JOURNAL OF VIROLOGY, Apr. 1988, p. 1388–1397
0022-538X/88/041388-10$02.00/0
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Vol. 62, No. 4

Multiple Viral Mutations Rather Than Host Factors Cause Defective Measles Virus Gene Expression in a Subacute Sclerosing Panencephalitis Cell Line

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Received 14 October 1987/Accepted 29 December 1987

A measles virus (MV) genome originally derived from brain cells of a subacute sclerosing panencephalitis patient expressed in IP-3-Ca cells an unstable MV matrix protein and was unable to produce virus particles. Transfection of this MV genome into other cell lines did not relieve these defects, showing that they are ultimately encoded by viral mutations. However, these defects were partially relieved in a weakly infectious virus which emerged from IP-3-Ca cells and which produced a matrix protein of intermediate stability. The sequences of several cDNAs related to the unstable and intermediately stable matrix proteins showed many differences in comparison with a stable matrix protein sequence and even appreciable heterogeneity among themselves. Nevertheless, partial restoration of matrix protein stability coul be ascribed to a single additional amino acid change. From an examination of additional genes, we estimated that, on average, each MV genome in IP-3-Ca cells differs from the others in 30 to 40 of its 16,000 bases. The role of extreme variability of RNA virus genomes in persistent viral infections is discussed in the context of the pathogenesis of subacute sclerosing panencephalitis and of other human diseases of suspected viral etiology.

Subacute sclerosing panencephalitis (SSPE), progressive rubella panencephalitis, kuru, and Creutzfeld-Jakob disease are the four prototypic chronic neurological diseases of humans produced by slowly replicating infectious pathogens (50). The etiologic agent of SSPE is measles virus (MV), a conventional virus recognized as the cause of the classical epidemic, acute, self-limited, exanthematous illness of children. In those rare individuals who develop SSPE, 5 to 10 quiescent years may elapse between the typical acute MV infection and the onset of subtle signs of intellectual or psychological dysfunction or both that signal the presence of persistent MV infection of brain cells. The subsequent overt phase of the disease lasts from months to years, proceeding inexorably through characteristic clinical stages of sensory and motor function deterioration, progressive cerebral degeneration, and ultimately death (46).

MV lacks the enzymatic machinery to reverse transcribe its RNA genome and thus cannot integrate DNA copies of its genomic sequences into host cellular DNA. Nevertheless, this virus survives in brain cells, escaping apparently normal or even exaggerated host defenses typified by exceptionally high MV antibody titers in both serum and cerebrospinal fluid, as well as by elevated interferon levels (11, 46). SSPE is distinguished by the failure of persistently infected brain cells to produce progeny virion particles. Cocultivation of SSPE brain material with permissive tissue culture cells only occasionally leads to the recovery of an infectious MV or to the emergence of a persistently infected SSPE cell line, indicating that the defect responsible for defective MV reproduction is not readily overcome (49). The inability to complete the viral infectious cycle has been ascribed either to restriction by cellular factors (11) or to defects in the structure or the formation of MV gene products. Initially, studies of the nature of MV defects in cases of SSPE focused on the matrix (M) protein (23). Defects in M gene transcription (3, 14), M mRNA translation (12), and M protein stability (42, 43, 51) have been described. Recently, however, detailed analysis of MV gene expression in brain tissue from different patients with SSPE has expanded the repertoire of recognized defects to include each of the MV envelope gene products: hemagglutinin (H), fusion (F), and M proteins (2, 3, 30).

Viral RNA-dependent RNA polymerases are notoriously error prone, presumably because they lack proofreading function (18, 24). Mutant genomes resulting from replicative errors committed during acute infections with RNA viruses tend to be rapidly eliminated, although genetic heterogeneity is maintained because of the high mutation rate (19, 45). In contrast, constraints imposed on the survival of variant genomes arising during persistent infections appear to be relaxed (25, 26, 36). These considerations suggest that replicative infidelity, as well as survival, propagation, and coexistence of multiple genomic variants, should characterize the long-standing persistent MV infections of brain cells and thereby account both for the diversity of MV gene expression defects observed and for the various levels at which the restriction in viral gene expression may be exercised (3, 43). And, indeed, that prediction is supported by the recent demonstration that 4 of 400 nucleotides differ in two M cDNA clones generated from the MV RNA present in the brain of an SSPE patient (15). We now report the results of studies of a viral genome derived from the brain tissue of an SSPE patient and subsequently propagated for many generations in a nonlytic, strictly cell-associated fashion (10). We show that genomic variants differing in several nucleotides are readily produced during this persistent MV infection. We provide evidence that accumulated genomic mutations are likely to account for the unstable M protein

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expressed by this SSPE cell line. Furthermore, we describe the rescue of a compromised, but nevertheless infectious, virus whose M gene shows nucleotide changes that probably specify partial restoration of M protein stability. By extrapolating from sequence data, we estimated that, on average, the coreplicating MV genomes present in this SSPE cell line differ one from another in 30 to 40 of their approximately 16,000 nucleotides. Finally, in the course of these studies, we have noted an error in the published sequence of the Edmonston strain MV nucleocapsid (N) gene (37) which we now correct.

MATERIALS AND METHODS

Cells and virus. The IP-3-Ca cell line was generated, characterized, and generously provided by T. Burnstein of Purdue University (10). It has been propagated for over 90 passages without detectable phenotypic variation. The monkey kidney cell lines BSc-1, CV-1, and Vero, as well as the human cell lines HeLa and HEp-2, were propagated as monolayer cultures in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and glutamine. The Edmonston vaccine MV strain was propagated in HeLa (S3) suspension cell cultures as described previously (47).

Isolation of virus 3-1 and analysis of its protein products. The infectious SSPE virus designated 3-1 was isolated from IP-3-Ca cells at passage 60. Its recovery relied on the capacity of ultrafiltrates (0.45-μm-pore membrane filter; Millipore Corp.) prepared from sonically disrupted IP-3-Ca cells to produce MV-like cytopathology in Vero cells. Such ultrafiltrates, after 10-fold dilution with Dulbecco modified Eagle medium (2% fetal calf serum), were allowed to adsorb for 2 h at 32.5°C to parallel, barely confluent Vero cell monolayers in 60-mm-diameter tissue culture dishes. The monolayers were washed with prewarmed medium, overlaid with Dulbecco modified Eagle medium, and incubated at 37°C. In one of multiple attempts, each involving Vero cells exposed to the ultrafiltrate of 10^8 sonicated IP-3-Ca cells, microscopic evidence of viral infection was noted. Cytopathology progressed to involve most of the monolayer after 14 days, when cells were collected, sonicated, and filtered through a 0.45-μm-pore membrane once again. The filtrate was blindly passaged onto Vero cells, in which another slowly progressing viral infection developed. Infectious virus was plaque purified, and a virus stock was developed by serial propagation in Vero cells at an input multiplicity of infection that never exceeded 0.001 PFU per cell. After eight passages in Vero cells, the titer of virus 3-1 achieved its maximum level of about 5×10^4 PFU per flask or about 10 new infectious virus particles from each initially infected cell.

