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# Natural history of restricted synthesis and expression of measles virus genes in subacute sclerosing panencephalitis

(hybridization tomography/*in situ* hybridization/slow virus infections)

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**ABSTRACT** **Subacute sclerosing panencephalitis (SSPE) is a slow infection caused by measles virus in which several years separate recovery from typical acute measles and the development of a slowly progressive neurological disease. We have investigated replication of measles virus in brain tissue obtained after the onset of neurological disease and in the terminal phase. With a hybridization tomographic technique that combines *in situ* hybridization with macroradioautographic screening of large areas of tissue, we analyzed the spatial and temporal distribution of virus genes *in vivo*, using region- and strand-specific probes for the nucleocapsid and matrix genes. We show that early in the course of SSPE there is a global repression in the synthesis and expression of the genome. In the final stage of SSPE most infected cells still have depressed levels of plus- and minus-strand viral RNA and contain nucleocapsid protein but lack matrix protein. These findings provide further evidence for a unified view of slow infections of the nervous system, where the general constraints on virus gene expression provide an explanation for persistence of virus in the face of the host's immune response, and the slow evolution of pathological change. In the final phases of SSPE the more specific block in virus replication accounts for the cell-associated state of the virus and the difficulty in virus isolation.**

In slow infections caused by conventional viruses, periods often of years separate acquisition of virus and overt symptoms, and, during the long incubation period, virus persists in the face of the host's immune response. Subacute sclerosing panencephalitis (SSPE), for example, is a rare complication of measles infections in which the afflicted child ostensibly recovers from measles, develops normally for several years, and then slowly succumbs to a neurological disorder. The neurological disease reflects destruction of cells in the brain harboring measles virus despite high levels of virus-specific antibody in serum and cerebrospinal fluid (1–3).

We have attributed the slow evolution of SSPE and the persistence of measles virus in this disease to restricted synthesis and expression of virus genes in the nervous system (4). These global constraints result in decreased levels of viral antigens in the cell and, *pari passu*, diminished effectiveness of immune surveillance in eradication of infected cells. Restricted gene expression also mitigates cellular injury accompanying virus replication, and tissue damage therefore accumulates slowly.

This interpretation of the pathogenesis of SSPE emerged from investigations of tissues obtained for diagnosis relatively early in the disease (4). In the terminal state a more specific block in virus replication has been identified (5–9). The viral matrix (M) protein required for maturation of measles virus is markedly decreased in brain and in cell cultures derived from a patient with SSPE. In the latter case, mRNA for the M protein is present but is not translated effectively *in vitro* (9).

In this article we report the results of a more detailed examination of measles virus gene expression in the course of SSPE. We have used newer methods of *in situ* hybridization with increased sampling power (10, 11) and gene- and strand-specific probes to measure the levels of nucleocapsid (NP) and M RNAs in individual cells in the brain. In conjunction with monoclonal antibodies to these gene products (12, 13), we define a progression in restriction in SSPE from an early global decrease in both minus- and plus-strand viral RNAs and no detectible antigen to a terminal phase in which the number of copies of viral RNA per cell remains low and NP antigen is detectible but M protein is not.

## MATERIALS AND METHODS

**Human SSPE Tissue and Infected Tissue Culture Cells.** Frozen tissues obtained by biopsy at the time of diagnosis of SSPE were from C. J. Gibbs, Jr., and have been described (4). Large frozen coronal sections obtained postmortem from two cases of SSPE were from the National Neurological Brain Bank. Vero cells were infected with a single plaque of measles virus and collected for hybridization studies 72–96 hr later, when viral cytopathic effects were evident in >90% of the monolayer.

