RNA Synthesis in BHK 21 Cells Persistently Infected with Vesicular Stomatitis Virus and Rabies Virus

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SUMMARY

Virus-induced RNA synthesis was studied in BHK 21 cells persistently infected with vesicular stomatitis virus (VSV) and rabies virus by labelling RNA synthesized in the presence of actinomycin D. During persistent infection the species of messenger RNA synthesized were similar in size and relative proportions to those seen during acute infection, but there were some minor differences. Full-sized B virion RNA was generally not detected during persistent infection, and new species (probably DI virion RNA) appeared.

INTRODUCTION

The ability of enveloped negative strand RNA viruses to establish persistent infection in vitro is well documented (for review, see Walker, 1964). The experiments of Chambers (1957) with arboviruses, and later of Wilcox (1959) and Henle (1963) with paramyxoviruses, provided preliminary experimental evidence that ‘incomplete’ viruses may be involved in persistently infected carrier cells. By the mid 1960s there was an extensive literature on persistent animal virus infection of cultured cells (see reviews by Ginsberg, 1958 and Walker, 1964), and autointerference and interferon were among the factors implicated. Later, in the first clear review and definition of DI particles, Huang & Baltimore (1970) proposed that defective viruses may generally be involved in acute and persistent virus disease processes. Using vesicular stomatitis virus (VSV) and rabies virus as model systems, Holland & Villarreal (1974) and later Kawai, Matsumato & Tanabe (1975) demonstrated a direct involvement of defective autointerfering viruses in in vitro persistent infection. In vivo experiments by Doyle & Holland (1973) showed that VSV defective particles can suppress or alter virus pathogenesis and it has been found that defective particles can replicate efficiently in baby mouse brains (Holland & Villarreal, 1975). At present little is known of the molecular mechanisms of persistence. Villarreal & Holland (1974) reported very low levels of transcribing virus RNP complexes in cells persistently infected with VSV or rabies (as compared to levels in acutely infected cells). Huang & Manders (1972) and Perrault & Holland (1972a, b) suggested that VSV defective particles interfere with replication of the B virion template RNA and not with transcription of virus mRNA. The precise mechanisms of DI autointerference remain to be established (for a review see Huang, 1973) but they are probably directly relevant to mechanisms of persistence (see previous paper; Palma & Huang, 1974; Holland, Villarreal & Breindl, 1976). The present study demonstrates greatly
reduced virus mRNA transcription in persistently-infected cells compared with acute infection, and shows that full-size virion RNA replication is even further reduced relative to mRNA transcription.

METHODS

Cells and viruses. BHK 21 cells were employed for virus production and assays. Cells were grown in Eagle's minimum essential medium (MEM) containing 7% calf serum. VSV was the Indiana serotype originally obtained from Dr John Mudd (Mudd & Summers, 1970). Rabies virus was the HEP-Flury strain provided originally by Dr Hilary Koprowski of the Wistar Institute and was propagated as described by Sokol et al. (1968). BHK 21 cells persistently infected with VSV (BHK 21 VSV_{inf} CAR4) were as previously described by Holland & Villarreal (1974). BHK 21 cells persistently infected with rabies were obtained in the same manner (by double infection with B virions and T particles at high multiplicity, and passage of surviving cells). Acute infection with rabies virus was achieved by adding to semiconfluent monolayers of BHK 21 cells a small volume of rabies inoculum (m.o.i. = 50) at room temperature for 1 h. Cells were incubated at 33 °C in MEM, split one to three and passaged when confluent (2 days after infection). Infected cells were labelled on days 3 and 5 post infection (p.i.). About 80% of the cells had detached from the glass by day 7.

