Characterization of the Non-permissive Infection of Rabbit Cornea Cells by Vesicular Stomatitis Virus

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SUMMARY

Rabbit cornea cells (RC-60) restrict the reproduction of vesicular stomatitis virus (VSV) (Thacore & Youngner, 1975). In cells infected with VSV alone, an inhibition in the synthesis of VSV genome RNA is observed. A number of parameters which could affect virus RNA synthesis have been examined: virus transcription and post-transcriptional modification, translation, modification of proteins and migration of the G protein to the surface of the cell; they all appear to be normal, although somewhat diminished, in the restricted system. In these cells, therefore, it is the replication of VSV RNA that is directly inhibited, although limited synthesis of both (+) and (−) strand genome length RNA does occur. When the cells are co-infected with rabbit poxvirus (RPV) as a helper virus, however, VSV production is normal. Our studies suggest that RPV plays a role in the maturation as well as in the replication of VSV RNA in RC-60 cells. Certain mutants of RPV have been found to lack the helper function and are unable to convert the RC-60 cells into a permissive host for VSV. These mutants should facilitate the elucidation of the mechanism by which RPV is able to overcome the restriction in these cells.

INTRODUCTION

Vesicular stomatitis virus (VSV), the prototype member of the rhabdovirus group, contains a single-stranded RNA genome of the negative strand sense and five structural proteins (L, G, NS, N and M) which account for virtually the entire coding capacity of the genome (Moyer et al. 1975; Rose & Knipe, 1975; Wagner, 1975). The mechanism of replication of the virus RNA is still not well understood, although it is thought to involve the synthesis of a full length RNA complementary to the genome (Soria et al. 1974) and to require continuous protein synthesis (Marcus et al. 1971; Wertz & Levine, 1973). The importance of a proper host cell environment in this process has been demonstrated by the discovery of several mammalian cell lines which will not support VSV growth. These cell lines include: human lymphoblastoid cells (Raji; Nowakowski et al. 1973); rabbit kidney cells (DRK3; Chen & Crouch, 1978; Crouch, 1978); and rabbit cornea cells (RC-60; Thacore & Youngner, 1973a, b, 1975).

Thacore & Younger (1975) found that the RC-60 cell line was non-permissive for the growth of VSV and they further demonstrated that co-infection of these cells with the orthopoxvirus, vaccinia, rendered the cells permissive for VSV reproduction. Treatment of the co-infected cells with actinomycin D at times early in the infection eliminated the ability of vaccinia to rescue VSV. However, co-infection of the cells in the presence of hydroxyurea,
an inhibitor of DNA synthesis, had no effect on the rescue of VSV. These results indicated that the vaccinia-mediated rescue of VSV requires transcription of the pre-replicative region of the vaccinia genome but does not require virus DNA replication. Thacore & Youngner (1975) also examined RC-60 cells singly-infected with VSV and showed that the level of intracellular 42S RNA was greatly reduced, although some virus messenger RNAs were produced.

Our studies have been directed at the further characterization of both the non-permissive VSV infection of RC-60 cells, and the productive mixed infection, in order to determine whether replication is directly affected or is impaired as a consequence of defects in other aspects of virus macromolecular synthesis. We have shown that a limited replication of the VSV genome does take place in the restricted infection, although hybridization data indicate that even this replication is abnormal. All other parameters examined in the non-permissive infection (transcription, translation and protein modification) appear to be normal, although reduced, so that the block in VSV RNA replication is direct and not a secondary effect of another lesion. In other experiments reported here, we have used the vaccinia-like virus, rabbit poxvirus (RPV), to promote the rescue of VSV in RC-60 cells and have also found mutants of RPV which are unable to serve as a helper virus.

**METHODS**

**Cell cultures and virus.** Rabbit cornea cells (RC-60; obtained from Dr J. S. Youngner, University of Pittsburgh, U.S.A.) were grown in modified Eagle’s medium containing Hanks’ balanced salt solution (Gibco, F-12) supplemented with 10% foetal calf serum and 1% glutamine. Mouse L cell monolayers were grown in modified Eagle’s medium containing Earle’s balanced salt solution (Gibco, F-11) supplemented with 5% foetal calf serum and 1% glutamine. Mouse L cells and baby hamster kidney cells (BHK 21) were grown in suspension as described previously (Banerjee et al. 1974). The procedures for the growth and purification of VSV (Indiana serotype) free of detectable defective interfering particles have been described previously (Banerjee et al. 1974). Rabbit poxvirus (RPV), Utrecht strain, was obtained from the American Type Culture Collection. RPV stocks were propagated and maintained on RC-60 cells. The white pock host range mutants of RPV (RPwhr mutants; Moyer & Rothe, 1980) were propagated and maintained as described by Fenner & Sambrook (1966).

