Interference of reovirus strains occurs between the stages of uncoating and dsRNA accumulation

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Interference of wild-type reovirus growth by some temperature-sensitive (ts) mutant viruses under non-permissive conditions or by other wild-type isolates has been demonstrated; however, the stage of the virus replication cycle at which interference occurs has not been defined. Examination of the time-course of the yields of T1 Lang (T1L) dsRNA in the progeny of mixed infections of T1L with T3 Dearing (T3D) or with a panel of T3D ts mutants at a non-permissive temperature revealed that interference takes place by 8–10 h post-infection and occurs prior to or at the same time as accumulation of reovirus dsRNA. Taken together with our previous results, these data indicate that interference occurs during a window between virus uncoating and synthesis of dsRNA in the reovirus replication cycle, probably at the stage of assembly of primary reovirus particles.

Mammalian reoviruses are non-enveloped viruses and their genomes consist of 10 dsRNAs enclosed in a double protein shell (reviewed in Schiff & Fields, 1990). One of the outer shell proteins, μ1, has been linked to the property of interference of wild-type reovirus isolates (Rozinov & Fields, 1994). Interference of reovirus wild-type isolate T3 Dearing (T3D) by certain T3D ts mutants (Ahmed et al., 1980; Ahmed & Fields, 1981; Chakraborty et al., 1979) prompted these authors to speculate that interference was a direct consequence of incorporation of the mutant product into mixed virus progeny (Ahmed et al., 1981; Ahmed & Fields, 1981). In other studies it has been proposed that interference was due to competition for the virus replication machinery (Huang & Baltimore, 1977; Maloy et al., 1994). In terms of their ability to interfere with the growth of other strains, reovirus isolates have demonstrated the following hierarchical order: T3D = T2 Jones (T2J) > T1 Lang (T1L) = T3 Abney (T3A) (Rozinov & Fields, 1994). In this work we focused on the question of which step in the reovirus replication cycle is the probable stage where interference occurs. Previously we have found that interference did not take place during the early steps of virus infection, adsorption and uncoating, and therefore interference appeared to be a later event in the virus replication cycle (Rozinov & Fields, 1994). Reovirus dsRNA segments can be resolved by SDS–PAGE (10% polyacrylamide) in Tris–glycine buffer (Laemmli, 1970; Ramig et al., 1977). Most of the corresponding dsRNAs from the different reovirus isolates have different electrophoretic mobilities (Ramig et al., 1977), allowing us to compare the yields of virus dsRNAs in single infection versus mixed infection with a second virus. L929 cell monolayers infected by a mixture of T1L plus T3D and T1L plus T3A or by single viruses T1L, T3D and T3A (initial m.o.i. of 10 for both single and mixed infections; Rozinov & Fields, 1994) were incubated at 37 °C for 8 or 10 h (including 1 h of adsorption) in the presence of 0.5 µg/ml actinomycin D, a concentration that inhibits cellular mRNA synthesis but not reovirus RNA synthesis (Acs et al., 1971), and [32P]orthophosphate. Unpurified virus lysates were incubated with 1% SDS (55 °C for 10 min) to release virus RNA and treated by DNase I followed by RNase A in 0.4 M-NaCl (in these conditions only dsRNA survives). We observed a reduction in

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the amount of all TIL dsRNAs in the mixed infection with T3D compared to dsRNAs of TIL in single infection (Fig. 1, compare lanes 2 and 5). The amount of TIL S1 dsRNA in the progeny of mixed infection with T3D was reduced by approximately 4.5-fold compared to that in single TIL infection, as evaluated by autoradiogram scanning. We had shown previously a similar reduction of S1 TIL dsRNA in a mixed infection with T3D grown for 43 h (4.3-fold; Rozinov & Fields, 1994). T3A did not interfere with the yield of TIL dsRNA in the mixed infection (Fig. 1, compare lanes 2 and 6) and this is also in accordance with our previous data (Rozinov & Fields, 1994). Consequently, interference of TIL by T3D occurred prior to or at the same time as dsRNA synthesis or accumulation. We were able to detect virus dsRNAs as early as 10 h post-infection (including 1 h of adsorption); prior to this the level of virus dsRNA was too low.