**Strand-Specific Probes for Measles Virus M and NP Genes.** cDNA clones specific for the NP and M genes of measles virus were obtained from S. Rozenblatt (14, 15). These recombinants were isolated from a library made by reverse-transcribing mRNA from measles virus-infected cells and inserting the transcripts into the *Pst* I site of pBR322. Plasmid DNA with the insert was purified, cleaved with *Pst* I, and electrophoresed in 1% low-gel-temperature agarose. The insert DNA band, visualized by staining with ethidium bromide, was excised from the gel and purified by phenol extraction after melting the agarose. The purified inserts were ligated into M13mp8RF digested with *Pst* I and used to transform *Escherichia coli* JM103. Recombinants with a lac$^-$ phe-

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Abbreviations: SSPE, subacute sclerosing panencephalitis; M, matrix; NP, nucleocapsid.

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notype were picked and propagated in small cultures. Single-stranded DNA prepared from the recombinants was labeled with [$^{32}$P]dATP by annealing a probe primer to the DNA, followed by extension with DNA polymerase (Klenow fragment). The labeled complementary strand was cross-linked to the M13 DNA with psoralen (16), and the labeled M13 was hybridized to oligo(dT)-selected mRNAs from infected Vero cells spotted on sheets of nitrocellulose. Clones hybridizing to mRNA contain inserts of the same minus-strand polarity as the measles virus genome. Clones containing inserts of the opposite polarity were identified by complementation test with M13 DNAs that did not hybridize to mRNA from infected cells (17).

**Labeling M13 Probes for *in Situ* Hybridization.** To prepare the small probes (range, 100–400 nucleotides) necessary for efficient hybridization *in situ*, the sequencing primer for M13 (18) was annealed at the 3′ side of the insert and extended with the Klenow fragment by using labeled dNTPs. For hybridization tomography, the probe was labeled to $6 \times 10^9$ dpm/$\mu$g with $^{35}$S-labeled dATP and $^{35}$S-labeled dTTP, each at 5 $\mu$M (700 Ci/mmol), and 5 $\mu$M $^{125}$I-labeled dCTP (2200 Ci/mmol). For *in situ* hybridization of productively infected tissue culture cells, $^3$H precursors were used for probes with specific activities of 3–5 $\times 10^8$ dpm/$\mu$g. After labeling and exclusion chromatography (Sephadex G-50), the probe was separated from the viral insert in M13 by sedimentation through an isokinetic alkaline sucrose gradient (19). This cDNA, opposite in polarity to the insert, was concentrated by precipitation with ethanol and stored in Tris·HCl, pH 7.4/1 mM $Na_4EDTA$/10 mM dithiothreitol.

**Hybridization Tomography.** Large coronal sections were embedded in 5% CM-cellulose. The frozen block was mounted on the LKB 2258 cryomicrotome, and 15- to 20-$\mu$m sections were cut and transferred to 3M 845 tape. After drying, the sections were fixed in cold ethanol (4°C) for 10 min and in ethanol at room temperature for 10 min. The dry sections were stored at room temperature. For hybridization, the tissue on the tape was treated to enhance diffusion of probe (10, 11), and the tape was pressed face down on a coated and acetylated glass slide. About $2 \times 10^6$ cpm of $^{125}$I- and $^{35}$S-labeled probe in 50 $\mu$l of buffered formamide with 10% dextran sulfate was injected with a 26-gauge needle between the tape and slide, and the hole was sealed with rubber cement. After hybridization at room temperature for 60 hr, the tapes were removed and washed extensively in 3 liters of hybridization medium lacking dextran sulfate. Tapes were exposed to x-ray film and subsequently coated with NTB-2 emulsion. After development, they were stained with hematoxylin/eosin. Soft laser densitometry was used to compare relative levels of hybridization of NP and M probes to tissues.

***In Situ* Hybridization.** Frozen sections of brain biopsies were cut, picked up with treated glass slides, and hybridized *in situ* as described (4). Infected Vero cells were deposited by cytocentrifugation on treated slides and hybridized with the dual-labeled probes or, for precise quantitation, with $^3$H-labeled probes [to obtain lower grain counts in the shortest exposure time (1.5 hr) compatible with drying the NTB-2 emulsion]. The specificity of hybridization was established by showing that the measles virus probes reproducibly bound to tissue and that only background levels of hybridization were obtained for sections pretreated with ribonucleases or hybridized to a probe specific for an unrelated virus (visna, a retrovirus of sheep).