**Preparation of labelled virus RNAs and proteins.** RC-60 cells (2 × 10⁷ cells/plate) were infected in 2.5 ml of supplemented medium containing 14 mm-Hepes, pH 7.5, but lacking foetal calf serum, with VSV at 20 p.f.u./cell in the presence or absence of RPV (5 to 10 p.f.u./cell). The cultures were maintained at 37 °C for 1 h and then diluted to 7.5 ml with medium containing 14 mm-Hepes, pH 7.5, and final concentrations of 2% foetal calf serum and 50 mm-hydroxyurea. For the isolation of mRNA from cultures infected with VSV alone, actinomycin D was added at a final concentration of 5 µg/ml. The infected cultures were labelled with ³H-uridine (50 µCi/ml) beginning at 1.5 h post infection (p.i.) for various times as indicated in the text. In order to label with ³²P-orthophosphate (500 µCi/ml), RC-60 cells were starved for 24 h before infection in phosphate-free minimal essential medium and then infected with virus in phosphate-free medium, as described above. Virus proteins were labelled with ³H-leucine (50 µCi/ml) in leucine-free minimal essential medium. Labelled virus was pelleted from the supernatant fluid at 24 h p.i. and virus was purified by density equilibrium sedimentation on a 7 to 52% (w/w) sucrose gradient as described previously (Moyer & Banerjee, 1975).

**Isolation and purification of virus RNAs.** The infected cells were harvested at 5.5 h p.i. by scraping them into the infection media with a rubber policeman followed by centrifugation.
The cell pellet was washed twice with Earle's balanced salt solution at 4 °C and resuspended at 4 × 10^7 cells/ml in RSB (10 mM-tris-HCl, pH 7.4, 10 mM-NaCl and 0.15 mM-MgCl₂) containing 100 µg/ml heparin and allowed to swell for 10 min at 4 °C. The cells were lysed with 0.5% NP40 and the nuclei removed by centrifugation at 800 g for 3 min. The supernatant was adjusted to 25 mM-EDTA and, in leucine-labelled preparations, 1 mM-phenylmethylsulphonyl fluoride in order to dissociate polysomes and inhibit protease activity, respectively.

For the subsequent isolation of virus nucleocapsids and mRNAs, a cell extract from 2 × 10⁷ cells was layered on to a 15 to 30% (w/v) sucrose gradient in NEBS buffer (50 mM-tris-HCl, pH 7.5, 10 mM-NaCl, 5 mM-EDTA) and sedimented at 19,000 rev/min for 17 h at 4 °C in the SW41 rotor. The fractions of the gradient corresponding to the VSV nucleocapsids and mRNPs were pooled separately. The RNA of each component was extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. The RNA was recovered by centrifugation, then denatured by heating in 90% DMSO at 37 °C for 30 min and, finally, reprecipitated with ethanol.

The polyadenylated mRNAs extracted from the virus mRNPs were purified by two cycles of binding to and elution from an oligo(dT)-cellulose column as described previously (Banerjee et al. 1974). Each RNA preparation was further purified by centrifugation on SDS sucrose gradients as described previously (Moyer & Banerjee, 1975).

**RNA hybridization.** Purified preparations of labelled nucleocapsid RNA were suspended in hybridization buffer (50 mM-tris-HCl, 4 mM-NaCl, 1 mM-Pr, 0.2% SDS and 60% formamide at a final pH of 8.0). Reaction mixtures (90 µl) containing a fixed quantity of labelled nucleocapsid RNA were prepared in the presence or absence of purified, unlabelled 42S virion RNA or the 31S and 12 to 18S mRNAs. The samples were then denatured by heating at 90 °C for 2 min followed by quick chilling. The salt concentration was adjusted to 0.4 M-NaCl and the samples (100 µl) were incubated at 37 °C for 24 h. The samples were then diluted to 1 ml with final concentrations of 2 × SSC, 50 µg/ml RNase A and 10 units/ml RNase T₁ and incubated for 30 min at 37 °C. Carrier transfer RNA (40 µg) and 2 ml of 20% TCA were added and the acid-precipitable, ribonuclease-resistant material collected on Millipore filters.

**Analysis of the 5'-termini of the mRNAs.** The samples of RNA were dissolved in 100 µl of 50 mM-sodium acetate, pH 6.0, with 20 µg carrier yeast tRNA, heated at 90 °C for 2 min, cooled and then digested with P₁ nuclease (200 µg/ml) for 16 h at 37 °C. This was followed by digestion with bacterial alkaline phosphatase (20 units/ml), pH 8.0, for 1 h at 37 °C. The digestion products were analysed by high voltage electrophoresis on Whatman 3MM paper in pyridine-acetate buffer, pH 3.5, at 2600 V for 2.5 h. The appropriate marker compounds were co-electrophoresed with the samples and were located by u.v. light absorption. The paper was cut in 1 cm strips and the radioactivity determined by liquid scintillation counting. The nucleotides were eluted and further digested with nucleotide pyrophosphatase as described previously (Rose, 1975) and the products analysed by high voltage electrophoresis.

