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Growth and Survival of Reovirus in Intestinal Tissue: Role of the L2 and S1 Genes

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Reovirus serotype 1 Lang can be recovered in high titer from the intestines of neonatal mice up to day 8 after peroral inoculation. By contrast, reovirus serotype 3 Dearing cannot be recovered from intestinal tissue past day 4 after peroral inoculation. This difference between the two reoviruses was mapped by using reassortants generated from nonmutagenized laboratory stocks. When the L2 and S1 genes of reovirus serotype 3 Dearing were present in reassortants, the reassortants behaved like serotype 3 Dearing in exhibiting a decreased capacity to be recovered from intestinal tissue. Likewise, viruses which contained the L2 and S2 genes from serotype 1 Lang exhibited an enhanced capacity to grow and survive, which is characteristic of serotype 1 Lang. Thus, the capacity of reovirus to survive in intestinal tissue was determined by the L2 and S1 genes.

The upper alimentary tract is the major portal of entry for a number of viruses. Little is known either about the factors in the gastrointestinal tract that influence the capacity of viruses to utilize this pathway or about the viral components that regulate the growth of viruses in the gastrointestinal tract. In our studies we have used the mammalian reoviruses as a model to define the genetic and biochemical factors influencing early events in the viral life cycle occurring in the gastrointestinal tract as well as events involving later virus-host interactions. The reoviruses have segmented double-stranded RNA genomes which, by readily allowing reassortants to be generated after mixed infection by two parents, provide a useful system for identifying viral genes involved in pathogenesis (reviewed in reference 22). The initial reassortants used in our studies were generated by crosses between mutagenized temperature-sensitive stocks of reovirus serotype 3 Dearing and wild-type stocks of serotype 1 Lang (13, 15, 19). These initial reassortants were used to show that a number of biologic properties, including type-specific neutralization and cell and tissue tropism, were determined by the S1 gene, which encodes the viral hemagglutinin (24–26).

In contrast to the qualitative differences between the parental viruses that mapped to the viral hemagglutinin, other viral properties differed in a quantitative fashion. For example, all reovirus strains examined have the capacity to inhibit protein synthesis or to grow in intestinal tissue following peroral inoculation. However, differences exist in the extent of inhibition of protein synthesis or the extent of viral growth in the gastrointestinal tract among different isolates of the same or different serotypes. In studies in which these quantitative differences were examined, the use of reassortants made from mutagenized temperature-sensitive parental stocks often gave results so ambiguous that no clear genetic link could be established. These findings led us to isolate a second series of reassortants, generated by crosses between nonmutagenized stocks of serotype 3 Dearing and either serotype 1 Lang or serotype 2 Jones (2, 4, 18). These reassortants were used in a number of studies which confirmed and extended the previous S1 mapping (see, for example, references 10 and 23) or which allowed properties to be mapped to genome segments other than S1 (2, 4, 5, 18).

The study of Rubin and Fields (17) is one in which a determinant of virulence other than S1 had been identified by using reassortants derived from the initial mutagenized stocks. In this study, growth in intestinal tissue was mapped to the M2 gene. However, inconsistencies in the data were described in the initial report which were thought to be due to aberrant M2 gene products present in the temperature-sensitive stocks. In a recent study involving the more recently isolated reassortants that were made from nonmutagenized stocks, Keroack and Fields (11) demonstrated that viral shedding from the intestines of neonatal mice and transmission between littermates both were determined by the L2 gene. This finding raised the question of whether the L2 gene might also play a role in growth and/or survival of reovirus in intestinal tissue. We thus reexamined the genetic basis for growth and survival of reovirus in intestinal tissue by using the reassortants generated from nonmutagenized stocks. In the present study we found that the L2 and S1 genome segments played a role in determining differences in the growth and/or survival of the two viruses on days 4, 6, and 8 postinoculation. Although both the L2 and S1 genome segments were very clearly implicated, the S1 segment contributed more significantly to survival than the L2 segment did.

