JOURNAL OF VIROLOGY, May 1993, p. 2442–2447
0022-538X/93/052442-06$02.00/0

Vol. 67, No. 5

Isolation and Genetic Characterization of Ethanol-Resistant Reovirus Mutants

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Received 9 December 1992/Accepted 28 January 1993

To better understand the mechanism(s) by which viruses respond to chemical or physical treatments, we isolated a series of mutant strains of reovirus type 3 Dearing that exhibit increased ethanol resistance. Following exposure to 33% ethanol for 20 min, the parental strain exhibited a 5 $\log_{10}$ decrease in infectivity. The mutant strains, however, exhibited a 2 to 3 $\log_{10}$ decrease in titer following identical treatment. Through the use of reassortant viruses, we mapped this increased ethanol resistance mutation to the M2 gene segment, which encodes a major outer capsid protein, μ1C. Sequence analysis of mutant M2 genes revealed that six of seven unique mutants possessed single-point mutations in this gene. In addition, the change in six of seven mutants caused a predicted amino acid change in a 35-amino-acid region of the gene product between amino acids 425 and 459. The identification of ethanol resistance mutations within a discrete region of this outer capsid protein identifies that portion of the protein as important in reovirus stability. The presence of viral particles possessing altered stability also suggests that subpopulations of viruses may possess altered environmental stability, which, in turn, could affect viral transmission.

In many disparate settings, general chemical and physical agents such as chlorine, UV light, and ethanol routinely are used as viral disinfectants. Numerous reports, however, indicate that different viruses react differently to such reagents (9–11, 13, 19, 26, 37, 41). It previously has been shown that two closely related strains of a single virus may exhibit different responses to inactivating agents (14). Few studies, however, have examined the biochemical basis of viral stability in response to these treatments. More definitive studies into the mechanism(s) of viral inactivation by chemical and physical agents may provide important clues into the nature of viral stability. In this report, we describe the isolation and genetic analysis of a series of reovirus mutants that possess increased ethanol resistance and discuss the role that these mutants may play in furthering our understanding of viral stability.

Studies by Drayna and Fields (14) showed that various laboratory strains of reovirus exhibit different degrees of resistance to inactivation by ethanol. By examining reassortant viruses, this strain-specific difference was found to be mediated by the M2 gene segment (14), which encodes a single polypeptide, μ1 (28, 30). A cleavage product of the μ1 protein, termed μ1C, is generated during virion assembly and constitutes a major reovirus outer capsid protein (25, 38, 43). Genetic and biochemical studies have shown an association between a number of reovirus properties and the M2 gene and/or the μ1 protein, including phenol resistance (14), in vivo protease resistance (34), pH optimum of in vitro viral transcriptase activation (16), and modulation of neurovirulence within a reovirus serotype (20). We have isolated and examined a series of reovirus type 3 Dearing (T3D) stability mutants in order to better understand how viral inactivation is mediated. Our results show that mutations conferring altered ethanol resistance map to the M2 gene segment. The M2 nucleotide sequence of these mutants indicates that single amino acid changes within a discrete region of the μ1 protein are important for reovirus resistance to ethanol.

Thus, our results show that ethanol acts on a specific region of the μ1C protein and have aided in the identification of a region of this protein important in reovirus stability. In addition, these findings suggest that spontaneous viral mutants possessing altered resistance to various chemical and physical agents may prove useful in the analysis of virus structure and stability. Finally, this set of novel reovirus stability mutants may be useful for examining various aspects of reovirus biology, such as viral uncoating and pathogenesis.

MATERIALS AND METHODS

Cells and viruses. Mouse L929 (L) cells adapted for growth in suspension and in monolayer culture were maintained in Joklik's modified Eagle minimal essential medium (MEM) (Irvine Scientific, Santa Ana, Calif.) supplemented with 2.5% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2.5% VSP neonate bovine sera (Biocell Laboratories, Carson, Calif.), 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μg of streptomycin (Irvine Scientific) per ml.

Reovirus strains type 1 Lang (T1L) and T3D are laboratory stocks. Fresh stocks were generated by twice plaque purifying virus in L cells and then amplifying the virus through two rounds of growth in tissue culture (P2 stock). Viral titers were determined by the plaque assay technique as previously described (18).

