Cellular Quiescence Modulates the Replication of $L^{ts}$ Mutants of Vesicular Stomatitis Virus

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SUMMARY

Infectious particle production by temperature-sensitive ($ts$) mutants of vesicular stomatitis virus (VSV) was measured in a variety of different host cell types maintained in a state of quiescence or stimulated to proliferate. At permissive temperatures, all $ts$ mutants and the wild-type virus replicated equally well and with the same kinetics in both quiescent and proliferating cells. At semi-permissive temperatures, however, $L^{ts}$ mutants, with temperature-sensitive virion polymerases, showed a delay of about 6 h in infectious particle production relative to wild-type virus in proliferating cells and greater than 16 h in quiescent cells. The effect was specific for the $L^{ts}$ class of mutants and was not seen for representative mutants in any of the four other complementation groups of VSV. Regarding cellular determinants, the effect was correlated only with the growth phase and not with the species of origin, interferon inducibility or with malignant transformation.

INTRODUCTION

The role of cellular physiology in determining the outcome of virus–cell interactions is of obvious importance in understanding phenomena such as virus persistence in vitro or in vivo, and virus pathology in vivo, yet it has received relatively little attention. Most molecular and genetic virological research has involved the use of rapidly proliferating undifferentiated cells whereas, in vivo, most cells are quiescent and in various states of differentiation. In view of the increasing interest in the use of vesicular stomatitis virus (VSV) as a model for persistent infection (Farmilo & Stanners, 1972; Holland & Villarreal, 1974; Youngner et al. 1976; Sekellick & Marcus, 1978), we felt it was important to investigate any changes in virus–cell interactions which might pertain to the virus mutations often implicated in persistence (Youngner et al. 1976; Stanners et al. 1977). The mutations of $T1026$, a double mutant of VSV capable of initiating persistent infection at relatively high input multiplicities (Stanners & Lam, 1978), were of particular interest. These are a $ts$ mutation, $L^{ts}$, in the virion RNA-dependent RNA polymerase activity, and a non-$ts$ mutation in P, the virus function which results in inhibition of total protein synthesis in infected cells (Stanners et al. 1977), both of which are required for establishing persistent infection at temperatures semi-permissive for the $ts$ mutation (Stanners & Lam, 1978). Recently, we and others have shown that mutations in the virus P function also result in a greatly increased ability to induce interferon (Sekellick & Marcus, 1979; Francoeur et al.
and that L MUT mutations potentiate this effect, a cellular response which is modified by cellular quiescence (Francoeur et al. 1980). We show here that the replication of L MUT mutants at semi-permissive temperatures is specifically influenced by cellular quiescence.

**METHODS**

**Virus strains and procedures.** All virus mutants and strains were of the VSV Indiana serotype. The origins of HR, the heat-resistant wild-type strain, the double mutant T1026 (L MUT, P-), its ts revertant R1 (L+, P-) and the L MUT (group I) mutant, T230, which is P+ at permissive temperatures and which has a cut-off temperature of 39 °C similar to that of the ts mutation in T1026, have been described in detail previously (Stanners et al. 1977; Stanners & Lam, 1978). Mutants representative of the other four complementation groups of VSV Indiana were obtained from Dr C. R. Pringle, prefix ‘G’ (Glasgow), or from Drs N. Genty or P. Marcus, prefix ‘O’ (Orsay), or from Drs D. Cormack and A. Holloway, prefix ‘W’ (Winnipeg).

Virus lysates were grown in HA cells from about five picked plaques, and were free of defective-interfering particles by electron microscopic observation and by velocity sedimentation analysis. The infectious particle production by infected cultures was measured by plaque assay at 34 °C of small samples of the culture medium. The plaque assay employed HA cells and a methyl cellulose overlay as described in detail previously (Farmilo & Stanners, 1972; Francoeur et al. 1980).

Cut-off temperatures for virus mutants were determined from the yields of infectious virus from HA cultures infected at 2 p.f.u./cell and maintained for 48 h at a series of temperatures. The temperature at which the yield of virus fell by 10³ to 10⁴ relative to the permissive yield was defined as the cut-off temperature.

**Cell strains and procedures.** HA cells were Syrian hamster embryo fibroblasts obtained from 13 to 14 day-old embryos and were used in the 3rd to 5th subculture after explantation. HA(Py) was a clonal line of polyoma virus-transformed HA cells denoted STR-T in Stanners et al. (1963). CHO-S cells were the wild-type strain referred to in Thompson et al. (1973). LR-73 was derived from CHO-S by selection for cells with more normal growth properties (Pollard & Stanners, 1979). Vero, a line of African green monkey kidney cells, was obtained from Dr P. I. Marcus.