MATERIALS AND METHODS

Cells and viruses. Mouse L cells were propagated in suspension in Joklik minimal essential medium as described previously (14). L-cell monolayers were used for virus plaque formation and for subsequent propagation of virus. Viral preparations inoculated into animals were used at the second passage level. Reovirus serotype 1 Lang and serotype 3 Dearing were standard laboratory stocks provided by Karen Byers.

Reassortants used in this study were prepared from nonmutagenized laboratory stocks of reovirus serotype 1 Lang

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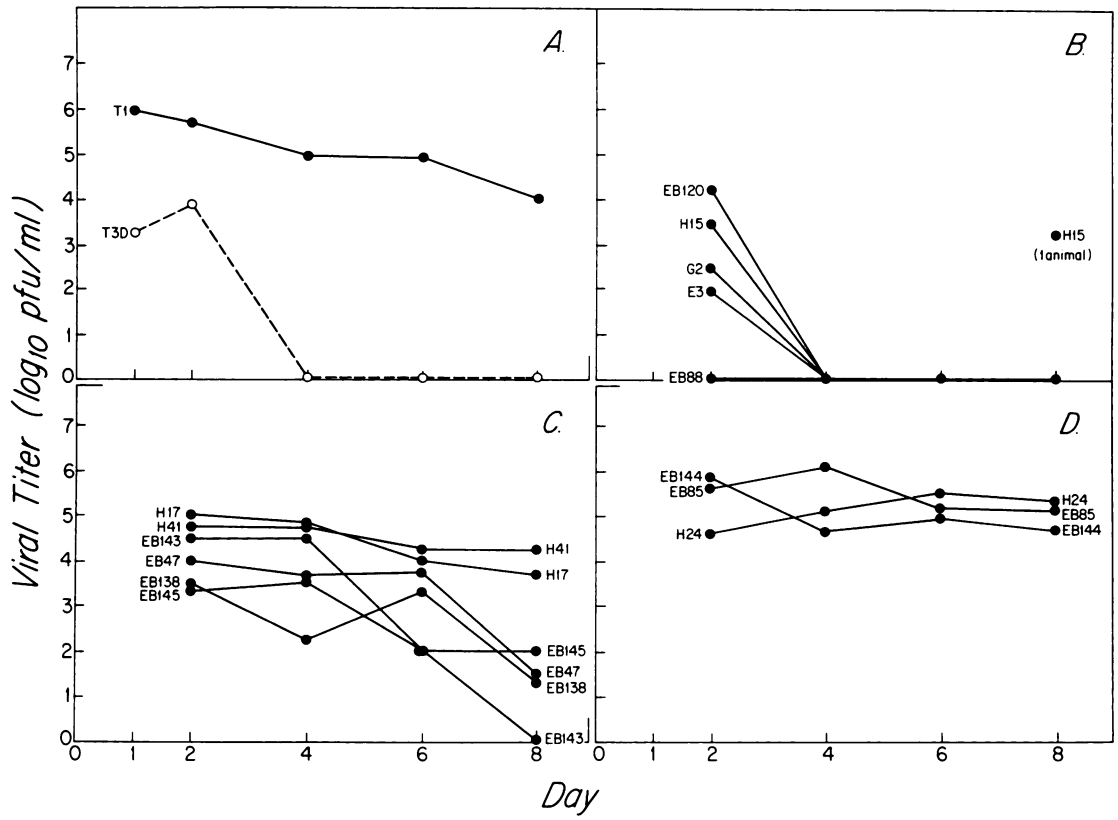

