H]$uridine was followed over 16 consecutive days by daily 6 h labelling periods. The cells were passaged on days 1, 6, 9, 11 and 14. Fig. 4(a) presents a daily follow-up of the cell densities in the Petri dishes: at days following cell passages (arrows), total $A_{259}/PD$ was low, increasing on the following day(s) until the next passage. Thus, the limit of 2.5 $A_{259}/PD$ chosen to discriminate between growing and confluent cells (see above), distinguished two groups of cells. Group I comprised growing cells which consisted mainly of cells analysed the day following a passage, at days 2, 7, 8, 10, 12 and 15 (six values). Group II comprised confluent cells analysed at days 1, 3, 4, 5, 6, 9, 11, 13, 14 and 16 (10 values). This separation was not perfect, since cells at day 3, classified as confluent, were clearly capable of further growth (increment of $A_{259}/PD$ from 3 to 6, Fig. 4a). It was nevertheless kept, since 2.5 $A_{259}/PD$ conveniently separated all the cell samples analysed before and after the passages. Moreover, inclusion of the day 3 cell sample in group I would not have changed our conclusion.

When the incorporation of $[^{3}H]$uridine into viral genome RNA (NC-RNA) was measured, the values obtained showed some variation (Fig. 4b), with mean values (m) of $[^{3}H]$uridine incorporation of 0.95 and 0.92 $[^{3}H}$ c.p.m. $\times 10^{-3}/A_{259}$ for growing and resting cells respectively. Application of a Mann–Whitney test (Meddis, 1975) to the ranked values of the two groups failed to show a statistical difference at the 1% level of confidence using a one-tail test ($P \gg 0.5$).
Fig. 4. Daily estimation of cell growth phase and of viral genome replication in BHK-Pi. Every day for 16 days, samples of BHK-Pi were labelled for 6 h with 10 μCi/ml \(^3\text{H}\)uridine in the presence of 2 μg/ml AMD. The cells were harvested, the intracellular nucleocapsids were purified on CsCl density gradients, and the total amount of RNA in the pellets of the CsCl gradients was measured. The incorporation of \(^3\text{H}\)uridine into NC-RNA was estimated as previously described (Roux & Waldvogel, 1981). (a) Amount of RNA present in the CsCl pellets estimated as A\(_{259}\). (b) Incorporation of \(^3\text{H}\)uridine in NC-RNA per A\(_{259}\) during 6 h labelling periods of (○) confluent or (●) actively growing BHK-Pi cells. Arrows indicate days at which cells were passaged.

Fig. 5. Daily estimation of viral genome template and of genome replication per template available. Along with the estimation of \(^3\text{H}\)uridine incorporation in NC-RNA presented in Fig. 4, aliquots of the purified nucleocapsids were pelleted (300000 g, 1 h, 4 °C); nucleocapsids were resuspended in gel electrophoresis sample buffer (Laemmli, 1970) and electrophoresed on a discontinuous 12.5% polyacrylamide gel. The Coomassie Brilliant Blue-stained gel was scanned in a Joyce-Loebl densitometer to estimate the amount of NP in each NC sample (Roux & Waldvogel, 1981). (a) Amount of nucleocapsid in the sample normalized to the amount of cell material (NCU). (b) Incorporation of \(^3\text{H}\)uridine as measured in Fig. 4(b) normalized to the amount of nucleocapsids in (○) confluent or (●) actively growing BHK-Pi. Arrows indicate days at which cells were passaged.

Therefore, no apparent influence of cell growth conditions on viral genome replication was observed in these experiments. It is noteworthy that without correction for the excess \(^3\text{H}\)uridine uptake in growing cells, the two groups of values would have been significantly different (m\(_{\text{growing}}\) = 3.36, m\(_{\text{confluent}}\) = 0.92 \(^3\text{H}\) c.p.m. × 10\(^{-3}\)/A\(_{259}\), P = 0.01; data not shown).

As the extent of viral genome replication depends on the availability of potential template, and as the amount of template may vary in the cells due to continuous cell division, it was of interest also to measure the amount of intracellular nucleocapsids and to relate the amount of viral replication to the template available. Here, the amount of viral nucleocapsid was determined as follows. An aliquot of nucleocapsid purified on a CsCl gradient was concentrated by centrifugation and electrophoresed on an SDS–polyacrylamide gel. The Coomassie Brilliant Blue-stained gel was scanned to evaluate the amount of NC protein (NP) present in the nucleocapsid sample. The amount of NP, directly proportional to the amount of viral genome
RNA (St or DI RNA), was taken as the amount of nucleocapsid (NC). It was further divided by the total A$_{259}$ measured in the CsCl gradient pellet from which the NC sample originated, and is expressed as nucleocapsid units (NCU). NCU represented a normalization to the number of cells so that different samples could be directly compared (see also Roux & Waldvogel, 1981). Indeed, the amount of NC varied during the time of analysis: it slowly increased during the first 10 days of analysis, peaked at day 11, and then decreased for the next 5 days (Fig. 5a). When the efficiency of viral genome replication, as measured in Fig. 4(b) ($[^{3}H]$ c.p.m. $\times 10^{-3}$/A$_{259}$), was normalized to the amount of nucleocapsid, and expressed as [$^{3}$H]uridine incorporated into NC-RNA per NCU (Fig. 5b), again no significant difference between the two groups of values could be found using a Mann–Whitney test ($m_{\text{growing}} = 0.44$, $m_{\text{confluent}} = 0.46$ $^{3}$H c.p.m. $\times 10^{-3}$/NCU, $P > 0.5$). In this case also, the uncorrected values showed a significant difference between the two groups of cells ($m_{\text{growing}} = 1.56$, $m_{\text{confluent}} = 0.46$ $^{3}$H c.p.m. $\times 10^{-3}$/NCU, $P < 0.001$; data not shown).

In conclusion, when the difference in [$^{3}$H]uridine uptake observed between growing and resting cells was taken into account, viral genome replication was not found to be influenced by the cell growth conditions. This was the case whether genome replication was expressed as [$^{3}$H]uridine incorporated into NC-RNA per amount of cells, or per amount of template available.

Our results do not necessarily contradict the data of Joseph et al. (1975) or Holland et al. (1978), since we have looked at the level of viral genome replication while these authors measured the production of viral particles. Taken together, these results might suggest that cell growth conditions are likely to limit viral production by blocking a step in viral assembly rather than by limiting viral genome replication. In view of reports showing that the cell surface expression of viral antigens is cell growth-dependent (Ehrnst, 1979; Roux & Waldvogel, 1983), we would suggest that, in resting cells, the insertion of the viral membrane antigens in the cell plasma membrane, a step which is a prerequisite for the budding of viral particles, may be restricted in such a way that maturation of viral particles is prevented. One could also argue that a study following viral genome replication in persistently infected cells mainly reflects estimation of DI genome replication (Roux & Holland, 1980). Therefore, a possible inhibition of standard virus genome replication in resting cells could have been missed. Such a selective inhibition could in turn affect viral particle production by limiting transcription and viral protein synthesis. This is unlikely, however, since DI genome replication depends on viral protein synthesis as well. Therefore, inhibition of protein synthesis, due to lack of template available for transcription, would have affected DI genome replication in our experiments.

This work was supported by grant No. 3.332-0.82 from the Swiss National Research Foundation. The authors wish to thank Benjamin Blumberg for critical reading of the manuscript.

REFERENCES


Short communication


(Received 23 March 1984)