To minimize experimental variation, large batches of frozen cells for each cell type were prepared and stored at −70 °C and samples thawed at a fixed time before experiments (2 to 3 weeks). All cells were cultured in α-MEM (Stanners et al. 1971) containing asparagine.H2O at 50 μg/ml and 10% foetal calf serum (FCS). HA, HA(Py), Vero and LR-73 were grown in monolayer culture as described previously (Stanners & Becker, 1971; Stanners et al. 1979). CHO-S cells were cultured in suspension using spinner flasks in temperature-regulated water baths as described previously (Stanners et al. 1978).

HA and LR-73 were found to be inducible for interferon while CHO-S, HA(Py) and Vero cells were non-inducible, as determined by Desmyter et al. (1968) for Vero cells or by us, using the plaque interferon assay of Francoeur et al. (1980) and P- mutants of VSV as inducers.

The growth cycles of normal HA cells, quasi-normal LR-73 cells and of transformed cell lines, CHO-S and HA(Py), have been described previously (Stanners & Becker, 1971; Pollard & Stanners, 1979; Stanners et al. 1979). HA and LR-73 are limited in stationary phase mainly by exhaustion of serum components, while CHO-S and HA(Py) are limited chiefly by components of the medium (Stanners et al. 1979). Stationary and exponential phases of Vero cells have not been characterized in detail in our system.

**Virus infection.** For the studies involving cellular quiescence, monolayer cultures of HA, HA(Py), Vero and LR-73 were grown in replicate 4 oz Brockway bottles seeded with
10⁶ cells in 10 ml α-MEM supplemented with 3% FCS for HA and LR-73 or with 2% FCS for Vero and HA(Py). The cultures were incubated for about 3 days at 37°C. At this time HA and LR-73 cultures had reached a serum-limited stationary phase and HA(Py) and Vero had reached a medium-limited stationary phase, as determined by the criteria of low rates of DNA synthesis and cell division described previously (Farmilo & Stanners, 1972). The (conditioned) medium was removed, virus was added at the indicated multiplicity in 1 ml conditioned medium and the cultures immersed in thermostatically controlled water baths at the indicated temperatures (±0.1°C). After 1 h incubation to allow virus adsorption, the cultures were given either 9 ml conditioned medium to give quiescent cultures, or 9 ml of stimulating medium [fresh α-MEM plus 10% FCS for HA and LR-73; or fresh α-MEM plus 2% FCS for Vero and HA(Py)] to initiate proliferation.

CHO-S cells grown in suspension at 2 x 10⁵ to 3 x 10⁶ cells/ml in α-MEM plus 10% FCS were concentrated by centrifugation and resuspended in the conditioned medium at 2 x 10⁶ cells/ml, then infected in replicate spinner flasks maintained in thermostatically controlled water baths with magnetic stirrers. After 1 h for virus adsorption, one-half of the replicate cultures were diluted with 9 vol. fresh α-MEM plus 10% FCS (proliferating cultures) while the remaining cultures received no further medium (quiescent cultures).

At 2 h intervals after infection, three replicate infected monolayer cultures in each growth state were removed, aliquots of the medium taken for virus titration and the cell number determined as described previously (Farmilo & Stanners, 1972). The p.f.u. data for monolayer cultures represents the mean of the total p.f.u. from the three replicate cultures. For the suspension cultures of CHO cells, aliquots were removed from two replicate infected cultures at 2 h intervals and virus and cell number determinations performed as above. The p.f.u. data for these cultures also represents the mean of the two replicate cultures.

RESULTS

Effect of cellular quiescence on replication of mutants of VSV

In preliminary experiments it was noted that the replication of the double mutant, T1026 (L⁺,P⁻), at the semi-permissive temperature of 38.5°C, was delayed in proliferating HA cells and strongly inhibited in quiescent HA cells. To investigate this phenomenon further, experiments were designed to test the role of both virus determinants and cellular determinants.

