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Temperature-sensitive Phenotype of a Mutant Sendai Virus Strain Is Caused by Its Insufficient Accumulation of the M Protein*

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We investigated the process interrupting the production of a temperature-sensitive mutant strain of Sendai virus, Cl.151, at the nonpermissive temperature (38 °C). The amount of virus M protein increased up to 6-fold when the cells persistently infected with Cl.151 strain at 38 °C are transferred to 32 °C, while the amount of nucleocapsid proteins did not alter. Cl.151 strain could restore virus production at 38 °C not only by the supplementation of M protein of wild type (Z) strain but also by the supplementation of M protein of Cl.151 strain. Neither the amount of M mRNA nor the rate of synthesis of M protein was altered by temperature in cells infected with the Cl.151 strain. However, we found that M protein of Cl.151 virus, which has 3-amino acid alterations from the wild type, was highly unstable at 38 °C when expressed under the control of an actin promoter. These results clearly show that Sendai virus M protein has a critical role in the production of virus particles without affecting virus gene expression.

Introduction of foreign genetic materials into tissue cells has become an important tool in medical research, and many virus vectors have been developed for this purpose (for review, see Friedmann (1989) and Miller (1992)). We previously reported that DNA encapsulated inside the envelope of Sendai virus could be introduced into cultured cells (Nakanishi *et al.*, 1985) and into the parenchymal hepatocytes of living rats (Kato *et al.*, 1991a, 1991b). These data encouraged us to develop a new virus vector based on Sendai virus.

Sendai virus is a prototype virus of the paramyxovirus family and contains a nonsegmented negative strand RNA genome (15,383 nucleotides), which codes eight proteins (NP, P/C/V, M, F, HN, and L). NP, P, and L proteins make up the nucleocapsid by tightly associating with the genomic RNA. The nucleocapsid is surrounded by a viral envelope, which is derived from the host cell plasma membrane and contains two glycoproteins (F and HN). The nucleocapsid and the envelop are connected by M proteins (Shimizu and Ishida, 1975; Yoshida *et al.*, 1976).

The persistent infection is an important requirement in developing virus vectors to express a foreign gene stably. Although certain paramyxoviruses can establish stable persistent infection (Randall and Russell, 1991), the detailed mechanism of this phenomenon was not well documented. In Sendai virus, persistent infection is caused by two types of mutant viruses, defective interfering (DI)[1] viruses (Tadokoro, 1958; Sokol *et al.*, 1964; Kingsbury *et al.*, 1970) and a temperature-sensitive (ts) mutant virus (Yoshida *et al.*, 1979). DI viruses have lost most of the genomic RNA of the wild type virus and can coinfect the cells persistently with the wild type virus. Because the short DI genomic RNA replicates more rapidly than wild type genomic RNA, persistent infection by DI viruses was thought to be caused primarily by the suppression of the replication of the wild type genome (Re, 1991). Roux and Waldvogel (1982) and Tuffereau and Roux (1988) reported the decline of the amount of M protein and HN protein due to their decreased stability in cells infected persistently with DI genome. However, both the relationship between the defects in the replication cycle of the virus and the instability of the virus proteins and the precise molecular mechanism of the instability of these proteins remain unclear.

On the contrary, defects in the replication of most of the ts mutant viruses with single genome RNA were thought to be caused by the mutation of a single gene (Portner *et al.*, 1974). A ts mutant of Sendai virus, Cl.151, which was isolated from a Sendai virus-carrier culture, is blocked in a late function required for virus assembly at the nonpermissive temperature (38 °C). This ts mutant can readily establish persistent infection in various cells without the aid of the DI virus (Yoshida *et al.*, 1982). By indirect immunofluorescent analysis of the cells infected with Cl.151 virus, Yoshida *et al.* (1979) demonstrated that the amount of the viral M protein was less at 38 °C than at 32 °C, whereas the amounts of the NP and P proteins were almost equivalent at either temperature. In addition, the pulse-labeled experiments of the infected cells showed that the M proteins synthesized at the nonpermissive temperature were not incorporated into virions produced after shift-down to the permissive temperature (32 °C) (Yoshida *et al.*, 1979). These data suggested that some defect(s) in M protein might interrupt the assembly of virus particles at the nonpermissive temperature.

In this article, we studied the factor that interrupted the virus production in the Cl.151 strain. We find that the alteration of amino acid residues in the M protein causes the decreased stability of the proteins and that this instability interrupts the production of virus particles without affecting the viral gene expression. Our result is an important step for

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[1] The abbreviations used are: DI, defective interfering; MEM, minimum Eagle's essential medium; NCS, newborn calf serum; PBS, phosphate-buffered saline; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate.

developing the stable gene transfer vectors based on Sendai virus.

MATERIALS AND METHODS

Virus and Cells—Sendai virus Z strain and Nagoya strain were grown in 10-day-old embryonated chicken eggs at 35.5 °C (Okada *et al.*, 1961). Sendai virus Cl.151 strain was grown as described previously (Yoshida *et al.*, 1979).

The recombinant vaccinia virus strain, RVV-T7, was a kind gift from Dr. Akihisa Takamizawa, the Research Foundation for Microbial Disease of Osaka University, Kanonji, Kagawa, Japan. Preparation of the vaccinia virus stock and the titration of the stock were carried out as previously described (Mackett *et al.*, 1985).