**Immunoprecipitation and immunofluorescence.** Rabbit anti-VSV sera was kindly provided by Dr D. F. Summers, University of Utah College of Medicine, U.S.A. Cytoplasmic extracts of RC-60 cells infected with VSV in the presence or absence of RPV and labelled with ³H-leucine were prepared as described above. VSV-specific proteins were precipitated with anti-VSV sera and isolated by adsorption of the protein:antibody complex to heat-inactivated Staphylococcus aureus (Cowan I strain) as described by Kessler (1975).

For the demonstration of VSV-specific antigens on the surface of infected cells, RC-60 cells were grown on glass coverslips until they were 50% confluent and then infected as described above. At 4.5 h p.i. the coverslips were washed twice with 2 to 3 ml of phosphate

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buffered saline (PBS), pre-warmed to 37 °C. The coverslips were placed in a humidified box and covered with 100 to 200 µl of a 1:100 dilution of rabbit anti-VSV antibody and incubated for 35 min at 37 °C. The coverslips were washed in 200 ml of PBS at 37 °C for 10 min. The cells were then covered with 100 to 200 µl of a 1:5 dilution of fluorescein conjugated sheep anti-rabbit antibody (Gibco) and incubated at 37 °C for 35 min. The coverslips were again washed with 100 to 200 ml of PBS at 37 °C for 30 min, drained and inverted into a drop of glycerol:PBS (1:1) on a microscope slide. The fluorescence was examined using a Zeiss fluorescence microscope with internal 35 mm camera.

**Polyacrylamide gel electrophoresis of proteins.** Samples for gel electrophoresis were adjusted to 1% SDS and 1% mercaptoethanol and heated at 100 °C for 3 min. Ten per cent polyacrylamide SDS slab gels were prepared and run as described by Laemmli (1970) and protein bands were identified by radiofluorography as described by Bonner & Laskey (1974). Eight per cent polyacrylamide tube gels containing 0.5 M-urea and 0.1% SDS were prepared and run as described by Obijeski et al. (1974). The gels were fractionated on a Gilson gel crusher and counted in 10 ml of ACS fluid (Amersham Corp., Arlington Heights, Ill., U.S.A.) in a scintillation spectrometer.

**Chemicals and enzymes.** Carrier-free 3P-orthophosphate was purchased from New England Nuclear (Boston, Mass.) and 5,6-3H-uridine (40 Ci/mmol) and 4,5-3H-leucine (61 Ci/mmol) from Schwartz/Mann (Orangeburg, N.Y.). Oligo(dT) cellulose Type T2 was purchased from Collaborative Research (Waltham, Mass.). Actinomycin D and hydroxyurea were purchased from Sigma Chemical Company (St Louis, Mo.). Enzymes were purchased as described previously (Moyer & Banerjee, 1976).

**RESULTS**

**Rescue of VSV by RPV — the effect of temperature on virus production**

To demonstrate the ability of RPV to rescue VSV in a non-productive infection of RC-60 cells, monolayers were infected with VSV in the presence or absence of RPV. The infected cultures were labelled with 3H-uridine and incubated at 37 °C for 24 h. All infections were carried out in the presence of 50 mM-hydroxyurea, which has been shown to inhibit the replication of poxvirus DNA and subsequent RPV late gene expression without affecting its ability to rescue VSV (Thacore & Youngner, 1975). At 24 h p.i. the supernatant was removed and any released virus was pelleted and analysed on a density equilibrium gradient as described in Methods. The data (Fig. 1a) demonstrate that at 37 °C in cells infected with VSV alone there was no observable production of virus, while RPV, like vaccinia, was able to catalyse significant synthesis of VSV. These results confirm the original observations of Thacore & Youngner (1975). In multiple experiments the residual amount of released virus observed during infection with VSV alone ranged from 0.1 to 1.0% of that from the mixed (VSV and RPV) infection.

Gibson et al. (1978) have demonstrated that the inhibition of VSV growth in a permissive cell line by the drug tunicamycin, an inhibitor that prevents glycosylation of the VSV G protein and also prevents its subsequent migration to the plasma membrane, could be partially overcome by growth of the virus at 30 °C rather than at 37 °C. They postulated that only at the lower temperature is the non-glycosylated G protein able to assume a configuration necessary for its migration to the plasma membrane which then allows virus maturation. To determine whether there might be any temperature dependence in the non-permissive VSV infection of RC-60 cells, we studied the effect of temperature on the production of VSV. VSV infected cultures at 30 or 37 °C were labelled with 3H-uridine at 1.5 h p.i., incubated for 24 h and the amount of released virus was determined as described above. The data (Fig. 1b) show that in the cells incubated at 30 °C there is a significant
production of intact virus representing about 13% of the virus released from a mixed infection at 30 °C. The productive mixed infection at 30 °C was reduced 45% compared to that at 37 °C. The observed temperature dependence, however, is not due to a block in the migration of the VSV G protein to the cell surface, since VSV antigens are present on the surface of cells infected with VSV alone (see below).