Virus inactivation. Absolute ethanol (Aaper, Shelbyville, Ky.) was diluted in gelatin-saline to a final concentration of 33 or 40%. Aliquots of these solutions were placed in a 37°C water bath. Virus isolated from cell lysates was diluted 1:10 into the ethanol–gel-saline mixture, vortexed, and maintained at 37°C. After 20 min, the treated virus was diluted 1:10 into gelatin-saline at 4°C. The sample then was stored at 4°C until the viral titer was determined.

Isolation of mutants. A P2 cell lysate stock of T3D was

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exposed to 33% ethanol at 37°C as previously described. This treated virus was plated onto 60-mm-diameter tissue culture dishes seeded with 2.2×10^6 cells, and a plaque assay was performed. Viral plaques were harvested and placed in 2-dram (ca. 7.4-ml) glass vials containing 0.5 ml of MEM; 2.5×10^5 cells were added to each vial, and the vials were incubated at 37°C until significant cytopathic effect was evident. The samples then were subjected to three rounds of freezing at −70°C to lyse the cells and create an amplified P0 stock.

The effect of 33% ethanol on the amplified P0 stocks was determined as previously described in a single assay. For stocks that appeared to possess an altered degree of ethanol resistance, the viruses were twice plaque purified on L-cell monolayers and then amplified by two rounds of growth in tissue culture in the absence of ethanol. The ethanol resistance phenotypes of these viral clones were retested.

Generation of viral reassortants. Two-dram vials were seeded with 2.5×10^5 cells in 0.5 ml of MEM. Following an overnight incubation, the medium was aspirated from the vials, and the cells were coinfected with T1L and 3a9, an ethanol-resistant mutant of T3D, at a combined multiplicity of infection of 10 in a ratio of 4:1 (T1L/3a9). After addition of the viruses, the cells were incubated for 1 h at 37°C, overlaid with 2 ml of MEM, and incubated at 37°C. At 28 h postinfection, the vials were subjected to three rounds of freezing at −70°C. This lysate was used to infect L cells in 60-mm-diameter tissue culture dishes, and a plaque assay was performed. Viral plaques were isolated, and amplified P0 stocks were grown as described above.

RNA was extracted from the samples, resuspended in Laemmli sample buffer, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10% gels for 400 mA · h at constant current as previously described (23). Gels were stained with ethidium bromide and visualized with UV irradiation. Reassortant clones were twice plaque purified and amplified twice in tissue culture. The electropherotypes of these viruses then were reconfirmed.

Sequence analysis. Genomic double-stranded RNA segments were isolated from purified virions and sequenced by using dideoxy sequencing reactions according to the procedure of Bassel-Duby et al. (4), with minor modifications. The dideoxynucleotide, reverse transcriptase, and RNase inhibitor concentrations were decreased by one-half. Deoxynucleotides, dideoxynucleotides, avian myeloblastosis virus reverse transcriptase, and human placenta RNase inhibitor were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); [^{35}S]dATP (1,000 Ci/mmol) was obtained from Amersham (Arlington Heights, Ill.). Oligonucleotide primers were made with an Applied Biosystems oligonucleotide synthesizer and purified by C-18 Sep-Pak column chromatography (Waters Associates, Milford, Mass.) (2). Following purification, the primers were diluted in H_2O to maximize the sequencing efficiency. Primers were synthesized corresponding to the reported T3D M2 sequence (23) at approximately 200-nucleotide intervals for both RNA strands.

RESULTS

Effects of ethanol on reovirus T1L and T3D. Drayna and Fields previously had shown the effects of ethanol inactivation on purified reovirions (14). To extend these findings, we examined the effects of ethanol inactivation on second-passage cell lysate stocks of reovirus T1L and T3D to various concentrations of ethanol as described in Materials and Methods. These two laboratory stocks of virus differed markedly in their degree of inactivation following certain treatments (Fig. 1). When treated with 33% ethanol for 20 min, T1L exhibited a 1 $\log_{10}$ decrease in infectivity. T3D, on the other hand, exhibited a 5 $\log_{10}$ loss in infectivity following similar treatment. When the ethanol concentration was increased to 40%, however, T1L and T3D exhibited similar and significant decreases in titer. To stop the inactivation process, the ethanol concentration was decreased 10-fold. At these lower concentrations of ethanol, there was no effect on the viral titer for extended periods of time (data not shown). These data are consistent with the earlier results of Drayna and Fields (14).

