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Mutation in NS2, a nonstructural protein of influenza A virus, extragenically causes aberrant replication and expression of the *PA* gene and leads to generation of defective interfering particles

(reassortant virus/*NS* gene sequence)

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ABSTRACT **Several consecutive undiluted passages of infectious virus are usually required to obtain defective interfering particles of influenza virus. In contrast, a reassortant (Wa-182) of influenza A/WSN, which we isolated, whose *NS* gene was replaced with the *NS* gene of A/Aichi, was readily converted to defective interfering form after only a single high-multiplicity infection. The defective interfering particles of Wa-182 were devoid of the *PA* gene (RNA segment 3) but possessed several species of subgenomic RNAs of the *PA* gene origin. Such aberrant replication of the *PA* gene was shown to be caused by an extragenic effect of the *NS* gene of Wa-182, because, when the *NS* gene of Wa-182 was singly transferred to the wild-type A/Ann Arbor/6/60 virus, the recipient showed exactly the same features. Analysis of nucleotide sequence demonstrated that the *NS* gene of Wa-182 contained three point mutations relative to the wild-type *NS* gene that resulted in two amino acid substitutions in the nonstructural protein NS2, suggesting that the mutation in NS2 protein affected the normal replication of the *PA* gene of Wa-182. The results also suggest that the NS2 protein plays an important role in the synthesis of intact genome RNAs.**

Defective interfering (DI) particles of influenza virus are produced by serial undiluted passages of a standard infectious virus (1). DI particles have a reduced amount of virion RNA (vRNA) segments accompanied by the acquisition of smaller RNA segments (DI RNAs), the amount of which correlates with the interfering ability of the particles (2–4). The majority of DI RNAs arise through simple internal deletion, retaining their original 3′- and 5′-terminal regions, and derived predominantly from polymerase genes (*PA*, *PB1*, or *PB2*) (5–7). Although the generation of DI RNAs may occur during the process of vRNA replication (8), its precise mechanism is not understood, probably because primary DI RNA products from their progenitor genes are unavailable.

The first step in replication on influenza virus RNA is the production of full-length transcripts of the vRNAs that serve as templates for synthesis of additional vRNAs. The second step is copying template RNAs into vRNAs. These steps are initiated without a primer and are not terminated at the poly(A) site used during viral mRNA synthesis (9, 10). The synthesis of template RNA and vRNA requires the synthesis of nucleocapsid protein not associated with nucleocapsids (10). However, the transition from template RNA to vRNA synthesis may require the synthesis of other virus-specific proteins, such as nonstructural protein (NS protein). In fact, some temperature-sensitive mutants of the *NS* gene of influenza A virus failed to produce vRNAs at the nonpermissive temperature, whereas they normally synthesized both the mRNAs and template RNAs at that temperature (11).

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By crossing influenza viruses A/WSN and A/Aichi, we have isolated a reassortant (Wa-182) that is readily converted to DI particles lacking the *PA* gene in a one-step growth process after a high-multiplicity infection. In the present report, we demonstrate that the deletion of the *PA* gene in the DI particles of Wa-182 was not attributable to the *PA* gene itself but to the mutated NS2 protein that is encoded by the smaller reading frame of genome RNA segment 8 (12, 13). This finding suggests that the NS2 protein is required for the replication of normal genome RNA. (The nucleotide sequence of *NS* genomic RNA is presented.†)

MATERIALS AND METHODS

Viruses and Tissue Culture. Influenza viruses A/WSN/33 (H1N1) wild-type (wt), A/Aichi/2/68 (H3N2) wt, and A/Ann Arbor/6/60 (H2N2) wt were employed. A reassortant, Wa-182, was made by coinfection with A/WSN and A/Aichi. Reassortants 182AA/23-2 and 182AA/30 were made by coinfection with Wa-182 and A/Ann Arbor. These reassortants were purified by three successive plaque-to-plaque passages and then propagated in Madin–Darby canine kidney (MDCK) cells cultured in a liquid maintenance medium [Eagle's minimal essential medium (MEM)/0.2% bovine serum albumin] containing acetylated trypsin (4 μg/ml) for the working stocks. MDCK cells were grown in Eagle's MEM supplemented with 10% (vol/vol) fetal calf serum.

