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Transduction of a Human RNA Sequence by Poliovirus

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Cells infected with poliovirus express a virally encoded polyprotein which undergoes self-mediated cleavage into structural and nonstructural viral proteins. Most of these cleavages are catalyzed by the 3C proteolytic domain of the polyprotein. Polyprotein synthesized in vitro from an RNA template containing a three-nucleotide insertion in 3C underwent proteolytic processing at all but one of the 3C-dependent cleavage sites. When transfected into HeLa cells, this RNA template displayed a lethal phenotype. We report here the isolation of two pseudorevertant progeny strains with restored protein-processing phenotypes, one of which appears to have arisen by transduction of a stretch of nucleotides from human 28S rRNA.

Eukaryotes have evolved elaborate means for splicing RNA, which represents a special case of the recombination of an RNA molecule with itself. RNA viruses also commonly undergo self-recombination to generate defective interfering particles. RNA recombination thus appears to be a commonly occurring natural process. Recombination between two separate RNA molecules having similar but not identical sequence also occurs in nature but is less commonly observed. Such homologous RNA recombination (i.e., events which yield neither insertion nor deletion of sequence at the recombination site [14]) was first described 30 years ago for poliovirus and has since been demonstrated for other picornaviruses, for the coronaviruses, and for other viral systems (10, 11, 14, 17, 19). Crosses between closely related poliovirus strains (intratypic crosses) exhibit recombination frequencies ranging from 2 to 20% when measured or extrapolated over the genome length of approximately 7,500 nucleotides (12, 14, 15, 17). Intertypic crosses, occurring between less closely related strains, show a significantly decreased recombination frequency (31), and interspecies crosses between picornaviruses do not yield viable progeny with a measurable frequency. Transduction of cellular RNAs, an example of nonhomologous recombination between unrelated RNA molecules, has never been described for the picornaviruses. This article reports the discovery of a nonhomologous recombination event which occurred in tissue culture between an engineered poliovirus genomic RNA containing a lethal lesion and the human host cell RNA.

MATERIALS AND METHODS

Mutagenesis, transfection, and virus isolation. *Hin*fI mutagenesis of poliovirus cDNA has been described (30). The construction of a T7 expression vector containing the *Hin*fI(5980) mutation has also been described (4). Full-length poliovirus RNA containing the *Hin*fI(5980) mutation was synthesized in vitro and introduced into HeLa cell monolayers by DEAE-dextran-mediated transfection as described before (4). For the isolation of μ10-1, transfected monolayers were overlaid with liquid medium (Dulbecco's modified Eagle's medium [DMEM]) and incubated at 33°C until cytopathic effects became apparent (approximately 4 to 5 days posttransfection). Monolayers and medium were then harvested, subjected to five cycles of freezing and thawing to release viral particles, diluted, and used to infect fresh HeLa monolayers. These second cultures (first passage) and subsequent passages were overlaid with agar-containing medium and incubated at 33°C. Several well-isolated plaques were subsequently picked. One of these plaques gave rise to the isolate labeled μ10-1. In a parallel experiment, monolayers were overlaid with agar-containing medium immediately posttransfection and incubated at 33°C, and plaques were picked when visible on day 5 posttransfection. One such plaque gave rise to the isolate designated μ10-51.

Nucleic acid sequence analysis and cDNA reconstructions. Sequence analysis of DNA from the *Hin*fI μ10-expressing T7 template and of RNA from plaque-purified virus was performed as previously described (4). A region of viral RNA from nucleotide (nt) 5805 to 6024 was sequenced, and no changes other than those reported were found. In order to rule out second-site changes in areas other than those sequenced, cDNA was synthesized in vitro from μ10-1 viral RNA, and a portion of the cDNA corresponding to the region of verified sequence and containing the known mutations was amplified by PCR (8) and used to reconstruct full-length templates de novo from the original wild-type-expressing T7 clone (of known sequence). Thus, the reconstructed full-length clone was entirely of known sequence. The reconstruction was performed essentially as detailed in reference 4. RNA transcribed from the reconstructed templates was then reintroduced into cells, and plaques picked from the transfection were expanded into viral stocks. The reisolated virus showed the same growth phenotype and protein-processing phenotype as the original isolates, making it unlikely that second-site changes were present.

