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Rapid degradation restricts measles virus matrix protein expression in a subacute sclerosing panencephalitis cell line

(persistent viral infection/chronic diseases/gene expression)

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ABSTRACT **Measles virus matrix protein expression is restricted in the persistently infected brain cells of patients with the chronic neurological disease subacute sclerosing panencephalitis (SSPE). Prior studies of the nature of this restriction have identified polyadenylylated matrix gene-encoded RNA transcripts unable to direct effective translation. The defective nature of these mRNAs readily accounted for the inability to detect matrix protein in these persistently infected cells and suggested that in SSPE the restriction of matrix protein expression is achieved by preventing its synthesis. Recently, however, we reported evidence that matrix protein is synthesized in at least one example of this persistent infection, the SSPE cell line IP-3-Ca. In this case, failure of matrix protein to accumulate normally accounted for its restricted expression [Sheppard, R. D., Raine, C. S., Bornstein, M. B. & Udem, S. A. (1985) *Science* 228, 1219–1221]. To clarify the nature of the restriction displayed by IP-3-Ca cells, the synthesis and fate of the matrix protein of this SSPE cell line were examined in detail. No evidence of constraints on the efficiency of matrix protein mRNA transcription or translation was found. Instead, the restricted expression proved to be the result of rapid posttranslational degradation of matrix protein. We suggest that matrix protein gene mutations incurred in the course of genome replication are likely to be responsible for the diversity of observed mechanisms restricting matrix protein expression. In that event, the nature and position of the nucleotide substitution(s) would be the determinants of the level at which restricted expression is achieved.**

Subacute sclerosing panencephalitis (SSPE) is a rare chronic disease whose clinical manifestations of progressive and ultimately fatal neurological deterioration are the result of persistent measles virus (MV) infection of brain cells (for review, see ref. 1). The persistent infection is initiated by virus acquired in an antecedent typical acute measles illness experienced months to years before the onset of neurological dysfunction (1, 2). That intervening period is silent, lacking any hallmarks to distinguish the rare individual who will later succumb to SSPE (1, 2). As a result, the factors mediating the transition from acute infection to this slow viral infection of the central nervous system have been inaccessible to study and thus remain enigmatic. Efforts to understand the nature of this persistent infection have been forced to rely on examination of materials obtained from patients after disease onset. Such studies have generated considerable and consistent evidence indicating that the expression of one specific MV gene product, the matrix protein, is defective in SSPE (3–9). This protein is thought to mediate the organization of viral structural components at the host-cell plasma membrane and their subsequent budding and release in the form of virion progeny (10–14). As such, matrix protein is vital to the successful completion of the MV reproductive cycle. Should the expression of matrix protein be constrained, maturation of virion progeny would prove abortive despite ongoing intracellular synthesis and accumulation of the other virus-specified macromolecules. These, in fact, are the very features that characterize the persistent infection of SSPE and distinguish it from the acute MV infection (1, 2, 5–9).

Previously, analyses of the matrix gene products found within persistently infected brain cells or derivative SSPE cell lines revealed only polyadenylylated matrix RNA transcripts unable to direct matrix protein synthesis either *in vivo* or *in vitro* (5, 15, 16). The defective nature of these RNA templates readily accounted for the recurrent failure to detect matrix protein in SSPE and created the impression that restriction of matrix protein expression is achieved by preventing its synthesis. It has since become clear, however, that matrix protein may be synthesized in SSPE. Recently, we reported the results of studies of the independently derived SSPE cell line IP-3-Ca, which revealed synthesis of a matrix protein that fails to accumulate normally (17). Furthermore, Norrby and coworkers' subsequent demonstration (18), by immunofluorescence microscopy, of matrix protein antigens in brain cells of several patients with SSPE indicates that matrix protein synthesis may occur in the persistently infected human host as well.