**Monoclonal Antibodies to M and NP Gene Products: Immunoperoxidase Methods.** Monoclonal antibodies to M, NP, and other measles virus gene products were prepared in mice (12, 13). The IgG fraction of ascitic fluid was isolated by precipitation with $Na_2SO_4$ and ion-exchange chromatography on DEAE- and CM-Sephadex. Antibodies diluted to 10–100 $\mu$g/ml in phosphate-buffered saline were allowed to react with fixed sections on tape or glass slides. Subsequent steps in the immunoperoxidase procedure followed protocols accompanying the avidinbiotin-peroxidase reagents from Vector Laboratories (Burlingame, CA).

## RESULTS

**Measles NP and M Genes and Gene Products in Terminal SSPE.** To evaluate virus gene expression in SSPE brain obtained postmortem, we needed to locate regions of the brain with viral RNA for comparison with unrestricted productive infections in tissue culture. To screen large areas of frozen tissue, we employed an *in situ* hybridization technique (10, 11) that combines detection of genes on a macroscopic scale with the fine cellular resolution of *in situ* hybridization. This panoramic view of infection is achieved by incorporating $^{125}$I and $^{35}$S into the hybridization probe. The $\gamma$-emission of $^{125}$I produces a macroscopic image on x-ray film, whereas the Auger and $\beta$ particles emitted by $^{125}$I and $^{35}$S, respectively, produce microscopic radioautographs with good resolution after the section has been coated with nuclear track emulsion. High sensitivity of detection obtains for both the macroscopic and microscopic ends of the scale; for x-ray film, amplifying screens and high specific activity of probes provide sensitivity; for microscopic radioautography, the high specific activity of probes and the high efficiency of latent image formation with the dual label (0.4 grain per disintegra-

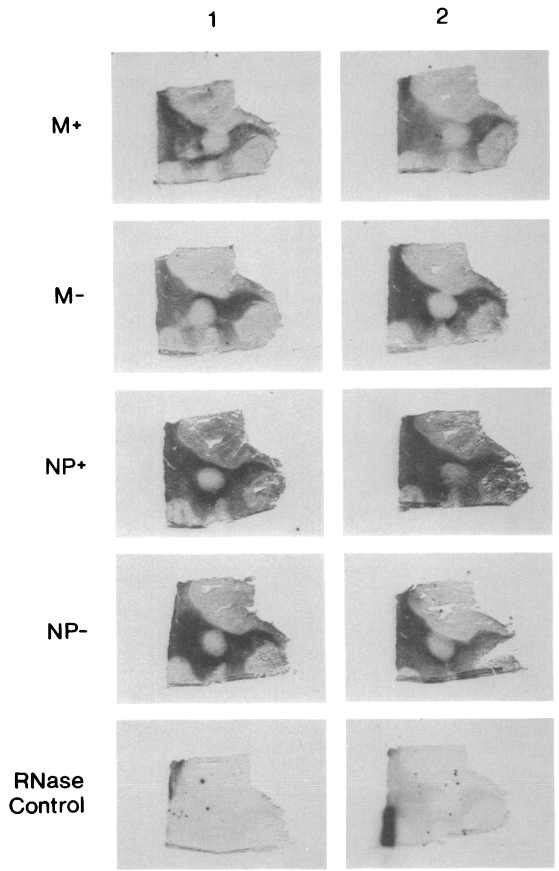


FIG. 1. Detection of measles virus genes by hybridization tomography. A block of frozen tissue obtained postmortem from a patient with SSPE was embedded in CM-cellulose. Sections 15- to 20-$\mu$m thick were cut at two levels, designated 1 and 2, and transferred to clear tape. After hybridizing to plus- or minus-strand probes (the designation + or − refers to the polarity of the probe) for the M and NP genes, the tapes were washed and placed against Kodak XAR-5 film for 4 days in cassettes with Cronex Lighting Plus intensifying screens.

tion) provide sensitivity. We refer to this method as hybridization tomography to emphasize the imaging of viral genes in multiple planes.

