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Biased Hypermutation and Other Genetic Changes in Defective Measles Viruses in Human Brain Infections

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Summary

We assessed the alterations of viral gene expression occurring during persistent infections by cloning full-length transcripts of measles virus (MV) genes from brain autopsies of two subacute sclerosing panencephalitis patients and one measles inclusion body encephalitis (MIBE) patient. The sequence of these MV genes revealed that, most likely, almost 2% of the nucleotides were mutated during persistence, and 35% of these differences resulted in amino acid changes. One of these nucleotide substitutions and one deletion resulted in alteration of the reading frames of two fusion genes, as confirmed by in vitro translation of synthetic mRNAs. One cluster of mutations was exceptional; in the matrix gene of the MIBE case, 50% of the U residues were changed to C, which might result from a highly biased copying event exclusively affecting this gene. We propose that the cluster of mutations in the MIBE case, and other combinations of mutations in other cases, favored propagation of MV infections in brain cells by conferring a selective advantage to the mutated genomes.

Introduction

Subacute sclerosing panencephalitis (SSPE) is among the most thoroughly studied persistent viral infections of the human central nervous system and serves as a model for analysis of the development of persistent viral infections known or suspected to cause several human syndromes, including multiple sclerosis (ter Meulen et al., 1983; Kristensson and Norrby, 1986; Dowling et al., 1986). SSPE generally develops 5 to 10 years after acute measles, starting with subtle signs of intellectual and psychological dysfunctions, continuing with sensory and motor function deterioration and progressive cerebral degeneration, and leading to death after months or years (ter Meulen et al., 1983). The measles inclusion body encephalitis (MIBE) clinical and virological manifestations are similar to those of SSPE, but the incubation time of MIBE can be shorter (Roos et al., 1981; Ohuchi et al., 1987; Baczko et al., 1988). Moreover, MIBE is found in immunosuppressed patients, whereas SSPE patients mount high antibody titers against all measles virus (MV) proteins except matrix (M protein; Hall et al., 1979). M protein is responsible for viral assembly, and it was postulated that silencing of M protein expression could account for lack of viral budding and favor persistence (Hall et al., 1979). Indeed initially, M protein could not be detected in brain tissue of SSPE patients (Hall and Choppin, 1981), but in recent studies using monoclonal antibodies, M protein was found in diseased human brains where the viral envelope proteins fusion (F) and hemagglutinin (H) could not be detected (Norrby et al., 1985; Baczko et al., 1986). Thus, defective M protein expression might not be the only viral determinant correlating with persistence.

The study of the molecular basis for defective MV gene expression in SSPE concentrated initially on the cell-associated, defective MVs that can be occasionally obtained by cocultivation of brain cells of SSPE patients with stable cell lines (Wechsler and Fields, 1978; Hall et al., 1979; Carter et al., 1983; Sheppard et al., 1986). However, the relevance of these observations for human brain infections remains to be established, since these viruses might not be truly representative of the viruses present in infected brains (Norrby et al., 1985). To assess the alterations of viral gene expression characteristic for MV persistence in diseased human brains, it appeared desirable to clone MV genes directly from brain tissue. Until now, this has been accomplished only for one M gene in one SSPE case, where it turned out that among many other alterations a point mutation introduced a stop codon at position 12 of the M reading frame (Cattaneo et al., 1986). In the present work, using a procedure allowing selective full-length cDNA cloning of MV RNAs (Schmid et al., 1987), and starting with only 2.5 μg of polyadenylated brain RNA, we achieved cloning of at least one transcript of all the viral genes, except the large polymerase gene, from two SSPE cases and one MIBE case. From examination of the three sets of nucleocapsid (N), phospho (P), M, F, and H genes, we determined that, on average, the MV genomes recovered from a single brain differ from each other in 20–30 of their 16,000 bases. We also estimated that in all three cases, 200–300 mutations have been fixed during the course of persistence. About 35% of these mutations resulted in amino acid substitutions; in one M and two F genes, reading frames were grossly or slightly changed, respectively. Remarkably, in the M gene of the MIBE case, but in no other gene, a cluster of transitions converted 50% of the U residues to C.

Results

Alterations of MV Proteins after Persistence

We have previously characterized MV gene expression in brain autopsies of two cases of SSPE (A and B) and one case of MIBE (C) by immunofluorescence analysis of brain sections with monoclonal antibodies and immunoprecipitation of MV proteins translated in vitro from brain RNA (Baczko et al., 1986, 1988). The N and P proteins, in-

volved in viral replication, were detected in all three cases. The M protein, responsible for viral assembly, was detected only in the brain of case A, the F protein only in case B, and the other envelope protein H only in case C. Since expression of the M, F, and H mRNAs is diminished in diseased brains (Cattaneo et al., 1987b), it is conceivable that the failure to detect the corresponding proteins could simply be because of low mRNA levels. To produce sufficient amounts of these RNAs, we cloned full-length cDNAs of these genes from the A, B, and C brains and from the reference MV Edmonston strain in an in vitro RNA expression vector (Experimental Procedures). Synthetic transcripts were then used to direct protein synthesis in a rabbit reticulocyte lysate.

Figure 1 is an analysis of the proteins produced from the N, M, F, and H synthetic transcripts of cases A, B, and C, as compared with the proteins produced from the synthetic transcripts of the Edmonston (E) strain. The M gene of case C produced only low levels of proteins considerably smaller than the Edmonston M protein (about 27 kd, 25 kd, and 17 kd instead of 36 kd). In contrast, the other proteins that had not been detected in brain autopsy materials (the F and H proteins of case A, the M and H proteins of case B, and the F protein of case C) were produced in amounts comparable to the Edmonston proteins and had approximately the expected size. This indicated that the reading frames of these genes were intact or only slightly modified. (Note that single amino acid substitutions could substantially change the mobility of a protein [Noel et al., 1979].) In the case of the F genes, differences in migration were greater than in other genes, amounting to apparently 4 kd between the most rapidly migrating protein (Figure 1, fusion gene, case B) and the one migrating most slowly (Figure 1, fusion gene, case C). This is at the upper limit of the differences in migration observed in the proteins of defective SSPE viruses (Hall et al., 1979).