Radioisotope Labeling of vRNAs and Proteins. *[^{32}P]vRNAs.* Cells were infected at either a high multiplicity of infection (moi) [10 plaque-forming units (pfu) per cell] or a low moi (6×10^{-4} pfu per cell) and incubated for 15 hr or 34 hr in a phosphate-free maintenance medium supplemented with $^{32}P_i$ at 2.96 MBq (80 μCi; 1 Ci = 37 GBq)/ml. [^{32}P]vRNAs were extracted from the purified virions as described (14).

[^{35}S]Methionine-labeled viral proteins. At various times after infection, virus-infected cells were pulse-labeled for 20 min with [^{35}S]methionine at 1.11 MBq (30 μCi)/ml (specific activity, 1123 Ci/mmol; NEN), and equal amounts of cell lysates were subjected to SDS/PAGE.

Hybrid Selection of Subgenomic RNAs (sgRNAs). Plasmids, Bluescript M13+ (pBS M13+; Stratagene), containing a full-length cDNA of the *PB2* or *PA* gene of Wa-182 were used for hybrid selection of sgRNAs that originated from the polymerase genes. The hybridization on a nylon membrane and elution of the hybridized RNAs from the membrane were carried out as described (15). The hybrid selected RNAs were then analyzed by SDS/PAGE.

Abbreviations: DI, defective interfering; vRNA and sgRNA, virion and subgenomic RNAs, respectively; pfu, plaque-forming unit(s); moi, multiplicity of infection; HA, hemagglutinin.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession no. M34829 for Aichi and M35094 for Wa-182).

PAGE. Purified [^{32}P]vRNAs were analyzed by SDS/PAGE using a 3.0% polyacrylamide gel containing 6 M urea in a Tris acetate buffer (pH 7.8) at 32°C, as described (16). To obtain a good separation of the RNA segments that code for the polymerase proteins, a 2.5% polyacrylamide gel containing 6 M urea was employed and electrophoresed for 24 hr at 35°C at 108 V.

Viral proteins were analyzed on a 13% polyacrylamide gel in 0.375 M Tris·HCl (pH 8.8) as described (17).

Cloning and Sequencing of the *NS* Gene. Double-stranded cDNAs were synthesized from purified A/Aichi wt and Wa-182 vRNAs by using reverse transcriptase and the synthetic oligonucleotide primer 5′-d(AGCAAAAGCAGG)-3′. Subsequently, the cDNAs were blunt-ended with T4 DNA polymerase and fractionated on 1.5% neutral agarose gel to isolate the DNA fragments copying the *NS* gene. NS cDNA fragments eluted from the agarose gel were digested with *Hpa* II and inserted into *Acc* I and *Sma* I sites of the M13 phage vectors mp18 and mp19 (18). Cloned NS cDNAs were sequenced by the modified T7 polymerase Sequenase (United States Biochemical) using an M13 universal primer, 5′-d(GTAAAACGACGGCCAGT)-3′. All sequence alignments of the *NS* gene were determined on both strands (vRNA and mRNA senses) of cDNA fragments.

RESULTS

Growth Characteristics of Reassortant Wa-182 and Its Subclones. Wa-182 is a single-gene reassortant that contained the *NS* gene derived from A/Aichi and the other seven genes derived from A/WSN (Fig. 1). This reassortant exhibited different growth behavior at a low moi and at a high moi (Fig. 2). When infected at a low moi, the reassortant showed a normal growth pattern in which high infectivity and high hemagglutinin (HA) titers increased in parallel (Fig. 2*L*). On the other hand, when infected at a high moi, this virus had a high HA titer but produced progeny with extremely low infectivity (Fig. 2*H*). Furthermore, the reassortant strongly interfered with the growth of homologous wt A/WSN (data not shown), indicating that Wa-182 is readily converted to DI form by only a single high-multiplicity infection.

To exclude the possibility that preexisting DI particles in the stock virus of Wa-182 were the main cause of the above phenomenon, the subclones of Wa-182 were isolated from the seed stock and their growth patterns after high- and low-multiplicity infections were compared (Table 1). All subclones (indicated as P-4 in Table 1) showed growth similar to that of Wa-182 at high moi and low moi. No subclones possessing the normal growth pattern of a standard wt virus were isolated from the stock virus of Wa-182. In addition, a subclone (182/A-1) isolated from the subclone 182/A also yielded a large amount of DI virus at a high moi, although this subclone was purified five times by plaque-to-plaque passage. These results, therefore, strongly suggest that the growth characteristics of Wa-182 were not due to the DI particles inherently associated with the stock virus but to the vulnerability of the genome(s) of Wa-182 to be converted to a DI form.