These recent observations and their implications led us to explore in greater detail the nature of the restriction of matrix protein expression displayed by the IP-3-Ca cells. Our studies have revealed no constraints on the efficiency with which matrix mRNA is transcribed or translated but have shown instead that, in IP-3-Ca cells, the newly synthesized matrix protein is unstable, undergoing rapid degradation at a rate approximating first-order reaction kinetics and with a calculated half-life of ≈75 min.

MATERIALS AND METHODS

Cells and Virus. The SSPE virus-carrier cell line IP-3-Ca was generously provided by T. Burnstein of Purdue University (19). BSc-1 cells, supportive of MV reproduction, were also employed. All cell lines were propagated and maintained at 37°C as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum and glutamine (complete medium).

The reference virus selected for these studies was the Edmonston strain of MV. Its propagation and the development of viral stocks have been described (20).

Metabolic Radiolabeling and Cell Lysate Preparation. All cell lines were grown to confluence in 35-mm tissue culture

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Abbreviations: SSPE, subacute sclerosing panencephalitis; MV, measles virus.

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dishes and radiolabeled using identical protocols. Acute Edmonston MV infections were initiated at an input multiplicity of infection of 3–5 plaque-forming units per cell, after which the infection was allowed to proceed at 37°C for ≈10 hr. Cultures of BSc-1 cells were always prepared and examined in parallel. Radiolabeling was preceded by a 30-min incubation at 37°C in methionine-free DMEM with 10% dialyzed serum in order to deplete the intracellular methionine pool. The depletion medium was then removed and replaced with fresh methionine-free DMEM containing [^{35}S]methionine (800 μCi/ml; ≈1000 Ci/mmol, Amersham; 1 Ci = 37 GBq), and incorporation of isotope was allowed to proceed at 37°C for times prescribed by the experimental design. In pulse-labeling studies, radiolabeling was performed for intervals of 10 to 90 min. Pulse–chase experiments were initiated with a 15-min period of [^{35}S]methionine incorporation at 37°C. The radiolabeling medium was then replaced with chase medium (complete medium containing 1 mM methionine), and the cultures were incubated at 37°C for periods of 15 min to 6 hr. In those experiments in which the flow of pulse-labeled MV proteins out of the cell was to be evaluated, serum was excluded from the chase medium. No adverse effects on the fate of the pulse-labeled MV proteins were produced by this elimination.

At selected times, incubation of cells was terminated by collecting the medium and rinsing the monolayers twice with ice-cold Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}. When the medium was to be analyzed for the presence of MV proteins, it was chilled rapidly to 4°C and the following reagents were added sequentially to the final concentrations indicated: 10 mM EDTA, 1% (wt/vol) Nonidet P-40 (NP-40), 0.5% (wt/vol) sodium deoxycholate, 0.3% (wt/vol) $NaDodSO_4$, and aprotinin (1000 kallikrein units/ml; Mobay Chemical Corporation; FBA Pharmaceuticals, New York). Cells were disrupted by exposing the monolayer to 1 ml of a lysis buffer [20 mM Hepes/500 mM NaCl/2 mM EDTA/1% NP-40/0.5% sodium deoxycholate/0.3% $NaDodSO_4$/aprotinin (1000 units/ml), pH 7.5]. Lysates were clarified by centrifugation for 30 min at 10,000 $\times$ g. The resultant supernatant, containing more than 90% of the total cellular protein, was collected. Aliquots were assayed for protein content and for trichloroacetic acid-precipitable [^{35}S]methionine-labeled protein. The remainder was then frozen and stored at −70°C for subsequent analyses.

