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Role of Neuraminidase in the Morphogenesis of Influenza B Virus

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When *ts*7, a temperature-sensitive (*ts*) mutant of influenza B/Kanagawa/73 virus, infected MDCK cells at the nonpermissive temperature (37.5°C), infectious virus was produced at very low levels compared with the yield at the permissive temperature (32°C) and hemagglutinating activity and enzymatic activity of neuraminidase (NA) were negligible. However, viral protein synthesis and transport of hemadsorption-active hemagglutinin to the cell surface were not affected. When the cell lysate was treated with bacterial NA, hemagglutinating activity was recovered but infectivity was not, even after further treatment with trypsin. It was found that *ts*7 was defective in transport of NA to the cell surface and formation of virus particles. Analysis of the genomes of non-*ts* recombinants obtained by crossing *ts*7 and UV-inactivated B/Lee showed that *ts*7 had the *ts* mutation only in RNA segment 6 coding for NA and the glycoprotein NB. Nucleotide sequence analysis of the RNA segment revealed that *ts*7 had four amino acid changes in the NA molecule but not in NB. We suggest that assembly or budding of influenza B virus requires the presence of NA at the plasma membrane, unlike influenza A virus.

ts (temperature-sensitive) mutants of influenza A viruses have been isolated in a number of laboratories and have provided a valuable tool in studying many aspects of influenza biology (8). Recently, we have isolated *ts* mutants of influenza B/Kanagawa/73 virus generated by mutagenesis with 5-fluorouracil (5 FU) and classified them into seven recombination groups (unpublished data). Among these mutants, three (*ts*5, *ts*7, and *ts*22) belong to the same recombination group and to none of six other groups. Our recent study has shown that *ts*5 possesses the mutation in the neuraminidase (NA) gene and lacks functionally active NA and hemagglutinin (HA) at the nonpermissive temperature (37.5°C) (12). Despite the low yield of infectious virus, large aggregates of numerous virus particles accumulated on the infected cell surface and were broken by bacterial NA treatment, with concomitant appearance of hemagglutinating activity in the culture fluid. These virus particles were converted to infectious form by further trypsin treatment. Similar results were obtained with *ts*22 (unpublished data). The preliminary experiment showed that *ts*7 also lacked functionally active NA and HA at the nonpermissive temperature, but unlike with *ts*5, the infectivity was not recovered by trypsin treatment. This finding led us to further characterize *ts*7.

MDCK cells were infected with either wild-type virus or *ts*7 at 5 PFU per cell and incubated in Eagle's minimum essential medium containing 0.1% bovine serum albumin at 32°C or 37.5°C for 16 h. Unadsorbed virus was neutralized by the addition of antiserum (a final dilution of 1:400) to the cultures for 15 min, 2 h after infection. Cultures were frozen and thawed and assayed for biological activities as previously described (12). Wild-type virus grew well at both temperatures, and the yield of *ts*7 at 32°C was comparable to that of wild-type virus, except that enzymatic activity of NA was markedly low (Table 1). In contrast, at 37.5°C infectious virus was produced at very low levels and hemagglutinating and enzymatic activities were negligible. However, all of the cells were hemadsorption-positive, indicating that HA is expressed at the cell surface at the nonpermissive temperature. When NA of *Clostridium perfringens* (Sigma) (1 U/ml) was added to the cultures to give 0.025 U/ml for 30 min at 37.5°C 16 h after infection, cell-associated hemagglutinating activity appeared (Table 1), suggesting that the lack of hemagglutinating activity is probably the secondary effect which results from the lack of enzymatic activity. Further treatment with trypsin (1 μg/ml) had little effect on infectivity titer, unlike with *ts*5-infected cells (12).