$LLCMK_2$ cells (Kan *et al.*, 1970) and HeLa S3 cells were grown in minimum Eagle's essential medium (MEM) containing 10% new born calf serum (NCS) in a CO_2 incubator at 37 °C. A cell line persistently infected with Sendai virus, LLC151Cl.9, was established by cell cloning from $LLCMK_2$ cells infected with Sendai virus Cl.151 at 38 °C.

Protein Purification and Antibodies—Sendai virus M protein was purified from virus particles as previously described (McSharry *et al.*, 1975). Sendai virus NP and P proteins were purified from the nucleocapsid of the virus by solubilizing in 6M guanidine HCl and column chromatographies on Toyopeal HW65, DEAE Toyopeal 650, and PhenylToyopeal 650 (Tosoh Inc., Tokyo, Japan).[2] The rabbits (2-month-old female, Japan white) were immunized with NP, P, or M proteins emulsified in Freund's adjuvant. Total serum IgG was purified with Avid AL gel (Bioprobe International, Inc., Tustin, CA) as described (Ngo and Khatter, 1990).

cDNA Cloning and Construction of Plasmids—pCAM1-Z was constructed by inserting the *Xho*I fragment of pM7, a cDNA encoding M protein of Sendai virus Z strain[3] cloned in the Okayama-Berg vector (Okayama and Berg, 1983), into *Xho*I site of pBY1. pBY1[4] contained cytomegalovirus enhancer/chicken β-actin hybrid promoter from pCAGGS (Niwa *et al.*, 1991) (a kind gift from Dr. Junichi Miyazaki), a unique cloning site (*Xho*I and *Bam*HI), and SV40 early gene poly(A) signal on the base of pGEM5Zf(+) (Promega Corp.). pCAM2-Z was constructed by removing the extra nucleotides of the 5′-untranslated region of pCAM1-Z by *in vitro* mutagenesis as described (Kunkel, 1985), with modification, using the oligonucleotide (5′TTCCTCCGCAGCCAGCCATGGCAGATATCTATAG3′).

The genomic RNA of Cl.151 and Nagoya strains of Sendai virus was prepared as described (Re and Kingsbury, 1986). The cDNA was synthesized by Molony murine leukemia virus RNase H^- reverse transcriptase (Life Technologies, Inc.) according to the protocol from the manufacturer, using the 30-mer primer (5′GGGGGAATTCACATAGAGTCACTGACCAAC3′), which could anneal to the P/C gene of Sendai virus genomic RNA. The cDNAs were digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI/*Bam*HI site of pGEM7Zf(−) (Promega Corp.), resulting in pM-Cl.151 for the Cl.151 strain and pM-Nagoya for the Nagoya strain. The cDNAs were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) after subcloning.

pCAM2-Cl.151 was constructed by replacing the *Eco*RV/*Bam*HI fragment of pCAM2-Z with the *Eco*RV/*Bam*HI fragment of pM-Cl.151. pCAM2-69Glu, pCAM2-116Ala, and pCAM2-183Ser were prepared by *in vitro* mutagenesis using pCAM2-Z as a template. The nucleotide sequences of all the mutagenized plasmids were confirmed by sequencing analysis. The oligonucleotides used in mutagenesis reaction were 5′ACAAACAACCAATCTAGAGAGCGTATCTGACTTG3′ for replacement of Gly^{69} with Glu, 5′ATCACGGTGAGGAGGGCTGTTCGAGCAGGAG3′ for replacement of Thr^{116} with Ala, 5′GGGGCAATCACCATATCCAAGATCCCAAAGAC3′ for replacement of Ala^{183} with Ser.

pT7-ø was constructed by inserting the *Eco*RV/*Bam*HI fragment of pET3b (Rosenberg *et al.*, 1987) containing T7 RNA polymerase termination signal into the *Bam* HI/NsiI site of pGEM7Zf(−) after filling the *Nsi*I site with T4 DNA polymerase. pT7M-Z was constructed by inserting the *Xho*I fragment of pCAM1-Z into the *Xho*I site of pT7-ø. pT7M-Cl.151 was constructed by inserting the *Eco*RI/*Bam*HI fragment of pM-Cl.151 into the *Eco*RI/*Bam*HI site of pT7-ø.

pGEM7-M-1 was constructed by inserting the *Stu*I/*Xho*II (238 base pairs) fragment of pCAM1-Z into the *Sma*I site of pGEM7Zf(−) after filling the *Xho*II end with T4 DNA polymerase.

Transfection—On day 0, 5×10^5 to 1×10^6 $LLCMK_2$ cells were seeded into 100 mm Petri dishes in MEM containing 10% NCS. On day 1, the cells were transfected with 1 μg of pSV2-neo (Southern and Berg, 1982) and 19 μg of pCAM2-Z, pCAM2-Cl.151, pCAM2-69Glu, pCAM2-116Ala, or pCAM2-183Ser by the calcium phosphate precipitation method as described (Graham and Van der Ed, 1973). The cells were cultured in the presence of G418 (300 μg/ml) from day 3. The cell lines expressing M protein, LLCZMCl.4, LLCZMCl.11, LLCZMCl.14, LLCZMCl.21, LLCZMCl.23, LLCZMCl.31, and LLC151MCl.N, were isolated by first screening with indirect immunofluorescent staining using polyclonal anti-M rabbit antibodies and then with the immunoblotting and the immunoprecipitation. The cells resistant to G418 were used, without cloning (pool culture), for the experiment in Fig. 7.