For protein analysis, monolayers infected with virus 3-1 at the maximal achievable multiplicity of infection were incubated for about 15 days with frequent medium changes, until about 25% of the monolayer displayed cytopathic effect. The infected culture was then metabolically labeled with medium containing [^{35}S]methionine (800 μCi/ml) for 48 h. Cell extracts were immunoprecipitated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the accumulated radiolabeled polypeptides were detected by fluorography (42, 43).

Isolation of intracellular MV RNPs for transfection. MV ribonucleocapsid particles (RNPs) were isolated from IP-3-Ca cells between passages 56 and 65, essentially according to Rozenblatt et al. (38). Cells were prelabeled with [^{3}H]uridine (20 μCi/ml) for 24 h prior to harvest to provide a tracer for purification. Cells (2×10^8 to 5×10^8) were collected from confluent monolayers, washed by repeated sedimentation and resuspension in ice-cold phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), suspended at a density of 2×10^7 to 3×10^7/ml in hypotonic lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$, 100-U/ml placental ribonuclease inhibitor), allowed to swell for 15 min, and disrupted by addition of Nonidet P-40 to 0.65% (wt/vol) and 20 strokes in a 0.0015-inch (0.0038-cm) tolerance Dounce homogenizer. These and all subsequent steps were performed at 4°C. Nuclei were removed by sedimentation at $1,000 \times g$ for 5 min, and the supernatant was saved, adjusted to 2 mM EDTA and 2% Nonidet P-40, and centrifuged in SW27.1 polyallomar tubes containing a discontinuous gradient of sucrose in 10 mM HEPES (pH 7.1), 1 mM EDTA, and 20-U/ml placental ribonuclease inhibitor. Gradients of sucrose layers (4 ml of 40% [wt/wt], 3.5 ml of 30%, and 2.5 ml of 20%) were centrifuged at 23,000 rpm for 2.5 h and fractionated, and the MV RNP band in the region of the 30 to 40% sucrose interface was identified by A_{260} and [^{3}H]uridine counts per minute. The corresponding fractions were pooled, and the protein content was determined (8). When necessary, the preparation was concentrated by centrifugation on a 60% sucrose cushion. Edmonston MV RNPs were prepared from cytoplasmic extracts of acutely infected HeLa suspension cells (48) by using the same protocol. The morphological integrity and relative purity of RNP preparations were confirmed by electron microscopic visualization of samples, spread and shadowed after brief fixation with glutaraldehyde (5% [wt/vol]; pH 7), and by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of their protein composition.

Transfection of permissive cultured cells with MV RNP and analysis of protein products. Coprecipitates of MV RNP and calcium phosphate were prepared and applied to barely confluent cell monolayers in 35-mm-diameter tissue culture dishes (22, 38). After 5 h of absorption, the precipitates were aspirated and the cultures were incubated in Dulbecco modified Eagle medium. Culture medium was collected every 3 to 4 days, stored at −70°C, and subsequently examined for the presence of infectious virus by plaque assay (47). Development of MV-induced cytopathic effect was monitored by phase-contrast microscopy. For MV protein analysis, cell monolayers washed extensively with ice-cold phosphate-buffered saline were disrupted and collected in gel sample buffer (27). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (43). Resolved proteins were electrophoretically transferred to a nitrocellulose membrane (0.2-μm pore size) which was then blocked with bovine serum albumin or nonfat dry milk (9). This template was probed with hyperimmune rabbit anti-MV serum (43), and the presence of reactive MV proteins was revealed with autoradiographic detection of [^{125}I]protein A bound to the antibodies (9).

RNA preparation and cDNA cloning. For cDNA cloning, RNA was prepared from IP-3-Ca cells amplified two additional passages (passage 62) beyond that at which virus 3-1 was recovered (passage 60). Virus 3-1 was propagated in Vero cells to passage 10 to generate a stock adequate to infect the cells necessary for RNA preparation. In both cases, cells were solubilized in guanidinium isothiocyanate and the total cellular RNA was collected by sedimentation through 5.7 M CsCl (17). The RNA preparations were

treated with RNase-free DNase and proteinase K (14), and poly(A) RNA was selected (28) by one passage over a column of oligo(dT) cellulose (type 3; Collaborative Research, Inc., Waltham, Mass.). The method used for selective full-length cDNA cloning was described in detail for line IP-3-Ca RNA (41) and was performed in an analogous way with RNA from virus 3-1-infected cells.

Plasmid DNA preparation and sequencing. To obtain good results on long-range chain termination sequencing of double-stranded plasmid DNA (16), a plasmid preparation protocol including RNase digestion and alkali treatment was used. For this, bacterial cultures (25 ml) were grown for more than 20 h with vigorous shaking. Bacteria were pelleted and resuspended in 400 μl of 50 mM Tris [pH 7.8]. Lysis was achieved by addition of 125 μl of 10-mg/ml lysozyme (freshly prepared in 50 mM Tris [pH 8.0]) followed by shaking and incubation for 10 min at 0°C, addition of 150 μl of 0.5 M EDTA followed by shaking and incubation for 10 min at 0°C, and, finally, addition of 2% Triton X-100 followed by shaking and incubation for 60 min at 0°C. After centrifugation for 1 h at 15,000 × *g* at 4°C, the supernatant was collected and extracted with phenol and then with chloroform. A total of 0.025 volumes (V) of 5 M NaCl and 0.8 V of isopropanol were added, and DNA was allowed to precipitate for 30 min at 0°C, pelleted, and dissolved in 125 μl of 10 mM Tris (pH 7.5)-1 mM EDTA (TE). This solution was treated with 50 μg of pancreatic RNase per ml for 40 min at 37°C, and DNA was precipitated by the addition of 0.3 V of 5 M NaCl and 0.3 V of 30% polyethylene glycol 6000 and allowed to stand overnight at 0°C. After the DNA was pelleted by centrifugation, the supernatant was carefully removed and the pellet was dissolved in 75 μl of TE. To ensure complete deproteinization, sodium dodecyl sulfate and pronase were added to 0.5% and 0.4 mg/ml, respectively, and the digestion mixture was incubated for 30 min at 37°C. To eliminate contaminating cellular DNA, an equal volume of pH 12.5 buffer (50 mM NaCl, 40 mM NaOH; made fresh) was added. The mixture was incubated for 10 min at room temperature and neutralized by adding Tris (pH 7.5) to 50 mM. NaCl was added to a concentration of 0.5 M, the mixture was extracted twice with phenol and once with chloroform, and DNA was precipitated by addition of 2.2 V of ethanol. The precipitate was kept for 30 min at −20°C and pelleted. The pellet was washed twice with 80% ethanol and suspended in TE. With the plasmid Bluescript derivatives used here (41), yields of 50 to 100 μg of pure plasmid DNA from 25-ml cultures were routinely obtained.