Immunoprecipitation and Analysis of MV Proteins. Immunoprecipitation of viral proteins was performed using established protocols (17). For any given pulse or pulse–chase experiment, parallel immunoprecipitation reaction mixtures were established, each containing an identical amount of extracted cell protein and a previously determined excess of anti-MV antibodies. The hyperimmune serum used for these studies was generated by repeated immunization of rabbits with highly purified, detergent-disrupted, Edmonston MV (20) and was shown to recognize the corresponding MV structural proteins specified by the SSPE genome within the IP-3-Ca cell (17). Reaction volumes were adjusted to 1 ml by the addition of 20 mM Hepes/50 mM NaCl/2 mM EDTA, pH 7.5, containing 1% NP-40, 0.2% ovalbumin, and 1000 units of aprotinin per ml. After incubation at 4°C for 16 hr, immune complexes were collected by binding to protein A-Sepharose. Sequential washes were performed to remove nonspecifically adsorbed radiolabeled species (17). The resultant immunoprecipitates were disrupted and solubilized at 100°C in Laemmli gel sample buffer (21). Portions were assayed by liquid scintillation to determine total immunoprecipitated radiolabeled MV proteins, and the remainder was subjected to electrophoretic resolution in $NaDodSO_4$/polyacrylamide gels [10% (wt/vol) acrylamide monomer] (21).

Following electrophoresis, gels were prepared for fluorography, dried, and exposed to photosensitized Kodak XAR film (17). The radiolabel present in individual MV structural proteins was quantified by densitometric scanning of appropriately exposed fluorograms. Occasionally the densitometric evaluation was corroborated by liquid scintillation counting of viral polypeptide bands excised from the gel, using the fluorogram as a template.

RESULTS

Initially, efforts to identify the basis for the markedly diminished matrix protein accumulation displayed by IP-3-Ca cells focused on the quantity and quality of the prerequisite matrix RNA transcript. These studies revealed that the steady-state levels of matrix mRNA in IP-3-Ca cells, relative to the other MV transcripts, were comparable to those found in tissue culture cells lytically infected with Edmonston MV. Moreover, this matrix mRNA proved capable of programming efficient *in vitro* translation of matrix protein (unpublished results). Compromised matrix protein accumulation, therefore, could not simply be ascribed to inadequate or inherently defective template. Nonetheless, the possibility remained that unknown host-cell factors created an intracellular environment in which translation of this MV transcript was selectively constrained without being ablated. This consideration was addressed by examining the rate at which each of the MV structural proteins was synthesized in the SSPE cell line. Similar assessment of BSc-1 cells acutely infected with Edmonston MV was performed as well. Selection of the BSc-1 cells for this comparative analysis was based on its parental relationship to the IP-3-Ca cells (19). Cells were exposed to [^{35}S]methionine for short intervals (range: 10–90 min), after which their radiolabeled MV proteins were immunoprecipitated and analyzed by $NaDodSO_4$/PAGE (Fig. 1). Ten minutes of isotope incorporation proved sufficient to reveal synthesis of all the major MV structural proteins in both the acute and the persistent infection. Longer labeling periods produced proportionally increased amounts of each radiolabeled viral polypeptide, their synthesis and accumulation apparently proceeding in parallel over the 90-min span of incorporation examined. In particular, the relative rate at which radiolabeled matrix protein appeared in IP-3-Ca cells and acutely infected BSc-1 cells seemed indistinguishable, suggesting that selective attenuation of matrix protein synthesis did not account for its restricted expression.

Quantification of these electrophoretic analyses confirmed this impression. In both infections, the incorporation of radiolabel into MV structural proteins was shown to be a linear function of the duration of exposure to the isotopic precursor, the amount of radiolabeled MV proteins immunoprecipitated after a 90-min pulse being ≈9 times that found after 10 min (Fig. 2). The rate at which newly synthesized matrix protein appeared in both IP-3-Ca cells and acutely infected BSc-1 cells completely paralleled the curve for incorporation into total MV proteins. Identical results were also found for the nucleocapsid protein (as shown) as well as for the other major MV structural proteins.