Infected MDCK cells were labeled for 1 h with [^{35}S]methionine (5 μCi/ml) 8 h after infection and subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and autoradiography as described previously (7). HA, NP, M, and NS1 of *ts*7 were detected at both temperatures but, NA, NB, and three P proteins were not visible even in wild-type virus-infected cells (Fig. 1A). At 10 h after infection, cells were processed for Western blot (immunoblot) analysis using rabbit antiserum to NA as described previously (12). Figure 1B shows that *ts*7 produces NA at both temperatures, though in smaller amounts than wild-type virus.

MDCK cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) at room temperature for 20 min, 10 h after infection, and stained with fluorescein isothiocyanate-conjugated antibody against NA as described previously (12). Bright fluorescence was seen at the surface of cells infected with wild-type virus at 32°C (data not shown) or 37.5°C (Fig. 2A) or with *ts*7 at 32°C (Fig. 2B). However, the surface fluorescence was not seen in cells infected with *ts*7 at 37.5°C (Fig. 2C), even in the presence of protease inhibitors such as phenylmethylsulfonyl fluoride and aprotinin, suggest-

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TABLE 1. Effect of bacterial NA and trypsin on biological activities of *ts*7 produced at 32 and 37.5°C

Virus	Temp (°C)	Prepn examined	Virus yields after 16 h under indicated conditions						
			No treatment			Bacterial NA		Bacterial NA + trypsin	
			HA	NA[a]	PFU/ml	HA	PFU/ml	HA	PFU/ml
*ts*7	32	Fluids	64	0.000	3.1×10^6	64	9.9×10^6	64	1.0×10^7
		Cells	256	0.030	1.2×10^6	256	5.3×10^6	256	5.0×10^6
	37.5	Fluids	<2	0.000	$<10^3$	<2	$<10^3$	<2	$<10^3$
		Cells	<2	0.000	3.1×10^3	32	9.3×10^3	32	7.0×10^3
ts$^+$	32	Whole[b]	128	>1.0	1.4×10^7	128	2.0×10^7		
	37.5	Whole	128	>1.0	1.0×10^7	128	9.0×10^6		

[a] Enzymatic activity of NA was measured by reading the optical density at 549 nm per 0.1 ml.
[b] Cells plus culture fluids.

ing that it is unlikely that the absence of NA at the cell surface results from the degradation of NA by protease. Surface fluorescence of *ts*7 HA was detected at both the permissive and nonpermissive temperatures (data not shown). When fixed cells were further treated with Triton X-100 in PBS for the internal staining, *ts*7 NA was detected at the perinuclear region of the infected cells at either temperature (Fig. 2E and F), as observed with wild-type virus-infected cells (Fig. 2D). Perinuclear and surface fluorescence were not detected in uninfected cells. The cells infected with *ts*7 at 37.5°C for 9 h were shifted to 32°C for 2 h. Cycloheximide (100 μg/ml) was added 30 min prior to and during the shift down to eliminate further protein synthesis. Weak fluorescence of NA appeared at the cell surface within 1.5 h (data not shown). The above results indicate that *ts*7 is defective in transport of NA to the cell surface at the nonpermissive temperature.

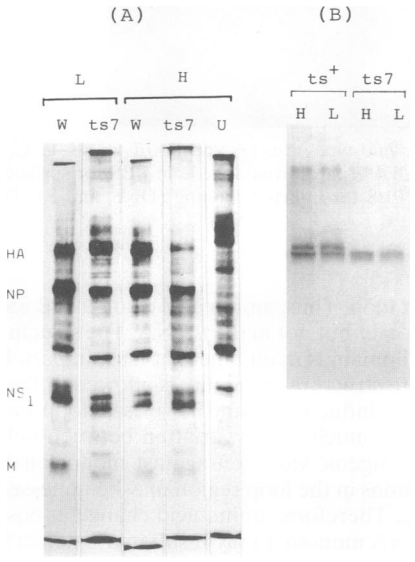

FIG. 1. (A) SDS-PAGE of [^{35}S]methionine-labeled proteins. MDCK cells infected with either wild-type virus (W) or *ts*7 were incubated at 32°C (L) or 37.5°C (H) for 8 h and labeled for 1 h with [^{35}S]methionine (5 μCi/ml). The cell extracts were subjected to SDS–10% PAGE and autoradiography. U, uninfected cells. (B) Western blotting of infected cell extracts using anti-NA serum. MDCK cells infected with either wild-type virus (ts$^+$) or *ts*7 at 32°C (L) or 37.5°C (H) were subjected to SDS–10% PAGE 9 h after infection and processed for Western blot analysis using antiserum against NA, as described previously (12).