The primers used for sequencing the M gene correspond to the following positions (see also Fig. 3): 2 to 22, plus strand; 241 to 257, plus strand; 683 to 704, plus strand; 744 to 723, minus strand; 925 to 943, plus strand; and 1467 to 1448, minus strand. The primers used for sequencing the N gene were the following (see also Fig. 5): 55 to 77, plus strand; 862 to 884, plus strand; 953 to 934, minus strand; and 1741 to 1723, minus strand. For sequencing the phosphoprotein (P), F, and H clones, commercial primers hybridizing flanking plasmid sequences were used. Sequencing was performed mostly with [α-^{35}S]dATP (650 Ci/mmol; Amersham International, England) and with the Klenow fragment of polymerase I (Boehringer GmbH, Mannheim, Federal Republic of Germany). The subsequent use of T7 DNA polymerase (Sequenase; U.S. Biochemical Corporation, Cleveland, Ohio) allowed the resolution of several positions which could not be defined by using the Klenow enzyme. To facilitate the evaluation of sequences, all the T elongation products obtained with the same primer on sibling clones were loaded side by side, as were the C, G, and A elongation products.

RESULTS

Transfer of IP-3-Ca genome into different host cells fails to alleviate the restriction of matrix protein expression. Unlike other examples of SSPE, the restricted M protein expression displayed by IP-3-Ca cells is not due to lack of M protein synthesis. Instead, it results from rapid degradation of an otherwise normally synthesized M protein (42, 43). Two mechanisms that might account for the instability of M protein expression were considered: first, that a host cell factor(s), such as an endogenous protease, selectively degrades an otherwise normal M protein, and second, that mutations incurred and accumulated within the M gene sequence in the course of persistent infection lead to an M protein that is inherently unstable or otherwise predisposed to proteolysis. To appraise the first possibility, the biologically active MV genome, present intracellularly in the form of an RNP, was removed from IP-3-Ca cells and transferred to other tissue culture cells in which reproduction of reference strains of MV is known to proceed efficiently. (From now on, we will refer to the MV genome originally propagated in IP-3-Ca cells as the IP-3-Ca genome and to its products as the IP-3-Ca proteins.) The calcium phosphate-mediated transfection technique of Rozenblatt et al. (38) was adopted for this purpose.

Western blot (immunoblot) analysis of the accumulation of the major viral structural protein, nucleocapsid (N), was used to evaluate the capacity of transfected Vero cells to support IP-3-Ca genome replication and viral gene product expression. Cells transfected with Edmonston MV RNP were examined in parallel. Results are shown in Fig. 1A. Both Edmonston and IP-3-Ca MV RNPs appeared to be transfected with comparable efficiency (see 0.5-day time points). Over the following 2 to 3 days, those cells transfected with Edmonston MV RNP supported the synthesis and accumulation of abundant amounts of N protein while experiencing extensive typical MV cytopathology (lanes on left). In contrast, cells transfected with IP-3-Ca MV RNP required 2 to 3 weeks to attain maximal levels of accumulated N protein, consistent with the slower and far less prolific development of small syncytia which failed to progress to cytolysis. The lysates derived from Vero cells 3 days after Edmonston MV RNP transfection and 15 days after IP-3-Ca MV RNP transfection were reexamined to appraise the capacity of these cells to support the synthesis and accumulation of other MV structural proteins. It is evident that the favorable intracellular environment provided to the transfected IP-3-Ca genome was sufficient to support the accumulation of both N and phospho- (P) proteins but failed to relieve the constraint imposed on M protein expression (Fig. 1B).

To confirm that defective M protein expression is specified by the IP-3-Ca genome, IP-3-Ca RNPs were introduced into four additional cell lines, HeLa, HEp-2, CV-1, and BSc-1. None of these transfected cell lines accumulated IP-3-Ca M protein (Fig. 2, right panel). In contrast, control transfections with Edmonston RNPs resulted in abundant M protein accumulation (Fig. 2, middle panel). As expected, no infectious virus was detected in the medium in which the IP-3-Ca RNP-transfected cells were cultivated, whereas abundant progeny virus was produced in Edmonston RNP-transfected cells (3 × 10^{3} to 5 × 10^{4} PFU/ml of medium).

IP-3-Ca matrix cDNA clones show many differences in comparison with the matrix gene of a lytic MV, and their

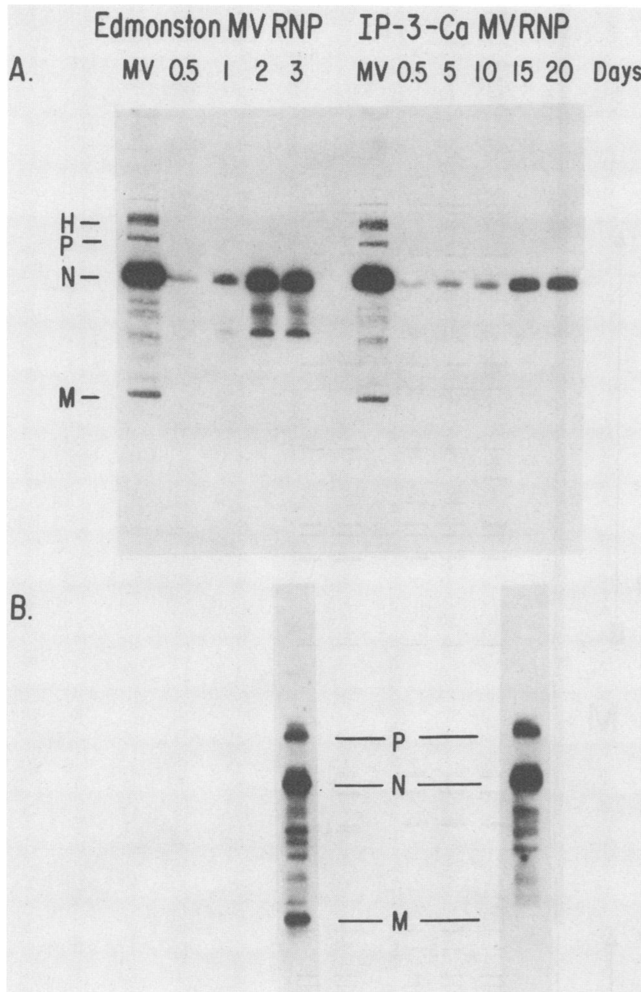


FIG. 1. Western blots of Vero cells transfected with Edmonston MV or IP-3-Ca MV RNP. (A) Accumulation of viral protein after transfection. A rabbit anti-nucleocapsid monospecific serum was used as a probe. Each culture was transfected with about 100 μg of crude RNP, and at various times thereafter, the cells were collected and about 100 μg of cell lysate was run on each lane. The control lanes (MV) contain about 20 μg of pure ^{35}S-labeled Edmonston strain virus. A prominent N band that has reacted with anti-nucleocapsid antibodies and [^{125}I]protein A and other bands corresponding to other MV proteins labeled with ^{35}S (H, P, and M, as indicated on the left) were detected. (B) Proteins produced by Edmonston MV RNP at day 3 after transfection and by IP-3-Ca RNP at day 15 after transfection. The same lysate as used for gels in panel A was run on a gel, blotted, and probed with rabbit anti-MV antiserum which recognizes all the major MV proteins on Western blots except for the H protein. The minor bands seen between the M and N proteins are degradation products of the N protein (43).