To confirm the effect of temperature on the growth of VSV in RC-60 cells, the production of infectious virus was also monitored. Monolayers were infected with VSV at a m.o.i. of 1.0, incubated at 30 or 37 °C and at 2 h p.i. the adsorption medium was removed and replaced with 10 ml of normal maintenance medium. At 24 h the supernatants were removed and titrated, along with the adsorption medium, on L cells. At 2 h p.i., 62% and 80% of the input virus had adsorbed to the cultures incubated at 30 and 37 °C, respectively. At 24 h p.i. there was a significant difference in virus production between the two cultures with the cells incubated at 37 °C showing a VSV yield of approx. 0.1 p.f.u./cell and the cells incubated at 30 °C yielding 83 p.f.u./cell, consistent with the results obtained by the radioactive labelling of progeny virus (see Fig. 1). While the yield at 30 °C was far below that obtained from infection of a permissive cell line, it did show almost a 3 log increase in virus production over the infection carried out at 37 °C.

Intracellular virus nucleocapsids and their component RNAs in infected RC-60 cells

In an effort to characterize in detail the intracellular events in the non-permissive VSV infection, to determine the nature of the block in virus replication, we first monitored the production of cytoplasmic VSV nucleocapsids which are the replicative intermediates. RC-60 monolayers were infected with either VSV or RPV or a combination of both viruses and labelled with ³H-uridine from 1.5 to 5.5 h p.i. Cytoplasmic extracts were prepared and the subviral components were separated on 15 to 30% sucrose-NEBS gradients as described.
Fig. 2. The effect of RPV on the formation of VSV nucleocapsids in RC-60 cells. RC-60 cells (2 x 10^7 cells) infected with VSV alone (m.o.i. 20; •—•), RPV alone (m.o.i. 10; △—△) or a mixture of both viruses (○—○) were incubated with 3H-uridine (50 μCi/ml from 1.5 to 5.5 h p.i. A cell extract was then prepared and analysed on a 15 to 30% sucrose-NEBS gradient as described in Methods.

in Methods. In the productive infection (VSV + RPV), there was the accumulation of a large VSV nucleocapsid peak which sedimented at 120S, as expected, while in cells infected with RPV alone there was no detectable material sedimenting at that position (Fig. 2). In the extract from cells infected with VSV alone, there was a small but detectable nucleocapsid peak which contained, under steady state conditions, about 15% of the radioactivity found in the corresponding peak from cells infected with both viruses. In different experiments there was some variation in the amount of the nucleocapsid material isolated from cells infected with VSV alone, but the average value was about 22% compared to material from doubly infected cells. Analysis of combined, differentially labelled cytoplasmic extracts from cells infected with VSV alone or VSV + RPV reveals that nucleocapsids from both sources have identical sedimentation characteristics (data not shown).

Since there was some de novo synthesis of the virus replicative intermediate in the restricted system, the RNA from the nucleocapsid region of the sucrose-NEBS gradients (Fig. 2) was extracted and further characterized. Samples of 3H-uridine-labelled nucleocapsid RNA from cells infected with VSV alone and 14C-uridine-labelled 42S RNA extracted from intact VS virions were mixed and analysed by gradient centrifugation. Fig. 3(a) shows that the VSV nucleocapsid RNA synthesized in the restricted infection was identical in size to virion RNA. VSV nucleocapsid RNA from an infection of RC-60 cells labelled with 32P
Fig. 3. Analysis of VSV RNA from RC-60 cells infected in the absence or presence of RPV by gradient centrifugation. (a) ^3^H-labelled nucleocapsid RNA from RC-60 cells infected with VSV alone was isolated, purified and analysed by SDS-sucrose gradient centrifugation as described in Methods and Fig. 2. •—•, ^3^H-nucleocapsid RNA; △—△, ^14^C-marker RNA from released virus. (b) ^3^H-VSV mRNA from RC-60 cells infected in the absence (•—•) or presence (○—○) of RPV was isolated, purified and analysed by SDS-sucrose gradient centrifugation as described in Methods.

Table 1. Hybridization of VSV nucleocapsid RNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(+)</th>
<th>Resistance (%)†</th>
<th>RNase (ct/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleocapsid RNA from VSV alone in RC-60 cells</td>
<td>(-)</td>
<td>(+)</td>
<td>Resistance (%)†</td>
</tr>
<tr>
<td>+ 42S RNA (0.15 μg)</td>
<td></td>
<td>861</td>
<td>36.1</td>
</tr>
<tr>
<td>+ 42S RNA (0.30 μg)</td>
<td>839</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>+ mRNA (0.15 μg)</td>
<td>1137</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>+ mRNA (0.30 μg)</td>
<td>1163</td>
<td>51.4</td>
<td></td>
</tr>
<tr>
<td>+ mRNA (0.75 μg)</td>
<td>1366</td>
<td>61.2</td>
<td></td>
</tr>
<tr>
<td>Nucleocapsid RNA from VSV + RPV in RC-60 cells</td>
<td>4791</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>+ 42S RNA (0.03 μg)</td>
<td>958</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>+ 42S RNA (0.15 μg)</td>
<td>959</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>+ 42S RNA (0.30 μg)</td>
<td>935</td>
<td>17.3</td>
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<tr>
<td>+ mRNA (0.30 μg)</td>
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<td>50.9</td>
<td></td>
</tr>
<tr>
<td>+ mRNA (0.60 μg)</td>
<td>3600</td>
<td>70.4</td>
<td></td>
</tr>
<tr>
<td>+ mRNA (1.20 μg)</td>
<td>4105</td>
<td>80.9</td>
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</tr>
<tr>
<td>24 h self-anneal</td>
<td>28094</td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td>48 h self-anneal</td>
<td>24401</td>
<td>41.5</td>
<td></td>
</tr>
</tbody>
</table>