Pulse-chase labeling with [^{35}S]methionine. Pulse-chase experiments were performed essentially as described before (4) with HeLa S3 suspension cultures infected with μ10-1. Infected-cell cultures were incubated in methionine-free medium (DMEM) at 33°C for 7.5 h and then exposed to labeled methionine for 5 min and chased with an excess of unlabeled methionine. Aliquots of the cultured cells were taken every 15 min during the chase period. These samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel.

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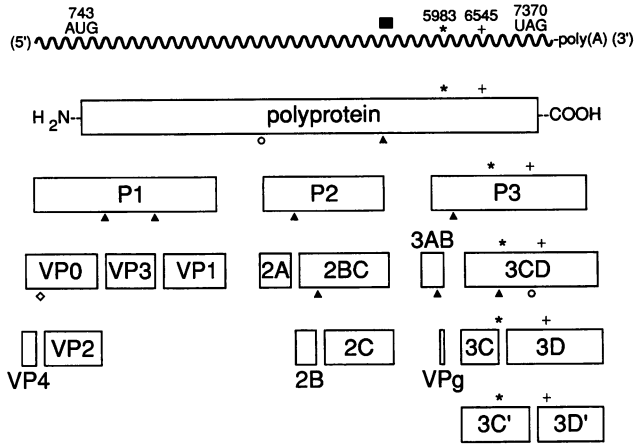

FIG. 1. Poliovirus polyprotein processing map. Full-length message-sense RNA (wavy line) contains a single long open reading frame beginning at the AUG at position 743 and extending to the stop codon at nt 7370 for poliovirus type 1 (Mahoney). The polyprotein precursor is autocatalytically cleaved by two viral proteinases: 3C, which cleaves at the sites indicated by solid triangles, and 2A, which cleaves at the sites indicated by open circles. An unknown proteinase carries out the cleavage marked by the open diamond during viral maturation. The products of P1 processing are structural proteins; the products of P2 and P3 cleavage are nonstructural proteins, including the two proteinases and the viral polymerase, 3D. Also shown above the RNA (and protein) are the positions of the mutations discussed in the text. The asterisk indicates the site of 3-nt (serine) insertion mutagenesis in the μ10 template and site of a further 15-nt (five amino acid) insertion in revertant virus μ10-1; the plus sign marks the location of the second-site change in revertant μ10-51. A region of rRNA sequence complementarity (see text) is also shown by the shaded box above the RNA.

RESULTS AND DISCUSSION

The poliovirus genome is a single-stranded, infectious (i.e., message sense) RNA molecule which encodes a single long open reading frame. It is translated in infected cells into a polyprotein, one domain of which (3C) contains the viral proteinase responsible for most of the proteolytic processing of the polyprotein (9) (Fig. 1). As part of ongoing studies to understand the function of 3C, Semler et al. (30) and Ypma-Wong et al. (32) engineered a 3-nt *Hin*fI fill-in insertion mutation (μ10) near the 3′ end of the 3C coding sequence in a cDNA clone of genomic RNA at nt 5981 (*Hin*fI site at nt 5980). Polyprotein synthesized in vitro from the mutated template demonstrated normal proteolytic processing at all 3C-specific cleavage sites except the one at the carboxy end of 3C itself (i.e., between the 3C proteinase and 3D polymerase). No evidence of processing at that site was observed.

RNA templates bearing the above mutation at nt 5981 were introduced into HeLa cell monolayers by transfection and were deemed nonviable, since no plaques were visible after 4 days of incubation under agar at either 33 or 37°C. Wild-type control plaques were visible 2 to 3 days after transfection. It has been our experience that plaques arising more than 3 days after transfection usually represent revertants rather than slow-growing mutants containing the original lesion. Indeed, plaques did become visible 5 days after transfection with mutated RNA at 33°C, and in parallel transfection mixtures overlaid with liquid medium, cytopathic effects were evident after 4 days at 33°C. Virus was isolated and plaque purified.