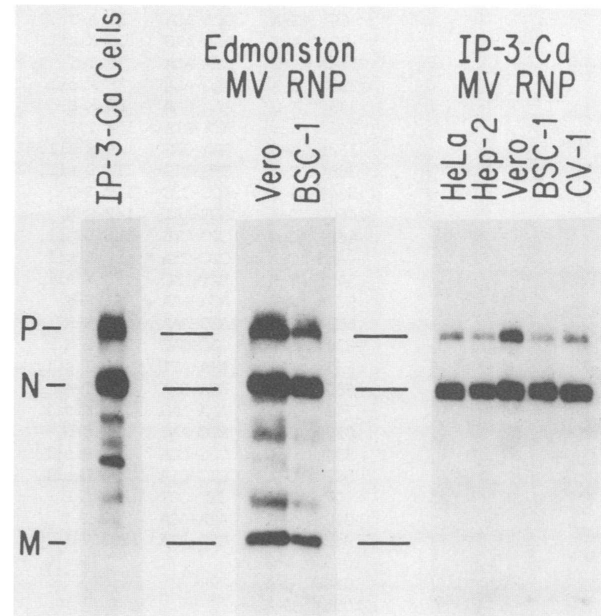
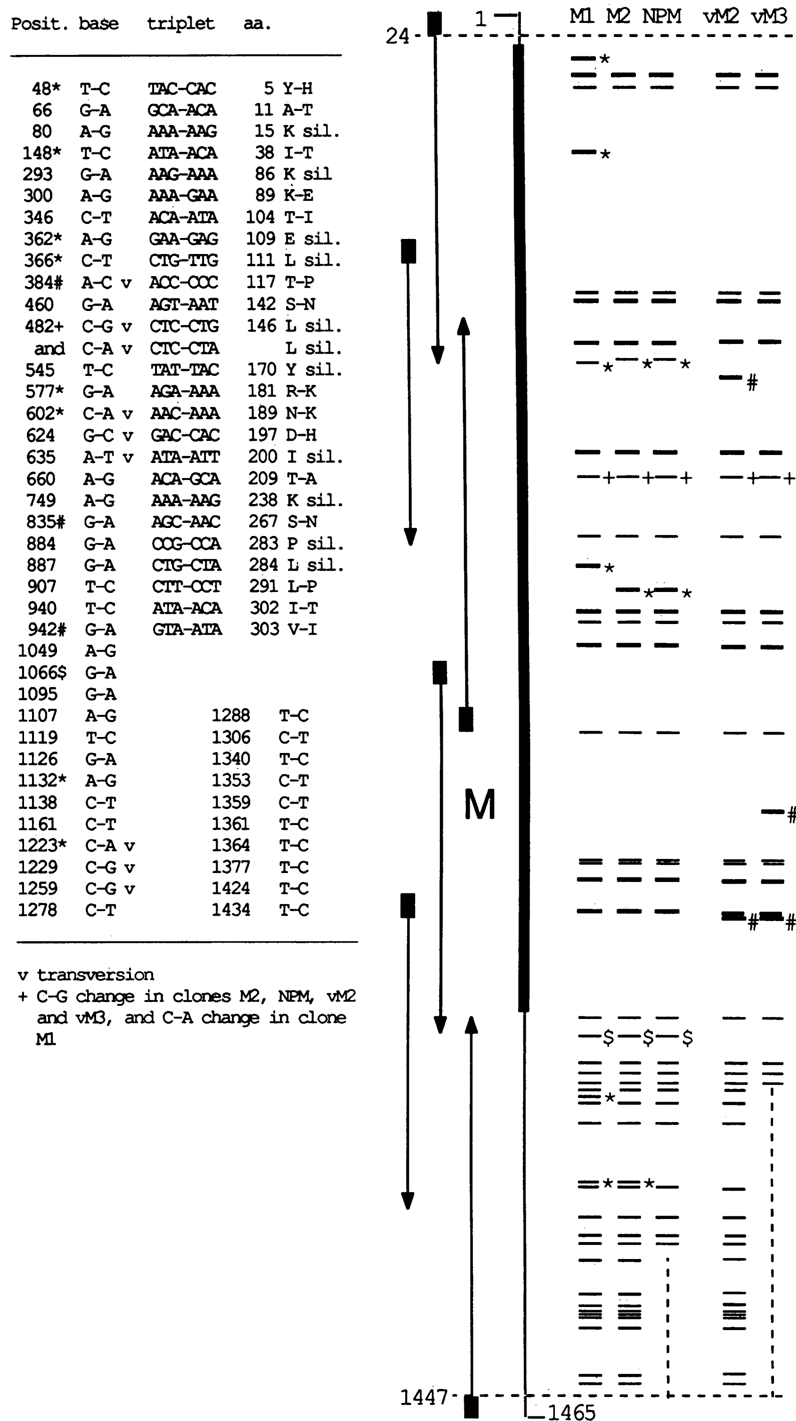


FIG. 2. Western blot of MV-specific proteins produced in different cell lines after transfection with Edmonston or IP-3-Ca MV RNP. Although the figure is divided into three panels, the results are all from the same gel. A lysate of IP-3-Ca cells was run as control in lane 1. In lanes 2 and 3, lysates of cells transfected with Edmonston MV RNPs, collected 3 days after transfection, were loaded. In the five lanes on the right, IP-3-Ca RNP-transfected cells harvested 2 weeks after transfection were loaded. The amount of total cell protein run on each lane was similar. The probe used was the rabbit anti-MV antiserum described in the legend to Fig. 1.

sequence is highly heterogeneous. Since the instability of the M protein synthesized in IP-3-Ca cells could not be ascribed to constraints imposed by the host cell, the possibility that the restricted expression is ultimately determined by matrix gene mutation(s) was explored. The sequence of the stable M protein from the Edmonston strain was recently published (5). To collect comparable data regarding the unstable IP-3-Ca M protein, the corresponding cDNA was cloned by using a protocol allowing selective full-length cDNA cloning (41). Since previous studies indicated that MV persistence in human brain is characterized by high genomic variability (15), three independent clones, termed M1, M2, and NPM, were analyzed. The sequences of these M cDNAs derived from RNA of IP-3-Ca cells are outlined in Fig. 3. (cDNAs vM2 and vM3 were derived from another RNA source and are discussed below.)