FIG. 1. (A) Recovery of reovirus serotype 1 Lang and serotype 3 Dearing in intestinal tissue following peroral inoculation (10^5 PFU) into neonatal mice. The virus was administered into the stomachs of neonatal mice following intragastric intubation. The intestines were harvested at days 1, 2, 4, 6, and 8, and the fluid contents were washed out as described previously (11). The intestinal tissue remaining after the wash was assayed for the presence of virus. Since the lowest dilution that could be assayed was a 10^{-2} dilution, the limit of detectability in this assay was 5×10^2 PFU. Each time point represents the mean of three or four samples. The standard deviation was less than 0.6 $\log_{10}$ at all time points. (B) Recovery of reassortants exhibiting an L2-S1 gene pair from serotype 3 Dearing in intestinal tissue following peroral inoculation (10^5 PFU) of neonatal mice. The standard deviation was less than 1.5 $\log_{10}$ for day 2 samples and was 0 (given a limit of detection of 5×10^2 PFU) for all samples on days 4, 6, and 8. (C) Recovery of reassortants exhibiting a heterologous L2-S1 gene pair in intestinal tissue following peroral inoculation (10^5 PFU) of neonatal mice. The standard deviation was less than 1.5 $\log_{10}$ at all time points. (D) Recovery of reassortants exhibiting an L2-S1 gene pair from serotype 1 Lang in intestinal tissue following peroral inoculation (10^5 PFU) of neonatal mice. The standard deviation was less than 0.5 $\log_{10}$ at all time points.

and serotype 3 Dearing. The reassortants used in this study were isolated and electropherotyped either by D. Drayna (4) or by E. Brown and M. Nibert (2). Reassortants were provided by M. Nibert.

Mice. Pregnant NIH Swiss mice were obtained from the National Cancer Institute. All adult mice were fed standard laboratory chow and water ad libitum. Neonatal mice were inoculated 2 days after birth by intragastric intubation (17) and placed into cages with one mother per eight suckling mice. At specified times after inoculation, pairs of neonatal mice were sacrificed by cervical dislocation. The abdomen of each mouse was opened, and the intestine was dissected from duodenum to anus. The intestinal contents were flushed as described previously (11). The intestinal tissue was placed in 1 ml of gelatin-saline and frozen at −70°C prior to titration.

Viral titer determination in mouse intestines. Mouse intestines were frozen and thawed three times and disrupted by sonication for 30 to 45 s with the microprobe of a Heat System–Ultrasonics W225R sonicator. Serial dilutions were made in gelatin-saline, and 100 μl of the 10^{-1} to 10^{-5} dilutions was used to inoculate L-cell monolayers. The plaque assay procedure has been described previously (3). Titers are expressed as $\log_{10}$ PFU per milliliter of suspension.

Wilcoxon rank sum distribution test. Titers from days 2, 4, 6, and 8 were used to rank the viruses (parental and reassortant) according to titer: 16 was the highest and 1 was the lowest. Rank sums for each gene from each parent were referred to a table of critical values (12).

RESULTS

Recovery of serotype 1 Lang and serotype 3 Dearing from intestinal tissue of neonatal mice. To reevaluate the genetic determinant(s) of recovery of reoviruses from intestinal tissue, we initially used conditions that mimicked those of the study of Keroack and Fields (11) in which it was demonstrated that the L2 gene determined transmission among littermates. Mice were given a peroral inoculum of 10^5 PFU of either serotype 1 Lang or serotype 3 Dearing, and their intestinal tissues were harvested at days 1, 2, 4, 6, and 8 after infection. The titers of virus recovered from intestinal tissue are shown in Fig. 1A. Serotype 3 Dearing could be recovered from intestinal tissue at day 2, but by day 4 and on subsequent days, virus was not detected. In

TABLE 1. Reovirus reassortants used to map the ability of reovirus to grow in intestinal tissue