One plaque isolate, μ10-51, was found to be highly temperature sensitive (*ts*) for growth at 39°C (data not shown). Polyprotein synthesized in cells infected by μ10-51, unlike polyprotein whose synthesis was directed in vitro by RNA transcripts of the original mutagenized cDNA (32), was processed normally at all 3C-specific cleavage sites (data not shown). As described in the legend to Fig. 2, μ10-51 virion RNA was sequenced and found to contain three changes from the parental sequence: (i) an exact reversion at the mutagenesis site (deletion of 3 nt), (ii) an A-to-U transversion at position 5983 immediately adjacent to the mutagenesis site (resulting in a serine to arginine change in 3C; see Fig. 2A), and (iii) an A-to-G transition at position 6545 in the polymerase gene (resulting in a methionine to valine change).

A second isolate, μ10-1, was slightly *ts*, executed normal proteolytic cleavages at all 3C-specific cleavage sites except at the 3C-3D junction, where processing was more efficient than in the wild type, and demonstrated a marked shift in the electrophoretic mobility of 3C and 3C-containing protein precursors (Fig. 3). When this viral RNA was sequenced, it was discovered to contain a 15-nt insertion precisely at the site of the original mutation (Fig. 2). A search of GenBank for the 15-nt inserted sequence identified numerous 14 of 15 or 15 of 15 nt matches to large-subunit rRNA from various species, including human (nt 4477 to 4492 of human 28S RNA and nt 2572 to 2586 of *Escherichia coli* 23S rRNA; refer to Fig. 2A). Equally good alignments were found in the coding sequences of the human and porcine Na^+/K^+ ATPase alpha subunit, mouse heat shock protein 86, chicken alpha globin, and equine herpesvirus DNA helicase. No sequence identity with poliovirus plus- or minus-strand RNA or with the T7 transcription vector was identified. A search of GenBank with the complement of the 15-nt insertion produced no identities.

Although the original *Hin*fI μ10 mutation is lethal in that no virus containing the mutation could be recovered, we believe that the RNA must be capable of some minimal amount of replication in order to produce revertant progeny. The lethal phenotype of the original *Hin*fI μ10 mutation could be a result of one of several defects. First, the insertion of 3 nt at nt 5981 could disrupt an RNA secondary structure necessary for substrate recognition by the replicase. This explanation is made less likely by the existence of the viable μ10-1 mutant RNA, which contains, at the same site, an extra 15 nt of RNA sequence in addition to the engineered 3-nt insertion. A second possibility is that the insertion could adversely affect the ability of 3C to function as a proteinase, thereby indirectly affecting the assembly of the replication complex. This explanation, too, is made unlikely by the fact that the original mutation does not affect proteolytic processing at any other site but the 3C-3D junction (32), implying that the proteolytic function of the mutated 3C is intact. Third, the original mutation, by disrupting the 3C-3D cleavage junction, could result in insufficient production of 3D. Consistent with this possibility is the absence of radiographically detectable 3D on polyacrylamide gels of polyprotein synthesized from in vitro transcription-translation reactions programmed with the original *Hin*fI μ10 cDNA and allowed to undergo self-processing in vitro (32). Both μ10-1 and μ10-51 produce detectable 3D in tissue culture. However, very little 3D is absolutely required for RNA replication, as attested to by the existence of mutants such as Se1-3C-02 (5), which are viable despite very inefficient cleavage at the 3C-3D scissile bond.

An additional explanation for the lethal phenotype of *Hin*fI μ10 is that the engineered 3-nt insertion could adversely affect the nonproteolytic functions of 3C. Although the role of 3C in replication has not been defined, there is genetic evidence that the 3C domain of 3CD or another incompletely processed 3C-containing precursor polypeptide may be important for