Infected MDCK cells were processed for scanning electron microscopy 10 h after infection as described previously (12). A large number of single virus particles were found on the surface of cells infected with wild-type virus at 32°C (data not shown) or 37.5°C (Fig. 3A) or with *ts*7 at 32°C (Fig. 3C). However, these virus particles could hardly be detected on the surfaces of cells infected with *ts*7 at 37.5°C (Fig. 3B).

Egg-grown B/Lee was inactivated with UV light to a survival level of less than 10^{-4}, and MDCK cells were mixedly infected with inactivated B/Lee and *ts*7. After incubation at 32°C for 16 h, non-*ts* recombinants in the culture fluids were isolated by plaque formation on MDCK cells at 37.5°C in the presence of anti-Lee serum (1:200 dilution). After further plaque purification, the genome compositions of the recombinants were examined by gel electrophoresis as described by Ueda et al. (13). The result showed that any RNA segment of the B/Lee genome did not comigrate with the corresponding RNA segment of *ts*7, allowing us to determine the origin of each RNA segment of non-*ts* recombinants. Table 2 shows that all non-*ts* recombinants tested shared RNA segment 6 from B/Lee and that recombinants 8-3-1, 8-2-1B, and 9-2-1M inherited all RNA segments from *ts*7 except for RNA segment 6, indicating that the *ts* defect of *ts*7 lies only in RNA segment 6.

The sixth RNA segments of genomes of influenza B viruses have two genes, one coding for NA and one for NB, unlike those of influenza A viruses, which possess only the NA gene. These proteins are translated from a bicistronic mRNA transcribed from RNA segment 6 by using overlapping reading frames (6, 10–12). The nucleotide sequence at positions 47 to 1454 of cDNA corresponding to the coding region of RNA segment 6 of wild-type virus has recently been reported in message sense (12). The nucleotide sequence of RNA segment 6 of *ts*7 was analyzed as described previously (12) and compared with that of wild-type virus (Table 3). Six nucleotide changes between wild-type virus and *ts*7 were found at positions 762 (T→C), 1236 (G→A), 1426 (T→C), 1436 (C→T), 1441 (C→A), and 1442 (T→C). A→G, G→A, and U→C transitions occur upon 5-FU treatment (8). Thus, nucleotide changes at positions 1436 and 1441 were thought probably to be due to a spontaneous mutation. The open reading frames of both viruses for translation that begin at the first ATG codon (47 to 49) terminate at nucleotide 349. No nucleotide change was found between the two viruses. These reading frames are identical in size to that of B/Lee cDNA encoding NB polypeptide of 100 amino acids (6, 11, 12). The second ATG (54 to 56) is separated from the first ATG by four nucleotides and followed by an open reading frame extending to nucleotide 1454, and these reading frames are identical in size to the NA gene of B/Lee (6, 11, 12). Five of six nucleotide changes