Clone	Origin of genome segment encoding[a]:										Rank no.[b]
	Outer capsid				Core						
	L2[c]	S1	S4	M2	L1	L3	M1	S2	M3	S3	
EB85	1	1	1	1	1	1	1	3	3	1	16
H24	1	1	3	1	1	1	1	1	1	1	15
T1	1	1	1	1	1	1	1	1	1	1	12.5
EB144	1	1	1	3	1	1	1	1	3	3	12.5
H17	3	1	1	3	3	3	1	3	3	3	12.5
H41	3	1	1	1	1	3	1	3	3	3	12.5
EB143	1	3	1	1	3	1	1	1	1	1	10
EB47	3	1	1	1	1	1	1	1	1	1	9
EB138	1	3	1	3	3	1	1	3	1	1	8
EB145	3	1	3	3	3	3	3	3	1	3	7
G2	3	3	1	1	1	1	1	1	1	1	3.5
E3	3	3	3	1	3	3	3	3	3	3	3.5
EB88	3	3	3	1	3	3	3	3	3	3	3.5
EB120	3	3	1	1	3	1	1	3	1	1	3.5
H15	3	3	1	3	1	3	1	3	3	3	3.5
T3	3	3	3	3	3	3	3	3	3	3	3.5
1/3	6/10	8/8	11/5	10/6	8/8	9/7	12/4	6/10	8/8	8/8	

[a] Genome segments from serotype 3 Dearing or from serotype 1 Lang are designated 3 or 1. The reassortants are listed from high to low according to their viral titers in intestinal tissue on day 4 (Fig. 1).

[b] The rank number of each reassortant is given in the right-hand column. Tied values received the mean of the rank numbers at the positions concerned. The significance levels attained by the rank sum for a given gene are shown in Fig. 2.

[c] L2, a gene encoding a core protein, is here listed as an outer capsid component simply to emphasize its association with S1.

contrast, we recovered serotype 1 Lang through day 8 after inoculation. These samples were also assayed for the presence of virus in intestinal washings, as described by Keroack and Fields (11; data not shown). We found that titers of virus in intestinal washes and in intestinal tissue were roughly equivalent. The 4- to 5-$\log_{10}$ PFU difference in the recovery of serotype 1 Lang and serotype 3 Dearing from intestinal tissue indicated that this phenotype was suitable for genetic mapping.

Recovery of reassortants from intestinal tissue. To identify the genes responsible for the difference between serotype 1 Lang and 3 Dearing, we tested reassortants for their capacity to grow and survive in intestinal tissue. Because of the report of Rubin and Fields (17) that the M2 gene plays a role in determining the differences in growth of reoviruses in intestinal tissue and the report of Keroack and Fields (11) that the L2 genome segment is responsible for differences in transmission of virus between neonatal mice, we initially assessed the independent contributions of the M2 and L2 genome segments to growth and survival in intestinal tissue. E3 (isolated by D. Drayna) and EB88 (isolated by E. Brown) were independent isolates containing the M2 gene from serotype 1 Lang and all other genes, including L2, from serotype 3 Dearing (Table 1). Both of these isolates were similar to serotype 3 Dearing in the extent to which they were recovered from intestinal tissue (Fig. 1B), indicating that the M2 gene from serotype 1 Lang did not confer on the reassortants the capacity to grow and survive in intestinal tissue. The opposite reassortant, EB144, which has an M2 gene segment from serotype 3 Dearing and an L2 gene segment from serotype 1 Lang (Table 1), grew like serotype 1 Lang (Fig. 1D), again indicating that under these conditions, the M2 gene did not determine the capacity of reovirus to grow in intestinal tissue. Thus, the use of these newer reassortants indicated that the M2 gene of serotype 1 Lang did not confer on reassortants containing other serotype 3 genes the capacity to grow in intestinal tissue.