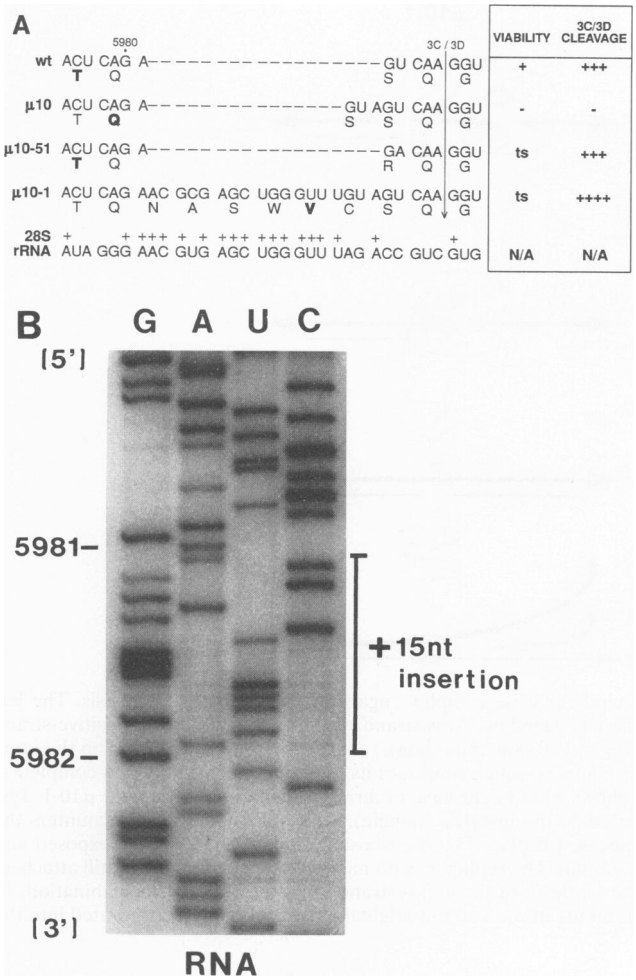

FIG. 2. RNA sequence analysis and comparison to rRNA sequences. (A) The top line marked wt is the nucleotide sequence of wild-type poliovirus type 1 (Mahoney) RNA in the region of interest (taken from reference 16). Mutagenesis of a *Hin*fI restriction enzyme site at nt 5980 of poliovirus cDNA resulted in the addition of 3 nt to the wild-type sequence and produced the RNA whose sequence is shown on the second line, marked μ10. The third and fourth lines show the RNA sequences of the mutants μ10-51 and μ10-1, respectively. The fifth line shows the sequence of human 28S rRNA (nt 4471 to 4503; taken from reference 7) where it aligns with the inserted nucleotides of μ10-1. The + signs above the rRNA sequence show nucleotide matches. Printed below each nucleotide sequence is the corresponding translated protein sequence in single-letter code. The P4 positions are shown in boldface. To the right of the sequences is a table indicating whether each RNA was nonviable (−) or led to the recovery of viruses that were viable (+) or *ts*; also shown is the relative degree of processing of the 3C-3D peptide bond (+, proteolytic products just detectable; ++, proteolysis less complete than wild type; +++, comparable to wild type; ++++, more complete than wild type). N/A, not applicable to the rRNA. (B) RNA sequence analysis of virus μ10-1. A portion of viral RNA sequence (oriented top to bottom in the 5′ to 3′ direction) was determined by the dideoxynucleotide method with reverse transcriptase. The numbers to the left of the lanes refer to wild-type poliovirus (type 1) RNA sequence numbers. The figure shows an autoradiogram of an 8% polyacrylamide–7 M urea gel.