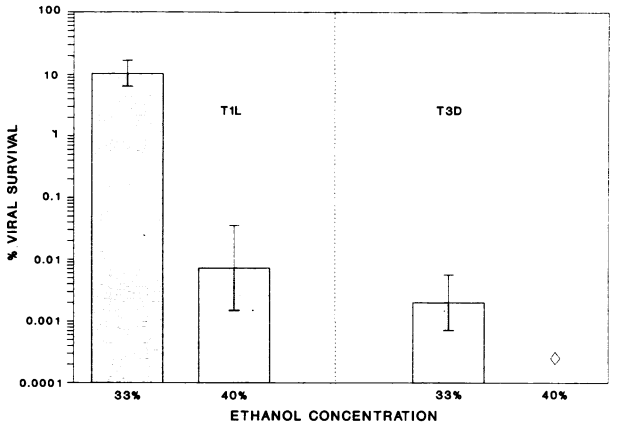

FIG. 1. Effects of ethanol on infectivity of T1L and T3D. Second-passage L-cell lysate stocks were diluted into ethanol mixed with gelatin-saline to a final ethanol concentration of 33 or 40%. Following a 20-min incubation at 37°C, virus was diluted into gelatin-saline and assayed for infectivity. Bars represent percent viral infectivity compared with control incubations in the absence of ethanol. The data points and error bars represent the averages of at least three separate determinations ± standard errors of the means. ◇, no PFU detected.

Isolation of ethanol-resistant mutants of T3D. To determine the molecular basis for the observed strain differences in ethanol resistance, we attempted to isolate mutants of reovirus T3D that possessed increased resistance to this reagent. Following exposure to 33% ethanol, some infectious T3D virions did remain. Viruses that escaped inactivation were isolated. These viruses were derived from three independent parental T3D stocks (termed a, b, and d) to minimize the possibility of isolating sibling mutants. The effect of 33% ethanol on amplified stocks of these isolates was tested (data not shown). In all assays, the relative degree of inactivation exhibited by the virus stocks was constant. The absolute values, however, varied in some experiments. As a result of this variation, the stocks were classified simply as ethanol resistant or ethanol sensitive. Approximately half of these isolates exhibited a 1 to 2 $\log_{10}$ loss in infectivity following exposure to ethanol and were classified as ethanol resistant. The remaining isolates exhibited a loss in infectivity of >3.0 $\log_{10}$ and were classified as ethanol sensitive. Thus, 53% (10 of 19) of the possible mutant strains were found to possess an ethanol-resistant phenotype. The effect of 33% ethanol on these T3D-derived clones was retested following plaque purification and amplification of these strains. Following ethanol treatment, all of the mutant clones exhibited a 1.5 to 3 $\log_{10}$ loss in infectivity, similar to that

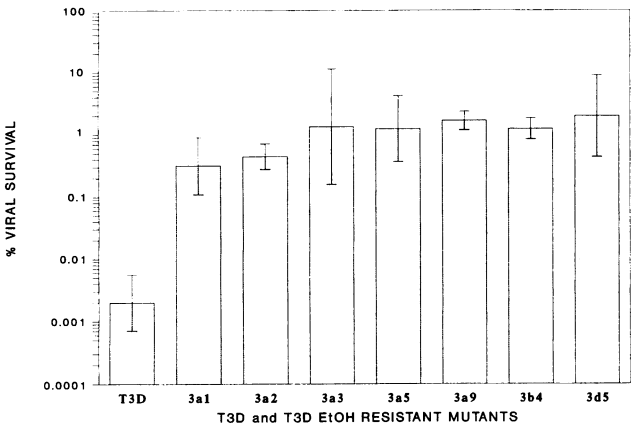

FIG. 2. Effects of ethanol on infectivity of T1L, T3D, and mutants. Second-passage L-cell lysate stocks were diluted into ethanol (EtOH) mixed with gelatin-saline to a final ethanol concentration of 33%. Following a 20-min incubation at 37°C, virus was diluted into gelatin-saline and assayed for infectivity. Bars represent percent viral infectivity compared with control incubations in the absence of ethanol. The data points and error bars represent the averages of at least three separate determinations ± standard errors of the means.