To ascertain whether the reading frames of these genes were intact, we sequenced the ends of each clone used for expression analysis; in all genes except the M gene of case C (see below) and the F genes of cases A and B (Figure 2A), the signals for initiation and termination of protein synthesis were intact. In the F gene of case A, deletion of one nucleotide at position 2153 resulted in a shift in the reading frame causing substitution of the last 27 amino acids of the Edmonston F protein by 11 other residues (Figure 2A, nucleotide and amino acid positions as in the convention of Richardson et al. [1986]; this deletion was confirmed in two clones). This explains the higher electrophoretic mobility of this protein as noted above. The apparent molecular weight of the F protein of case B was even lower than that of case A (Figure 1, fusion gene, cases A and B). This was due to the introduction of a new stop codon (UAA) by a C to U mutation at position 2161, resulting in the expression of an F protein shortened by 24 amino acids (Figure 2A). Thus, two of the three F genes examined expressed an F protein with a mutated C terminus.

The active F protein of paramyxoviruses is liberated from its inactive precursor by endoproteolytic cleavage after a stretch of basic amino acids (Figure 2B, center), giving rise to a unique hydrophobic domain (Figure 2B, right; Varsanyi et al., 1985; Richardson et al., 1986; Glickman et al., 1988). It was demonstrated recently for the paramyxoviruses, as well as for influenza A virus and human immunodeficiency virus, that full expression of viral infectivity depends on the efficiency of proteolytic cleavage at this site (Webster and Rott, 1987; Glickman et al., 1988; McCune et al., 1988). To identify possible sequence alterations causing inefficient F protein cleavage and thus leading to the loss of lytic function typical for MV infections of human brains, we analyzed the region of the F cDNAs coding for the cleavage/activation site. However, this site was completely conserved in all three brain-derived F genes (Figure 2B, bottom).

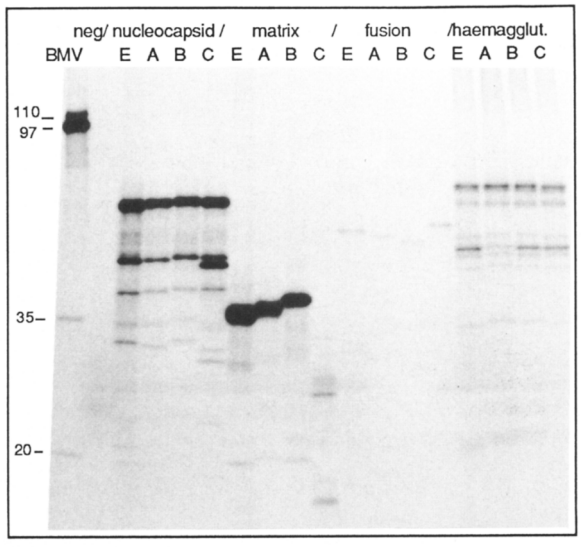


Figure 1. Analysis of the In Vitro Translation Products of Synthetic RNAs

About 200 ng of synthetic MV transcripts was translated in a rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of ^{35}S-labeled methionine (in vivo cell labeling grade, more than 1000 Ci/mmol, Amersham International, England). Equal amounts of the products of these reactions were loaded onto a protein gel (Laemmli, 1970), which was soaked in sodium salicylate, dried, and autoradiographed (Chamberlain, 1979). The RNAs translated were (from left to right): Brome mosaic virus RNA (BMV), yielding marker proteins of 110 kd, 97 kd, 35 kd, and 20 kd; no RNA (neg.): the transcripts of the genes and cases are indicated on the top. The apparent sizes of the N, M, F, and H protein products of the Edmonston strains are the expected ones for proteins translated in the system, 59 kd, 36 kd, 52 kd, and 69 kd, respectively (Hasel et al., 1987). Proteins of higher mobility were detected in addition to the full-size N and H proteins, as observed in a previously study (Hasel et al., 1987).

50% of U Residues Mutated to C in a Matrix Gene

To define the mutations introduced during persistence, ideally the sequences of the lytic viruses that infected the three children investigated should be available for comparison. It is however impossible to retrace these viruses, and comparison with the Edmonston strain sequence would not be valid since this virus has undergone numerous passages in chick embryos and cultured cells during the process of attenuation (Enders et al., 1960; Schwartz,

```
A.  Edm.  511 PALICCCRGRCNKKGEQVGMSRPGLKPDLTGTSKSYVRSL 550
    Case A      ..............R.NKLVCQDQA 535
    Case B      ................ 526
    Case C      ..................................... 550

B.  Paramyxovirus              carboxyterminus of F2 / aminoterminus of F1
                                          .         .         .
    Measles virus              RRHKR / FAGVVLAGAALGVATAAQITAGIAL
    Simian virus 5             ..RR. / .....IGL.........V..AV..
    Sendai virus               VPQS. / .F.A.IGTI......S.........
    Parainfluenza virus type 3 P.TKR / .F.G.IGTI......S.....AV..
    Newcastle disease virus    ..Q.. / .I.AIIG.V...........

    Measles virus - case A     ..... / ........................
    Measles virus - case B     ..... / ........................
    Measles virus - case C     ..... / ........................
```

Figure 2. Sequences from the F Protein of Paramyxoviruses

(A) Sequence of the carboxyl terminus of the F_1 protein of the Edmonston strain and of cases A, B, and C. The one-letter amino acid code was used. Dots indicate amino acids identical to the Edmonston sequence.

(B) Sequences of the cleavage-activation site of the F proteins of five paramyxoviruses and of cases A, B, and C. References as in Varsanyi et al. (1985) and Glickman et al. (1988). For details see text.

1962). To overcome this limitation as well as possible, we compared our data with a consensus M gene sequence, indicated in Figure 3 as PRE, which comprises the nucleotides represented most often in nine sequences determined experimentally (three sequences of lytic MVs and six of persistent MVs), as detailed in the legend to Figure 3. We will be calling the deviations from this consensus "mutations," although this term is not actually accurate.

As mentioned above, the M gene of case C was of particular interest because its protein product had an apparent molecular weight considerably lower than expected (Figure 1, matrix gene, case C). From the sequence analysis presented in Figure 3, it is immediately evident that mutations in this gene are more abundant than in other M genes and that U to C transitions account for a large majority of mutations. In fact, 132 of the 266 U residues encoded in the PRE sequence (that is about 50%) are changed to C in the case C sequence. This phenomenon resulted in the alteration of the M protein initiation signal (Figure 3, positions 37 and 186–188). Moreover, in clone pcM1, used for production of the synthetic RNAs, a nucleotide deletion at position 756 created a frameshift resulting in the introduction of a stop codon (TAG at position 793–795). Taken together, these two events should lead to the production of an altered M protein with a molecular mass of about 23 kd. Indeed, a major product of 25 kd is detected (Figure 1, matrix gene, case C).