Several conclusions can be drawn from these sequence data. First, the M gene sequence of each clone differs from that specifying the Edmonston M protein, resulting in amino acid differences in 11, 9, and 9 positions of M1-, M2- and NPM-encoded M proteins, respectively. Any or all of these amino acid differences may be responsible for the instability of the IP-3-Ca protein. Second, a striking feature of the three IP-3-Ca M gene sequences is high heterogeneity; clone M1 differs from M2 in eight positions and from NPM in nine positions. Clones M2 and NPM differ in one position. These differences correspond to 18 changes over 3,935 nucleotides when compared pairwise. Thus the IP-3-Ca M gene sequence variability is about 0.45%, highly exceeding the variability noted previously in clones derived from Edmonston strain virus stocks separated by many serial passages (15). Third, only about 50% of the nucleotide differences in the M-coding regions of the IP-3-Ca and Edmonston genomes are silent. This is similar to the ratio of silent mutations to expressed mutations defined in the M-protein-coding region of vesicular stomatitis virus mutants (VSV; a nonsegmented negative-strand RNA virus like MV; 21). Finally, although the differences between the Edmonston and the IP-3-Ca M sequences are distributed over the entire gene, they are concentrated in the untranslated region. This suggests that selective pressures to preserve at least some M protein function(s) remain in effect, a suggestion supported by the observation that IP-3-Ca cells have retained the



Posit.	base	triplet	aa.
48*	T-C	TAC-CAC	5 Y-H
66	G-A	GCA-ACA	11 A-T
80	A-G	AAA-AAG	15 K sil.
148*	T-C	ATA-ACA	38 I-T
293	G-A	AAG-AAA	86 K sil
300	A-G	AAA-GAA	89 K-E
346	C-T	ACA-ATA	104 T-I
362*	A-G	GAA-GAG	109 E sil.
366*	C-T	CTG-TTG	111 L sil.
384#	A-C v	ACC-CCC	117 T-P
460	G-A	AGT-AAT	142 S-N
482+	C-G v	CTC-CTG	146 L sil.
and	C-A v	CTC-CTA	L sil.
545	T-C	TAT-TAC	170 Y sil.
577*	G-A	AGA-AAA	181 R-K
602*	C-A v	AAC-AAA	189 N-K
624	G-C v	GAC-CAC	197 D-H
635	A-T v	ATA-ATT	200 I sil.
660	A-G	ACA-GCA	209 T-A
749	A-G	AAA-AAG	238 K sil.
835#	G-A	AGC-AAC	267 S-N
884	G-A	CCG-CCA	283 P sil.
887	G-A	CTG-CTA	284 L sil.
907	T-C	CTT-CCT	291 L-P
940	T-C	ATA-ACA	302 I-T
942#	G-A	GTA-ATA	303 V-I
1049	A-G		
1066$	G-A		
1095	G-A		
1107	A-G	1288	T-C
1119	T-C	1306	C-T
1126	G-A	1340	T-C
1132*	A-G	1353	C-T
1138	C-T	1359	C-T
1161	C-T	1361	T-C
1223*	C-A v	1364	T-C
1229	C-G v	1377	T-C
1259	C-G v	1424	T-C
1278	C-T	1434	T-C

v transversion

+ C-G change in clones M2, NPM, vM2 and vM3, and C-A change in clone M1

FIG. 3. Differences between five M cDNAs from cell line IP-3-Ca or virus 3-1 and the Edmonston strain matrix gene sequence. Clones M1, M2, and NPM were derived from RNA of IP-3-Ca cells, and clones vM2 and vM3 were derived from RNA of Vero cells infected with virus 3-1. The M gene is drawn to scale in the middle; the M-coding region is indicated by a thick line, and the 5′ and 3′ noncoding regions are indicated by thin lines. On the left, all the positions at which one or more of the IP-3-Ca and virus 3-1 clones differed from the Edmonston M sequence (5) are listed. In the middle, the map of the M gene and the sequencing strategy is outlined, and on the right, the positions at which IP-3-Ca clones M1, M2, and NPM, as well as virus 3-1 clones vM2 and vM3, differed from the Edmonston strain sequence are indicated with bars. Symbols: ——, silent mutations; ▬▬, amino acid substitutions; *, differences not present in all three IP-3-Ca clones; $, IP-3-Ca mutation not present in the virus 3-1 M gene sequence; #, additional nucleotide changes in virus 3-1 M sequences; ■, sequencing primers; →, the extent of the sequence read from each primer. Clones M1, M2, and vM2 contained full-length copies of the M gene. Clones NPM and vM3 contained the whole M-coding region but lacked part of the 3′ untranslated region, as indicated by an interrupted line. Clone NPM contained the entire N and P sequences but was colinear with the M gene only until position 1303, and clone vM3 was colinear to the M gene only until position 1115. Not all the nucleotides could be unambiguously defined in all five clones examined: in clones M1, M2, NPM, and vM2, we could not define 13, 17, 3, and 1 positions, respectively. In clone vM3, all positions were defined. The nondefined positions, corresponding to strong stops in the sequencing reactions, were considered as showing no variation. In our convention, the M gene begins with the C of the CUU (on the plus strand) intercistronic trinucleotide. Nucleotide 1 in reference 5 corresponds to nucleotide 4 in our convention. The Edmonston M gene sequence was corrected in the 3′ untranslated region by the substitution of a GC dinucleotide by an A nucleotide at positions 1419 and 1420 (14). Posit., Position; aa., amino acid; sil., silent.

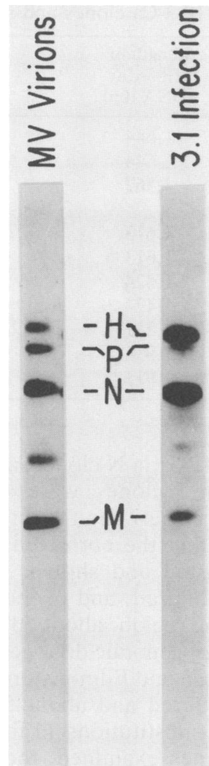


FIG. 4. Immunoprecipitate of SSPE virus 3-1-infected Vero cells and of Edmonston strain virions. In the first lane, labeled proteins from Edmonston strain virions were loaded as a control. In the second lane, an immunoprecipitate of Vero cells infected with virus 3-1, labeled with [^{35}S]methionine for 48 h, was loaded. The proteins of virus 3-1 migrated to positions slightly different from those of the proteins of the Edmonston strain, as is the case for the IP-3-Ca proteins (43). Proteins from SSPE strains often migrate to positions slightly different from those of the Edmonston MV proteins (23).

capacity to produce an unstable M protein throughout their tissue culture passage history. Residual M protein functions might include stabilization of the viral envelope proteins (31, 34) and regulation of transcription (4).