M protein of Z strain or Cl.151 virus was also expressed transiently by using pT7M-Z, pT7M-Cl.151, and T7 RNA polymerase encoded in recombinant vaccinia virus RVV-T7 as described (Fuerst *et al.*, 1986) (Fig. 3). LLC151Cl.9 cells were washed once with MEM containing 2.5% NCS and then were infected with RVV-T7 with an infection multiplicity of ~5 in the same medium. 45 min later, the cells were incubated with 10 μg of DNA and 50 μg of synthetic cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mannheim) in 4 ml of Opti-MEM (Life Technologies, Inc.) for 8 h at 38 °C. The amount of the M proteins expressed transiently was determined by immunoblotting with polyclonal anti-M rabbit antibodies. Both pT7M-Z and pT7M-Cl.151 could express almost the same amount of M protein as stable cell line LLCZMCl.31 (data not shown).

RNA Analysis—Total RNA were extracted from cells with guanidine thiocyanate (Han *et al.*, 1987). RNA was denatured in formamide and formaldehyde and was analyzed by electrophoresis in a denaturing agarose gel (Lehrach *et al.*, 1977). RNA was electrically transferred from agarose gel to nylon membrane (GeneScreen Plus, Du Pont-New England Nuclear) (Ishihara and Shikita, 1990). Single strand RNA probe for detecting the M mRNA was synthesized with SP6 RNA polymerase (Melton *et al.*, 1984) using pGEM7-M-1 digested with *Eco*RI as a template and [α-^{32}P]UTP (Bresatec Ltd., Thebarton, South Australia ~3000 Ci/mmol). The membranes were hybridized with RNA probe (1×10^6 cpm/ml) at 55 °C and washed as described (Church and Gilbert, 1984). The membranes were then exposed to the Fuji RX-H x-ray film at room temperature for 16 h. The amount of M mRNA was determined by densitometry.

Immunoblotting—The cells were harvested in 1 ml of ice-cold PBS and centrifuged at 3000 rpm for 10 s. The pellets were suspended in 200 μl of sample buffer (60 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.001% Bromphenol Blue, and 50 mM dithiothreitol), boiled for 2 min after brief sonication, then centrifuged at 15,000 rpm for 10 min at 20 °C to remove the insoluble materials. The samples containing 200 μg of protein were separated by discontinuous SDS-polyacrylamide gel electrophoresis (9%) (Laemmli, 1970) and transferred onto the polyvinylidene difluoride membrane (Bio-Rad) (Towbin *et al.*, 1979). The membrane was washed twice with PBS containing 0.05% Tween 20 and 3% nonfat milk at room temperature for 30 min. The membrane was then treated with a rabbit polyclonal antibody (3 μg/ml protein) to NP, P, or M protein for 2 h at room temperature, followed with ^{125}I-labeled protein A (50 nCi/ml, >30 mCi/mg), (Amersham), and washed twice with PBS containing 0.05% Tween 20 and 3% nonfat milk for 5 min at room temperature and then once with PBS for 5 min at room temperature. The membranes were then exposed to Fuji RX-H x-ray film at room temperature for 24 h to detect NP and P protein and for 72 h to detect M protein. The amount of viral proteins was determined by densitometry.

Immunoprecipitation—To examine the efficiency of synthesis of M protein (Fig. 5), the LLC151Cl.9 cells (5×10^5) were grown in 60-mm dishes with 5 ml of MEM containing 10% NCS at 32 °C or 38 °C for 24 h. After the cells were washed and incubated with 1 ml of MEM lacking methionine for 15 min at either temperature, the medium was replaced with 1 ml of prewarmed MEM with 5% NCS, lacking cold methionine and containing 500 μCi/ml [^{35}S]methionine
(Expre ^{35}S-protein labeling mix, >1000 Ci/mmol, Du Pont-New England Nuclear) to 20 min at either 32 or 38 °C.

For pulse-chase experiments (Figs. 6 and 7), the cells expressing M protein (5×10^5) were grown in 60-mm dishes with 5 ml of MEM containing 10% NCS at 32 °C for 16 h. After the cells were incubated with 1 ml of MEM lacking methionine for 15 min at 32 °C, the medium was replaced with 1 ml of prewarmed MEM with 5%

[2] M. Nakanishi, manuscript in preparation.
[3] N. Miura, unpublished observations.
[4] M. Nakanishi, unpublished observations.

NCS lacking cold methionine and containing [^{35}S]methionine (LLCZMCl.4, 50 μCi/ml; LLC151MCl.N and the cells in the experiment of Fig. 7, 500 μCi/ml) for 1 h at 32 °C. Then the cells were washed twice and incubated with 5 ml of MEM containing 5% NCS and 0.5 mM cold methionine at either 38 or 32 °C for various periods before harvest.

The cells were harvested in 1 ml of ice-cold PBS, and suspension was centrifuged 3000 rpm for 10 s. The pellets were suspended in 200 μl of lysing buffer (0.1 M NaCl, 10 mM Na-Na phosphate buffer (pH 7.2), 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin, and 100 μg/ml E64) and chilled on ice for 5 min. The suspension was then centrifuged for 3 min, 5000 rpm at 4 °C in microcentrifuge tubes, and the supernatant was recovered. 10 μl of the extract was saved for examining trichloroacetic acid-insoluble radioactivity. The remainder of the extract was mixed with 1 ml of dilution buffer (0.5 M NaCl, 10 mM Na-Na phosphate buffer (pH 7.2), 5 mM EDTA, 0.5% Zwittergent 314 (Calbiochem), 0.15% SDS, 10 mM dithiothreitol, 5 mg/ml bovine serum albumin, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM leupeptin, and 10 μg/ml E64). Then an amount of radiolabeled M protein was determined by immunoprecipitation as described previously (Nakanishi *et al.*, 1988) with 25 μg of polyclonal rabbit anti-M IgG.