With this technique we were able to quickly identify regions of brain with measles virus genes, such as the one shown in Fig. 1, at two depths designated 1 and 2. We quantitated by densitometry the relative amount of plus- and minus-strand probe that had hybridized to these sections and found essentially equivalent levels of hybridization for the genome (minus) strand M and NP RNAs (NP/M = 1.01).

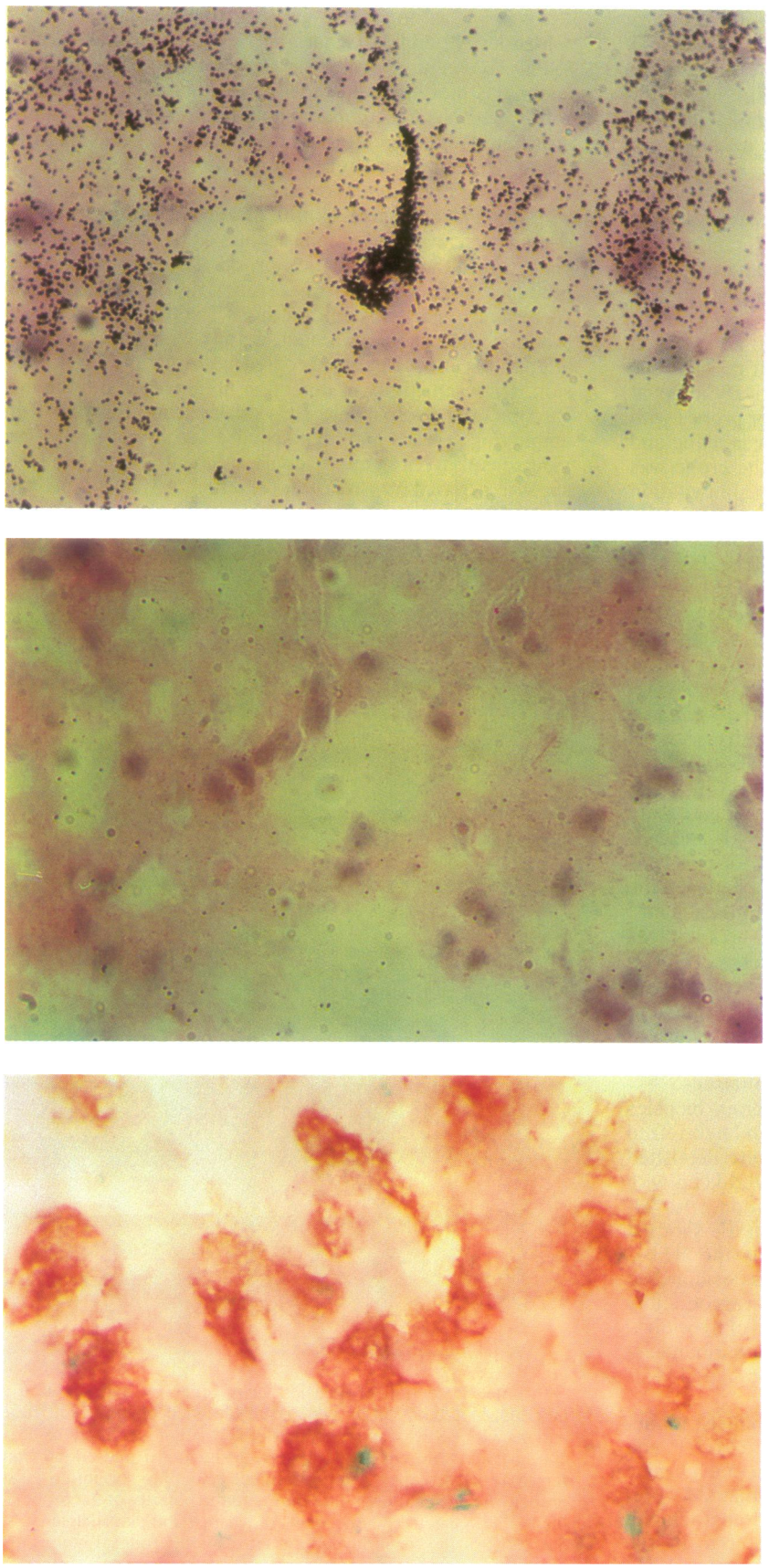

FIG. 2. Detection of measles virus genes and gene products *in situ* in terminal SSPE. After coating the hybridized tapes with NTB-2 emulsion and exposing at 4°C for 10 days, the tapes were developed and stained with hematoxylin/eosin. Regions with higher levels of hybridization were located by tracing the darker areas in the x-ray film onto the slide. Cells with correspondingly higher levels of hybridization of probe were readily identified in these areas. (*Top*) Neuron with NP minus-strand RNA in the cell body and axon. Viral RNA is also present in cellular processes throughout the neuroparenchyma, reflected in the increased number of grains between cells compared to a section