Rescue from cell line IP-3-Ca of a compromised but infectious virus producing a matrix protein with partially restored stability. The preceding sequence analyses revealed 12 amino acid differences between the M protein(s) of the IP-3-Ca cells and that specified by the Edmonston MV genome. Presumably, one or more of these differences is ultimately responsible for the rapid posttranslational degradation by which M protein expression is restricted. However, which of these amino acid substitutions lead to the instability of this protein cannot be determined from an inspection of their positions and their chemical characters. We have attempted to identify amino acid difference(s) crucial for M protein stability by examining the sequence of the matrix gene of a rare, in fact singular, revertant infectious virus recovered from this otherwise nonproductive SSPE cell line (Materials and Methods). This revertant isolate was called virus 3-1. Although it produced lytic infection of permissive tissue culture cells, it had not truly reverted to a wild-type phenotype. Instead, its lytic infection proceeded slowly over a 14- to 21-day period and led only to limited production of virion progeny (less than 10 new infectious particles from each initially infected cell). Despite its replicative limitations, however, this revertant displayed one crucial feature that distinguished it from the parental IP-3-Ca cell: it specified the synthesis of an M protein whose stability had been partially restored (Fig. 4, right lane).

Precise determination of the half-life of the virus 3-1 M protein proved impossible because of low virus titers and slow growth. Nevertheless, an estimate of its stability relative to the M proteins of IP-3-Ca and Edmonston MV was derived from a comparison of the degree to which each of these M proteins accumulated in infected cells. Densitometric quantification of autoradiograms such as those shown in Fig. 4 indicated that approximately 3 to 5% of the total MV structural proteins accumulating in virus 3-1-infected cells was M protein. In contrast, the amount of M protein accumulating in IP-3-Ca cells is below the level of detection (42, 43), whereas the Edmonston MV matrix protein represents about 15 to 20% of the total MV protein accumulating in acutely infected cells (42, 43), as well as in purified virions (Fig. 4, left lane). We conclude, therefore, that the emergence of virus 3-1 from the IP-3-Ca cell line coincided with the appearance of a genomic variant encoding a matrix protein whose stability is intermediate between those of the stable Edmonston protein and the unstable IP-3-Ca M protein.

To establish the sequence of virus 3-1 M protein, we cloned the corresponding cDNA from RNA of virus 3-1-infected cells (Materials and Methods). Two virus 3-1 M cDNA clones were completely sequenced by using the method outlined in the legend to Fig. 3, and their sequences (vM2 and vM3) are presented on the right of Fig. 3. vM2 and vM3 cDNAs shared almost all those differences that distinguished the IP-3-Ca from the Edmonston MV M gene sequence (only one silent mutation, marked with $, was not detected in the virus 3-1 clones). It is also evident that the escape of virus 3-1 from the IP-3-Ca cells was associated with the appearance of additional nucleotide changes in the virus 3-1 M sequences (marked with #), two found in the vM2 clone and two in the vM3 clone and all encoding amino acid substitutions. Since only one amino acid change is shared by both clones (the rather conservative valine-to-isoleucine mutation at amino acid 303, caused by a G-to-A transition), it seems likely that this single mutation was sufficient to restore M protein stability. Interestingly none of the mutations observed in virus 3-1 resulted in a reversion to the sequence of the Edmonston MV M protein. Therefore virus 3-1 is not a true revertant, but a compromised virus carrying additional amino acid change(s) partially compensating for the defects established by previous mutations. Similarly, a high incidence of pseudoreversions has been recently monitored in phenotypic revertants of VSV M protein mutants (29). Finally, vM2 and vM3 differed in 2 of 1,091 comparable nucleotides, corresponding to an internal variability of about 0.2%.

Mutations and divergence of genes other than matrix. Recent examination of the MV gene products found in brain cells of different patients with SSPE has shown examples of restricted expression of the viral F and H proteins, as well as of M protein (3, 30). In contrast, N, P, and L proteins, the three proteins required for transcription and replication and therefore for the survival of infecting genomes, were always detected. The simplest explanation for this observation is that the constraints imposed in persistent infections on M, F, or H MV genes are relaxed since each encodes a nonvital viral function. However, when the MV replication machinery introduces important nucleotide changes in the N, P, or L sequence, the resultant mutant genomes cannot survive.