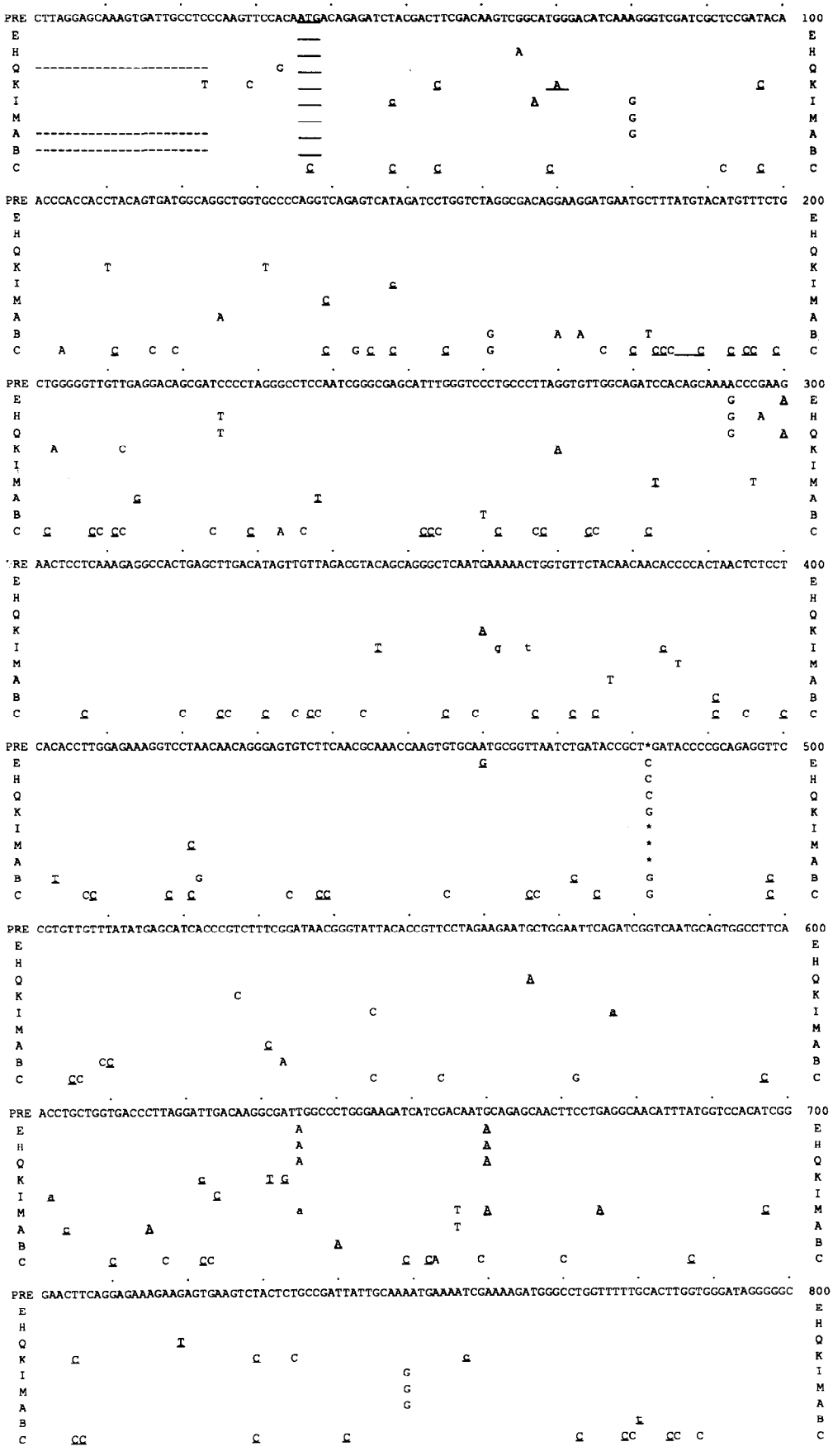
It is also of interest that several minor proteins were produced by the synthetic pcM1 transcripts. This was probably the result of initiation of translation on downstream AUG and upstream non-AUG codons, characterizing translation of genes possessing a "weak" AUG, like the one at position 186–188 of the M gene of case C (Kozak et al., 1983; Curran and Kolakofsky, 1988). The other four sequenced clones of this M gene did not contain nucleotide deletions, and their major protein products migrated approximately at the position of the M proteins of the other cases (data not shown). This was expected since the loss of 50 amino acids from the amino terminus should be compensated by the gain of 40 at the carboxyl terminus, the gain being due to the substitution of the termination signals at position 1041–1043 by a new signal at position 1161–1163 (both underlined in Figure 3).

Given the very high level of U to C transitions detected in the M gene of case C, we predicted that if these mutations had progressively accumulated during persistence, other genes of case C would also have accumulated similar transitions. To test this hypothesis, we sequenced the complete N gene and one third to one half of the P, F, and H genes. We also analyzed the corresponding genes of the A and B cases and compared them with consensus sequences as defined above (Figure 4 indicates the genomic areas sequenced). As shown in Table 1, in the M gene of case C the level of U to C transitions exceeded by a factor of at least 20 all other kinds of mutations, whereas, surprisingly, the levels of U to C transitions in all other genes of case C were comparable to the levels of the other mutations. To further investigate the distributions of U to C mutations in the MV genome, we also sequenced clones covering the whole M and part of the flanking genes (pcM4 and pcM6, legend to Figure 3). From the graphical representation of these analyses (Figure 4), it is evident that, whereas in the N, P, and H genes two or fewer transitions have been introduced per group of 20 Us, in the M gene between 4 and 16 changes have occurred. Interestingly, the switch between high and low levels of U to C transition was abrupt at the P–M gene junction but more gradual at the M–F gene junction; in the first 20 Us of the F gene, distributed over not less than 455 nucleotides, five U to C mutations were detected, whereas in the following groups of 20 Us, first three and then two or fewer mutations per group were detected. Thus, the limits of the genomic regions with high levels of U to C mutations roughly coincide with the limits of the M gene.

From Table 1, it is also evident that in the M gene of case C, the level of A to G mutations, corresponding to U to C mutations in the other MV genomic strand, was not enhanced. This indicates that the transitions must have been introduced exclusively in one strand, an event that could arise theoretically either by sequential, strand-specific cycles of localized and biased mutations or, more plausibly, by a single hypermutation event. By sequencing five sibling but not identical clones of the M gene of case C, we also noted that in the few positions that were variable between sibling clones (small letters in sequence C of Figure 3), we could not detect any U to C mutation, that is 132 of the 132 U to C transitions were conserved in all five M cDNAs of case C. We thus conclude that a single event, rather than a continuous, progressive introduction of U to C transitions, must account for the amazingly high level of U replacements.