Labelling RNA with 3H-uridine. Duration of labelling and time after infection at which label was added is indicated in the text. Thirty min prior to adding 3H-uridine (5 min for rabies) actinomycin D (Calbiochem) was added to a final concentration of 5 µg/ml. 3H-uridine (Schwartz/Mann) was added to a final concentration of 25 µCi/ml (1 ml per 2 x 10^6 cells) at indicated times. After incubation, the medium was poured off and the cells rinsed with cold saline. RNA was extracted with 1:1 phenol:chloroform with an equal vol. of 6 M-urea-99% formamide polyacrylamide slab gels. Denaturing RNA slab gels were run as described by Villarreal, Breindl & Holland (1976). Briefly, 2.8% (w/v) polyacrylamide slab gels containing 6 M-urea in 99% formamide solvent were employed. The gels were polymerized directly on to the back of a sheet of Whatman no. 1 filter paper to increase mechanical strength and allow handling for fluorography.

Quantitative fluorography. Preparation of gels for fluorography was done according to the method of Bonner & Laskey (1974) with modifications as described by Villarreal et al. (1976). Briefly, after formalin fixation, gels were exchanged several times with DMSO and then with 11% PPO in DMSO. PPO was precipitated in the gel with water and DMSO exchanged out with 50% ethanol/water, after which the gels were dried and exposed to Kodak RP 'Royal' X-Omat film. The film had been pre-exposed according to the methods of Laskey & Mills (1975) to allow for quantitative tritium detection. The films were exposed for the appropriate times to assure that overexposure did not occur in any band, and the developed film was scanned with a Gilford linear transport device on a Gilford model 2000 spectrophotometer at 550 nm.

RESULTS

Patterns of virus RNA synthesis in BHK 21 cells acutely and persistently infected with VSV

Rose & Knipe (1975) were the first to separate VSV-induced RNA species under denaturing conditions. Knipe, Rose & Lodish (1975) using in vitro translational systems identified the gene products of four out of the five mRNAs. Fig. 1 demonstrates the profile of intracellular
Virus RNA in persistence

Fig. 1. Denaturing polyacrylamide gel electrophoretic analysis of VSV RNA species induced during acute infection of BHK 21 cells at high multiplicity with Indiana serotype. At 2.5 h p.i. 5 μg/ml actinomycin D was added and RNA was labelled with "H-uridine from 3.0 h to 4.5 h. RNA was extracted and analysed on 6 M-urea, 99% formamide polyacrylamide slab gels as described in Methods. 40000 ct/min were loaded in each well with parallel wells containing marker RNA (ribosomal RNA and B virion and DI genome RNA). After electrophoretic separation of RNA species, X-ray film fluorograms were developed and optical density of the film image was determined in a Gilford spectrophotometer equipped with a linear transport device at a wavelength of 550 nm and a scan rate of 0.5 cm/min.

Virus-induced RNA synthesized during acute VSV B virion infection in the presence of actinomycin D as visualized on denaturing formamide-acrylamide slab gels. Five resolvable bands are seen with apparent mol. wt. of 3.5 × 10^6, 1.2 × 10^6, 0.63 × 10^6, 0.50 × 10^6 and 0.29 × 10^6 corresponding to peaks 1 to 5 respectively. The relative amounts of these RNAs have been reported (Villarreal et al. 1976). Peak 1 co-migrates with VSV B virion RNA and peaks 2 to 5 represent virus specific mRNAs (Knipe et al. 1975). No actinomycin D resistant large RNAs became labelled in uninfected control cells under our labelling conditions, only 4S RNA.

BHK 21 cells persistently infected with VSV as described by Holland & Villarreal (1974) and passaged in vitro for over 2 years (see previous paper, Holland et al. 1976) were examined for their ability to synthesize RNA in the presence of actinomycin D. The kinetics of "H-uridine incorporation after a 5 min actinomycin D pre-treatment show (Fig. 2) a biphasic profile with the first plateau occurring between 20 to 30 min after label addition, and the
second after 2 h. The time and amount of label seen for the first plateau corresponds to the residual 4S actinomycin D-resistant RNA labelling seen in uninfected BHK 21 cells.