Other Assays—Hemagglutinating activity was determined as described (Salk, 1944). Protein was determined as described (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

RESULTS

Insufficient Accumulation of the Virus M Protein Interrupts Virus Particle Assembly in the Temperature-Sensitive Mutant Sendai Virus Cl.151—To investigate the relationship between the level of each viral protein and the production of Cl.151 virus, we first examined the amount of M, NP, and P proteins in the LLC151Cl.9 cells, a cell line persistently infected with Cl.151, by quantitative immunoblotting. The cells were maintained at 38 °C and then at 32 °C for 3 days. The cells were harvested every 24 h, and the amount of viral protein was determined. As shown in Fig. 1*A*, the amount of M protein increased about 6-fold after the culture temperature was shifted to 32 °C for 24 h from 38 °C, but the amounts of NP and P proteins did not alter at either temperature. The increase in the amount of M protein in the cells at 32 °C was followed by the release of virus particles into the culture medium 48 h after temperature shift-down (Fig. 1*B*).

In a separate experiment, we examined whether the expression of wild type M protein could complement the defect of Cl.151 virus. We made an expression vector in which the coding sequence for the wild type M protein of Z strain was under the control of the strong chicken β-actin/CMV hybrid promoter, transfected it to $LLCMK_2$ cells, and obtained 4 cell lines expressing the M protein stably at a high level (LLCZMCl.4, LLCZMCl.14, LLCZMCl.21, and LLCZMCl.31) (Fig. 2*B*, *lanes 6*, *8*, *9*, and *11*). These cells were indistinguishable from normal cells in every aspect. This is in clear contrast to vesicular stomatitis virus, whose M protein has a strong cytopathic effect when expressed in the cells (Blondel *et al.*, 1990). The cells expressing M protein were infected with Cl.151 virus and cultured at 38 °C for 3 days, and the amount of virus particles in the culture medium was determined. As shown in Fig. 2*A*, virus particles were detected in the medium of all four cell lines expressing the wild type M protein infected with Cl.151 virus at the nonpermissive temperature (38 °C). These results strongly suggested that the production of Cl.151 virus at the nonpermissive temperature was interrupted by defect(s) in the M protein and that the production of the virus might depend on the amount of M protein in the persistently infected cells.

Although the primary structure of the M gene of Cl.151 virus had not been determined, some alterations of amino

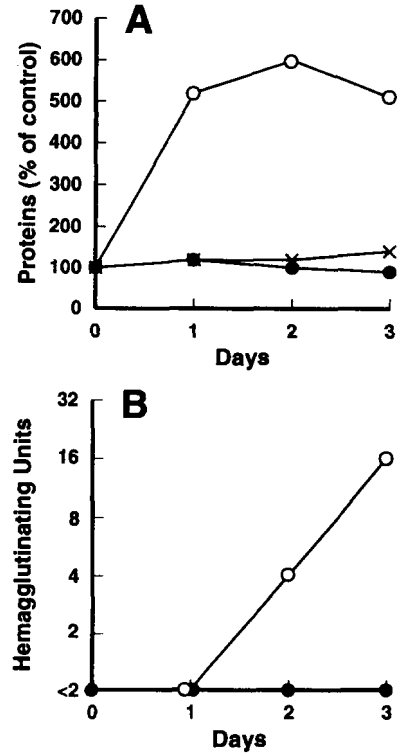

FIG. 1. **Effect of temperature on the amount of viral protein in the LLC151Cl.9 cells.** LLC151Cl.9 cells were maintained at 38 °C as described under "Materials and Methods." On the day before the experiment, the cells were set up at 2×10^6/100-mm dish in MEM containing 10% NCS at 38 °C. After incubation for 24 h at 38 °C (day 0), the medium was replaced with 10 ml of fresh medium. Then the cells were cultured at 32 °C except (●) in *panel B*. The cells were harvested every 24 h (days 1–3), and 200 μg of the total cellular proteins were separated by SDS-polyacrylamide gel electrophoresis. Then the amounts of each viral protein were determined by immunoblotting using each respective antibody as described under "Materials and Methods." The relative amount of each protein was indicated as percentages with the amount at day 0 as 100%. At the same time, hemagglutinating activity in the culture medium was assayed as described under "Materials and Methods." *Panel A*, the amount of NP (●), P (×), and M (○) protein in the cells. *Panel B*, hemagglutinating activity in the culture medium; ○, 32 °C; ●, 38 °C.

acid residues in the M protein of Cl.151 virus were suggested from the electrophoretic behavior as shown in Fig. 2*B* (also see Yoshida *et al.*, (1979)). Since the temperature-dependent phenotype might be caused by these alterations, we cloned the M genes of Cl.151 strain and Nagoya strain, the parental strain of Cl.151 strain, including 5′ and 3′ noncoding regions of the gene and determined their nucleotide sequences. Neither deletion nor insertion of nucleotides was detected in either the coding or the noncoding regions of M genes of Cl.151 strain and Nagoya strain, compared with the M gene of Z strain. Also no alteration of nucleotides was detected in the noncoding region (data not shown). Table I summarizes the comparison of amino acid residues in M proteins deduced from the nucleotide sequences of three wild type strains (Z, Nagoya, and Harris) and the Cl.151 strain. We found the alteration at 7 amino acid residues among the 4 virus strains. Among those, 3 alternated amino acid residues at no. 69, no. 116, and no. 183 were found only in the M protein of Cl.151 virus. These 3 altered amino acid residues of the M protein were considered the probable causes of the interruption of Cl.151 virus production at the nonpermissive temperature.