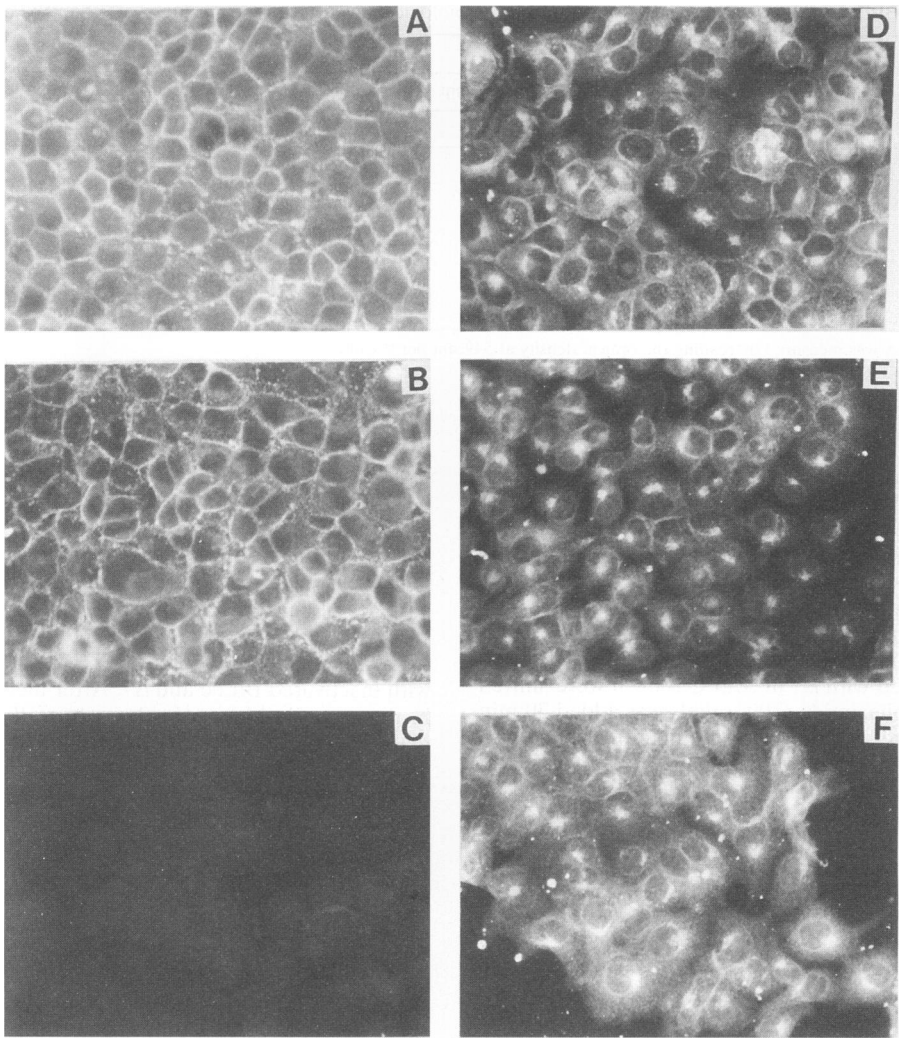

FIG. 2. Immunofluorescent staining of NA antigen. MDCK cells infected with either wild-type virus (A and D) or *ts*7 (B, E, C, and F) were incubated at 32°C (B and E) or 37.5°C (A, D, C, and F) for 8 h. The cells were fixed with 4% paraformaldehyde in PBS for surface staining (A, B, and C). The fixed cells were permeabilized by treatment with 0.1% Triton X-100 in PBS for internal staining (D, E, and F). The cells were stained with fluorescein-conjugated antibody to NA.

resulted in the amino acid substitutions at positions 237 (Try→His), 395 (Ala→Thr), 458 (Ile→Thr), and 463 (Ala→Asp). Four potential glycosylation sites (Asp-X-Ser/Thr) and 18 cysteine residues found in the open reading frame of wild-type virus were conserved in *ts*7.