Virus RNA species synthesized in the carrier cells are very similar to those observed during acute VSV infection, but a few differences are clearly observed (Fig. 3a). B virion-sized RNA is generally not detectably synthesized and there is an over-abundance of peak 3 and possibly peak 2 relative to acute infection. When actinomycin D is not used, ribosomal RNAs are predominantly labelled and no distinct virus peaks are seen (Fig. 3b). During one labelling period immediately preceding a rare episode of cytopathology in the carrier cells we observed very high levels of virion RNA synthesis in these carrier cells but this fortuitous labelling prior to rare c.p.e. has not been repeated as yet.

Because these carrier cells were originally established using the temperature sensitive mutant (ts G31) of Pringle (1970), it was of interest to determine if virus RNA synthesis was ts after 1 to 2 years of persistence. Table 1 shows that actinomycin D-resistant RNA synthesis was about 0.8% of total cellular RNA synthesis at 37 °C and 1.9% at 33 °C, but much of this difference was due to higher levels of host RNA synthesis at 37 °C. The absolute level of increase in resistant synthesis at 33 °C was only about 30%. This indicates that temperature sensitivity is not a major factor controlling levels of B virion directed RNA synthesis. Levels of actinomycin D-resistant RNA synthesis varied from preparation to preparation of carrier cells, with lower values of 0.7% and upper values of about 3%. The above studies were done with carrier cells at between 1 and 2 years of persistent infection but recently (after 2 years of persistence) the carrier cells have stabilized (see previous paper). They now exhibit very little c.p.e. or virus release, and have been exhibiting only about 1/5 to 1/10 the above levels of actinomycin D-resistant RNA synthesis (Table 1). Virus-induced RNA synthesis is now generally only barely detectable in gels (not shown).
Fig. 3. Denaturing polyacrylamide gel analysis of virus induced RNA synthesized in BHK 21 cells persistently infected for 2 years with VSV. Conditions were as for Fig. 1 except that cells were labelled (a) for 4 h at 37 °C after 30 min pre-incubation with 5 μg/ml actinomycin D or (b) for 4 h at 37 °C in the absence of actinomycin D.

Table 1. Levels of RNA synthesis in cells persistently infected with VSV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>3H, ct/min incorporated</th>
<th>Actinomycin D-resistant synthesis* (％)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier cells between 1 and 2 years after establishment of resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No drugs</td>
<td>37</td>
<td>697000</td>
<td>—</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>37</td>
<td>5760</td>
<td>0.82†</td>
</tr>
<tr>
<td>No drugs</td>
<td>33</td>
<td>464200</td>
<td>—</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>33</td>
<td>8750</td>
<td>1.9</td>
</tr>
<tr>
<td>Carrier cells between 2 and 2·5 years of passage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No drugs</td>
<td>37</td>
<td>720647</td>
<td>—</td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>37</td>
<td>1900</td>
<td>0.26</td>
</tr>
</tbody>
</table>

* 2 × 10⁶ persistently infected cells were used for each data point. Actinomycin D was added 6 min prior to addition of 3H-uridine (5 μCi/ml). Incubations were for 1 h at indicated temperatures. Incorporation was stopped by addition of SDS to 1 % concentration and assayed as ct/min precipitated by 10 % trichloroacetic acid (TCA).