MV Genomic Variability, Selective Pressures, and Fixation of Mutations

Previous studies indicate that during persistence, mutations are continuously introduced and fixed in MV genomes (Cattaneo et al., 1986, 1988). This phenomenon is


PRE CTTAGGAGCAAAGTGATTGCCTCCCAAGTTCCACAATGACAGAGATCTACGACTTCGACAAGTCGGCATGGGACATCAAAGGGTCGATCGCTCCGATACA 100
PRE ACCCACCACCTACAGTGATGGCAGGCTGGTGCCCCAGGTCAGAGTCATAGATCCTGGTCTAGGCGACAGGAAGGATGAATGCTTTATGTACATGTTTCTG 200
PRE CTGGGGGTTGTTGAGGACAGCGATCCCCTAGGGCCTCCAATCGGGCGAGCATTTGGGTCCCTGCCCTTAGGTGTTGGCAGATCCACAGCAAAACCCGAAG 300
PRE AACTCCTCAAAGAGGCCACTGAGCTTGACATAGTTGTTAGACGTACAGCAGGGCTCAATGAAAAACTGGTGTTCTACAACAACACCCCACTAACTCTCCT 400
PRE CACACCTTGGAGAAAGGTCCTAACAACAGGGAGTGTCTTCAACGCAAACCAAGTGTGCAATGCGGTTAATCTGATACCGCT*GATACCCCGCAGAGGTTC 500
PRE CGTGTTGTTTATATGAGCATCACCCGTCTTTCGGATAACGGGTATTACACCGTTCCTAGAAGAATGCTGGAATTCAGATCGGTCAATGCAGTGGCCTTCA 600
PRE ACCTGCTGGTGACCCTTAGGATTGACAAGGCGATTGGCCCTGGGAAGATCATCGACAATGCAGAGCAACTTCCTGAGGCAACATTTATGGTCCACATCGG 700
PRE GAACTTCAGGAGAAAGAAGAGTGAAGTCTACTCTGCCGATTATTGCAAAATGAAAATCGAAAAGATGGGCCTGGTTTTTGCACTTGGTGGGATAGGGGGC 800

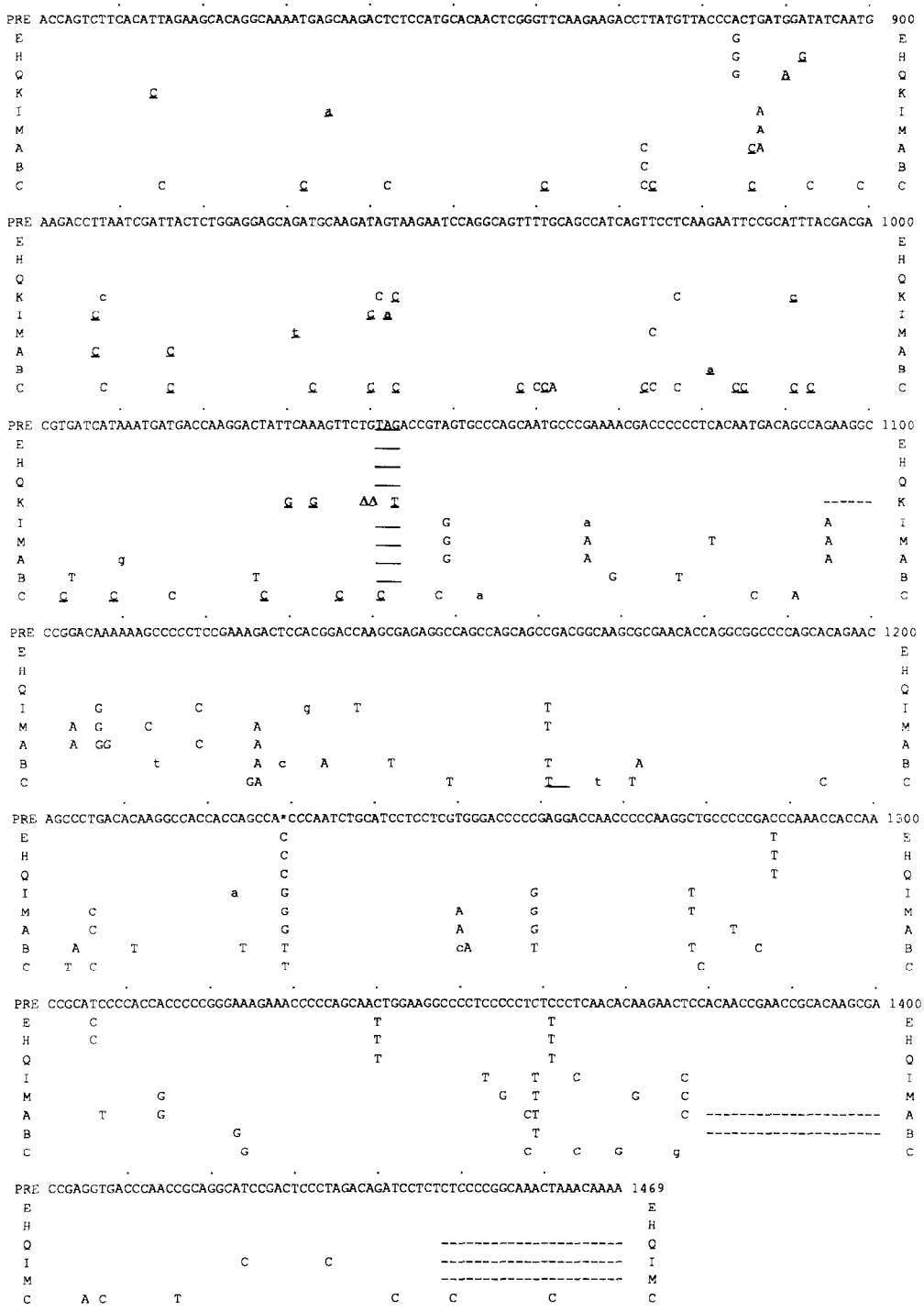


Figure 3. Sequences of the M Genes of Cases A, B, and C in Comparison with Other M Gene Sequences