NA spikes are formed as tetramers of four identical subunits and have a mushroom-shaped structure with a stalk and a head. The active site of the enzyme is located at the surface of the head of each monomer (4, 5). The monomeric head of NA is made up of six β-sheets, each containing four antiparallel strands connected by loops of variable sizes (4, 5). *ts*5 NA monomer has four amino acid changes, at positions 238, 429, 431, and 432 within its head domain. These mutations are located at two β-sheets (β 3S2 for position 238 and β 6S2 for positions 429 and 431) and a loop (position 432) between strands β 6S2 and β 6S3 in the three-dimensional structure of NA of influenza virus strain B/Beijing/1/87, in the nomenclature of Burmeister et al. (4). Two of four mutations of the *ts*7 NA monomer are located at two β-sheets (β 3S2 for position 237 and β 5S4 for position 395), and the other two are located at a different loop. Thus, amino acid change in β 5S4 lies in the *ts*7 NA monomer but not in the *ts*5 NA. The structural integrity of the head domain is maintained, at least in part, by β-sheets, whereas more structural flexibility is allowed in the connecting loops. In both influenza A and B viruses, loop mutations are responsible for much of the variation between field strains as well as for antigenic variation against monoclonal antibodies (4, 5). Mutations in the loop region may be nonessential for the *ts* phenotype. Therefore, amino acid change at position 395 in β 5S4 of *ts*7 NA monomer may result in a *ts* defect in transport of NA to the cell surface.

*ts*7 belongs to the same recombination group as *ts*5 and *ts*22, and all of them lacked hemagglutinating and enzymatic activities at the nonpermissive temperature. However, the major difference in *ts* phenotype was found between them. *ts*5 NA was transported at the nonpermissive temperature to the cell surface, where numerous noninfectious virus particles with uncleaved HA accumulated in large aggregates (12). This was the case with *ts*22 (unpublished data). In contrast, *ts*7 was defective in both transport of NA to the cell surface and virus