† This value fluctuates somewhat from one preparation to another and values have been observed as high as 3 %.
Table 2. Nucleic acid hybridization studies on RNA from cells persistently infected with VSV

<table>
<thead>
<tr>
<th>Source of ³H-labelled RNA for annealing*</th>
<th>Source of unlabelled RNA</th>
<th>Annealing time (h)</th>
<th>Ribonuclease resistance (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D-resistant RNA from carrier cells</td>
<td>VSV B virion RNA, 3 μg/ml</td>
<td>24</td>
<td>75</td>
</tr>
<tr>
<td>None</td>
<td>VSV B virion RNA, 3 μg/ml</td>
<td>48</td>
<td>73</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>—‡</td>
<td>10</td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Source of ³H-labelled RNA for annealing*</th>
<th>Source of unlabelled RNA</th>
<th>Annealing time (h)</th>
<th>Ribonuclease resistance (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D-resistant RNA from carrier cells</td>
<td>VSV B virion RNA, 3 μg/ml</td>
<td>24</td>
<td>67</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>Actinomycin D-resistant RNA from acute B infection</td>
<td>VSV B virion RNA, 3 μg/ml</td>
<td>24</td>
<td>90 ‡</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>24</td>
<td>5 ‡</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>—‡</td>
<td>1</td>
</tr>
</tbody>
</table>

* Annealing reactions were carried out in 0.48 M-sodium phosphate buffer, pH 6.8, at 70 °C for the times indicated (1000, cpm ³H-RNA).
† Ribonuclease resistance was determined by the percentage bound to DEAE cellulose filters after digestion at 37 °C for 45 min with 20 μg/ml pancreatic ribonuclease and 10 units/ml T1 ribonuclease (Blatti et al. 1970).
‡ Not annealed. Heated to 100 °C and cooled quickly.

Annealing studies showed (Table 2) that the actinomycin D-resistant RNA synthesized by VSV carrier cells is able to hybridize to 75 % with VSV B virion RNA, and it exhibits self-annealing to a level of 35 %. In other experiments this actinomycin D-resistant RNA self-annealed to as much as 42 % and annealed to B virion RNA up to 67 %. However, in many experiments we found that the actinomycin D-resistant RNA was predominantly + or messenger strand in polarity, with variable but significant amounts of virus minus strand (defective ?) RNA also present in these carrier cells.

RNA synthesis during acute and persistent rabies virus infections

Koprowski (1974) has reported that rabies virus production is inhibited by enucleation with cytochalasin B while VSV is not inhibited. Reports by Sokol & Clark (1973) and Villarreal & Holland (1974) showed that purified rabies virions lack transcriptase but did induce primary transcription in infected cells in the presence of actinomycin D, suggesting a difference between VSV and rabies RNA synthesis. Table 3 shows the results of ³H-uridine pulse labelling of RNA in the presence of actinomycin D on various days after acute rabies infection. Actinomycin D-resistant RNA synthesis was 2-9 % of total cellular RNA synthesis on day 1 following infection with a high multiplicity of rabies virus, and reached a maximum of 13 % on day 6. During a 7-day period, total RNA synthesis fell steadily to 20 % of that seen on day 1 as the % virus directed RNA synthesis increased. Surprisingly, the kinetics of this virus RNA synthesis (Fig. 4) indicate that incorporation continues only for 30 to 40 min after actinomycin D addition. These kinetics are observed both early in infection (Fig. 4a) and late in infection (Fig. 4b). Pre-incubation of the infected cells with actinomycin D for 30 min and longer always reduced actinomycin D-resistant RNA synthesis to uninfected background levels (Fig. 4c). These results suggest basic differences between the transcriptase requirements of VSV and rabies.

Gel analysis of rabies virus RNA species during acute infection is shown in Fig. 5. During extraction of rabies virus-induced labelled RNA, as much as 90 % of the label was often lost during phenol: chloroform: SDS extraction, and much of the recovered RNA would
Table 3. Levels of RNA synthesis during acute rabies infection