To evaluate the variability of MV genes other than the M

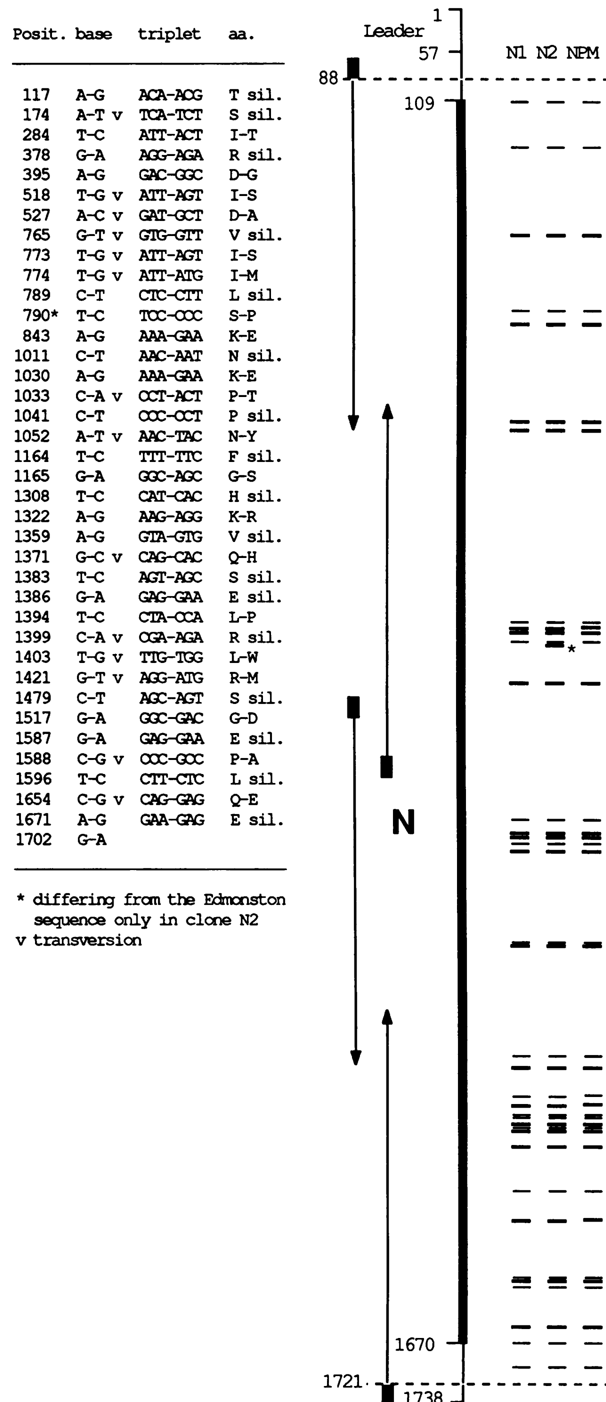


FIG. 5. Differences between three N cDNAs from cell line IP-3-Ca and the Edmonston strain nucleocapsid gene sequence. Symbols are as in the legend to Fig. 3. Position 1 corresponds to the first nucleotide of the MV minus-strand RNA (7). For convenience, the Edmonston strain N sequence is as given by Rozenblatt et al. (37), but we note the following: at position 1598 in our convention (position 1489 in the convention of Rozenblatt et al. [37]), we have monitored two Gs instead of one. This nucleotide insertion was detected in the Edmonston strain N gene and in the N genes of IP-3-Ca, of another SSPE case, and of the neurotropic MV strain CAM (K. Baczko and A. Schmid, unpublished results). The addition of this G modifies the Edmonston strain N protein sequence starting with the serine residue at position 497, which becomes an arginine. The last 27 amino acids should be deleted and substituted by the following 29 amino acids (one-letter code): RLQAMAGISE'EQ GSDTDTPT'VYNDRNLLD. It is noteworthy that with this modification, six of the last nine amino acids of the MV Edmonston strain N sequence become homologous to the last nine amino acids of the canine distemper virus Onderstepöort N sequence (37). In the previously published MV and canine distemper virus N sequences, none of the last nine amino acids was homologous (37). It should also be mentioned that not all the nucleotides were unambiguously defined in all IP-3-Ca N clones: in clones NPM, N1, and N2, we could not define 8, 51, and 47 positions, respectively. The nondefined positions, corresponding to strong stops in the sequencing reactions, were considered as showing no variation. Posit., Position; aa., amino acid; sil., silent.

TABLE 1. IP-3-Ca clones and sequences

Clone	Length of IP-3-Ca cDNA (bp)	No. of bases defined
N1	1,644	1,593
N2	1,644	1,597
NPM	4,567	1,655 in N
		1,296 in M
P1	1,613	433
P2	1,613	433
M1	1,424	1,411
M2	1,424	1,407
F1	2,331	390
H1	1,911	517
H2	1,911	517

gene, three complete IP-3-Ca N clones, as well as the ends of two P, two H, and one F clones, were sequenced (Table 1). In Fig. 5, the differences between the three IP-3-Ca N clones (N1, N2, and NPM) and the corrected Edmonston MV N gene sequence are listed and shown. About 2.3% of the nucleotide positions differed, and of the 37 nucleotide substitutions in the coding region, about 50% led to amino acid changes. This degree of genomic divergence is similar to that of the M gene of IP-3-Ca and Edmonston MV, in which 3.2% of the nucleotides differed and about 50% of the changes encoded amino acid substitutions (Table 2). In the other three IP-3-Ca MV genes examined, the 5′ and 3′ terminal regions also showed 2.5 to 3.3% nucleotide differences from the corresponding Edmonston MV gene sequences, and again, about half of these produced amino acid substitutions (Table 2).

These data only tell us that each MV gene experienced a similar degree of selective pressure to maintain its sequence as the Edmonston and IP-3-Ca MV genomes diverged from their common ancestor. Such comparisons of distantly related MV strains, however, fail to address the question of the extent to which variation of individual MV genes is permitted while replicating in a persistently infected cell. Ideally, the nucleotide sequence of these IP-3-Ca cDNA clones should be compared with the genomic sequence of the virus that initiated the infection of brain cells from which the IP-3-Ca cell line was derived. Unfortunately, neither this virus nor, of course, its sequence is available. Instead, we have appraised the degree to which the individual IP-3-Ca MV genes vary by comparing the nucleotide sequences of sibling cDNA clones, i.e., M1 to M3, M1 to NPM, M2 to NPM, etc. Table 3 shows the results of the comparison of the level of heterogeneity manifested by the four IP-3-Ca genes analyzed. Although these sequence data are far from exhaustive and may be biased by the clones selected for analysis,

TABLE 2. Differences between Edmonston MV strain and the SSPE defective-virus IP-3-Ca genomes

Gene[a]	No. of base pairs compared/no. of differences (% difference)[b]	No. of base pairs in coding region/no. of differences (% difference)[c]	No. of base pairs in noncoding region/no. of differences[d]	No. of replacement sites/no. of silent sites	No. of transitions/no. of transversions
N	1,644/38 (2.3)	1,562/37 (2.3)	82/1	20/17	24/14
P	436/14 (3.2)	347/11 (3.2)	89/3	5/6	12/2
M	1,424/46 (3.1)	1,008/23 (2.3)	414/23	12/10	36/8
F	390/13 (3.3)	71/1	319/12	0/1	9/4
H	517/13 (2.5)	460/9 (2.0)	57/4	5/4	9/4

[a] The sequences of the P, F, and H Edmonston genes are from Bellini et al. (6), Richardson et al. (33), and Alkhatib and Briedis (1).
[b] Total difference is 2.8%.
[c] Total difference is 2.3%.
[d] Total difference is 4.5%.

the results do suggest that the degree of MV gene variability decreases in the order M > H > N > P, consistent with the prediction that genes encoding nonvital functions are subject to lower selective pressures to maintain their sequence than are those encoding functions required for genome transcription and replication. A similar observation has been made previously in a VSV persistent infection (36). Furthermore, by averaging the total of 22 nucleotide differences found in the sibling clones over the 9,668 nucleotides compared, we conclude that, on average, each of the MV genomes coreplicating within IP-3-Ca cells probably differs from the others in 30 to 40 of its 16,000 bases.

DISCUSSION

Mutations and restriction of matrix protein expression. The M protein of negative-strand RNA viruses serves as the organizing element for virion morphogenesis, mediating both viral assembly and budding from the cell membrane (4). When M protein expression is compromised, as occurs in persistent MV infections of brain cells of patients with SSPE, neither budding particles nor infectious virion progeny are detected (46, 49). Similarly, restricted M protein expression and the consequent failure to consummate the MV reproductive cycle characterize the derivative SSPE line IP-3-Ca. These parallels between the persistent MV infection of IP-3-Ca cells and of SSPE brain cells in vivo indicate that the defective MV genome derived by cocultivation of SSPE brain cells with BSc-1 cells (10) is likely to be representative of the MV genomes present in diseased brains and therefore support our view that the IP-3-Ca cell line provides a valid model of SSPE for detailed study.