Two possible mechanisms are considered for the ts phenotype of the M protein of Cl.151: first, mutant M protein loss

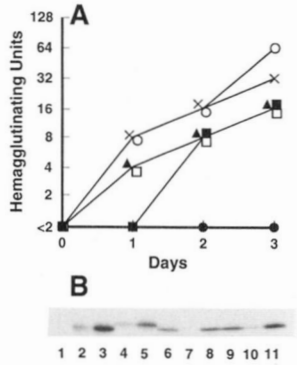

FIG. 2. **Effect of the expression of wild type M protein upon production of Cl.151 strain.** *Panel A*, $LLCMK_2$ cells expressing M protein of Z strain were obtained and maintained at 37 °C as described under "Materials and Methods." On the day before the experiment, the cells expressing M protein of Z strain were set up at 5×10^5/100-mm dish in 10 ml of MEM containing 10% NCS at 37 °C. After 24 h (day 0), the cells were infected with Cl.151 virus at a multiplicity of infection of ~40 and were cultured at 38 °C except (■) for 3 days as described under "Materials and Methods." 1 ml of the medium was harvested every 24 h, and the hemagglutinating activity was determined as described under "Materials and Methods." The cells used were: □, LLCZMCl.4; ×, LLCZMCl.14; ○, LLCZMCl.21; ▲, LLCZMCl.31; ●, mock-transfected cells cultured at 38 °C; ■, mock-transfected cells cultured at 32 °C. *Panel B*, the extract from $LLCMK_2$ cells expressing the M protein of Z strain (LLCZM clone series) and from $LLCMK_2$ cells infected with Sendai virus was prepared and separated on SDS-polyacrylamide gel electrophoresis (9%) as described under "Materials and Methods." The amount of M protein in cell extract (200 μg of protein) was determined by immunoblotting using polyclonal anti-M rabbit antibodies as described under "Materials and Methods." The cells used were: *lane 1*, mock-transfected cells; *lane 3*, cells infected with Z strain at a multiplicity of infection of ~40 and cultured for 24 h at 37 °C; *lane 4*, LLC151Cl.9 cells cultured for 24 h at 38 °C; *lane 5*, LLC151Cl.9 cells cultured for 24 h at 32 °C; *lane 6*, LLCZMCl.4; *lane 7*, LLCZMCl.11; *lane 8*, LLCZMCl.14; *lane 9*, LLCZMCl.21; *lane 10*, LLCZMCl.23; *lane 11*, LLCZMCl.31. 200 ng of purified M protein of Z strain was run in *lane 2* as a standard.

TABLE I

Comparison of amino acid residues of M protein

Amino acid residues deduced from the nucleotide sequence of M gene of four different Sendai virus strains were compared with each other. Only the amino acids different from that of Z strain were illustrated. The nucleotide sequences corresponding to each amino acid residue were as follows: no. 69, Gly (GGG), Glu (GAG); no. 116, Thr (ACT), Ala (GCT); no. 129, Ser (TCG), Leu (TTG); no. 140, Arg (AGG), Lys (AAG); no. 167, Leu (CTC), Phe (TTC); no. 183, Ala (GCC), Ser (TCC); no. 277, Asn (AAT), Thr (ACT).

Strain	Position of amino acid residue						
	69	116	129	140	167	183	277
Z	Gly	Thr	Ser	Arg	Leu	Ala	Asn
Nagoya			Leu	Lys	Phe		Thr
Harris					Phe		Thr
Cl.151	Glu	Ala				Ser	Thr

of function, for example, such as the binding activities to the envelope glycoproteins and the the nucleocapsid at the nonpermissive temperature; second, mutant M protein is insufficiently accumulated in the cells due to its instability at the nonpermissive temperature. Though the 6-fold increase in the amount of M protein observed in the LLC151Cl.9 cells at the permissive temperature (Fig. 1*A*) strongly suggested the latter possibility, this finding is not strong evidence. If the M protein of Cl.151 virus has its normal function even at the nonpermissive temperature, the defect in replication of Cl.151 virus should be complemented by the supplementation of excess amounts of M protein of Cl.151 virus at the nonpermissive temperature. For this complement experiment, we made an expression vector that could express the M protein of Cl.151 virus under the control of a strong promoter as we did for the wild type M protein and transfected this expression vector to LLCMK2 cells as before. We got several cell clones that expressed the M protein of Cl.151 virus stably, but the expression was much less at the nonpermissive temperature (38 °C) as compared with the expression of wild type M protein (see below). These cell lines restored the production of Cl.151 virus partially at 38 °C (data not shown). However, we could not reach a conclusion because of the low accumulation of the M protein at 38 °C.