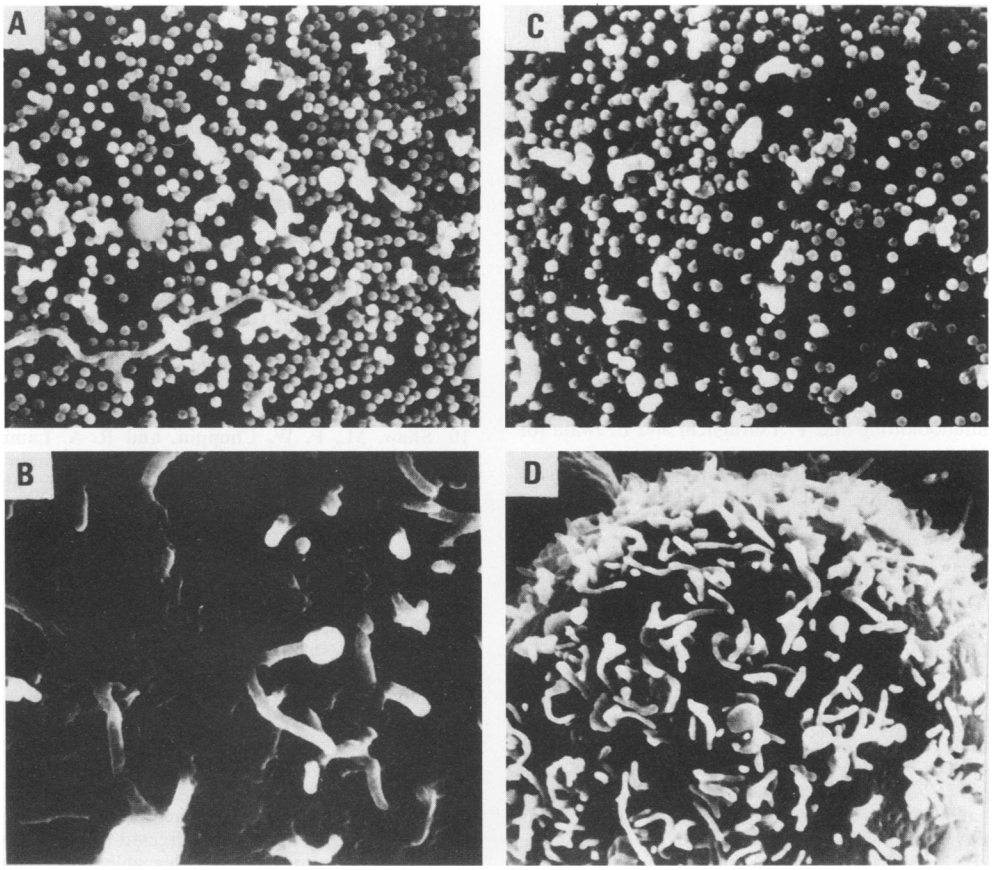

FIG. 3. Scanning electron microscopy. MDCK cells infected with either wild-type virus or *ts*7 were incubated at 32°C or 37.5°C for 10 h and then processed for scanning electron microscopy, as described previously (12). Cells infected with *ts*7 at 32°C (C) or 37.5°C (B) or with wild-type virus at 37.5°C (A) and uninfected cells (D) are shown. Magnification: panels A, B, and C, ×20,000; panel D, ×8,000.

morphogenesis. Since *ts*7 has a *ts* mutation only in the NA gene, we suggest that the presence of NA at the plasma membrane is essential for assembly or budding of influenza B virus.

It has been reported that influenza A viruses produce virus particles, despite the absence of NA at the cell surface, with NA *ts* mutants of A/WSN (1) or A/FPV (2) and a reassortant (H7N2) between A/FPV (H7N7) and A/Hong Kong (H7N2) (3). It has also been reported that although HA *ts* mutants of A/WSN are defective in transport of HA to the plasma membrane at the nonpermissive temperature, the mutants produce virus particles without HA spikes (9). These authors have suggested that with influenza A viruses, the presence of NA or HA at the plasma membrane is not necessarily obligatory for virus assembly or budding. A major difference between the influenza A and B viruses is the finding of NB glycoprotein in influenza B viruses. NB is an integral membrane protein expressed at the infected cell surface and is not in virions (14). Although its role in virus replication is still unknown, a role in organizing proteins at the cell surface to form patches of viral proteins or in the budding process would seem likely. It will be of interest to study whether assembly or budding of influenza

TABLE 2. Genome composition of ts^+ recombinants obtained by crossing between *ts*7 and wild-type B/Lee/40

ts^+ recombinant	Origin of indicated RNA segment[a]							
	1	2	3	4	5	6	7	8
R23	L	L	L	K	K	L	K	K
9-2-3B	K	L	L	K	K	L	L	L
9-2-5	K	K	K	K	K	L	L	K
8-3-1	K	K	K	K	K	L	K	K
8-2-1B	K	K	K	K	K	L	K	K
9-2-1M	K	K	K	K	K	L	K	K

[a] K, RNA segment derived from *ts*7 mutant; L, RNA segment derived from wild-type B/Lee strain.

TABLE 3. Mutation of open reading frame of RNA segment 6 of *ts*7

Region of gene	Nucleotide sequence		Amino acid sequence	
	Position	Substitution	Position	Substitution
NB start	47–49	ATG		
NA start	54–56	ATG	1	
NB end	347–349	TAG		
	762	TAT→CAT	237	Tyr→His
	1236	GCT→ACT	395	Ala→Thr
	1426	ATT→ACT	458	Ile→Thr
	1436	GGC→GGT	461	Silent
	1441	GCT→GAC	463	Ala→Asp
	1442			
NA end	1452–1454	TAA		

B viruses requires interaction between NA and NB at the cell surface.

At the nonpermissive temperature, *ts*7-infected cells, because of the lack of NA, still have the receptor for HA as well as HA at their surface. Therefore, freezing and thawing of the cells would generate the cell membrane fragments and vesicles of various sizes which expose the receptor and HA at their surface. One possible explanation for the appearance of cell-associated HA activity upon treatment with bacterial NA is that the HAs exposed at the surface of membrane vesicles interact with the receptor at the surface of other membranous structures and the membrane vesicles cannot hemagglutinate unless the receptor for HA is removed from the membranous structures by treatment with bacterial NA.

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