These experiments clearly established that the synthesis of matrix protein in IP-3-Ca cells proceeds efficiently and, consequently, that its expression must be compromised by processes ensuing thereafter. The nature of these posttranslational events was evaluated by pulse–chase studies focusing on the fate of the viral proteins synthesized within this SSPE cell line. Once again, parallel examination of BSc-1 cells infected with Edmonston MV was performed for comparison. A representative analysis of the fate of the MV proteins synthesized within IP-3-Ca cells is shown in Fig. 3*B*. Over the first 30 min of chase, no change in the intensity of each radiolabeled MV protein or in the relative distribution of these polypeptides was seen. Subsequently, however, the amount of radiolabeled matrix protein remaining within these

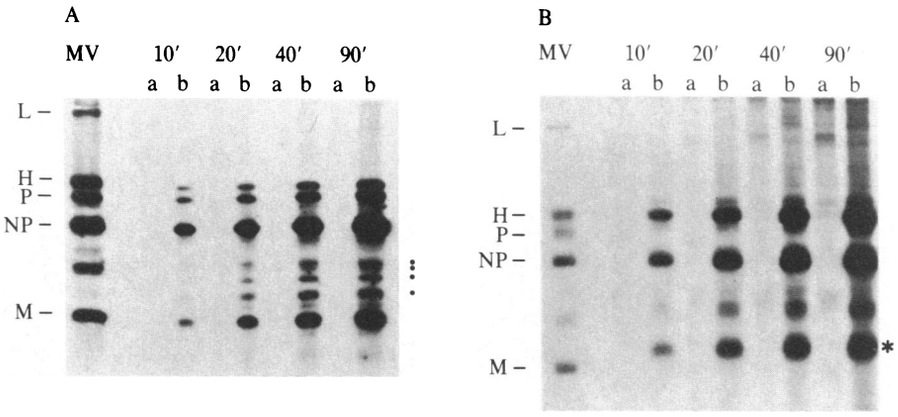

FIG. 1. Representative NaDodSO$_4$/PAGE analysis of synthesis of matrix protein in acutely infected cells and in the SSPE cell line. (*A*) BSc-1 cells infected with Edmonston MV and mock-infected cultures were labeled for various times. Lysates derived from each were exposed to hyperimmune anti-MV serum, and the resultant immunoprecipitates were subjected to electrophoresis. Structural proteins of purified Edmonston MV are shown in the far left lane. To its right and in sequence follow the immunoprecipitates obtained after labeling uninfected (lanes a) and infected (lanes b) cells for 10, 20, 40, or 90 min. (*B*) IP-3-Ca cells were labeled as above. In this case, parallel immunoprecipitations of each lysate were performed with preimmunization serum (lanes a) and with the hyperimmune anti-MV serum (lanes b). Note that the matrix protein synthesized in IP-3-Ca cells (*) migrates more slowly (38 kDa) than that of Edmonston MV (36 kDa). In all other respects, this electrophoretic analysis is organized and presented identically to that in *A*. The nomenclature and approximate mass of the Edmonston MV structural proteins shown are (20): L, 200 kDa; H (hemagglutinin protein), 80 kDa; P (phosphoprotein), 70 kDa; NP (nucleocapsid protein), 60 kDa; M (matrix protein), 36 kDa. The large subunit of the MV fusion protein (F_1, 41 kDa) is not denoted in this and subsequent figures because it is obscured by the commonly observed proteolytic cleavage products of NP (20, 22), whose positions are indicated (●) at right in *A*.

cells diminished rapidly. With this autoradiographic exposure, only trace amounts of matrix protein were found after 3 hr, and none could be detected at the time the experiment was terminated. The rate at which matrix protein disappeared was discordant with that of the other major MV structural proteins, significant levels of the latter radiolabeled species still being present after 6 hr.

Results of the parallel pulse–chase of acutely infected cells are seen in Fig. 3*A*. Although the duration for which the acute infection could be followed was limited by the development of marked cytopathology, the chase period was sufficient to reveal a significant difference between the fate of the MV proteins synthesized during the acute and the persistent infections. This difference lay in the abundant amounts of pulse-labeled MV structural proteins, including matrix protein, found in the acutely infected cells after 3 hr of chase and, though somewhat diminished, still present after 5 hr.