* ^35^P-labelled nucleocapsid RNA was purified and annealed in the presence of unlabelled VSV 42S genome RNA or a mixture of 31S and 12 to 18S mRNAs as described in Methods.

† The values were corrected for RNase resistance (2.2%) at zero time.
was purified and its strand sense determined by hybridization with either unlabelled virion RNA or unlabelled VSV mRNA as described in Methods. The data (Table 1) show that 80% of the RNA from the productive infection was rendered RNase-resistant by hybridization to mRNA and 17.8% was protected by the (−) strand virion RNA. The nucleocapsid RNA in the mixed infection is, therefore, 80% of the (−) strand sense and approx. 20% of the (+) strand sense, a ratio similar to the value reported by Soria et al. (1974) for a VSV infection of a permissive cell line. The nucleocapsid RNA from cells singly infected with VSV, however, was only 60% protected by the mRNA, while virus RNA protected 36% from RNase digestion. Therefore, in the restricted infection, the synthesis of nucleocapsid RNA was reduced and the relative ratio of the (+) and (−) strand RNAs was altered.

The observation of Thacore & Youngner (1975) that a reduced level of 42S genome sized RNA was formed in the non-permissive infection of RC-60 cells by VSV is consistent with our finding of a reduced steady state level of intracellular VSV nucleocapsids under the same conditions (Fig. 1, 2). From these results one might have predicted that replication was inhibited at the step of the synthesis of genome (−) strand RNA leading to the accumulation of the (+) strand intermediate. Our data show that this is not the case and inhibition seems to occur at a subsequent, as yet undefined, step in replication. It is, however, by no means clear that replication is directly affected, since any block in either the synthesis or post-transcriptional modification of virus RNA or protein could also block replication as a secondary consequence. We have therefore characterized many different aspects of macromolecular synthesis in VSV infected RC-60 cells in order to clarify this question.

**Characterization of virus mRNA**

We have examined the transcripts of VSV in detail to determine whether the normal post-transcriptional modification of VSV mRNA occurs in RC-60 cells. Virus-specific mRNA from RC-60 cells infected with VSV in the presence or absence of RPV was extracted and purified by oligo(dT)-cellulose column chromatography as described in Methods. In both the non-permissive and complemented infections, about the same proportion of the RNA bound to the column and thus contained poly(A) sequences (data not shown). The poly(A)-containing mRNA was then analysed on an SDS-sucrose gradient which permits the separation of the 17S, 14.5S and 12S virus mRNA species. The data show (Fig. 3b) that all three size classes of mRNA were present in the cells infected with VSV alone and that they were synthesized in the same relative ratios as in the cells infected with both viruses. The cells infected with VSV alone contained approx. 43% of the 12 to 18S mRNA found in the doubly infected cells. Since the doubly infected culture did not contain actinomycin D, while the VSV infected cells did, this value may represent an underestimate of the relative percentage of VSV-specific mRNA.

Since the virus mRNAs were synthesized in the restricted system in apparently proper ratios, we determined whether they were correctly modified by capping and methylation. The 12 to 18S 32P-labelled VSV mRNA from both the single and mixed infections was purified by two cycles of oligo(dT) cellulose chromatography. The RNA was extensively digested with P1 nuclease and bacterial alkaline phosphatase (BAP) and analysed by high voltage paper electrophoresis as described in Methods. This treatment should digest all of the RNA to the component nucleotide monophosphates except for the major 5' terminal cap structure, m7G(5'ppp(5'))N6mA, which, upon electrophoresis, should migrate between the markers pA and pG. Fig. 4(a) shows the products of this digestion. A peak of radioactivity, indicated by the brackets, migrated like the expected cap structure. The amount of label recovered in the cap from the restricted system was about 40% of that from the productive infection, consistent with the relative amounts of RNA in each system (Fig. 3b). The other two peaks of radioactivity were shown to be incompletely digested RNA since, when this material was re-digested with P1 and BAP and analysed by electrophoresis, all
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Fig. 4. Analysis of the 5' terminal cap structure of VSV mRNA by high voltage electrophoresis. \(^{32}P\)-labelled VSV mRNA from RC-60 cells infected in the absence (■) or presence (□) of RPV was isolated and purified as described in Methods and Fig. 3(b). The RNA was digested with P1 nuclease and bacterial alkaline phosphatase, and analysed by electrophoresis on Whatman 3 MM paper (a). The material indicated by the bar was eluted, redigested with P1 nuclease and bacterial alkaline phosphatase, and analysed by electrophoresis (b). The peak material was eluted and digested with nucleotide pyrophosphatase and analysed by electrophoresis (c).