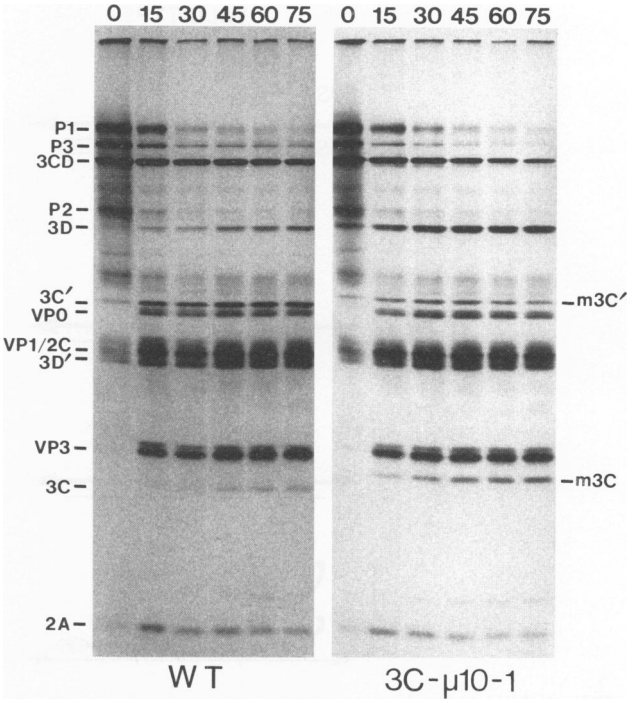

FIG. 3. Protein processing in HeLa cells infected with μ10-1. A pulse-chase labeling experiment with [^{35}S]methionine was performed essentially as described before (4) with HeLa S3 suspension cultures infected with μ10-1. The figure displays an autoradiogram of an SDS–12.5% polyacrylamide gel. The right panel shows protein from μ10-1 infections; the left panel shows protein from wild-type (WT) poliovirus (PV1 Mahoney) infections. Numbers above each lane indicate minutes into the chase. To the left of the lanes are the designations of the virus-specific proteins produced in infected cells.

replication (1, 6). The existence of the μ10-1 phenotypic revertant argues against this hypothesis, however, since 3C from μ10-1 RNA carries out its nonproteolytic functions despite the addition at the same site of six amino acids not present in the wild-type 3C. Finally, it is possible that the engineered mutation in 3C adversely affects the folding or function of the adjacent 3D amino acid sequences in the context of 3CD or other precursor polypeptides (without affecting the functions of 3C, as argued above).

Proteinase 3C of poliovirus is highly specific in its choice of protein substrates. The poliovirus polyprotein undergoes 3C-mediated cleavage at nine sites between glutamine and glycine amino acid residues. However, not all glutamine-glycine linkages in the polyprotein are cleaved. Thus, in addition to the Q-G amino acid pair, other determinants of 3C substrate recognition must exist. The majority of poliovirus 3C polyprotein cleavage sites contain a small hydrophobic amino acid as the fourth amino acid N-terminal to the site (the P4 position) (for a review, see reference 18). While these amino acid sequences alone are neither necessary nor sufficient to determine completely a site of 3C cleavage, changes away from the consensus have been found to decrease the efficiency of cleavage at the altered site (27, 33).

In experiments which changed the P4 residue of the 3C cleavage site at the VP3-VP1 junction, Blair and Semler (2) demonstrated that cleavage was most efficient when the P4 residue was alanine, the P4 residue found at the wild-type VP3-VP1 site. Many of the 3C cleavage sites in the poliovirus polyprotein as well as the predicted 3C cleavage sites in the polyproteins of coxsackievirus B3 and human rhinovirus 14 do, in fact, contain an alanine as the P4 amino acid (3, 18, 20). The poliovirus polyprotein 3C-3D cleavage site, however, is differ-