<table>
<thead>
<tr>
<th>Day p.i.</th>
<th>Total ct/min incorporated</th>
<th>Actinomycin D-resistant synthesis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>698,000</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>464,600</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>256,700</td>
<td>4.5</td>
</tr>
<tr>
<td>5</td>
<td>206,890</td>
<td>12.3</td>
</tr>
<tr>
<td>6</td>
<td>153,450</td>
<td>13.2</td>
</tr>
<tr>
<td>7</td>
<td>140,000</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Monolayers of $2 \times 10^6$ cells were used for each data point. Cells were labelled with $^3$H-uridine (5 μCi/ml) for 90 min at 33 °C. Actinomycin D was used at 5 μg/ml with a 5 min pre-incubation. Incorporation was stopped by adding SDS to 1% final concentration and assayed as ct/min precipitable by TCA. Although total actinomycin D-resistant synthesis declined steadily (to 20% of the day 1 level on day 7), virus-directed synthesis remained at much the same level throughout. Since many cells were dying and detaching (about 80% by day 7), virus-directed synthesis per cell was actually increasing.

Fig. 4. Kinetics of actinomycin D-resistant RNA synthesis in cells acutely infected with rabies virus at m.o.i. = 50. (a) Kinetics of $^3$H-uridine incorporation into TCA-precipitable material 2 days p.i. $^3$H-uridine was added 5 min after 5 μg/ml actinomycin D was added, and conditions were as for Fig. 2. (b) Kinetics at 7 days p.i. (c) Decreased rabies virus directed RNA synthesis with increasing times of actinomycin D pre-treatment. At two days after rabies virus infection, 5 μg/ml actinomycin D was added and at the indicated times 5 μCi/ml $^3$H-uridine was added for a 1 h labelling period.

not enter the 6 M-urea formamide-polyacrylamide gel. These recovery and gel migration problems were not seen with VSV-induced RNA in either acute or persistent infection. With some preparations of rabies-induced RNA we found that extraction was more efficient (20 to 30%) and the label did enter the gel. One such preparation is shown in Fig. 5. It exhibits a rather complicated pattern of virus RNA with the most abundant band having an apparent mol. wt. of $0.62 \times 10^6$. In all, at least 7 species of rabies virus-induced RNA were observed during acute infection. The largest species, apparently virion RNA, migrates slightly faster than VSV B virion RNA marker. The smallest species observed had an apparent mol. wt. of $0.31 \times 10^6$. Although generally reproducible, these profiles vary somewhat with time of labelling and method of extraction. Probably some of the variable large RNA peaks represent DI RNA (see previous paper). Crick & Brown (1974) first reported T particles of rabies virus. We have repeated their observation and have even obtained
Fig. 5. Denaturing polyacrylamide gel electrophoretic analysis of virus RNA synthesized in BHK 21 cells on the sixth day after infection with HEP Flury rabies virus. Conditions were as for Fig. 1, except that $2 \times 10^6$ infected cells were labelled at $33^\circ C$ beginning five min after addition of $5 \mu g/ml$ actinomycin D.

Table 4. Levels of RNA synthesis in cells persistently infected with rabies virus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature (°C)</th>
<th>ct/min incorporated</th>
<th>Actinomycin-resistant incorporation* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drugs</td>
<td>33</td>
<td>76000</td>
<td></td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>33</td>
<td>9650</td>
<td>12.5</td>
</tr>
<tr>
<td>No drugs</td>
<td>39</td>
<td>145000</td>
<td></td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>39</td>
<td>10110</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* About $1 \times 10^6$ cells were labelled with $^3H$-uridine ($5 \mu Ci/ml$) for 1 h at the temperature indicated. Assays were otherwise as described in Table 3.

DI late in the infection cycle with clonal pools as inoculum (see previous paper). Kawai et al. (1975) observed cyclic production of T particles in cells persistently infected with rabies. We established a persistently infected BHK 21 rabies carrier culture nearly 2 years ago (Holland & Villarreal, 1975) and the cells have shed B virions and DI particles since then.