The radioactivity migrated as inorganic phosphate (data not shown). As shown in Fig. 4(b), when the material initially identified as the cap structure was re-digested with P1 and BAP it again migrated between pA and pG. When the cap peaks were eluted and digested with nucleotide pyrophosphatase, which will hydrolyse the cap structure into its component nucleotide monophosphates, the products were \(\text{p'}\text{mG}\) and \(\text{pA}^\text{m}\), present in an approx. 1:1 ratio in mRNA from both the restricted and complemented infections (Fig. 4(c)). These
Table 2. VSV protein synthesis in RC-60 cells infected in the presence or absence of RPV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>L</th>
<th>G</th>
<th>N</th>
<th>NS</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV + RPV</td>
<td>9761</td>
<td>61282</td>
<td>415910</td>
<td>21885</td>
<td>8593</td>
</tr>
<tr>
<td>VSV alone</td>
<td>3581</td>
<td>34172</td>
<td>173688</td>
<td>8458</td>
<td>5745</td>
</tr>
<tr>
<td></td>
<td>(36.7%)</td>
<td>(55.8%)</td>
<td>(41.6%)</td>
<td>(38.6%)</td>
<td>(66.9%)</td>
</tr>
</tbody>
</table>

* The ³H-leucine-labelled antibody precipitated proteins were analysed by SDS–polyacrylamide slab gel electrophoresis. Protein bands were located by fluorography and the bands excised and counted in scintillation fluid.

experiments demonstrate that, in the non-permissive RC-60 cells, VSV is capable of properly transcribing and modifying its mRNA at both the 5’ and 3’ ends in the absence of the helper poxvirus.

**VSV protein synthesis**

Since virus replication could also be inhibited as a secondary result of the inhibition of protein synthesis, the functionality of the mRNAs synthesized in the restricted system was tested by measuring virus protein synthesis in the presence or absence of RPV. The total VSV proteins present in infected cells were isolated from cytoplasmic extracts by immunoprecipitation with anti-VSV sera and analysed by electrophoresis on SDS–polyacrylamide slab gels. The protein bands were identified by fluorography, eluted and quantified by liquid scintillation spectroscopy. The results of this analysis (Table 2) show that all five VSV proteins were synthesized in the restricted system and, when compared to the co-infected cells, were found in amounts approximately proportional to the level of their mRNAs. Furthermore, we have shown that virus nucleocapsids labelled with ³H-leucine from 1.5 to 5.5 h p.i. in the restricted infection appeared to be properly assembled since they had the proper sedimentation rate and contained all three nucleocapsid proteins (L, NS, N) in identical ratios to those found in nucleocapsids from the rescued infection (data not shown).

To determine whether the NS protein, a putative regulatory protein, synthesized in the restricted infection was modified by phosphorylation, as in a normal permissive infection, the proteins were labelled with both ³H-leucine and ³²P from 1.5 to 5.5 h p.i. and isolated by immunoprecipitation as described above. The virus proteins were quantified on SDS–urea polyacrylamide gels and the NS proteins synthesized in both the restricted and complemented infection contained approximately the same relative amount of ³²P (data not shown). Therefore, NS was phosphorylated under non-permissive conditions and was not apparently altered by RPV.

Another modification of the VSV proteins found in normal infections is the glycosylation of the G protein. Analysis of the G protein from RC-60 cells infected with VSV in the presence or absence of RPV showed that these proteins had identical mobilities when compared by SDS–polyacrylamide gel electrophoresis. By this criterion, the G protein synthesized in the restricted system was apparently glycosylated. In order to determine whether the G protein in cells infected with VSV alone migrated to the plasma membrane, RC-60 cells grown on coverslips were infected with VSV in the presence or absence of RPV at 37 °C. At 4.5 h p.i. the presence of VSV antigens on the surface of these cells was monitored by indirect immunofluorescence. The results of these experiments are shown in Fig. 5. In cells infected with VSV plus RPV (Fig. 5b), there was a strong positive fluorescence at the surface of the cells and a noticeable c.p.e. as the cells began to round up and come off the glass. In cells infected with VSV alone (Fig. 5a) there was, again, a strong positive fluorescence, indicating the presence of VSV antigens (the G protein) on the cell surface; however, the
Fig. 5. Indirect immunofluorescent stain of RC-60 cells infected with VSV in the absence or presence of RPV. Cells grown on coverslips were infected with (a) VSV (m.o.i. 20) alone or (b) VSV plus RPV (m.o.i. 10). At 4.5 h p.i., coverslips were washed, incubated with rabbit anti-VSV antiserum, washed again and then incubated with fluorescein-conjugated sheep anti-rabbit antiserum as described in Methods. Control cells infected with VSV alone in the presence of cycloheximide (100 μg/ml), showed no fluorescence (data not shown).
Table 3. Virus production in RC-60 cells infected with VSV in the presence or absence of RPV host range mutants