Role of virus determinants

To investigate the involvement of each of the mutations of T1026 in the phenomenon, replicate serum-starved quiescent HA cultures were infected at 5 p.f.u./cell with wild-type HR (L⁺,P⁺), RI (L⁺,P⁻), T230 (L⁺,P⁺) and T1026 (L⁺,P⁻) itself. Half of the infected cultures were then stimulated to enter the growth cycle by the addition of serum. The results are shown in Fig. 1. At 37°C, a temperature which is fully permissive for the L⁺ mutations of both T1026 and T230 (Stanners & Goldberg, 1975; C. P. Stanners & T. Lam, unpublished observations), all four virus mutants replicated equally well and with the same kinetics in both the quiescent and serum-stimulated cultures (Fig. 1a). All cultures showed extensive c.p.e. by 10 h after infection. At 38.5°C, however, the production of infectious virus by the two mutants bearing an L⁺ mutation was delayed by 6 h in the serum-stimulated cultures and by at least 16 h in the quiescent cultures (Fig. 1b). No c.p.e. was detected in the latter cultures. Similar results were obtained with six other independent Toronto L⁺ mutants and for L⁺ mutants G11, W14 and W135 at their semi-permissive temperatures (data not shown). These results show that cellular quiescence
Further experiments on virus determinants employed proliferating and quiescent CHO cells grown in suspension, as these were easier to sample in kinetic experiments and to maintain at the many temperatures required for work with different ts mutants. First, it affects L* mutations and not P- mutations, and that the depression of replication of mutants with L* lesions is temperature-dependent.
was shown that the magnitude of delays observed for replication of the L<sup>ts</sup> mutants at input multiplicities of 5 p.f.u./cell were the same for T1026 at input multiplicities of 0.05, 0.5 and 20 p.f.u./cell, so that the effect was not multiplicity-dependent (data not shown).

Second, delays were measured at 5 p.f.u./cell for at least two independent ts mutants in each of the five complementation groups of VSV corresponding to virus proteins L, NS, M, N and G. In these experiments it was first necessary to find a suitable semi-permissive temperature for each mutant. This was established as 0.5 °C below the cut-off temperature (see Methods). A kinetic curve for the production of infectious particles in both proliferating and quiescent CHO cells was then obtained for each mutant at its semi-permissive temperature as in Fig. 1 and the delay in virus replication was measured. The results are shown in Table 1. None of these mutants showed appreciable delay in either proliferating or quiescent CHO cells, except for the L<sup>ts</sup> mutants. None of the mutants, including the L<sup>ts</sup> mutants, showed any delays whatsoever at more permissive temperatures. The effect is thus specific for L<sup>ts</sup> mutations at semi-permissive temperatures.

### Role of cellular determinants

To explore the role of cellular determinants in replication delay of L<sup>ts</sup> mutants, a series of cell lines which were normal (HA), quasi-normal (LR-73) or transformed [HA(Py), CHO and Vero]; which could be induced to produce interferon (HA, LR-73) or could not be so induced [HA(Py), CHO and Vero]; and which showed quiescent states with different properties (see Methods) were infected with HR (L<sup>+</sup>,P<sup>+</sup>), RI (L<sup>+</sup>,P<sup>-</sup>), T230 (L<sup>ts</sup>,P<sup>+</sup>) and T1026 (L<sup>ts</sup>,P<sup>-</sup>) at 5 p.f.u./cell. The kinetics of production of infectious virus particles were measured in proliferating and quiescent cultures for each cell type and mutant as in Fig. 1, and the delays in replication were measured. The results shown in Table 2 indicate that replication delay was observed for L<sup>ts</sup> mutants in every cell system used, although the extent of the delay varied from system to system and the ratio of p.f.u. produced in proliferating to quiescent cultures late in infection varied from 10<sup>2</sup> for HA cells (Fig. 1) to 10<sup>3</sup> to 10<sup>4</sup> for HA(Py) cells (data not shown). Usually only a small amount of infectious virus was released by infected quiescent cells after the indicated delays and no c.p.e. was apparent. These results show that the chief cellular determinant of replication delay of L<sup>ts</sup> mutants is cellular quiescence, and that cellular transformation, the ability to induce interferon, the cell species or the method of inducing quiescence have no consistent effect.

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### Table 2. Role of cellular determinants in replication delay of L<sup>ts</sup> mutants