The T residues in these cDNAs correspond to Us in the MV transcripts. Sequence E is from the Edmonston strain (Bellini et al., 1986), sequence H from the street virus HU2 (Curran and Rima, 1988), sequence Q from the strain CAM (this paper, see Liebert and ter Meulen [1987] for a description of this strain), sequence K from SSPE case K (Cattaneo et al., 1986), sequence I from SSPE cell line IP-3-Ca (Cattaneo et al., 1988), and sequence M from SSPE cell line MF (this paper, see Cattaneo et al. [1987a] for a description of case MF). The PRE sequence is a consensus comprising the nucleotides represented most often in the nine sequences obtained experimentally. The positions differing from the PRE sequence are indicated with capital letters (positions diverging in all clones of the same case), or small letters (positions diverging only in some sibling clones). The translation start and stop codons are underlined, as are the mutations leading to amino acid changes. An asterisk in PRE indicates a variable position for which no consensus could be defined. Position 482 was variable not only within cases but also within sibling clones: it corresponded to G or A in cases I and A and to G or C in case M. A 2 nucleotide deletion at position 1039–1040 in case K is indicated with two deltas. M clones resulting from the elongation of non-M 3′ primers hybridizing semispecifically to the GC-rich 3′ nontranslated region of the M gene were obtained and completely sequenced: clones paM1, paM2, paM3, pbM1, pbM2, pbM3, pcM1, pcM2, and pcM3 coded for M genes, respectively, 172, 172, 90, 90, 185, 396, 190, 632, and 99 nucleotides shorter at their 3′ end. Clones pcM6, encoded the whole M gene and additional 90 nucleotides of the neighboring F gene, and pcM4 encoded the whole M gene and part of the N and P genes. About 1% of the positions could not be defined because of "strong stops" in the sequencing reactions, and these positions were considered as showing no variation from the PRE sequence. The GenBank accession number of this sequence is J03175.

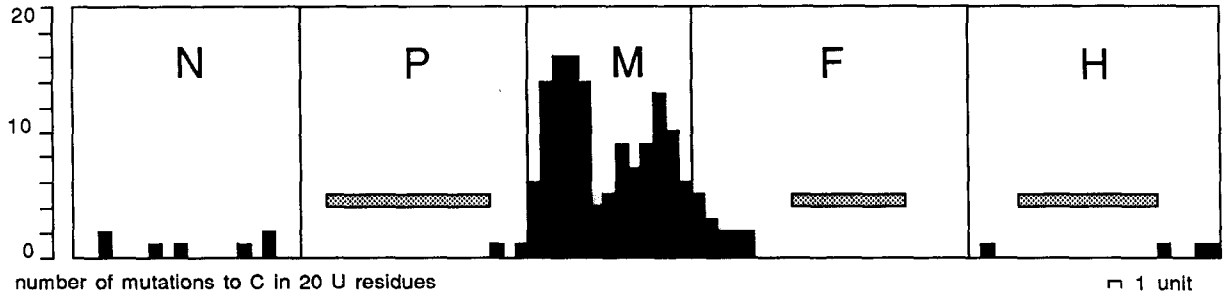


Figure 4. Distribution of U to C Changes in the MV Genome of Case C

About 9200 of the 16,000 nucleotides of the MV genome are represented on the horizontal axis, starting with the N gene and terminating with the H gene. Gene junctions are indicated by a vertical line. Groups of 20 consecutive U residues, corresponding in general to 60–120 nucleotides, have been considered as a unit and are represented with a standard width. The number of U residues mutated to C in each unit is indicated by the height of the corresponding black box and can be read on the scale on the left. Gray boxes indicate unsequenced parts of the genome.

Table 1. Quality of the Mutations from the Consensus (PRE) Sequence in Different MV Genes of Cases A, B, and C

		Transitions				Transversions							
Case	Gene	U→C	C→U	G→A	A→G	U→A	A→U	G→C	C→G	U→G	G→U	A→C	C→A
C	N	8	1	6	2	0	0	0	0	1	2	2	1
	P	2	2	2	0	0	0	0	0	0	0	0	0
	M	132	5	5	6	0	0	2	1	0	0	1	3
	F	13	14	7	6	0	0	0	0	1	0	1	2
	H	5	3	3	3	0	0	0	0	0	0	0	0
A	M	11	3	5	7	1	0	0	1	0	0	0	1
B	M	7	10	6	5	0	1	1	0	0	2	0	2

The PRE sequence of the M gene is shown in Figure 3; the other PRE sequences were constructed using the A, B, and C genes and the genes of case IP-3-Ca and of the Edmonston strain (Cattaneo et al., 1988, and references therein).

most likely based on one hand on the low fidelity of RNA replication (Domingo et al., 1978; for review see Steinhauer and Holland, 1987) and on the other hand on the low selective pressure exerted on viral genomes in nonlytic infections (Holland et al., 1979; Rowlands et al., 1980). In an attempt to quantify the level of internal variability of MV genomes in the human brain, we compared the sequences of three overlapping M clones of cases A and B and of five overlapping M clones of case C. Internal variability was 0.16% for the three M clones of case A (six differences over 3825 comparable nucleotide pairs), 0.18% for case B (six differences over 3361 pairs) and 0.06% for case C (seven differences over 11,217 pairs). Most of these changes are probably due to the MV polymerase itself rather than to the reverse transcriptase used for cloning, since clones obtained with the same technique from another RNA source differed in less than 0.02% of their positions (K. Baczko, unpublished data). It should be noted that the variability of the MV genomes in case C was lower than in the other two cases, which could be explained by the shorter time of viral persistence in the case of MIBE. This result reinforces the suggestion that at the final stage of the MIBE infection the MV polymerase was at least as precise as in the two SSPE infections. A variability of 0.15% will result in 20–30 differences between any two MV genomes with a length of 16,000 nucleotides, which is a high number even for an RNA virus (Steinhauer and Holland, 1987; Cattaneo et al., 1988).

To assess the variability in strains of lytic viruses, and to estimate the number of mutations introduced during persistence, we counted the differences of lytic and persistent viruses from a consensus as defined above. The lefthand panel of Figure 5 represents the mutations from the PRE consensus (Figure 3) detected in the M coding regions of the three lytic viruses, Edmonston (E), HU2 (H), and CAM (Q), and of the six persistent viruses, K, I, M, A, B, and C. In genes from lytic infections, 6–9 differences from the consensus were detected, whereas in genes from SSPE persistent infections, 15–25 differences were monitored (130 differences in the MIBE case C). Thus, we estimated that two to three times more mutations accumulated in the M coding regions of viruses implicated in persistent infections. From Table 2, it is clear that this holds true for all the others genes examined, with the exception of the N gene (legend to Table 2, note [c]). If we assume that the lytic viruses that initiated the three persistent infections had accumulated a number of differences from the PRE sequence similar to that of the three lytic strains studied, we can extrapolate that 50–70% of the mutations scored in cases A, B, and C accumulated during the persistent phase of these infections (this may be an underestimation, see the end of this section). Knowing that in the three persistent infections 442 mutations from the consensus sequence have been detected over 17,610 nucleotides compared (calculated from Table 2, first column), and assuming that 50%–70% of these mutations have