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>VSV production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ct/min  (%)</td>
</tr>
<tr>
<td>VSV + RPV-wt</td>
<td>89048 100.0</td>
</tr>
<tr>
<td>VSV + RP/hr28</td>
<td>99734 112.0</td>
</tr>
<tr>
<td>VSV + RP/hr7</td>
<td>49332 55.4</td>
</tr>
<tr>
<td>VSV + RP/hr23</td>
<td>2675  3.0</td>
</tr>
<tr>
<td>VSV + RP/hr31</td>
<td>1487  1.7</td>
</tr>
<tr>
<td>VSV</td>
<td>607   0.7</td>
</tr>
</tbody>
</table>

* RPV host range mutant nomenclature is based on the scheme of Moyer & Rothe (1980). Mutant stocks were initially isolated by Fenner & Sambrook (1966).

c.p.e. was not as great and the typical morphology of the cells was still visible. In control cells, cycloheximide was added at zero time to the infection and there was no detectable fluorescence at 4.5 h p.i. (data not shown).

Non-complementing mutants of RPV

In our study of the VSV:RC-60 system we have chosen to use RPV as the complementing poxvirus, rather than vaccinia, because of the availability of a large number of host range mutants of RPV (Fenner & Sambrook, 1966). The individual mutants differ from one another in the number of RPV genes expressed in a given non-permissive cell line. We have examined four of the host range mutants of RPV (RP/hr mutants: Moyer & Rothe, 1980) for their ability to complement VSV in RC-60 cells. Although all the mutants differ from one another in terms of their expression in non-permissive cells, two mutants, RP/hr7 and RP/hr28, synthesize normal amounts of RPV DNA but are blocked at some step of virus assembly. These will be termed ‘late’ RP/hr mutants. The other two mutants, RP/hr23 and RP/hr31, are blocked at a much earlier stage, prior to RPV DNA replication. These will be termed ‘early’ RP/hr mutants. The results of these experiments are shown in Table 3. Both the late mutants can complement VSV growth in RC-60 cells. The mutant RP/hr28 has as full a potential to complement as wild-type RPV for VSV, whereas RP/hr7 can complement only half as well. However, the early mutants, RP/hr23 and RP/hr31, both fail to complement VSV growth. Thacore & Youngner (1975) showed that vaccinia can fully complement VSV in this system under conditions that inhibit poxvirus DNA synthesis and hence its late gene expression. Therefore, good complementation by the late RP/hr mutants which are blocked at a stage after DNA synthesis is not surprising. The failure of either early RP/hr mutant to yield any observable complementation allows us to eliminate the genes that these two mutants express under these conditions as being involved in the process of complementation. Furthermore, a study of the genes which are expressed by these and other early mutants should allow a further ‘bracketing’ of the RPV gene(s) involved in the complementation process.

The ability of RPV to rescue other strains of VSV in RC-60 cells and VSV from persistently infected cells

We have also determined whether the ability of RPV to rescue VSV from a restricted infection is limited to a single virus serotype or cell line. Infection of RC-60 cells with VSV-NJ (Glasgow) in the absence or presence of RPV revealed a characteristic pattern of restriction and rescue, respectively, similar to the results observed with VSV-Ind (Table 4). RPV seems to rescue VSV-NJ in RC-60 cells only to the extent that the VSV-NJ grows in a permissive cell line. Therefore, the rescue by RPV in RC-60 cells is not limited to a single
Table 4. Comparison of virus production with VSV-Ind and VSV-NJ in different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment</th>
<th>VSV production*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC-6o</td>
<td>VSV-Ind + RPV</td>
<td>ct/min</td>
</tr>
<tr>
<td></td>
<td>69400</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>VSV-Ind alone</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>VSV-NJ + RPV</td>
<td>22409</td>
</tr>
<tr>
<td></td>
<td>VSV-NJ alone</td>
<td>176</td>
</tr>
<tr>
<td>sBHK</td>
<td>VSV-Ind alone</td>
<td>431362</td>
</tr>
<tr>
<td></td>
<td>VSV-NJ alone</td>
<td>177727</td>
</tr>
<tr>
<td>CAR-4</td>
<td>VSV-NJ alone</td>
<td>49059</td>
</tr>
<tr>
<td></td>
<td>VSV-NJ + RPV</td>
<td>22526</td>
</tr>
<tr>
<td></td>
<td>VSV-Ind alone</td>
<td>8574</td>
</tr>
<tr>
<td></td>
<td>VSV-Ind + RPV</td>
<td>1981</td>
</tr>
<tr>
<td></td>
<td>RPV alone</td>
<td>1774</td>
</tr>
</tbody>
</table>

* VSV infected cells were labelled with 3H-uridine and released virus was determined as described in Methods.