hybridized to a visna virus-specific probe (*Middle*). (*Bottom*) Demonstration of NP antigen in terminal SSPE. Subjacent sections to those used for hybridization were allowed to react sequentially with dilute horse serum, murine monoclonal antibody to the NP antigen, biotinylated anti-murine IgG, and avidin-biotin-peroxidase. Endogenous peroxidase activity was blocked with methanol/$H_2O_2$ prior to addition of the latter reagent. The sites of peroxidase deposition were revealed by reaction with diaminobenzidine in $H_2O_2$, and the nuclei of cells were stained green with methyl green/$CuSO_4$. Sections were washed with phosphate-buffered saline between steps. Brown diaminobenzidine reaction product is evident in most cells in this focus. (Original magnifications, ×500; final magnifications, ×300).

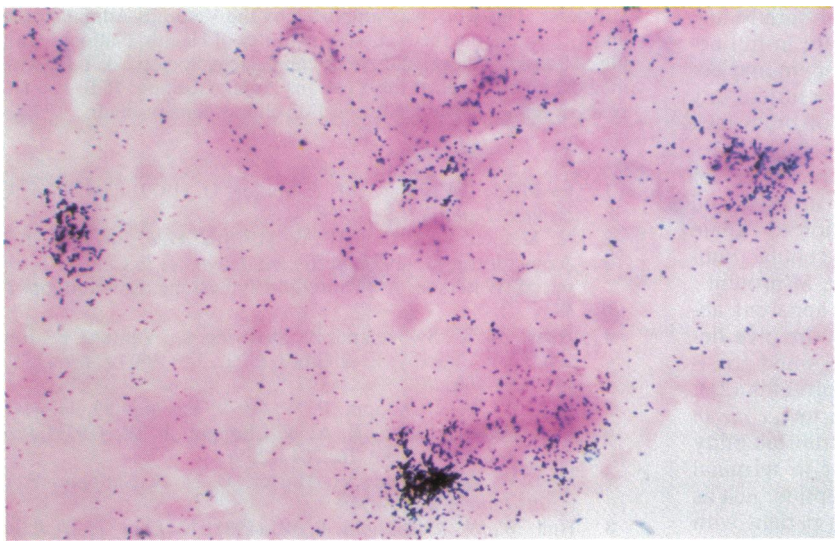

FIG. 3. Detection of measles virus-specific RNA by *in situ* hybridization in preterminal SSPE. Hybridization with NP probes to 10-$\mu$m sections of frozen brain from a biopsy of a patient with SSPE; 7-day exposure. (Original magnification, ×500; final magnification, ×300).

