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.

The phenomenon of virus interference or the inhibition of virus growth by another virus has been well known for a long time; however, the molecular mechanisms of this phenomenon remain obscure (for review see Whitaker-Dowling & Youngner, 1987). Interference has been described in two different systems: (i) serial passage of viruses at high m.o.i., leading to accumulation of defective-interfering viruses (Huang, 1973; Huang & Baltimore, 1977; Lazzarini et al., 1981; Von Magnus, 1954); and (ii) mixed infections of wild-type viruses with certain temperature-sensitive (ts) mutants (Chakraborty et al., 1979; Cooper, 1965; Jordan et al., 1989; Keranen, 1977; Youngner & Quagliani, 1978). Recently, we described a third situation where interference occurs: co-infection of cells with different wild-type virus isolates (Rozinov & Fields, 1994).

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. L292 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
Fig. 1. SDS-PAGE (10% polyacrylamide) autoradiogram of dsRNAs yield of 32P-labelled viruses (T1 L, T3D and T3A) in single and mixed infections after 10 h post-infection at 37 °C. L, M and S to the right of the gel designate the three size groups of reovirus dsRNAs: large (L1, L2 and L3), medium (M1, M2 and M3) and small (S1, S2, S3 and S4), respectively.

The amount of all T1L dsRNAs in the mixed infection with T3D compared to dsRNAs of T1L in single infection (Fig. 1, compare lanes 2 and 5). The amount of T1L S1 dsRNA in the progeny of mixed infection with T3D was reduced by approximately 4.5-fold compared to that in single T1L infection, as evaluated by autoradiogram scanning. We had shown previously a similar reduction of S1 T1L 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 T1L 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 T1L 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 T1L 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 μ1 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 T1L and T3D tsA324 under non-permissive conditions (39 °C) the mutant T3D μ1 protein is functionally defective and, thus, interference of T1L is expected to be reduced significantly; however, shifting to the permissive temperature (31 °C) will restore the interfering function of the mutant μ1 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 T1L 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 T1L 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 T1L 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 T1L dsRNAs in the mixed virus progeny were analysed by gel electrophoresis. The best markers of interference were the T1L S1 and M2 dsRNAs, owing to their good resolution from the corresponding T3D dsRNAs (Fig. 2). The amounts of T1L 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 T1L dsRNAs in control single infection at 31 °C for 68 h (Fig. 2, lane 1). Thus, the interference of T1L 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 T1L dsRNAs in single T1L 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 T1L and T3D described above. However, the interference pattern of T1L 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 (μ1, λ2 and σ2) 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
Fig. 2. Time-course of the yield of T1L dsRNAs in a mixed infection of T1L and tsA324 T3D mutant at down-shift temperature conditions (39 °C to 31 °C). SDS-PAGE (10% polyacrylamide; ethidium bromide staining) of dsRNAs prepared from unpurified virus lysates. T1L and T3D (m.o.i. of 10) single infections grown at 31 °C for 68 h are shown in the two left lanes. Mixed infections were performed at an m.o.i. of 10 for both viruses. Growth was at the non-permissive temperature for 0–22 h as indicated, followed by a shift to permissive conditions and continued incubation to 68 h. The two right lanes represent mixed infection of T1L and tsA324 T3D mutant grown at permanent non-permissive temperature (39 °C) for the times shown.

Fig. 3. DsRNAs yield of mixed infections of T1L and T3D with delaying of T3D infection until the times indicated. SDS–PAGE of unpurified virus lysates (10% polyacrylamide; ethidium bromide staining). Single infections (lanes 1, 2, 8 and 9) and mixed infections (all other lanes) were performed at an m.o.i. of 10 for both T1L and T3D except lanes 8 and 10–14, where the m.o.i. of T1L was 20. All incubations continued at 37 °C for 43 h.

indicated that interference occurred by 8–10 h post-infection (see above), we determined if the yield of T1L 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 T1L 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 (Lubin-Landris 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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