VSV serotype. In other experiments, we tested the ability of RPV to rescue VSV from a normally permissive cell line that had become persistently infected with VSV-Ind (CAR-4 cells; Holland et al. 1976). These cells normally produce very low levels of VSV and are resistant to superinfection with the homologous virus. Infection of persistently infected CAR-4 cells with RPV alone or RPV plus the homologous VSV serotype (Ind) did not result in the rescue of VSV (Table 4). These experiments demonstrate that the mechanism of restriction of VSV replication in RC-6o cells is different from the restriction in a persistently infected cell line and that RPV is only capable of overcoming the first type of restriction.

**DISCUSSION**

Our analysis of the non-permissive infection of RC-6o cells with VSV has demonstrated that, with the exception of virus replication itself, other parameters which could indirectly affect the process of replication appeared to be completely normal. All five virus messenger RNAs were synthesized in cells infected with VSV alone and all post-transcriptional modifications appeared normal, since the mRNAs were properly capped and methylated at the 5' ends and polyadenylated at the 3' termini. Furthermore, these mRNAs were biologically functional since all five VSV proteins were synthesized in approximately normal ratios. The analysis of nucleocapsids from cells infected with VSV alone showed that all three expected nucleocapsid proteins were present. The fact that L, N and NS proteins can interact with the RNA to form this subviral structure suggests that these proteins are also structurally functional.

Several types of post-translational protein modifications in the restricted infection have been examined and each appeared to be normal. First, analysis of VSV proteins showed that only the NS protein was phosphorylated in both the restricted and rescued infections. Several forms of the phosphorylated NS protein have been recently described (Clinton et al. 1978, 1979), but we have not yet determined if these multiple forms are also found in VSV infected RC-6o cells.

Secondly, the total G protein isolated from both permissively and non-permissively infected RC-6o cells had the same apparent mol. wt. as the G protein from progeny virus of the mixed infection. Normal glycosylation of the G protein, therefore, seems to occur in the restricted system. Immunofluorescent staining of RC-6o cells infected with VSV alone
demonstrated the presence of VSV antigens (i.e. G protein) on the surface of the infected cells. These data indicate that the G protein is able to migrate to the cell surface. The normal movement of the G protein to the plasma membrane is important in view of our observation that the degree of restriction is reduced at lower temperatures (Fig. 1b). The growth of VSV is inhibited by the drug tunicamycin which acts to block the movement of the G protein from the rough endoplasmic reticulum to the plasma membrane (Leavitt et al. 1977; Gibson et al. 1978). This inhibition was also shown to be temperature dependent, being much more effective at 37 than at 30 °C. Since we observe a normal movement of the G protein to the plasma membrane at 37 °C, the causative interaction leading to the thermolability in our system must reside elsewhere.

Since all other parameters of macromolecular synthesis that we have measured appeared unaffected, it is likely that the process of VSV RNA replication is the primary site of inhibition in RC-60 cells. We have measured the amount and the strand sense of the RNA from the nucleocapsids synthesized in infected RC-60 cells. Cells infected with VSV alone show the formation of some nucleocapsids containing genome length (42S) RNA, although at a level only 20% of normal. The RNA found within the nucleocapsids consisted of both (+) and (−) strands, indicating that the transition to (−) strand genome RNA synthesis did occur. However, the ratio of (−) to (+) strand 42S RNA found in the restricted infection (60:40) was different from the ratio (80:20) found either in the RPV rescued VSV infection or in the VSV infection of a permissive cell line (Soria et al. 1974). These findings are interesting in the light of recent work by Simonsen et al. (1979a, b) who characterized the polarity of VSV nucleocapsid RNA synthesized at different times during the course of a permissive infection. They observed a biphasic synthesis of total RNA, with peaks at 3.5 h and 6.5 h p.i. Analysis of the polarity of the genome length RNA synthesized at early and late times p.i. revealed (−) to (+) strand sense ratios of 60:40 at 2 h and 80:20 at 6 h. These data suggest that replication may be a two-step process. The ratio that we observe for genome length VSV RNAs synthesized in the RC-60 system may, therefore, reflect a block in the transition from the early to the late stage of replication.

Although we believe RNA replication to be the primary process blocked in RC-60 cells, limited nucleocapsid formation and insertion of virus proteins into the host cell plasma membrane both take place in the restricted infection, yet no virus particles are released. It seems likely, therefore, that there is also a secondary defect that involves the maturation of those nucleocapsids which do form into mature virions.

The means by which RPV converts RC-60 cells into a permissive host for VSV remains obscure, although these studies will certainly be facilitated by our discovery of a class of host range RPV mutants that fail to permit VSV growth. Since individual host range mutants of RPV can express differing amounts of the poxvirus genome, an application of these and other similar RPV mutants should permit a biochemical dissection of the RPV gene expression and facilitate the elucidation of the poxvirus gene product(s) that allow VSV growth in the normally non-permissive RC-60 cells.

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