The nature of the M-protein defect of IP-3-Ca is protein instability (43). Unstable M proteins have been noted in other persistent paramyxovirus infections, including a persistent Sendai virus infection of BHK cells (35) and a HeLa cell line persistently infected with Edmonston MV (51). In neither of these instances was the basis for M protein instability further explored. In our study, IP-3-Ca genomes, in the form of RNPs, were transfected into five cell lines capable of supporting efficient MV replication. In no case was the constraint on M protein expression relieved, and thus we conclude that the rapid M protein degradation displayed by IP-3-Ca is caused by mutations in the viral genome. To pursue and consolidate this point, a phenotypic revertant of the IP-3-Ca genome was sought and isolated. The single viral isolate recovered (virus 3-1) was not, in fact, a true revertant. Rather, it was a severely debilitated mutant that replicated slowly and produced a less unstable M protein. Comparison of the sequences of two M gene cDNA clones of this virus with those of IP-3-Ca M gene revealed only one nucleotide change conserved in both virus 3-1 clones. It is likely that this single-base change accounts not only for the restored stability of the M protein but also for the reversal of the IP-3-Ca cell-associated phenotype. We cannot exclude the possibility, however, that additional changes in other MV genes may have influenced M protein stability, thereby facilitating virus maturation and virion progeny production.

Mutations and RNA virus persistence. Persistence of RNA viruses in cultured cells has been associated with the production of defective interfering particles or the generation of heterogeneous populations of conditional lethal mutants or both (25, 26, 36; for a review, see reference 24). In the SSPE cell line examined here, defective interfering particles were never detected (data not shown), but the analyses of both IP-3-Ca and virus 3-1 genomes revealed a very high internal-sequence variability. Similar observations were made in a less-extensive survey of MV genomes present in the brain of an SSPE patient (15). By extrapolating from the IP-3-Ca MV sequence data, we estimated that, on average, the coreplicating MV genomes in this cell line differ from one another in 30 to 40 of their approximately 16,000 nucleotides.

This extraordinary high level of variation must be quantitatively compared with the degree of variability experienced by the genomes of other lytic procaryotic and eucaryotic RNA viruses, whose RNA polymerases lack proofreading capacity and thus are error prone. Early studies of bacteriophage Qβ indicated that the error frequency per genome doubling at given bases can be as high as 10^{-3} to 10^{-4} (19). Generally speaking, however, the mutated genomes produced during lytic Qβ infections are more or less rapidly eliminated, leading to the appearance of relative genomic stability (19). Direct sequence evidence for very high error frequencies experienced by animal virus genomes has been

TABLE 3. Differences within overlapping IP-3-Ca clones

Clones compared	No. of bases	No. of differences[a]
N1 and N2	1,593	1
N1 and NPM	1,593	0
N2 and NPM	1,597	1
M1 and M2	1,407	8
M1 and NPM	1,266	9
M2 and NPM	1,262	1
P1 and P2	433	0
H1 and H2	517	2

[a] Total difference is 0.2%.

obtained for VSV and for foot-and-mouth disease virus (a picornavirus). During VSV lytic infections, the average frequency of base substitutions incurred by a clonal population is between 10^{-3} and 10^{-4} (44), and in the case of foot-and-mouth disease virus, it is estimated that each infectious RNA in the population differs in two to eight positions from the average parental genome (about 8,000 nucleotides in length; 44). These studies suggest that the genomic sequence of lytically replicating RNA viruses should be viewed as a consensus, rather than as a defined, fixed sequence (see reference 18 for a review) and that each individual genome in populations of lytic viruses as taxonomically distant as VSV, foot-and-mouth disease virus, and the bacteriophage Qβ differs, on average, by 1 to 10 nucleotides from the parental genome sequence.

The level of heterogeneity found among the MV genomes persistently infecting the SSPE cell line described here is yet another order of magnitude higher than that found in Qβ, VSV, and foot-and-mouth disease virus lytic infections, attaining instead the 0.1 to 1.2% estimate for the degree of heterogeneity displayed by the genomes of two picornaviruses, poliovirus and Theiler virus, during persistent infections in the animal (39, 40). How this extreme level of genomic variability develops remains to be determined. Certainly, the relaxed selective pressure experienced by a viral genome which needs to conserve only a fraction of its encoded functions can account for the survival of mutations incurred and accumulated within nonvital gene sequences. But whether the rate of MV genomic mutations in the SSPE cell line is the same as that experienced during acute MV infection or is exaggerated by a mutated and consequently more-error-prone IP-3-Ca viral polymerase is yet unknown (see also reference 15 for a discussion of these hypotheses).

Possible role of MV mutations in the pathogenesis of SSPE. These studies on the MV genomes replicating in cell line IP-3-Ca clearly show that, once established, persistent MV infection is characterized by the continuous elaboration of genomic mutations and that nucleotide substitutions alone can account for MV-gene expression defects found in SSPE. In contrast, the factors, events, and processes that mediate the development of persistent infections by this otherwise typically cytolytic virus remain obscure. Our view is that these determinants are the province of the host. The possibility that host defense mechanisms may play a role in the establishment of persistent infections was raised by early studies showing that anti-MV antibodies can modulate viral antigen expression in infected cells (20, 32) and more-recent experiments showing that the development of persistence by an infectious SSPE virus strain is facilitated by exogenously introduced recombinant interferon (11). Furthermore, the recent demonstration of marked attenuation of MV transcription in SSPE brain cells suggests that determinants encoded by the parasitized cell may influence its propensity to develop and support persistent MV infection (13). Acting alone or in concert, these host factors may lead to viral persistence, both by attenuating the transcription of the MV genome and by confining those MV gene products expressed to the interior of the cell. The infected cell will thus be spared from recognition and destruction by MV-specific immunological defenses. During subsequent rounds of replication, the MV genome will incur and accumulate mutations. In general, these mutations will further compromise transcriptional and replicative efficiencies, ultimately leaving the previously infected cell only with vestiges or with no detectable evidence of MV genes or gene products. On rare occasions, however, the mutations that arise will permit viral replication to proceed in a sufficiently competent fashion for the persistent infection to survive. The MV infection could then spread to contiguous cells by occasional cell fusions, as occurs with IP-3-Ca cells, or may even be disseminated to neuroanatomically distant areas of the brain through axonal transport. Finally, after months to years, this slow, continuous propagation of mutated, cell-associated MV genomes and their gene products will declare its presence with the onset of the symptoms and signs of the chronic and ultimately fatal neurological disease, SSPE. Similar sequelae of events could characterize other viral infections known or suspected to be the cause of several human syndromes, like, for example, the more prevalent neurological disease multiple sclerosis.

ACKNOWLEDGMENTS

We are indebted to Sharon Pifko-Hirst for expert technical assistance, to Markus Aebi for synthesis of oligonucleotides, and to Knut Baczko for communicating unpublished information. We thank Marshall Horwitz, Scott Hawley, Laurent Roux, and Esteban Domingo for critical reading of the manuscript.

These studies were supported by National Multiple Sclerosis Society grant RG1241; Public Health Service grants AI20532, from the National Institutes of Health, and P30 CA13330-12, from the National Cancer Institute; Schweizerische Nationalfonds grant 3.141-085; and by the Kanton Zurich. S.A.U. is a recipient of an Irma T. Hirschl Trust research career award.

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