The kinetics of RNA synthesis in BHK 21 cells persistently infected with rabies virus was
virtually identical to that seen during acute infection (not shown). The level of actinomycin D-resistant rabies RNA synthesis in these persistently infected cells (Table 4) was 12.5% at 33 °C and 7.0% at 39 °C. The difference again was mainly due to increased host synthesis at 39 °C since the absolute level of virus induced synthesis was greater at 39 °C. Gel analysis of this RNA (Fig. 6) indicates five resolvable species with mol. wt. of $1.65 \times 10^6$, $0.98 \times 10^6$, $0.59 \times 10^6$, $0.51 \times 10^6$, and $0.45 \times 10^6$. It was often observed, as in acute infection, that a considerable amount of labelled RNA did not penetrate the gel, and the nature of this apparently large RNA has yet to be determined. As in acute infection difficulty was encountered during RNA extraction from persistently infected cells using phenol:chloroform and SDS. Often the majority of the label was not recovered from the aqueous phase even after heating the extraction mixture to 70 °C. To determine the total level of rabies virus-directed RNA synthesis we precipitated with 20% TCA.

DISCUSSION

Transcription by the virion associated polymerase of VSV is known to be totally resistant to actinomycin D both in vivo and in vitro (Baltimore, Huang & Stampfer, 1970). The use of actinomycin D to inhibit host RNA synthesis without affecting virus induced RNA synthesis is therefore a useful method for studying virus-directed RNA synthesis whenever host cell synthesis is not shut off by the virus. The RNA species thus labelled during acute VSV
infection were identical, within experimental error, to those described by Rose & Knipe (1975) and by Grubmann et al. (1975). The same is true for the RNA species seen in persistently infected cells except for the additional band presumed to be DI RNA.

The above observations and the previous paper are consistent with a direct involvement of defective virus particles in persistent VSV infection. The work of Perrault & Holland (1972b), Huang & Manders (1972), Roy & Bishop (1972), Emerson & Wagner (1972) and Reichmann et al. (1974) suggest that defective particles do not inhibit VSV virion associated transcriptase but do inhibit B virion replication, which seems to be happening in the carrier cells also.

Studies of Holland et al. (1976) and Holland & Villarreal (previous paper) show that during acute rabies infection an array of defective particles is generated that change in distribution and size as infection proceeds. This changing array of defective particles probably accounts for the rather complex profile of actinomycin D-resistant RNAs synthesized during acute rabies infection. A study using cloned rabies 'B' virions that do not generate defectives in one growth cycle would be more definitive but we have as yet been unable to obtain such a rabies stock, perhaps because of the great length of the rabies growth cycle.

We have at present no explanation for the kinetics of actinomycin D-resistant RNA synthesis during rabies infection. The shut-off of RNA synthesis in 30 to 40 min is surprisingly rapid and may imply the existence of an actinomycin D-sensitive step in rabies transcription (i.e. initiation). This might also be related to the inability to detect a rabies virion associated transcriptase (Sokol & Clark, 1973; Villarreal & Holland, 1974). Koprowski (1974) in fact reported that rabies virus cannot replicate in enucleated cells, suggesting a greater host involvement for rabies than for VSV.

We are also unable as yet to explain the difficulty encountered during extraction of rabies induced RNA or the observation that much of the RNA does not enter these gels. Aggregation is unlikely in view of the extremely denaturing conditions used (99 % formamide, 6 M-urea, heating to 70 °C). It could be that this RNA is of a very large mol. wt. The existence of extremely long filamentous forms of rabies RNP in the cytoplasm of infected cells by Matsumoto (1963) and Hummeler, Koprowski & Wiktor (1967) might relate to this phenomenon. Alternatively, the apparently large RNA may be due to some crosslinkage or other covalent joining of replicating or newly replicated molecules, or to some end-to-end annealing that is anomalously resistant to denaturation under these conditions. We are investigating these alternatives.

Overall, the above results show that VSV and rabies persistent infections involve a reduced level of virus transcription relative to acute infection and strong suppression of full-size virion RNA replication (except at rare times of cytopathic 'crisis'). These results (together with those of the previous paper) strongly implicate DI as regulators of virus RNA synthesis during persistence.

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