The results of these, as well as similar, experiments were quantified and selected features are displayed graphically in Fig. 4. For purposes of comparison, the fate of a given viral structural protein was expressed as the fraction of radioactive label remaining in that polypeptide species at each chase interval sampled. In both the acute and the persistent infection, a gradual diminution of radiolabeled nucleocapsid protein occurred at similar, if not identical, rates. This decrease of 20–25% can be completely explained by dilution with newly synthesized unlabeled nucleocapsid protein. In contrast, the amount of pulse-labeled matrix protein remaining within IP-3-Ca cells decreased far more rapidly. When plotted semilogarithmically, its disappearance was found to proceed with first-order reaction kinetics revealing an estimated half-life of about 75 min. The matrix protein synthesized during acute infection also disappeared significantly faster than nucleocapsid protein. Nonetheless, ≈50% of this pulse-labeled matrix protein was still present within these cells after 5 hr of chase, and most, if not all, of the remainder could be recovered from the chase medium, where it appeared either as free matrix protein or in the form of viral particles. Since the matrix protein synthesized by IP-3-Ca cells never appeared extracellularly, we concluded that its restricted expression was achieved exclusively by posttranslational degradation within the cell.

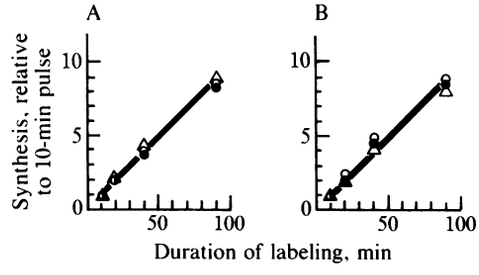

FIG. 2. Rate at which MV proteins are synthesized during the acute (*A*) and the persistent (*B*) infection. Incorporation of radiolabel into total as well as individual viral structural proteins was quantified from several pulse-labeling experiments, including that shown in Fig. 1. To facilitate comparison, data from each infection were normalized by expressing the amount of radiolabel incorporated into viral proteins in a given labeling interval relative to that recovered after a 10-min pulse. ○, Radiolabel in total immunoprecipitated measles virus structural proteins; ●, radiolabel in nucleocapsid protein (NP); △, radiolabel in matrix protein.

DISCUSSION

Matrix protein expression is restricted in SSPE (5–9). As a result, the events and processes leading to this constraint have been actively sought. Until recently, most of the evidence derived from such explorations indicated that the determinants of restriction act to prevent successful matrix protein synthesis. For example, monocistronic matrix mRNAs incapable of programming translation of matrix protein either *in vivo* or *in vitro* have been identified in one example of SSPE (15). In yet another instance, the sequence encoding matrix protein was shown to be contained, largely if not exclusively, in a bicistronic polyadenylylated RNA transcript derived from the contiguous *P* (phosphoprotein) and *M* (matrix protein) genes (16)—an RNA template that similarly fails to direct matrix protein synthesis.

Studies of the independently derived SSPE virus-carrier cell line IP-3-Ca provided an indication that restricted matrix protein expression may be achieved by mechanisms other than the transcription of defective matrix RNA templates. This example of SSPE differed from those described above in its capacity to specify the synthesis of all MV structural