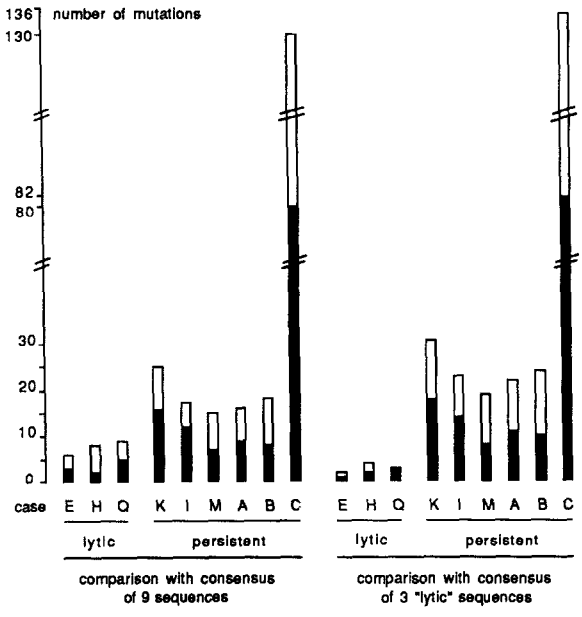


Figure 5. Expressed (Solid Boxes) and Silent (Open Boxes) Mutations from M Consensus Sequences in the Coding Regions of Three Lytic and Six Persistent MV Strains

The left part of the figure shows the comparison with the PRE sequence, constructed from all nine sequences available. The right part of the figure shows another comparison, this time with a consensus obtained only from the three lytic sequences (E, H, and Q, same symbols as in Figure 3). The M protein encompasses 335 amino acids.

been fixed during persistence, we can also extrapolate that 200–300 mutations have been introduced in the MV genome (16,000 nucleotides) during persistence. Using the cDNAs described here, it should be possible in principle to establish complementation assays to test the effect of single mutations on gene function, and thus to assess if the point mutations introduced during persistence resulted in slight alterations, gross distortions, or disruption of viral protein functions.

Examination of the MV proteins found in brain cells of different SSPE patients has shown examples of restricted expression of the F and H proteins, as well as of the M protein (Norrby et al., 1985; Baczko et al., 1986). In contrast, N and P proteins, the two proteins required together with the polymerase for MV transcription and replication, were always detected. The most straightforward explanation for these observations is that the constraints imposed in persistent infections on the M, F, and H genes are relaxed, since they encode viral functions generally presumed to be dispensable for replication (Rosenblatt et al., 1979). If this was the case, we would expect fixation of more mutations causing amino acid changes (replacement site mutations) in the viral envelope protein genes than in the N and P genes. As shown in Table 2 (first column), the levels of mutations accumulated in all genes were fairly similar (about 2%), except for the M gene of the MIBE case. In the F and H coding regions, respectively, 38% and 24% of the mutations resulted in amino acid changes, a similar

Table 2. Differences between the Genes of Cases A, B, and C and of the Edmonston Strain (E) and the PRE Genes

Gene	Case	Base Pairs Compared/ Differences (% Differences)	Replacement/Silent Mutations (Mutations in Noncoding Region)	% Mutations in Coding[a]/Noncoding[b] Regions
N	A	1655/14 (0.8)	4/9 (1)	0.8/1.3
	B	1655/25 (1.5)	7/16 (2)	1.5/2.6
	C	1655/23 (1.4)	8/15 (0)	1.4/0.0
	E	1655/22[c] (1.3)	15/6 (1)	1.3/1.3
P	A	616/14 (2.3)	4/5 (5)	1.9/5.6
	B	616/11 (1.8)	4/3 (4)	1.3/4.5
	C	616/6 (1.0)	0/3 (3)	0.6/3.4
	E	616/4 (0.6)	2/1 (1)	0.6/1.1
M	A	1356[d]/33 (2.4)	9/7 (17)	1.6/4.9
	B	1356[d]/38 (2.8)	8/11 (19)	1.9/5.6
	C	1469/155 (10.6)	80/50 (25)	12.9/5.4[e]
	E	1469/10 (0.7)	3/3 (4)	0.6/0.9
F	A	1483/25 (1.7)	4/7 (14)	1.3/2.1
	B	1483/26 (1.8)	3/5 (18)	1.0/2.7
	C	1483/44 (3.0)	6/9 (29)	1.8/4.3
	E	1483/11 (0.7)	0/2 (9)	0.2/1.3
H	A	741/9 (1.2)	1/7 (1)	1.2/1.8
	B	741/10 (1.3)	5/4 (1)	1.3/1.8
	C	741/14 (1.9)	1/11 (2)	1.8/3.6
	E	741/5 (0.7)	1/2 (2)	0.4/3.6

For this computation, two variable positions in the PRE sequence (marked with an * in Figure 3) were not considered.

[a] Total differences in the coding regions of the A, B, and C cases: N gene, 1.2%; P gene, 1.3%; M gene, 5.5% (1.7% if case C is not considered); F gene, 1.5%; and H gene, 1.4%.

[b] Total difference in all the noncoding regions of the three cases: 3.6%.

[c] The sequence of the Edmonston N gene diverges from the consensus in about twice as many positions as the sequences of the other Edmonston genes. It is remarkable that 18 of the 22 differences were concentrated in the last 700 bases.

[d] In these genes, 90 bases of the 3′ noncoding region and 23 of the 5′ noncoding region could not be determined.

[e] The relatively low incidence of mutations in the untranslated region of the M gene of case C is due to the scarcity of Us.

(in fact somewhat lower) percentage to the N and P coding regions (32% and 42%, calculated from the results presented in Table 2, second column). It should also be noted that, even without considering case C, the M genes had the highest percentage of replacement site mutations, that is 48%. The low number of replacement site mutations found in the H genes was reflected by the identical migration behavior of all H proteins (Figure 1, hemagglutinin genes), and the high number of amino acid changes found in the M genes by the relatively large differences in migration of the M proteins (Figure 1, matrix genes). These numbers suggest that the selective pressure acting on the genes directly involved in viral replication was not very different from that acting on the viral envelope genes during persistence. The fact that the differences from the consensus sequence were about twice as frequent in the untranslated regions of all the genes compared with the respective coding regions (Table 2, third column and notes [a] and [b]) reinforces the suggestion that selective pressures to preserve protein functions remained in effect.