Since Keroack and Fields (11) demonstrated, by using the reassortants generated from nonmutagenized stocks, that the L2 genome segment determined the transmission of reovirus, we further evaluated the role of the L2 gene in intestinal growth. To do this it was necessary to choose reassortants with an L2 gene from one parent and as many genes as possible from the other parent. EB47 contains an L2 gene segment of serotype 3 Dearing against a background of nine serotype 1 Lang genes (Table 1). EB47 was recovered in significantly higher titer from intestinal tissue than serotype 3 Dearing was, but it did not grow as well as serotype 1 Lang, indicating that L2 played some role in the growth and survival of the virus but did not convert the virus from a serotype 1 Lang level of growth to that of serotype 3 Dearing (Fig. 1B). G2, however, was identical to serotype 3 Dearing in its inability to be recovered from intestinal tissue (Fig. 1B). Since G2 contains both the L2 and S1 of serotype 3 Dearing against a serotype 1 background (Table 1), the comparison of G2 with EB47 indicated that although L2 played a role in viral survival in intestinal tissue, both L2 and S1 were necessary to confer on a reassortant the inability to survive in intestinal tissue that is characteristic of serotype 3 Dearing.

To confirm that the L2 and S1 genome segments determined viral growth and survival in intestinal tissue and to assess whether other genes played a role, we analyzed a larger collection of reassortants (Table 1; Fig. 1B to D). The recovery of these reassortants from intestinal tissue is shown in Fig. 1B to D. In each case, the serotype 3 Dearing L2-S1 gene pair conferred on all reassortants an inability to survive in intestinal tissue (Fig. 1B). Reassortants with the serotype 1 Lang L2-S1 gene pair grew as well as serotype 1 Lang (Fig. 1D), whereas reassortants with a heterologous L2-S1 gene pairing exhibited intermediate levels of recovery (Fig. 1C), indicating that both the L2 and S1 genes of serotype 1 Lang were necessary to achieve a serotype 1 Lang level of growth in intestinal tissue.

Relative contributions of L2 and S1 genes to intestinal growth. Reovirus genetic studies have classically yielded data in which the reassortants fall into two classes clustered about the parental phenotypes. In those studies, in which the association of two discontinuous factors are examined, we have used chi-square analysis to confirm that the association of a gene with a phenotype is statistically significant. In the present study, the levels of growth and survival in intestinal tissue that were exhibited by the reassortants did not fall into two classes clustered about the parental phenotypes, but, rather, exhibited a continuum. This distribution would best be described as discontinuous (T1 or T3 gene) versus continuous (range of growth) and is more appropriately analyzed by the Wilcoxon rank sum distribution test. The viral titers in intestinal tissue for each reassortant from days 2, 4, 6, and 8 were used to rank the reassortants from highest to lowest (Table 1). The only two genes whose rank sums consistently fell outside a level of $P > 0.02$ or less were L2 and S1 (Fig. 2). It can be seen that, as predicted from the curves in Fig. 1A, the most statistically significant data were generated from the day 4, 6, and 8 titers. It was clear that by days 6 and 8, S1 was the most significant determinant of the recovery of reovirus from intestinal tissue following peroral inoculation (Fig. 2). Although genes (M3, S3, and M1) other than L2 and

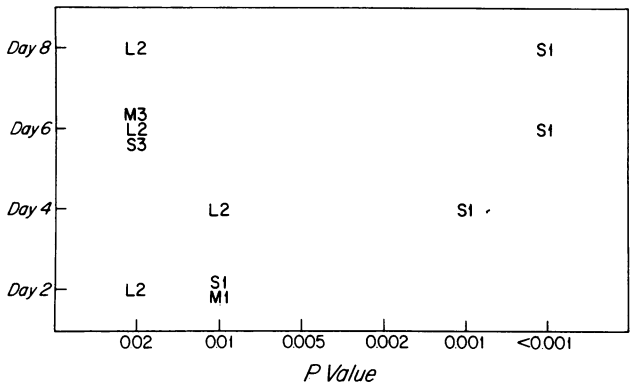

FIG. 2. Relative contributions of the 10 reovirus segments as estimated by the Wilcoxon rank sum distribution test. The average titers from days 2, 4, 6, and 8 were used to rank each reassortant. The rank sums for each gene were determined for days 2, 4, 6, and 8. When the sum of ranks fell outside the limits at a given P value, the next highest P value was examined, until the P value was identified within which the sum of ranks fell. The confidence limits for each gene are given along the x axis. Only genes which attained P values of 0.02 or less are shown.