To overcome this problem, we synthesized the M proteins in the cytoplasm using the much stronger expression system of T7 RNA polymerase encoded by the recombinant vaccinia virus. LLC151Cl.9 cells were superinfected with the recombinant vaccinia virus RVV-T7. 45 min after superinfection, the M protein expression vector (pT7M-Z and pT7M-Cl.151), which encoded the coding sequences of M protein under the control of T7 phage RNA polymerase promoter, was transfected. The cells were cultured at 38 °C for three days in a medium containing cytosine arabinoside (40 μg/ml), which was added to inhibit the replication of vaccinia virus (Cochran *et al.*, 1985). A portion of the medium was harvested every 24 h to examine the amount of virus particles. Cl.151 virus was produced at the nonpermissive temperature (38 °C) if supplemented with a large amount of the M protein of either Z strain or Cl.151 strain (Fig. 3). These results clearly demonstrated that the M protein of Cl.151 strain retained the function of the wild type protein even at the nonpermissive temperature and that the defect of the production of Cl.151 virus was simply due to the failure of M protein to accumulate in the cells sufficiently.

Alteration of Amino Acid Residues in the M Protein of Cl.151 Virus Made the Protein Unstable at the Nonpermissive Temperature—As described above, the M protein of Cl.151 virus could not accumulate in the cytoplasm at the nonpermissive temperature (38 °C). There were three possible explanations

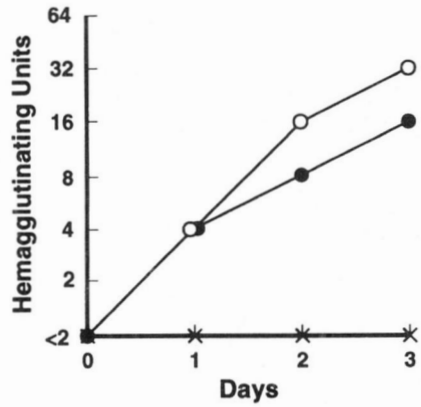

FIG. 3. **Effect of M protein expressed by using T7 RNA polymerase upon production of Sendai virus Cl.151 strain.** LLC151Cl.9 cells were set up at 1×10^6/100-mm dish in MEM containing 10% NCS. After being cultured at 38 °C for 24 h (day 0), the cells were infected with recombinant vaccinia virus RVV-T7 and then were cultured with 4 ml of Opti-MEM containing 50 μg of DOTAP and 10 μg of pT7M-Z, pT7M-Cl.151, or pUC19 as described under "Materials and Methods." After incubation at 38 °C for 8 h, the medium was replaced with MEM containing 10% NCS and cytosine arabinoside (40 μg/ml), and the cells were cultured at 38 °C for 3 days. 1 ml of medium was removed every 24 h, and the hemagglutinating activity was determined as described under "Materials and Methods." DNAs transfected were: ○, pT7M-Z; ●, pT7M-Cl.151; ×, pUC19.

for this phenomenon. First, the amount of messenger RNA (mRNA) encoding the M protein of Cl.151 virus was decreased at 38 °C due to the suppression of transcription or due to the decrease in stability of the mRNA; second, the mRNA encoding the M protein of Cl.151 virus was not translated efficiently at 38 °C; third, the M protein of Cl.151 virus was unstable at 38 °C.

To investigate these possibilities, we first examined the amount of M protein and the amount of mRNA encoding M protein in the LLC151Cl.9 cells. The cells were maintained at 38 °C and then were cultured at 32 °C for 3 days. The cells were harvested every 24 h, and the amount of M protein and mRNA was determined by protein and RNA blottings, respectively. As shown in Fig. 4, the amount of mRNA encoding M protein did not alter significantly either at 38 or at 32 °C, while the amount of the M protein was increased up to 6-fold during the incubation at 32 °C. Next we examined the rate of synthesis of M protein in the LLC151Cl.9 cells. The cells were maintained at 38 °C and then were incubated for 24 h either at 32 or 38 °C. The cells were pulse-labeled with [^{35}S] methionine for 20 min at either temperature, and the amount of M protein synthesized during this period was determined by immunoprecipitation. As shown in Fig. 5, the M protein of Cl.151 virus was synthesized at the same rate, either 38 or 32 °C. These data strongly suggested that the temperature-dependent alteration of the amount of M protein in the LLC151Cl.9 cells was due to the alteration in the stability of the M protein.

To examine the stability of the M protein directly, we performed pulse-chase experiments using cell lines stably expressing the M protein of Cl.151 strain (LLC151MCl.N) (see above). The LLCZMCl.4 (see Fig. 2) or LLC151MCl.N cells were cultured at 32 °C overnight, then labeled with [^{35}S] methionine for 1 h at 32 °C, and chased either at 32 or 38 °C with cold methionine for various times. The amount of the radiolabeled M proteins remaining after chasing were examined by immunoprecipitation. As shown in Fig. 6, the M protein of Z strain was stable either at 32 or 38 °C. On the contrary, the M protein of Cl.151 virus was stable at 32 °C but after a short lag degraded rapidly at 38 °C. These results

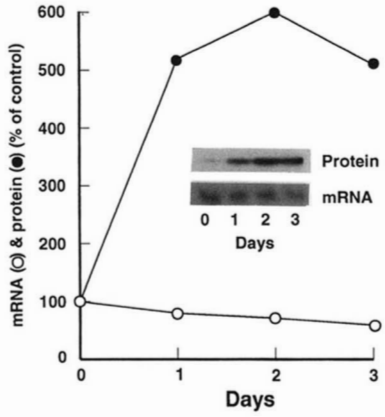

FIG. 4. **Effect of temperature on the amount of M protein and M mRNA in LLC151Cl.9 cells.** LLC151Cl.9 cells were set up at 2×10^6/100-mm dish in 10 ml of MEM containing 10% NCS. After being cultured for 24 h at 38 °C (day 0), the cells were fed with fresh medium and incubated at 32 °C for 3 days. The cells were harvested every 24 h to determine the amount of M protein and the amount of M mRNA in the cells by immunoblotting and RNA blotting as described under "Materials and Methods" (see *inset*). The relative amounts of M protein and M mRNA were indicated as percentages with the amount at day 0 being 100%. Symbols used were: ●, M protein; ○, M mRNA.