On the basis of the sequence comparisons presented in Figure 3, the three lytic viruses fall in a separate subclass from the six persistent viruses. If an M gene consensus sequence is constructed by considering only the lytic viruses, each of the "lytic" M coding regions differs only in 2–4 positions from the "lytic consensus," in contrast with the persistent viruses differing in 19–31 positions (136 differences in case C; right panel of Figure 5). This suggests that the number of mutations introduced during persistence, which was estimated above to 50%–70% of the total differences from the PRE sequence, might in fact be as high as 80%–90%.

The definition of a "lytic consensus" different from the PRE sequence implies that lytic viruses can be distinguished from persistent viruses on the basis of their sequences at characteristic sites. This observation, if confirmed on a larger sample of genes, might have important practical applications: diagnostic differences might be applied for tracing the source of viruses causing measles epidemics or persistent infections. Moreover, vaccine strains could be selected on the basis of their sequences, and finally, safer vaccines possessing all genomic characteristics defined as favorable in different strains could be engineered.

Discussion

Mutations and Defective Expression of Measles Virus Genes in Persistent Infections

Previously, the occurrence of viral mutations in SSPE cases has been documented (Cattaneo et al., 1986, 1988). However, it was never clarified whether certain mutations constitute a prerequisite for the development of the disease. Mutations might simply be a corollary phenomenon, to be explained by the release of selective pressure exerted on viral genomes that need only replicate and spread from cell to cell but that do not have to provide all the functions necessary for the assembly of infectious virus particles. Although the present study still does not directly establish a causal relationship between mutations and disease, two experimental findings presented here strongly support this hypothesis: first, the mechanism of M gene function inactivation by hypermutation in the MIBE case; and second, the very extensive and apparently directed drift separating all three persisting MV genomes analyzed from the infecting viruses.

Defective expression of M protein has been previously revealed in SSPE cases (Wechsler and Fields, 1978; Hall et al., 1979; Carter et al., 1983). In particular, both complete absence of M protein (Hall and Choppin, 1981) or presence of nonfunctional (i.e., unstable) M protein have been reported (Sheppard et al., 1986). In the present study, both of these possibilities were found in the three cases analyzed. In case B, we could monitor the efficient in vitro production of an M protein of approximately correct size from synthetic transcripts of a cDNA clone, in spite of the fact that such a protein could not be detected in the brain autopsy of this patient (Baczko et al., 1986), an observation that can be explained by postulating rapid M protein degradation in vivo. In case C, no M protein could be produced: the proteins synthesized inefficiently in vitro from the synthetic M mRNAs had grossly altered termini and dozens of mutated amino acids. The particular interest of this case resides in the mechanism of M inactivation; the analysis of the M and four other genes of this case indicated that a single, biased hypermutation event was most likely responsible for the selective silencing of M gene function. Since a lytic virus with intact M function must have been at the origin of the MIBE infection, we must conclude that the hypermutation event did not severly affect the efficiency of this genome to replicate. Instead, this event must have conferred a selective advantage for the spread of the mutated genome in the brain, because only mutated genomes were detected at death. Thus, for case C, our study provides a direct correlation between M function silencing and mutational change, which in this case came about by a probably unique and grossly distorting event. In other words, it seems very likely that the propagation of this lethal infection in the human brain originated from a single genomic clone of MV.

Nevertheless, M function silencing might not be obligatory for persistence, as suggested by the detection of M proteins in some SSPE cases (Norrby et al., 1985; Baczko et al., 1986) including case A reported here, where an M protein of approximately the correct size was detected both in vitro and in vivo. However, we do not know whether these M proteins are functionally competent. On the other hand, in case A, the carboxyl terminus of the F protein has been structurally altered. A similar alteration of the F protein, mediated by a different mutation, was identified in case B. This indicates that F protein function might be slightly or severely impeded in both cases A and B. In summary, gross alterations have been found so far only in M proteins, less severe modifications in F proteins. It remains to be seen whether all these changes, and/or more subtle changes in these and other viral proteins, might not also contribute to propagation of MV persistent infections in brain cells.

The second argument in favor of the view that some mu-

tations are instrumental for the development of brain infections is provided by the features of the populations of viral genomes present in brains. In RNA virus populations maintained at constant selective pressures, genomic variability is high, but a stable consensus sequence is established that usually changes minimally (Domingo et al., 1978; Holland et al., 1979). In contrast, when selective pressures change, viral RNA genomes do not maintain the consensus, but rapidly evolve by selection of the fittest (Holland et al., 1979; Rowlands et al., 1980). In cases A and B, the populations of viral genomes show an internal variability about ten times lower than the estimated number of changes acquired during persistence (20–30 variable positions versus 200–300 acquired changes). In case C, the internal variability is even lower and the number of acquired changes is of the same order as in the other cases if the changes introduced by the hypermutational event are disregarded. This strongly supports the argument that mutated genomes must have been selected one or more times, conferring a direction to the evolution of the system. Thus, viral mutations might indeed favor viral persistence if, instead of compromizing propagation of infection, they promote it in the particular environment of brain cells. Obviously, such evolved viral genomes can never become manifest as new viral strains because they are unable to propagate beyond the life span of their host.

The fact that SSPE and MIBE arise so rarely might indicate that combinations of mutations favoring propagation of persistent infections are infrequent. Moreover, it may well be that persistent infections can be established only in cases where some host defense mechanisms fail (Fujinami and Oldstone, 1979; Carrigan and Kabacoff, 1987). This is also suggested by the fact that MIBE, a complication typical for immunosuppressed individuals, arises more frequently in such patients than SSPE in untreated individuals. Similar considerations might apply for other viral infections known or suspected to be the cause of several human syndromes (Wolinsky and Johnson, 1980).

Mechanism of Delimited Hypermutation: A Biased RNA Polymerase?

We are not aware of any other documented case where genetic information involving an entire gene has been distorted so drastically, most likely in a single event. The recently described extensive editing of kinetoplastid mitochondrial transcripts by uridine addition and deletion results in a spectacular modification of the mRNAs, but not in alteration of the gene (Shaw et al., 1988; Feagin et al., 1988). It must be mentioned, however, that a similar exclusive mutation of one type of nucloetide to another has been described, albeit in a much more restricted region, in the related vesicular stomatitis virus (VSV). In that instance, analogous A to G transitions (14 of 29 positions considered) were detected in a short region (51 nucleotides, intrinsically very rich in A residues) of a defective interfering (DI) genome (O'Hara et al., 1984; note that our U to C mutations, as written in plus strand polarity, might have been introduced in the minus strand genome as A to G transitions).