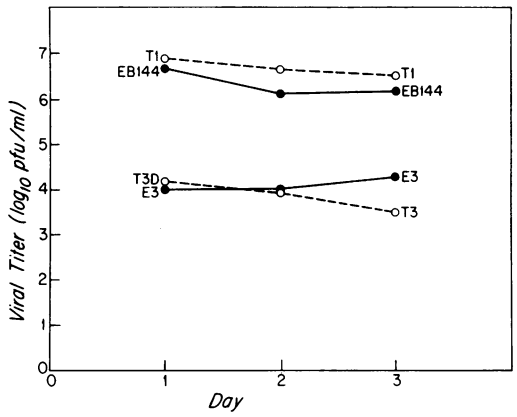

FIG. 3. Growth of reovirus T1 (Lang), T3 (Dearing), and two reassortants at days 1, 2, and 3 following inoculation with a higher dose (10^7 PFU) of virus to neonatal mice. The electropherotypes of the reassortants are given in Table 1. Procedures were as described for Fig. 1. The standard deviation was less than 0.5 $\log_{10}$ at all time points.

S1 appeared to show statistical significance on days 2 and 6, they did not appear to consistently contribute to the phenotype, and therefore their role has not been explored further.

Growth of reassortants given a high-dose inoculum. The study of Rubin and Fields (17) implying that M2 plays a role in the growth of reovirus in intestinal tissue differed from the present study not only in that the reassortants used were generated from mutagenized stocks but also in the dose of virus that was used and the times at which the intestinal tissues were harvested. Therefore, it was possible that the discrepancy in the genetic assignment in the two studies was due to the differences in the dose of virus or time of harvesting between the studies. Therefore, we repeated the evaluation of growth of reassortants generated from nonmutagenized stocks by using doses that mimicked the previous conditions as much as possible. Mice were given a peroral inoculum of 10^7 PFU, and their intestines were harvested at days 1, 2, and 3, as done by Rubin and Fields (17). Only reassortants which segregated the M2 gene (Table 1, EB144 and E3) were examined. Again, as in the experiments performed with a low dose of virus, it was seen that M2 did not play a role in determining the capacity of reoviruses to grow in intestinal tissue (Fig. 3). Thus, when the newer collection of reassortants was used to study the growth of reovirus serotype 1 Lang and serotype 3 Dearing in intestinal tissue, the L2 and S1 genome segments, rather than the M2 segment, were responsible for determining the capacity of the reassortants to grow to maximal titers.

DISCUSSION

In the present study we have found that the difference in the capacity of reovirus serotype 1 Lang and reovirus serotype 3 Dearing to be recovered from intestinal tissue following peroral inoculation was determined primarily by the S1 gene segment and, to a lesser extent, by the L2 segment. For these studies, reassortants were used that were generated from crosses of nonmutagenized parental virus stocks. Previous studies in which the analysis of growth of reovirus in intestinal tissue had been performed by using reassortants generated from mutagenized temperature-sensitive parental stocks suggested that the difference between reovirus serotype 1 Lang and reovirus serotype 3 Dearing is due to the M2 gene (17). We now believe that this earlier report is incorrect, most probably as a result of mutations present in the original reassortant stocks. Although the presence of mutations in our initial collection of reassortants was noted (3, 17), it was not thought to affect the assignment of viral intestinal growth to the M2 gene. The earlier collection of reassortants has not been used for genetic studies since 1980, and thus our recent assignments have all been based on reassortants generated from nonmutagenized stocks.

We would like to note two differences between our study and that of Rubin and Fields other than the reassortants used. Rubin and Fields used BALB/c mice, whereas we have used NIH Swiss mice. In addition, in the previous study reassortants were purified by CsCl gradient centrifugation prior to being inoculated into mice, whereas in the present study we inoculated stocks consisting of cell lysates. It is unlikely that the genetic determinant(s) of reovirus growth in intestinal tissue is specific to a particular mouse strain, since other mapping experiments that were done with BALB/c mice (24) have been reproducible with NIH Swiss mice (10). Moreover, in recent biochemical studies (D. K. Bodkin et al., manuscript in preparation), we did not detect any difference in the recovery of virus from the intestine following inoculation into mice either as purified virus or as cell lysates.