To examine the time-course of interference by a different approach, we measured interference of TIL by T3D ts mutants in temperature-shift experiments. We had shown previously that: (i) the phenotype of interference between reovirus isolates mapped to the M2 gene (Rozinov & Fields, 1994); and (ii) the $\mu_1$ protein, product of the M2 gene, was phenotypically mixed in the virus progeny of mixed infection (Rozinov & Fields, 1996). The rationale for temperature down-shift experiments was as follows: in the mixed infection of TIL and T3D tsA324 under non-permissive conditions (39°C) the mutant T3D $\mu_1$ protein is functionally defective and, thus, interference of TIL is expected to be reduced significantly; however, shifting to the permissive temperature (31 °C) will restore the interfering function of the mutant $\mu_1$ protein and interference will occur again. Consequently, we would expect that the longer the incubation time at the non-permissive temperature before transfer to permissive conditions, the higher the yield of TIL in mixed infection. In addition, if the step in the reovirus replication cycle in which interference occurs happens during incubation at the non-permissive temperature, then increasing the incubation time will not increase the TIL yield. Thus, these experiments allow us to determine the time-course of interference. In the down-shift experiments, L929 cell monolayers were infected with a mixture of TIL and T3D tsA324 (laboratory collection) viruses (m.o.i. of 10 for each virus) and incubated under non-permissive conditions for 2-22 h followed by transfer to the permissive temperature at 2 h intervals for the remainder of the 68 h period of infection. Subsequently, the amounts of TIL dsRNAs in the mixed virus progeny were analysed by gel electrophoresis. The best markers of interference were the TIL S1 and M2 dsRNAs, owing to their good resolution from the corresponding T3D dsRNAs (Fig. 2). The amounts of TIL S1 and M2 dsRNAs progressively increased, reaching a plateau when cells were incubated for at least 8-10 h or longer at 39 °C before shifting to the permissive temperature. However, these yields were still less than those of TIL dsRNAs in control single infection at 31 °C for 68 h (Fig. 2, lane 1). Thus, the interference of TIL by T3D tsA324 at the non-permissive temperature was reduced but not completely lost compared to that under permissive conditions. It should be noted that the yield of TIL dsRNAs in single TIL infections did not depend on the growth temperature in the range 31-39 °C (data not shown). These results demonstrated that the interfering events occurred by 8-10 h post-infection at 39 °C, consistent with the time-course of interference of wild-type viruses TIL and T3D described above. However, the interference pattern of TIL at the non-permissive temperature was the same with two other T3D mutants: tsB271 (L2 gene) and tsC447 (S2 gene) (data not shown). Neither the L2 gene nor the S2 gene was linked to the phenotype of interference (Rozinov & Fields, 1994). Because these L2 and S2 mutants behaved in the same manner as the M2 gene mutant and because the mutant gene segments encode capsid proteins, this suggests that interference may be exerted at the level of capsid assembly or function. It is important to note that the corresponding proteins ($\mu_1$, $\mu_2$ and $\alpha_2$) have never been shown to possess any functions outside of assembled virions.

To examine the time-course of interference between wild-type viruses, we delayed infection of the interfering virus for various times up to 8 h. Because two kinds of experiments...
indicated that interference occurred by 8–10 h post-infection (see above), we determined if the yield of TIL would still be inhibited after delay of infection of dominant-interfering parent T3D for a comparable time. If it was, interference could occur later, indicating that there is another step in the replication cycle of TIL where interference could take place. Mixed
infections of T1L and T3D at an m.o.i. of 10 for each virus were
done, with T3D infection delayed for 2–8 h post-infection.
The strongest interference was observed with T3D infection
delayed up to 6 h (Fig. 3, lanes 1–6), a time-course consistent
with the ts mutant experiments described above. However,
even when the T3D infection was delayed as long as 8 h, the
T1L dsRNA yields were somewhat reduced (Fig. 3, compare
lanes 1 and 7). Similar results were obtained when the m.o.i.
of T1L was increased twofold over T3D (m.o.i. of 20 and 10,
respectively; Fig. 3, lanes 8–14). These data showed that the
major interference occurred by 8 h post-infection but partial
interference can occur later in the replication cycle of T1L.

We report here that the majority of the interfering events
took place by 8–10 h post-infection, prior to or at the same
time as dsRNA accumulation. Our previous data showed that
interference was not involved in the early infection steps such
as adsorption and uncoating (Rozinov & Fields, 1994). Taken
together, these results define a window in the reovirus
replication cycle, between virus uncoating and synthesis of the
second strands of virus RNAs, during which interference can
occur. This period in the replication cycle includes the events
of core-derived plus-strand RNA transcription, virus protein
synthesis and assembly of primary virus particles. Reovirus
assembly presumably proceeds via entry of plus-strand virus
ssRNAs into nascent virions followed by synthesis of dsRNAs
(Acs et al., 1971; Joklik, 1974). Support for assembled virus
particles as a site of interference is provided by our recent
finding of phenotypic mixing of the μ1 protein in the progeny
of mixed reovirus infections (Rozinov & Fields, 1996). We
assume that interference could be a consequence of differential
viability of the phenotypically mixed primary virus particles
that represent the first step of virus assembly in the infected
cell. Although the possibility that interference could act at
stages other than assembly (e.g. RNA transcription or
translation) cannot be excluded, there is no proof that μ1
protein is involved in these stages. Rather, μ1 protein has
properties that suggest a role in the interaction of assembled
reovirus with cellular membranes (Lucia-Jandris et al., 1993;
Nibert & Fields, 1992; Nibert et al., 1991). We speculate that
the dominant μ1 protein on the outer shell surface of
phenotypically mixed virus particles determines the differential
survival of particles. This is in accordance with our current
knowledge that μ1 forms a critical protein network involved in
assembly of the virion outer shell and thus μ1 can control
assembly (Dryden et al., 1993). Another possibility is selection
for differential entry of isolate-specific ssRNAs into nascent
virions via functioning of either the dominant μ1 protein or
M2 ssRNA itself; however, there is no clear evidence for the
interaction of μ1 protein with virus ssRNAs either alone or in
complex with other reovirus proteins (e.g. non-structural) or
host proteins. The fact that delaying infection with the
interfering wild-type T3D (in the mixed infection with T1L) for
as long as 8 h still caused partial inhibition of the yield of T1L
shows that part of the interfering events can take place later via
another step in the replication cycle of T1L. These two steps
may share common features, possible candidates being primary
and secondary assembled virus particles (Acs et al., 1971;
Joklik, 1974). Nevertheless, two different mechanisms may
account for the early and late interference observed with wild-
type isolates. Finally, further studies of the mechanisms of
interference and the key role of μ1 protein (or the M2 gene
segment) may shed light on our understanding of reovirus
assembly.

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