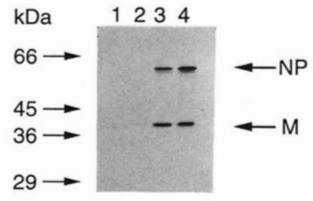

FIG. 5. **Effect of temperature on the rate of synthesis of M protein in LLC151Cl.9 cells.** The cells were set up at 5×10^5/60-mm dish in 5 ml of MEM containing 10% NCS at 38 or 32 °C. After 24 h, the cells were labeled with [^{35}S]methionine (500 μCi/ml) for 20 min either at 38 or 32 °C, and M protein synthesized during 20 min was determined as described under "Materials and Methods." The cells used were: *lane 1*, mock-infected LLCMK$_2$ cells; *lane 2*, cells expressing M protein of the Cl.151 strain (LLC151MCl.N); *lane 3*, LLC151Cl.9 cells cultured at 32 °C; *lane 4*, LLC151Cl.9 cells cultured at 38 °C. M_r standards were indicated.

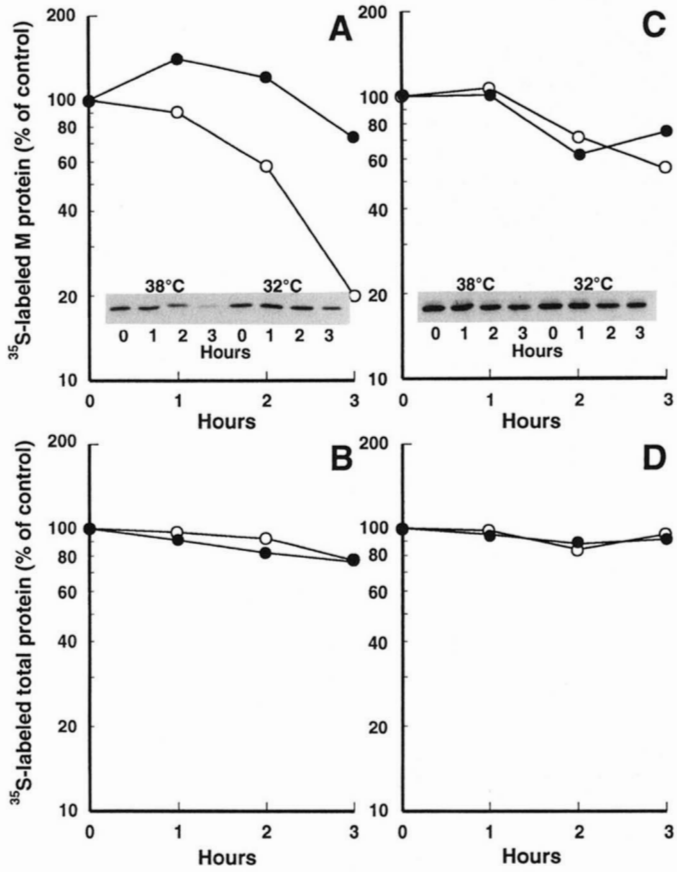

FIG. 6. **Effect of temperature on the rate of turnover of M proteins in the cells stably expressing M proteins.** LLCZMCl.4 cells (expressing wild type M protein) and LLC151MCl.N cells (expressing Cl.151 M protein) were set up at 5×10^5/60-mm dish in 5 ml of MEM containing 10% NCS and cultured at 32 °C for 24 h. Then the cells were labeled with [^{35}S]methionine (LLCZMCl.4, 50 μCi/ml; LLC151MCl.N, 500 μCi/ml) for 1 h at 32 °C and chased with 0.5 mM cold methionine either at 38 °C (○) or at 32 °C (●) as described under "Materials and Methods." After incubation for the indicated time, the cells were harvested to determine the amount of radiolabeled M proteins by immunoprecipitation (see *inset*) as described under "Materials and Methods." The rate of turnover of total cellular protein was determined by precipitation with 10% trichloroacetic acid as described under "Materials and Methods." The amount of radiolabeled proteins was indicated as percentage with the amount at day 0 being 100%. *Panel A*, turnover of M protein of Cl.151 strain in LLC151MCl.N cells. *Panel B*, turnover of total cellular protein in LLC151MCl.N cells. 100% control value was 1.2×10^6 cpm. *Panel C*, turnover of M protein of Z strain in LLCZMCl.4 cells. *Panel D*, turnover of total cellular protein in LLCZMCl.4 cells. 100% control value was 1.2×10^5 cpm.

clearly demonstrated that the stability of the M protein of Cl.151 virus was altered in a temperature-dependent manner and strongly suggested that the instability of M protein at high temperature interrupts the production of Cl.151 virus.