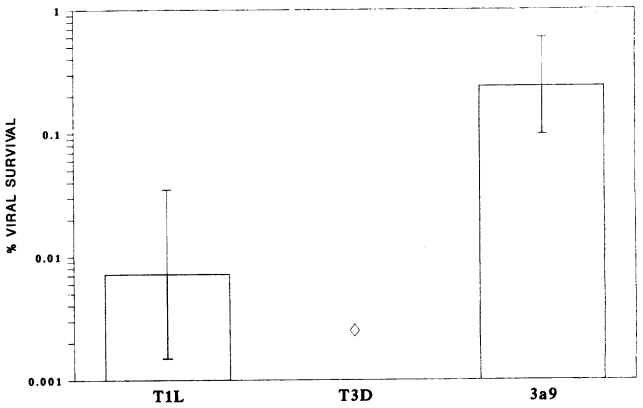

FIG. 3. Effects of ethanol on infectivity of T1L, T3D, and 3a9. Second-passage L-cell lysate stocks were diluted into ethanol mixed with gelatin-saline to a final ethanol concentration of 40%. Following a 20-min incubation at 37°C, virus was diluted into gelatin-saline and assayed for infectivity. Bars represent percent viral infectivity compared with control incubations in the absence of ethanol. Under these conditions, the mutants (represented by 3a9) exhibit a phenotype different from that exhibited by T1L. The data points and error bars represent the averages of at least three separate determinations ± standard errors of the means. ◇, no PFU detected.

exhibited by T1L but in marked contrast to the large decrease in infectivity exhibited by T3D (Fig. 2). To ensure that the presumptive mutants were T3D-derived viruses, the electropherotypes of these viruses were analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). All seven unique ethanol-resistant mutants possessed a T3D-like electropherotype, thereby confirming that these viral clones were T3D-derived ethanol-resistant mutants.

Because treatment of T1L and the T3D-derived mutants with 33% ethanol for 20 min resulted in similar degrees of inactivation, these inactivation conditions could not be used to screen reassortant viruses and map the genetic lesion causing altered ethanol resistance. We therefore attempted to identify ethanol treatment conditions that would differentiate T1L from the T3D-derived mutants. The effects of increased ethanol concentrations and increased periods of exposure were studied. When exposed to 40% ethanol for 20 min, T1L exhibited a 4.5 $\log_{10}$ loss in titer. The mutants, however, exhibited a 1.5 to 2.5 $\log_{10}$ loss in infectivity, as shown with mutant 3a9 (Fig. 3).

Genetic mapping of mutations. To identify the genetic basis for the ethanol resistance phenotype, reassortant viruses were generated by crosses of T1L and the T3D-derived mutant 3a9 as described in Materials and Methods. The electropherotypes of the reassortants used for the genetic mapping are presented in Table 1.

Our previous results showed that T1L and the mutant viruses exhibited different ethanol resistance phenotypes when exposed to 40% ethanol for 20 min. When the reassortant viruses were exposed to identical ethanol inactivation conditions, they exhibited two distinct phenotypes (Fig. 4). The titers of several of the reassortants decreased at least 4 $\log_{10}$, while the rest of the reassortants exhibited a 2 to 3 $\log_{10}$ loss in infectivity. Thus, the ethanol resistances of the reassortant viruses were divided into two categories: T1L-like and 3a9-like. All of the reassortants exhibiting a T1L-like phenotype possessed a T1L-derived M2 gene segment, and all reassortants exhibiting a 3a9-like phenotype possessed a 3a9-derived M2 gene segment. No other gene segment correlated with the observed phenotypes, thus indicating that a mutation(s) in the M2 gene segment is important for the phenotype of reovirus resistance to ethanol. Second-passage stocks of several of the reassortant viruses (Et89, Et90, Et112, and Et116) possessed titers approximately 1.5 $\log_{10}$ lower than those of the other virus stocks. Because of these low initial titers, no infectious virus was detected for these four isolates following ethanol treatment in our standard plaque assay at the lowest possible dilution, indicating a loss of infectivity of greater than 3 $\log_{10}$. On the basis of this significant loss in infectivity, these reassortant viruses were classified as T1L-like.