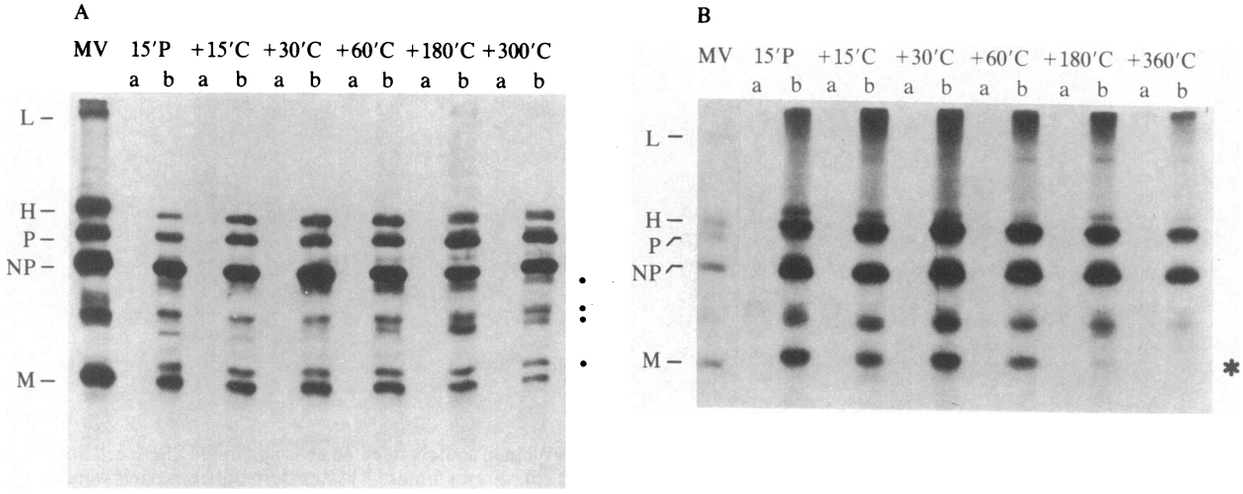

FIG. 3. Electropherograms of a typical pulse–chase experiment used to examine the fate of the matrix protein synthesized during the acute infection and in the SSPE cell line. (*A*) Edmonston MV-infected and mock-infected BSc-1 cells were pulse-labeled for 15 min and then chased for increasing lengths of time as described in *Materials and Methods*. Lysates of these cells were treated with hyperimmune anti-MV serum, and the immunoprecipitated radiolabeled viral structural proteins were separated by $NaDodSO_4$/PAGE. Data are organized in the same fashion as in Fig. 1, with the Edmonston virion structural proteins in the far left lane (MV), the 15-min pulse samples to its right, and the immunoprecipitates obtained after increasing periods of chase following sequentially (lanes a, uninfected; lanes b, infected). (*B*) IP-3-Ca cells were pulse-labeled and chased as above. Immunoprecipitates were obtained by treatment with preimmunization serum (lanes a) and with anti-MV serum (lanes b) and then subjected to $NaDodSO_4$/PAGE.

proteins including matrix protein (17). Yet, by failing to accumulate this protein, it too manifested restricted matrix protein expression (17). To identify the basis for this mode of restriction, the synthesis and fate of the IP-3-Ca matrix protein were examined in detail. No evidence of constraints on the efficiency with which matrix mRNA was either transcribed or translated was found. Instead, the restricted expression proved to be the result of the rapid degradation of matrix protein.

The mode of restriction displayed by IP-3-Ca cells is not unique to this example of a persistent MV infection (23). Quite to the contrary, it appears to be representative of a class of mechanisms by which matrix protein expression can be compromised following its synthesis. Rapid degradation of matrix protein during persistent Sendai virus infection of BHK cells has been described (24), suggesting that posttranslational constraints on matrix protein expression may be common to persistent paramyxovirus infections. Recently acquired evidence indicates that similar, if not identical, mechanisms may be operative in the persistently infected brain cells of patients with SSPE as well. Matrix protein antigens were detected when sections from brains of four different SSPE patients were probed with a battery of anti-matrix monoclonal antibodies and examined by immunofluorescence microscopy (18). Unfortunately, the static nature of this immunocytochemical assessment precluded an evaluation of the integrity, stability, or fate of the matrix protein visualized. Nonetheless, this study leaves little doubt that matrix protein synthesis may occur in SSPE *in vivo*, and that in such cases its restricted expression is likely to involve posttranslational processes.