To determine the amino acid residue(s) responsible for the instability of the M protein of Cl.151 virus, we constructed M protein expression vectors in which each of the three amino acid residues of the wild type M protein was converted to that found in the M protein of Cl.151 virus by *in vitro* mutagenesis. We obtained pooled cultures expressing three mutant M proteins by transfection and examined the stability of M protein as above. The relative stability of the protein at 38 °C was indicated as the percentage of the amount of radiolabeled M protein remaining after 3 h of chase at 38 °C compared with that remaining at 32 °C. As shown in Fig. 7, the M protein of Cl.151 virus and all the mutant M proteins was degraded more rapidly at 38 °C as compared with the wild type M protein. Two mutant M proteins, M69Glu in which glycine 69 was replaced with glutamic acid and M116Ala in which threonine 116 was replaced with alanine, were as unstable at 38 °C as the M protein of Cl.151 virus. These results demonstrated that each of these alterations of amino acid residues contributed to the instability of the M protein at 38 °C.

DISCUSSION

In this article, we studied the interruption of virus production in the ts mutant strain of Sendai virus, Cl.151. We observed the following. 1) The amount of M protein altered 6-fold depending on the temperature without affecting the gene expression of the virus. 2) Defects in the production of virus particles were repaired not only by the supplementation of wild type (Z strain) M proteins but also by the supplementation of mutant M protein of the Cl.151 virus (Figs. 2 and 3). 3) The amount and rate of translation of M mRNA were not altered by temperature. 4) The M protein of the Cl.151 virus contained 3 altered amino acid residues that were not found in the protein of 3 wild type Sendai viruses. 5) Turnover of the M protein of the Cl.151 virus in the cells stably expressing this protein was largely accelerated at 38 °C. All of this evidence supports our conclusion that the failure in the accumulation of M protein in Cl.151 virus at the nonpermissive temperature interrupts the production of virus particles.

An understanding of the reproduction mechanism of this virus was greatly facilitated by the use of ts mutants. The ts mutants of paramyxovirus have been studied mainly by classification of the mutants, complementation, and biochemical analysis of proteins and RNA (for review, see Pringle (1991)). Our study using a combination of the molecular biological, and biochemical approach is the first clear demonstration that M protein of Sendai virus has a critical role in the production of virus particles but not in the expression of virus genes. This conclusion is consistent with our previous *in vitro* study (Yoshida *et al.*, 1976) that the nucleocapsid could interact with purified virus envelope glycoproteins only in the presence of purified M protein.

The importance of the M protein in production of paramyxovirus was also suggested by the studies of subacute sclerosing panencephalitis viruses, mutant measles viruses that infected persistently in neuronal cells as well as the cultured cells. Subacute sclerosing panencephalitis viruses quite frequently lack or have a reduced amount of M protein (for review, see Billeter and Cattaneo (1991)). The definitive evidence was not presented, however, that failure in the accumulation of the M protein could explain all the features of the establishment of persistent infection.

Development of the vectors based on animal viruses requires deep insight into the replication mechanism of the virus. Sendai virus has many interesting and well established features that make this virus an attractive candidate virus vector, including the establishment of the complete primary structure of the genome RNA (Shioda *et al.*, 1986). The results described here demonstrated that we could control the production of the virus particles with the amount of M protein. This finding will contribute to our knowledge required for developing a virus vector based on Sendai virus.

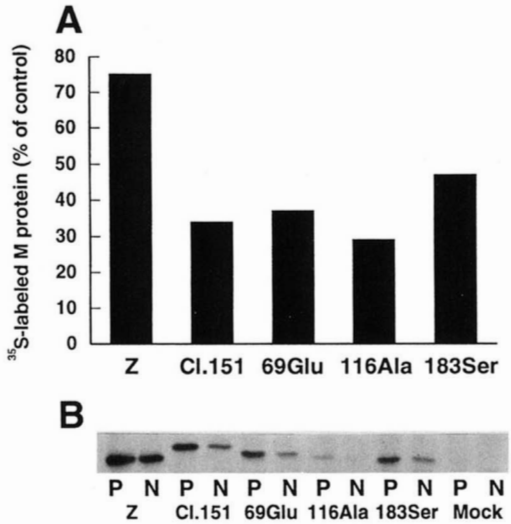

FIG. 7. **Effect of temperature on the rate of turnover of mutant M proteins.** $LLCMK_2$ cells were transfected with the expression vector for M protein, and the pool cultures expressing M proteins were prepared as described under "Materials and Methods." The cells were set up 5×10^5/60-mm dish in 5 ml of MEM containing 10% NCS. After culturing for 24 h at 32 °C, the cells were labeled with [^{35}S]methionine (500 μCi/ml) and chased with 0.5 mM cold methionine as described under "Materials and Methods." After being cultured with cold methionine for 3 h either at 38 °C (*lane N*) or at 32 °C (*lane P*), the cells were harvested to determine the amount of radiolabeled M protein by immunoprecipitation and densitometry as described under "Materials and Methods" (*Panel B*). The amount of M protein that remained at 38 °C was indicated as a percentage with the amount of M protein that remained at 32 °C as 100% of control (*Panel A*). The DNAs transfected were: *Z*, pCAM2-Z; *Cl.151*, pCAM2-Cl.151; *69Glu*, pCAM2-69Glu; *116Ala*, pCAM2-116Ala; *183Ser*, pCAM2-183Ser; *Mock*, pUC19.

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