The question remains as to how the mutational cluster in the MV genome of the MIBE case could have arisen. In principle, mutations of this kind could be introduced either by chemical mutagens or by imprecise polymerases. The MIBE patient had been subjected to a large variety of immunosuppressive and cytostatic drugs, including potential mutagens (Roos et al., 1981). However, the level of mutations observed here is much higher than the level of mutations induced by any chemical mutagen (Singer and Kusmierek, 1982). Even assuming that a chemical mutagen in a living cell could induce mutations leading to the replacement of 50% of the U residues, it would be very difficult to explain how these mutations could have selectively affected a defined region of a nonsegmented genome. To account for this, homologous recombination of an MV "standard" genome with a hypermutated MV genome or an mRNA that coexisted in the same cell would have to be invoked. Homologous recombination involving breakage and joining between preexisting strands as with DNA is not documented for RNA. The apparent recombination events common in positive strand RNA viruses probably take place by a copy choice mechanism, in which the viral RNA polymerase switches template during RNA replication (King et al., 1982; Kirkegaard and Baltimore, 1986; Keck et al., 1988). In contrast, in negative strand RNA viruses, nonhomologous recombination is less common, and homologous recombination has not yet been reported (Jennings et al., 1983; O'Hara et al., 1984; for review see Steinhauer and Holland, 1987).

A much more plausible explanation of the observed phenomenon is that one particular part of a genome is synthesized by a biased MV RNA polymerase complex nonselectively incorporating U or C residues when copying an A. Two prerequisites have to be met in this model: first, biased MV polymerase complexes must occasionally occur in an infected cell; and second, biased and faithful polymerase complexes must act in succession during the synthesis of one progeny RNA on one RNA template. The polymerase complex of nonsegmented negative strand RNA viruses is composed of the large polymerase itself and a phosphoprotein, tightly associated with each other and with the genomic (or antigenomic) ribonucleocapsids, that is RNAs enwrapped with nucleocapsid protein molecules (Banerjee, 1987). It has been shown that the polymerases and phosphoproteins are distributed in discrete clusters on cytosolic ribonucleocapsids (Portner and Murti, 1986; Portner et al., 1988), and it is conceivable that these clusters correspond to polymerase complexes reorganizing during replication (or transcription), possibly by exchanging parts of their components. RNA polymerase complexes giving rise to biased errors could arise because they are constituted from genetically altered subunits, because normal subunits assemble in a defective fashion, or because normal RNA polymerase complexes can temporarily assume a distorted conformation. Evidence for the existence of conformationally "perturbed" RNA polymerase complexes, introducing either C or U residues when copying an A after a triggering error, but

then returning to the normal fidelity, was obtained from a VSV DI genome; when rare RNA molecules were analyzed in which a particular misincorporation had occurred, it was found that in a position situated two nucleotides downstream of the misincorporated nucleotide, in 20%–50% of the molecules, a C residue was incorporated instead of a U (Steinhauer and Holland, 1986). Remarkably, all other nucleotides, including more downstream U residues, were incorporated with normal precision. The stabilization of a "perturbed" conformation of the polymerase complex could result in nucleotide transitions in short (O'Hare et al., 1984) or longer (case C) genomic stretches of negative strand RNA viruses.

In an alternative version of this model, the coexistence of normal and genetically altered RNA polymerases on a single template, and a relay race of several polymerase complexes during replication is postulated. In this view, the growing end of the replicating MV genome might occasionally be taken over by an entirely new strand elongation complex, or single components of the complex might be exchanged. Such events could well constitute an intrinsic property of the polymerase reaction during the transcription mode of ribonucleocapsids, that is during the formation of single mRNA molecules from antigenomic templates where a stop-start mechanism of the polymerase at gene junctions has been postulated (for discussion see Gupta and Kingsbury, 1985). During replication, mode analogous exchanges might occur, either regularly at gene junctions or occasionally by mistake. It should be possible to ascertain such a patchwise mode of polymerization with in vitro transcription-replication systems.

Experimental Procedures

Patients

Patients A and B (patients 1 and 2 in Baczko et al., 1986) were 9- and 10-year-old children who showed the first SSPE symptoms years after primary MV infection and died 3 and 6 months later, respectively. Patient C was a 3-year-old child who died of MIBE 22 months after the diagnosis of leukemia, 4 months after clinical measles, and 2 months after the first symptoms of neurological disease (Roos et al., 1981).

Cloning

For selective full-length cDNA cloning of five MV-specific genes using specific primers (Schmid et al., 1987), 2.5 μg of polyadenylated brain RNA prepared as described (Cattaneo et al., 1987b) were used. Clones in pBluescript were identified by colony hybridization and restriction and amplified as described (Cattaneo et al., 1988). A large majority of the N, P, F, and H clones were full-length, but some of the M clones were incomplete at their 3′ end (legend to Figure 3). Another unexpected finding was that E. coli–containing plasmids in which the H gene was cloned downstream of the *lac* promoter of pBluescript grew reproducibly slower, reaching lower densities, and yielding only small quantities of plasmid DNA. This is most likely due to a deleterious effect of H protein for E. coli; when the H insert was in the "antisense" orientation as compared with the *lac* promoter, normal growth occurred.

In Vitro Transcription

In vitro transcription, in the presence of the cap analog diguanosine triphosphate (G(5′)ppp(5′)G; Pharmacia, Uppsala, Sweden) and minute amounts of [^{32}P]GTP to quantify synthesis, was accomplished with T3 polymerase according to the instructions of the supplier (Genofit, Geneva, Switzerland). In general, about 2 μg of transcripts were obtained from 1 μg of template using 0.5 mM concentrations of ribo-ATP, -CTP, and -TTP, 0.125 mM of ribo-GTP, and 0.625 mM G(5′)ppp(5′)G. This corresponds approximately to a 1–6 molar ratio of template to product. The products were about 90% full-length, as judged by gel electrophoresis.

Sequencing

Chain termination sequencing of alkali-denatured plasmid DNA was performed using deoxyadenosine 5′[^{35}S]thiotriphosphate (650 Ci/mmol, Amersham International, England) and T7 DNA polymerase (Sequenase™, United States Biochemical Corporation, Cleveland, OH) basically according to the protocol of the supplier. The primers used for sequencing the M and N genes were the same as those used in Cattaneo et al. (1988). Since one primer did not hybridize efficiently to the M clones of case C, it was substituted by primer (−) 772–753 (positions as in Bellini et al. [1986]). For sequencing the ends of the P, F, and H clones, commercial primers (New England Biolabs, Beverly, MA) hybridizing with flanking plasmid sequences were used. To sequence over the large F gene 5′ untranslated region, the AUG, and the F2/F1 processing site, two primers (+) 322–338 and (+) 625–640 were used (positions as in Richardson et al. [1986]).

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