Both regions of the genome were expressed but NP plus-strand RNA was 1.35 times as abundant as M plus-strand RNA.

We used the macroscopic radioautographs as a guide to the areas in the section that should be examined microscopically to define the cellular distribution of the M and NP genes. After the sections had been coated with nuclear track emulsion and subsequently developed and stained, they were aligned with the macroscopic radioautographs, and the heavily darkened areas on x-ray film were traced onto the section. We found in these areas the expected higher levels of viral RNA in cells identified morphologically as glial cells or neurons—e.g., a neuron with viral RNA in the cell body and axonal process is evident in Fig. 2 *Top*. We also detected viral RNA diffusely distributed throughout the neuroparenchyma (Fig. 2 *Top*). The silver grains between cells reflect specific hybridization of the measles probe, since a homologous probe for an unrelated retrovirus of sheep, visna, does not bind to the subjacent section (Fig. 2 *Middle*). We used monoclonal antibodies to NP and M and the most sensitive of current methods for detection of antigens in individual cells (20) to evaluate synthesis of measles gene products in terminal SSPE. Cells in the subjacent sections to those that had both NP and M plus-strand RNA contained NP antigen (Fig. 2 *Bottom*) but lacked detectible M antigen.

**Measles Genes and Gene Products at an Earlier Stage in SSPE.** In preterminal SSPE, in biopsy material taken for diagnosis, we also detected by *in situ* hybridization both plus- and minus-strand NP and M RNA. The number of copies of viral RNA in foci like the ones shown (Fig. 3) were similar to those in terminal SSPE; in both cases copy numbers were reduced by more than two orders of magnitude compared to permissive infections (Table 1). The most striking difference between the terminal stage and the earlier stages of SSPE is accumulation of antigen. In multiple sections from four cases, cells that contained plus-strand M and NP RNA lacked detectible levels of both antigens (Table 1).

## DISCUSSION

This *in vivo* analysis of the measles virus life cycle in single cells provides direct evidence for altered synthesis and expression of the measles virus genome in SSPE. These changes historically progress from a global restriction in synthesis of minus-strand and plus-strand RNAs and in production of antigens to a terminal phase in which only NP antigen can be demonstrated. In both the early and late stages of

Table 1. Synthesis and expression of measles virus genes in SSPE

| Type of infection | Relative concentration of measles virus RNAs | | | | Measles virus proteins | |
|---|---|---|---|---|---|---|
| | NP − | M − | NP + | M + | NP | M |
| Permissive infection of tissue culture cells (Vero monkey kidney) | 100 | 100 | 100 | 100 | + | + |
| SSPE | | | | | | |
| Terminal | 0.3 | 0.2 | 0.2 | 0.07 | + | − |
| Preterminal | 0.4 | 0.7 | 0.2 | 0.09 | − | − |