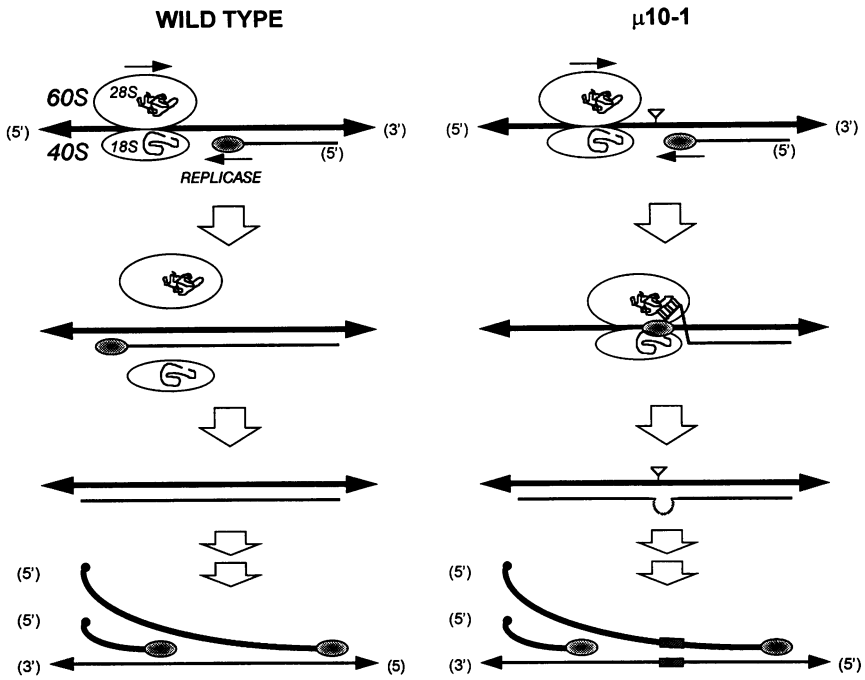

FIG. 4. Model depicting interaction between translating ribosomes and viral replicase complex engaged in minus-strand synthesis. The left panel portrays what might normally occur when a leftward-moving viral replicase engaged in minus-strand synthesis (thin line) on a positive-strand viral RNA template (heavy line) encounters a rightward-moving ribosome (60S and 40S subunits shown) engaged in protein synthesis on the same RNA template. The ribosome is displaced from the template by the replicase complex, which continues its movement leftward. Upon completion of the minus-strand synthesis, plus-strand synthesis ensues. The right panel shows what might have occurred to generate the mutant μ10-1. Just as it is approaching the site of the mutagenesis lesion (3-nt insertion, indicated by the inverted triangle), the replicase complex encounters the ribosome. What follows is a template switching event in which the viral replicase (displaced by the ribosome?) begins copying an exposed and accessible segment of the 28S rRNA which forms a part of the 60S ribosomal subunit. The replicase, with nascent minus-strand RNA still attached, then returns to the original or to another viral RNA template to complete the synthesis of the minus strand. As a result of the recombination, 15 nt of rRNA sequence (shown as a mismatch bubble between the recombinant minus strand and the original plus strand) are incorporated into the minus strand and then into progeny plus strands.

ent in that a threonine residue is present at the P4 position. Accordingly, processing at the 3C-3D site has been observed to be less efficient than at other polyprotein cleavage sites.

In the original *Hin*fI μ10 mutation, the 3 nt inserted at nt 5981 introduced a single amino acid between the original P4 (threonine) position of the 3C-3D cleavage site and the scissile bond. The mutation resulted in the occupation of the P4 position by a glutamine residue, which could explain the failure to observe any 3C-3D cleavage in in vitro assays. By acquiring 15 nt of exogenous RNA, five extra amino acids were gained in addition to the one inserted by mutagenesis. Consequently, a valine occupied the P4 position instead of the glutamine encoded by the original mutation (refer to Fig. 2). Valine, unlike glutamine, is one of the hydrophobic amino acids that occurs naturally at the P4 position of some 3C cleavage sites in the poliovirus polyprotein. In fact, the 3C-3D cleavage site of the μ10-1 polyprotein was actually processed by 3C more efficiently than in the wild-type polyprotein. This effect can be seen in the autoradiogram of a protein gel depicted in Fig. 3. The left panel of Fig. 3 shows the extent of processing, at 15-min intervals, of proteins that were synthesized in a 5-min period during which cells infected with wild-type virus were pulse labeled with [^{35}S]methionine. The right panel displays a similar analysis of protein from μ10-1-infected cells. Note the more rapid appearance and enhanced accumulation of 3C and 3D and the more rapid and complete disappearance of the P3 precursor in cells infected by the mutant virus than in cells infected by wild-type poliovirus. Enhanced proteolysis was not observed at any other sites, indicating that the mutant 3C is not simply a more efficient proteinase.