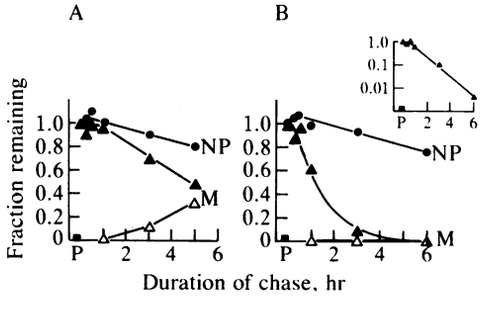

FIG. 4. Quantitative representation of the fate of newly synthesized matrix protein. The amount of radiolabeled matrix protein found in either the acutely infected BSc-1 cells (*A*) or in the IP-3-Ca cells (*B*) after the 15-min pulse (P) and at each chase interval thereafter was quantified as described in *Materials and Methods*. These data were expressed as the fraction of the radiolabel incorporated into newly synthesized matrix protein (M) in the 15-min pulse (▲). Similar analysis of the nucleocapsid protein (NP) was performed for comparison (●). The fraction of pulse-labeled matrix protein appearing extracellularly was assessed by immunoprecipitation of the chase medium with anti-matrix protein monospecific antibodies (△). (*Inset*). Semilogarithmic plot of disappearance of pulse-labeled matrix protein in IP-3-Ca cells, from which a half-life of 75 min was calculated.

Clearly, the restriction of matrix protein expression can be achieved by various means. Constraints imposed at levels preceding as well as following translation have now been found. To reconcile this diversity of mechanisms, we (17) and others (25) have proposed that each instance of restricted expression is ultimately specified by mutation(s) within the matrix gene sequence. Considerable infidelity characterizes RNA virus genome replication (for review, see ref. 26). Further, such mutant genomes have a propensity to survive during persistent viral infections, particularly when the mutations occur in genes encoding nonvital functions (26). The matrix protein is the product of such a gene. It plays no role in virus-specified transcription or replication or in cell-to-cell propagation of the viral infection. Consequently, the occurrence of matrix gene mutation—in fact, the accumulation of multiple mutations within the matrix gene—would be predicted for a long-standing persistent viral infection as that of SSPE. The nature and position of the mutation(s) would then predicate the mechanism of restriction. Thus nucleotide substitutions encoding amino acid changes might be expected to lead to the synthesis of a matrix protein that is functionally incapacitated, inherently unstable, or predisposed to degradation by endogenous host-cell proteases, whereas mutations producing new nonsense codons would determine premature termination of matrix polypeptide elongation and the resultant absence of an identifiable matrix protein. Nor is it necessary for mutations

to be confined to sequences encoding the matrix protein. Defective matrix RNA transcripts might also arise from nucleotide substitutions affecting noncoding regions required for ribosome binding or translational efficiency. Even mutations incurred outside the boundaries of the matrix gene itself could lead to similarly compromised templates if, for example, the transcription-termination recognition signal of the 3′ proximal *P* gene were altered—perhaps the way in which the unusual *P-M* bicistronic transcript described above arose.

Of course, the occurrence, much less the role, of MV genomic mutation in SSPE remains to be proven. Nucleotide sequence analyses of matrix genes found in the brain cells of patients with SSPE or in derivative SSPE cell lines will be needed to affirm or deny this hypothesis.

Note Added in Proof. Cattaneo *et al.* (27) recently generated cDNA clones of those MV matrix gene sequences present in the brain cells of one patient with SSPE. Subsequent nucleotide sequence analysis showed that multiple and diverse point mutations had been accumulated within this matrix coding region. One of the point mutations incurred created a premature termination signal at the twelfth codon, accounting for the restricted matrix protein expression displayed by this example of SSPE.

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