The concentrations of NP and M plus- and minus-strand RNAs in SSPE were compared to the terminal and preterminal stages of permissive infections in tissue culture as follows. (*i*) Vero cells were infected with one plaque of the Lec strain of measles virus isolated from a patient with SSPE. At 72 hr when the cytopathic effect involved 90% of the monolayer the cells were removed by trypsinization, collected by centrifugation, washed once with phosphate-buffered saline, and deposited with a cytocentrifuge onto treated glass slides. The productively infected cells were hybridized *in situ* with strand-specific probes with $^{3}$H-labeled dNTPs with a specific activity of $4 \times 10^{8}$ dpm/$\mu$g. After washing to remove unreacted probe, the slides were coated with NTB-2 emulsion, developed after 8 hr, and stained with hematoxylin/eosin. The average number of grains per cell in excess of a control of uninfected Vero cells was determined for 100 cells. The average number was normalized to a 24-hr exposure and assigned a value of 100. (*ii*) Sections from SSPE were hybridized with strand-specific probes labeled with $^{125}$I and $^{35}$S and average grain counts were determined for cells in the maximal regions of hybridization such as those evident in Fig. 1. The values were normalized to a 24-hr exposure and the different specific activities and efficiencies of grain development (the $^{125}$I and $^{35}$S probes had specific activities about four times those of the $^{3}$H-labeled proteins and four times the efficiency of $^{3}$H in forming latent images in NTB-2 emulsion). These assumptions were validated by directly comparing in infected Vero cells the radioautographic signal generated by the probes with $^{3}$H or with $^{125}$I or $^{35}$S label. The validity of comparing tissue culture cells and sections was also evaluated in hamsters succumbing to acute measles virus infection. The grain counts found in cells in sections in productive infections *in vivo* closely approximated those in infected tissue culture cells. (*iii*) The ratio of the normalized grain counts in SSPE and Vero cells was determined. (*iv*) The content of virus protein in individual cells in the last column of the table was evaluated with monoclonal antibodies and immunoperoxidase staining. Sections were evaluated by three independent observers to score cells stained with diaminobenzidine (designated +) or lacking stain (designated −).

SSPE, infected cells contain the plus strands for both M and NP RNAs but lack M antigen. These results are in full accord with observations made by Hall and Choppin and coworkers that patients succumbing to SSPE have little if any antibody to the M polypeptide, and extracts of brain lack significant quantities of M antigen (5–8). This specific restriction in the virus life cycle provides an explanation for the highly cell-associated state of measles virus in SSPE and the requirement for cocultivation or explantation to isolate virus; these maneuvers presumably furnish cellular substrates to repair the defect in production of the M protein.

The molecular mechanisms underlying the apparent absence of M polypeptide in terminal SSPE may involve decreased transcription and translation of M mRNA and possibly diminished stability of paramyxovirus M proteins (21). Our observations *in vivo* parallel those of Carter *et al. in vitro* (9) and are consistent with a defect in the translatability of M mRNA. Plus-strand M RNA is present in terminal SSPE, in cells that lack demonstrable M polypeptide, just as RNA extracted from a cell line derived from a patient with SSPE contains M mRNA that is not translated efficiently. However, the number of copies of M plus-strand RNA *in vivo* is also greatly reduced compared to productive infections (Table 1); decreased transcription therefore may contribute as well to the decreased concentrations of M protein in SSPE.

At an earlier stage in the development of SSPE, we confirmed the global decrease in viral RNA content documented previously with probes containing sequences from all regions of the genome and of both polarities (4). By using strand-specific probes, we know now that the block in gene expression in preterminal SSPE applies to the synthesis of both plus- and minus-strand RNA. We also confirmed the rarity of cells that contain detectible NP or M polypeptides using an immunoperoxidase technique that is 40 times more sensitive than immunofluorescence (20). Because the number of copies of plus-strand NP RNA is about the same in preterminal and terminal SSPE, but NP is readily detectible only in the latter stage, we surmise that the synthesis of antigens is a slow cumulative process. Eventually stable antigens such as the NP protein do accumulate in infected cells.

These results continue to suggest that the central nervous system (CNS) is peculiarly susceptible to slow infections because of the limited replication of many viruses in cells of this system (4, 22). The low levels of viral antigens in this situation provide a mechanism for the infected cell to escape detection and eradication by immune surveillance. At the same time the pathological effects associated with virus replication evolve at a commensurately slow tempo. This reconstruction encompasses the observations in SSPE (4), visna (23), and other slow infections (24–26) and the discovery of measles, herpes simplex, and polio virus nucleotide sequences in the brains of normal individuals and those with neurological diseases such as multiple sclerosis (5, 27, 28). Although the role of viruses in disease is still unclear, the discovery of an indigenous viral flora in the CNS is sufficiently provocative in our view to justify a larger search for viral genomes, using approaches illustrated in this article.

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