There are several possible explanations that could account for the origin of the 15-nt insertion in μ10-1. First, it is possible that the 15-nt insertion was present prior to its introduction into HeLa cells by transfection, although the T7 template from which the transfected RNA was derived was sequenced in the region of the mutagenesis site and found to contain only the predicted original 3-nt insertion. A second possibility is that the inserted 15 nt were added randomly following RNA transfection of HeLa cells in a template-independent fashion by the viral RNA polymerase. This seems unlikely, particularly in light of the considerable evidence that wild-type poliovirus polymerase is strictly RNA template dependent (29). A third possible origin of the 15-nt insertion is recombination between viral and cellular RNA. Of the several GenBank matches that were identified, those that pertain to genes of species such as mouse, pig, chicken, and equine herpesvirus can be dismissed as irrelevant. The large-subunit rRNAs are highly conserved as a group among organisms as diverse as eukaryotes, prokaryotes, and archaebacteria, and numerous rRNA sequence matches were found. Of the rRNA matches, only human 28S rRNA and possibly *E. coli* 23S rRNA are considered relevant to this discussion. A 14 of 15 match with human 28S rRNA was identified, as shown in Fig. 2A; the inserted sequence aligned with *E. coli* 23S rRNA in 15 of 15 positions. Finally, the search

identified a 14 of 15 match with a portion of the human Na^+/K^+ ATPase alpha subunit. We favor the hypothesis that the 15-nt insertion in μ10-1 arose by RNA recombination with human 28S RNA because rRNA is much more abundant than any mRNA (including that of the Na^+/K^+ ATPase alpha subunit). In addition, there are compelling reasons to expect that viral RNA synthesis and protein synthesis occur in proximity to each other within the cytoplasm of the infected cell (see below).

With the exception of retrovirus transduction of cellular proto-oncogene sequences, recombination between viral RNA and host RNA has only been demonstrated in a few other cases (13, 21, 23–25). The present case of recombination involving poliovirus enabled the recombinant μ10-1 virus to escape lethality, probably by restoring the P4 cleavage determinant at the 3C-3D junction. Such a recombination event suggests proximity in the cell between replicating viral minus-strand RNA and rRNA. In this regard, it should be noted that viral plus strands serve as templates for both protein synthesis and minus-strand RNA synthesis. This scenario is depicted in the model shown in Fig. 4. If both processes were to occur at the same time, the replication complex traveling in a 3′ to 5′ direction with respect to viral plus strands and a ribosome moving in a 5′ to 3′ direction would at some point have to occupy the same space, presumably resulting in the displacement of either the ribosome or the replication complex. That the inserted rRNA sequences in μ10-1 are of positive polarity is consistent with a copy choice model (15, 28), in which replicase, during the synthesis of minus strands, dissociated from the plus-strand viral RNA template and switched to the rRNA, producing a negative-sense recombinant template. This is in accord with the available evidence, which suggests that homologous recombination in poliovirus-infected cells usually occurs during minus-strand RNA synthesis (12, 15). It is also in accord with protection and chemical cross-linking studies of large-subunit rRNA, which indicate that the 3′-most nucleotides of the 15-nt segment of the rRNA template (i.e., the start point of RNA synthesis on the rRNA template following template switching) may normally be exposed and available for base pairing (26). Two of these bases, U_{2584} and U_{2585} (numbers refer to nucleotide positions in *E. coli* 23S rRNA), form part of the peptidyltransferase center of the ribosome and normally make contacts with the 3′ end of tRNA. Thus, it is possible that translating ribosomes contact and prematurely displace replication complexes engaged in minus-strand synthesis, perhaps facilitating recombination events (see model, Fig. 4).

An additional observation that suggests a physical association between poliovirus RNA and 28S rRNA was reported by McClure and Perrault (22). These investigators found that poliovirus plus-strand RNA shares an extended region of sequence complementarity with 28S rRNA (poliovirus nt 5075 to 5250; 28S rRNA nt 1097 to 1200) which may have enabled an interaction between the two RNA molecules. It must be emphasized that the recombination itself did not involve these sequences. Indeed, sequence comparisons of the 28S rRNA sequence and the region of poliovirus RNA immediately surrounding the recombination site did not disclose any sequence similarities, indicating that a nonhomologous recombination event had occurred. While we cannot presently account for the precise mechanism which produced the nonreciprocal RNA sequence exchange reported in this study, the ability of RNA viruses to recombine with host RNA provides yet another mechanism of sequence diversification available to these already proven masters of variability and adaptation.

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