Thus, it would appear that the prior mapping of intestinal growth to the M2 gene represents an instance of an incorrect genetic assignment as a result of the use of mutagenized stocks for preparing viral reassortants. Earlier experiments with reassortants generated from mutagenized stocks mapped the inactivation of reovirus in vitro by chymotrypsin to M2 (17). This result was subsequently confirmed in a genetic analysis of transcriptional activation (which correlates with loss of infectivity) by using the reassortants generated from nonmutagenized stocks (5). Other studies by Hrdy et al. (8) mapping differences in neurovirulence within a serotype to M2 involved reassortants generated from nonmutagenized stocks. As noted in the introduction, earlier studies with reassortants generated from mutagenized stocks

involving the mapping of properties to the S1 gene have been confirmed in a number of subsequent studies.

These findings underscore potential problems arising from the high frequency of mutation of RNA viruses and illustrate the need to minimize the generation of mutations in viral stocks that might alter biologic properties of the viruses in question. The interpretation of data from experiments performed with reassortants generated from mutagenized stocks may be difficult because the reassortants may contain mutations not present in the original, nonmutagenized viruses. Using bunyaviruses, Rozhon et al. (16) also found that temperature-sensitive stocks generated by chemical mutagenesis contain non-temperature-sensitive mutations which influence viral virulence. Further, given the high mutation rate of RNA versus DNA viruses (7), the problem of silent mutations which influence biologic properties may be particularly acute for RNA viruses. The fact that the reported cases of non-temperature-sensitive mutations which influence viral virulence involve RNA as opposed to DNA viruses is consistent with this hypothesis.

The data presented here, combined with other studies performed in this laboratory, imply that it is relatively late in the infection that the L2 and S1 genes determine the differences in the recovery of reovirus serotype 1 Lang and serotype 3 Dearing from intestinal tissue. Upon entry into the intestinal lumen, the intact virus of the input inoculum is converted to intermediate subviral particles identical to those generated in vitro with chymotrypsin (D. K. Bodkin, M. Nibert, and B. N. Fields, unpublished data). In these particles the σ3 protein, (the S4 gene product) has been removed and the μ1c protein, (the M2 gene product) has been cleaved. However, this early cleavage event does not determine the differential growth of serotypes 1 Lang and 3 Dearing, since the genetic analysis presented in this study indicates that the differences in recovery of the virus after day 4 between reovirus serotype 1 Lang and serotype 3 Dearing are governed by the L2 and S1 genes rather than by the S4 and M2 genes.

How might the L2 and S1 genes be operating to determine the difference in survival and possibly growth of serotypes 1 Lang and 3 Dearing? One possibility is that it is cleavage of the viral σ1 protein (the S1 gene product) that accounts for the difference between serotype 1 and serotype 3. Early studies implied that the serotype 3 Dearing σ1 may be cleaved in vitro (9, 20). Nibert (unpublished data) has confirmed that the T3 Dearing σ1 protein is cleaved in vitro by chymotrypsin, whereas Sturzenbecker et al. (21) demonstrated that the serotype 1 Lang hemagglutinin is more resistant to proteolytic digestion. Given the role of the λ2 protein (the L2 gene product) as an anchor to the viral attachment end of the σ1 protein (1, 6), it is possible that the L2 and S1 genetic compounds we have identified involve accessibility to a cleavage site on the hemagglutinin to intestinal or cellular proteases. Evaluation of the process by which serotype 3 Dearing loses infectivity in the intestine may further elucidate the interaction of λ2 and σ1 in reovirus and provide insights into factors determining the capacity of reovirus to grow and survive in intestinal tissues.

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