Sequence analysis of mutant M2 gene segments. To determine the molecular basis of ethanol resistance in reovirus,

TABLE 1. Genetic mapping of ethanol-resistant mutants

Virus	Electropherotype									
	S1	S2	S3	S4	M1	M2	M3	L1	L2	L3
Ethanol-resistant clones										
3a9	M	M	M	M	M	M	M	M	M	M
Et1	1	1	1	1	1	M	1	M	M	M
Et4	M	M	M	M	1	M	M	M	M	M
Et8	1	1	1	1	M	M	M	M	M	M
Et30	1	1	M	1	1	M	M	1	1	1
Et44	1	1	1	1	1	M	M	M	M	M
Et60	1	1	1	1	1	M	M	1	M	M
Et64	1	M	1	1	M	M	M	M	M	M
Ethanol-sensitive clones										
T1L	1	1	1	1	1	1	1	1	1	1
Et15	1	1	1	1	M	1	M	M	M	M
Et43	M	M	M	M	M	1	M	M	M	M
Et89	1	1	1	1	1	1	1	M	M	M
Et90	1	1	M	1	M	1	1	M	M	1
Et112	M	M	M	M	M	1	1	1	M	M
Et116	M	M	1	1	1	1	M	M	M	M

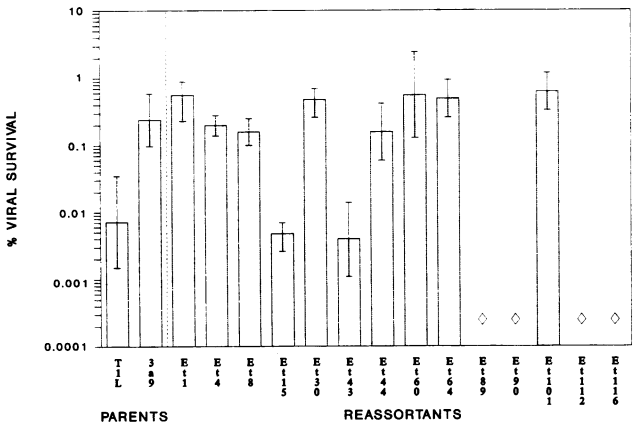

FIG. 4. Effects of ethanol on infectivity of T1L, 3a9, and reassortants. Second-passage L-cell lysate stocks were diluted into ethanol mixed with gelatin-saline to a final ethanol concentration of 40%. Following a 20-min incubation at 37°C, virus was diluted in gelatin-saline and assayed for infectivity. Bars represent percent viral infectivity compared with control incubations in the absence of ethanol. Reassortants are classified as either ethanol resistant (3a9-like) or ethanol sensitive (T1L-like). The data points and error bars represent the averages of at least three separate determinations ± standard errors of the means. ◇, no PFU detected.

the complementary strands of the M2 gene segments of the mutant viruses and the T3D parent virus were sequenced by using the double-stranded RNA sequencing procedure of Bassel-Duby et al. (4). Oligonucleotide primers were generated from the T3D M2 sequence (23) at approximately 200-bp intervals on the gene. The full-length sequence of each strand was determined. The sequence of the M2 gene segment of the parent T3D virus was identical to the M2 sequence reported by Tarlow et al. (40). To determine what mutations were responsible for the observed ethanol resistance phenotype, the M2 gene sequences of the 10 mutant viruses were compared with the M2 gene sequence of the parental T3D virus. On the basis of the sequence data, seven unique ethanol-resistant mutants of T3D were identified. Six of the seven mutant viruses possessed single nucleotide changes in the M2 gene segment, while the remaining virus possessed two changes (Table 2).

All of the observed nucleotide changes resulted in changes in the deduced amino acid sequence of the M2 gene product, μ1. Furthermore, the amino acid change in six of the unique mutants occurred within a 35-amino-acid region of the protein, between amino acids 425 and 459, suggesting that this region of the protein represents a site of ethanol action. The seventh mutant possessed a change at amino acid 319 of this protein.

TABLE 2. Sequence analysis of ethanol-resistant mutants

Clone	Amino acid position	Amino acid change
3a1	233	Pro → Ser
	442	Ile → Val
3a2	454	Pro → Leu
3a3	454	Pro → Arg
3a5	319	Ala → Glu
3a9	459	Lys → Gln
3b4	440	Gln → Leu
3d5	425	Val → Phe

DISCUSSION

Despite the widespread use of ethanol as a virucidal agent, little is known about the mode of action of this chemical. Numerous studies have examined the relative stability of viruses to various chemical and physical agents, such as ethanol (see reference 5 for a review). Few studies, however, have provided a molecular basis for these findings. Previously, Drayna and Fields (14) have shown that different reovirus strains possess different degrees of resistance to ethanol and that this phenotypic difference is associated with the M2 gene segment. In this set of experiments, we have extended these initial findings and identified single nucleotide changes in the M2 gene segment of reovirus T3D that greatly alter the degree of ethanol resistance displayed by this virus.