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell phenotype</th>
<th>Growth state</th>
<th>HR (L&lt;sup&gt;+&lt;/sup&gt;,P&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>RI (L&lt;sup&gt;+&lt;/sup&gt;,P&lt;sup&gt;-&lt;/sup&gt;)</th>
<th>T230 (L&lt;sup&gt;ts&lt;/sup&gt;,P&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>T1026 (L&lt;sup&gt;ts&lt;/sup&gt;,P&lt;sup&gt;-&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Normal</td>
<td>Prolif.</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IF ind&lt;sup&gt;++&lt;/sup&gt;</td>
<td>Quiescent</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>HA(Py)</td>
<td>Transformed</td>
<td>Prolif.</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IF ind&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Quiescent</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>LR-73</td>
<td>Quasi-normal</td>
<td>Prolif.</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IF ind&lt;sup&gt;++&lt;/sup&gt;</td>
<td>Quiescent</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>CHO</td>
<td>Transformed</td>
<td>Prolif.</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
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<tr>
<td></td>
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<td>0</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Vero</td>
<td>Transformed</td>
<td>Prolif.</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IF ind&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Quiescent</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>HA</td>
<td>Normal</td>
<td>Prolif. + Ara C&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IF ind&lt;sup&gt;++&lt;/sup&gt;</td>
<td>Quiescent + Ara C</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>24</td>
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</table>

* IF ind<sup>++</sup>, Interferon inducible by VSV, determined by PIF assay (Francoeur et al. 1980).
† Ara C, Cytosine arabinoside used at 10<sup>-5</sup> M in growth medium lacking nucleosides.
To determine whether a particular phase of the cell cycle was required for replication of L\textsuperscript{ts} mutants, which could explain the delay in virus replication in cells emerging from quiescence and the extended delay in cells left quiescent, Ara C was added to serum-stimulated quiescent HA cells infected with T\textsubscript{1026} (5 p.f.u./cell) at a concentration sufficient to inhibit strongly the initiation of DNA synthesis. The result shown in Table 2 indicates that the kinetics of replication of T\textsubscript{1026} in serum-stimulated cultures was the same in the presence or absence of Ara C. Thus progression around the cell cycle \textit{per se} is not required for the replication of L\textsuperscript{ts} mutants.

**DISCUSSION**

We show here that the replication of L\textsuperscript{ts} mutants of VSV is strongly dependent on the physiology of the host cell. At permissive temperatures, L\textsuperscript{ts} mutants replicated equally well in quiescent or proliferating cells, as did the wild-type HR. At semi-permissive temperatures, however, the replication of L\textsuperscript{ts} mutants was delayed for about 6 h in proliferating cells and was severely depressed in stationary phase cells. This effect is specific for L\textsuperscript{ts} mutants in that no such inhibition of infectious virus production was detected at permissive or semi-permissive temperatures for representative mutants in the four other complementation groups of VSV. This evidence for cellular modulation of virus function through the activity of the virus transcriptase has also been inferred from the work of Pringle (1978) and of Simpson \textit{et al.} (1979) on host-range mutants of VSV. It is of special interest that L\textsuperscript{ts} mutants have been implicated in virus persistence (Youngner \textit{et al.} 1976; Stanners \& Lam, 1978); our observation that virus replication of such mutants is responsive to cellular proliferation may be relevant to the maintenance of persistence \textit{in vivo} where cells exist in growth states ranging from complete quiescence to rapid proliferation.

The molecular basis for the observed cellular modulation of the activity of the virus L protein deserves comment. First, we have recently reported that P\textsuperscript{−} mutants of VSV, unlike wild-type VSV, are able to induce interferon and that L\textsuperscript{ts} mutations as well as cellular quiescence can enhance the induction (Francoeur \textit{et al.} 1980). However, the fact that replication delays of identical magnitude for T\textsubscript{1026} were observed for three cell lines that cannot be induced to produce interferon, and the fact that RI (L\textsuperscript{+},P\textsuperscript{−}), a potent inducer of interferon, showed no replication delay, argues strongly against the role of interferon in the phenomenon. Second, the fact that the same replication delay was observed in serum-stimulated cells in the presence of Ara C makes it unlikely that events in the cell cycle subsequent to the initiation of DNA synthesis are involved. Third, whatever the cellular factors involved, they are common to transitions between proliferating and quiescent cell types from different animal species and for cell types with very different cell culture characteristics. The stationary growth phases of the transformed cells used in this study are very different biochemically from those of normal cells (Stanners \textit{et al.} 1979). One interesting common feature of the quiescent states, however, is that they all show a depression in the initiation of translation (Stanners \textit{et al.} 1979). One possible but entirely speculative model for the effect, therefore, is that cellular factors required for the initiation of translation can modulate virus replication by affecting the activity of the L protein. Since transcription is required for the production of virus proteins, cellular modulation of the activity of the virion transcriptase would be expected to affect the synthesis of all virus proteins. Such an interaction could have evolved to allow persistent infections \textit{in vivo}, an obvious advantage to the virus as it permits survival of its animal host (Huang \& Baltimore, 1970).
VSV replication in quiescent cells

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REFERENCES


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