In this study, we isolated mutants of T3D that exhibit increased resistance to inactivation caused by ethanol. A genetic analysis of one of these mutants indicates that the mutations in the ethanol-resistant mutants of T3D reside on the M2 gene segment. Furthermore, an analysis of the M2 sequences of these mutants shows that six of seven unique mutants possess single nucleotide changes in the M2 gene, while the seventh mutant possesses two nucleotide changes in this gene. These nucleotide mutations all result in changes in the deduced amino acid sequence of the primary M2 gene product, μ1. Furthermore, the changes in six of the seven mutants cluster in a 35-amino-acid region of the protein between amino acids 425 and 459, while the remaining mutant possesses a change at amino acid 319.

The primary M2 gene product, μ1, has been shown to undergo a series of cleavage events in vivo and in vitro. The first cleavage event, which probably occurs during assembly of the virion (43), generates a 4.2-kDa amino-terminal fragment (μ1N) and a 72.1-kDa carboxy-terminal fragment (μ1C) (23, 32, 42). Both of these polypeptides, along with some copies of uncleaved μ1, are present in mature virions. Recent evidence has shown that the common amino terminus of μ1 and μ1N is N myristoylated (32). Treatment of virions with exogenous endoproteases causes a second cleavage event to occur in vitro near the carboxy terminus of μ1 and μ1C, resulting in the generation of the large μ1δ and δ polypeptides, respectively (7, 24, 35) and Φ, the smaller carboxy-terminal fragment (31). This cleavage event results in the formation of a partially uncoated form of the virus, referred to as the infectious subviral particle. A similar or identical cleavage event occurs in cultured cells infected with reovirus (8, 36, 39) and in the intestine of neonatal mice following peroral inoculation with reovirus (3, 6). The amino acid changes associated with increased ethanol resistance all reside within the δ portion of the primary polypeptide. More extensive in vitro digestion of the viral particles results in the formation of core particles, which lack all μ1 moieties (38).

By isolating ethanol-resistant mutants of T3D, we have shown that viral strains with increased resistance to such a common virucidal agent do exist. Numerous reports have documented the existence of viral strains exhibiting altered resistance to specific antiviral drugs such as acyclovir (12) and amantadine (33). Additional reports have described the emergence of guanidine-resistant viral mutants (1). Few reports, however, have documented the existence of viral mutants with increased resistance to chemical virucidal agents. The existence of such viruses may represent a major

concern. In our experiments, ethanol treatment decreased the titer of T3D 5 $\log_{10}$. Of the remaining infectious virus, approximately one-half possessed a true altered ethanol resistance phenotype, indicating that ethanol-resistant viruses exist at a relatively high frequency in the laboratory population of reovirus. It is thus likely that treatment of virus-contaminated areas with ethanol would result in the selection of viruses possessing increased ethanol resistance.

Through the use of T1L/3a9 reassortant viruses, we have mapped the ethanol resistance mutation to the M2 gene segment. This genetic mapping is in agreement with the previous mapping of reovirus strain differences in ethanol resistance to the M2 gene segment (14). Furthermore, the sequence analysis of these viruses indicates that ethanol treatment of reovirus affects a specific region of the M2 gene product. Six of seven unique mutants possess an amino acid change between amino acids 425 and 459. Biochemical studies of virus inactivation suggest that ethanol inactivates reovirus by denaturing the outer capsid (15). We conclude from our data, then, that alterations in a 35-amino-acid region of μ1 can make the μ1 protein more resistant to denaturation.

If these ethanol-resistant mutants resist denaturation, then one may hypothesize that the mutations strengthen some protein-protein interaction. Possibly, the region of the μ1 protein from amino acids 425 to 459 is involved in some inter- or intramolecular interaction. In addition, the region of the molecule encompassing amino acid 319 may be involved in the same or a second interaction. The existence of an ethanol resistance mutation at this location (mutant 3a5) suggests that this portion of the protein also may affect viral stability. We also cannot rule out the effects of changes in the region of the protein encompassing amino acid 233 on ethanol resistance. Although mutant 3a1 possesses a mutation in the amino acid 425 to 459 region, this mutant also possesses an amino acid change at position 233. Because of current limitations in reovirus molecular biology, the relative effects of these two changes individually cannot be addressed. Our data do indicate, however, that these two amino acid changes in concert do not result in ethanol resistance greater than that conferred by a single mutation in the amino acid 425 to 459 region of the protein. Interestingly, preliminary results indicate that the double mutant (3a1) exhibits a substantial decrease in neurovirulence when injected into neonatal mice. Perhaps the amino acid 233 change is involved in this observed attenuation. Work currently is in progress to address this possibility.

Isolation and genetic characterization of additional ethanol-resistant reovirus mutants may reveal the relative importance of changes in these three regions of the μ1 protein to strengthened capsid interactions. An analysis of additional T3D mutants and T1L mutants may provide insight into this problem. In addition, the characterization and sequence analysis of reovirus natural isolates may aid in this endeavor. Possibly, natural isolates will display a range of sensitivities to ethanol. By sequencing selected regions of the M2 gene segment of these isolates, a determination of the importance of various regions of the μ1 protein on capsid stability may be possible. Finally, it may be interesting to isolate mutants of these mutant viruses that exhibit an even greater degree of ethanol resistance.

Although these T3D-derived mutants exhibit a degree of ethanol resistance more like that of reovirus T1L than that of T3D, the observed amino acid changes do not result in a more T1L-like μ1 polypeptide. In fact, every amino acid position in which an ethanol resistance mutation has been observed represents an amino acid conserved among the three major laboratory strains of reovirus (T1L, T3D, and type 2 Jones) (42). Indeed, a sequence comparison of these three virus strains shows that the μ1 proteins of these viruses exhibit approximately 97% identity at the amino acid level (42). Given the important role that this protein presumably has during viral attachment, entry, and penetration, such sequence constraints are understandable. We hypothesize that most alterations in this sequence, such as those that we have identified, may affect dramatically the normal functioning of this molecule. Preliminary results support this hypothesis.

The outer capsid of reovirus is composed primarily of equal numbers of σ3 and μ1C (23). The σ1 protein is a minor surface protein (38) that is located near the λ2 core spikes at the vertices of the icosahedron (25). Both σ3-μ1C (21, 25) and μ1C-μ1C (38) complexes have been identified. Possibly, the mutation resulting in increased ethanol resistance strengthens one of these interactions or strengthens an as yet unidentified interaction. A more thorough understanding of the secondary structure of the μ1 protein, in conjunction with the sequence data, will provide additional information on the structure of the reovirus outer capsid.

These mutants also represent a novel set of reovirus M2 gene mutants. As a result, they may be useful in examining viral properties that previously have been associated with the M2 gene or the μ1 protein. In addition to mapping ethanol resistance to M2, Drayna and Fields (14) mapped reovirus strain differences in resistance to phenol and the pH optimum of viral transcriptase activation (16) to this gene. Hrdy et al. (20) determined that the M2 gene can modulate the neurovirulence of a reovirus serotype. As mentioned previously, the M2 gene product undergoes a series of endoproteolytic cleavages and is involved in the uncoating of the virus in vitro and in vivo. In addition, this gene has been associated with reovirus strain differences in the release of ^{51}Cr from preloaded cells, suggesting that μ1 may be capable of interacting with the cell membrane (27).

Finally, mutations in a class of reovirus temperature-sensitive mutants (*tsA*) have been mapped to the M2 gene (29). Sequence analysis of a representative *tsA* mutant indicates that a change at amino acid 315 of μ1 causes the temperature-sensitive phenotype and that a change at amino acid 429 or 445 can result in a pseudoreversion to the wild-type phenotype (22). Although the secondary structure of the protein is not known, the linear proximity of the *tsA* pseudoreversion mutations and the ethanol resistance mutations is noteworthy and offers further evidence that this region of the μ1 protein is important in viral stability and structure.

Since isolating this set of novel reovirus T3D M2 mutants, we have begun studying the functional properties associated with the mutations. Preliminary studies indicate that these mutations may affect viral uncoating and virulence. These findings suggest that alterations in viral stability may confer pleiotropic biological changes on the virus. Possibly, these results are indicative of the effects of stability alterations in other viruses. Thus, such findings suggest that the isolation of virus stability mutants may represent a novel means of isolating biologically interesting viral variants.

ACKNOWLEDGMENTS

We thank Elaine Freimont for expert technical assistance; Dinah Bodkin, Terence Dermody, and Kevin Coombs for helpful discussions; and Terence Dermody for careful review of the manuscript.

This work was supported by Public Health Service grant 5R37AI13178 from the NIAID. We gratefully acknowledge the support of the National Science Foundation (D.R.W.) and the Shipley Institute of Medicine (B.N.F.).

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