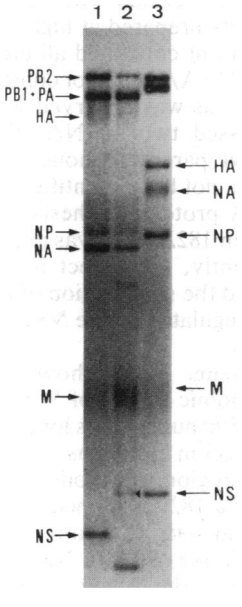

FIG. 1. PAGE of [^{32}P]vRNAs extracted from the virions grown at high moi. The labeled vRNAs were electrophoresed on a 2.8% polyacrylamide gel containing 6 M urea for 41 hr at 26°C. Lanes: 1, A/WSN/33; 2, Wa-182; 3, A/Aichi/2/68. Nomenclature of the viral proteins encoded by the RNA segments of A/WSN and A/Aichi were derived from Massicot *et al.* (19). The arrowhead (lane 2) indicates the gene derived from A/Aichi.

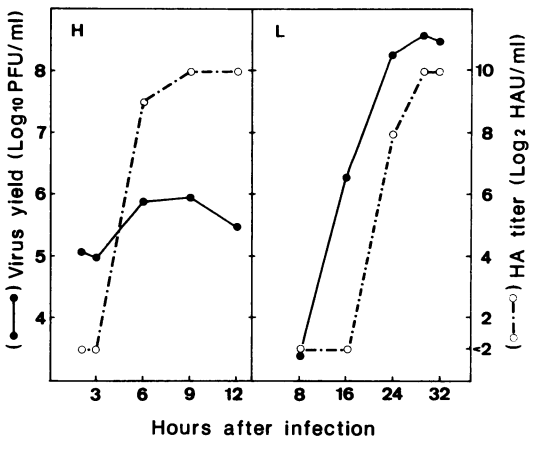

FIG. 2. Growth curves of Wa-182 at high moi (10 pfu per cell) (*H*) and low moi (6×10^{-4} pfu per cell) (*L*). At various times, culture medium from infected cells was harvested after one cycle of freezing and thawing. Infectivity of the progeny virus was determined by a plaque assay on MDCK cells and the HA titer was determined by a standard microtiter method using 0.5% chicken erythrocytes.

Synthesis of Viral Proteins in Infected Cells. The kinetics of viral protein synthesis of Wa-182 is shown in Fig. 3. When infected at a high moi (Fig. 3*A*), polymerase (PB1 and PB2), nucleocapsid (NP), matrix (Ml), and NSl proteins were detected 2 hr after infection and HA and NS2 proteins appeared 4 hr after infection. Synthesis of these proteins gradually increased with time. However, the band for the PA protein was not detected even when all other viral proteins were definitely visible. In contrast, when infected at a low moi, Wa-182 normally synthesized all of the proteins including PA (Fig. 3*B*).

vRNAs Prepared at Low or High Moi. Fig. 4*A* shows vRNA patterns of the infectious progeny of Wa-182 propagated at a low moi (N virion) and the DI progeny propagated at a high moi (DI virion). In the N virions, all eight RNA segments were obviously detected (lane N). However, the DI virions

Table 1. Growth characterization of Wa-182 subclones at a low moi and a high moi

Clone	Plaque passage	pfu/HA ratio	
		Low moi	High moi
Wa-182	P-3	4.2×10^5	1.1×10^3
182/A	P-4	7.6×10^4	2.7×10^3
182/D	P-4	4.2×10^5	2.3×10^3
182/F	P-4	5.0×10^4	6.2×10^2
182/G	P-4	2.0×10^5	2.2×10^3
182/I	P-4	1.4×10^5	2.7×10^3
182/J	P-4	9.8×10^4	6.3×10^1
182/A-1	P-5	6.3×10^5	2.3×10^3
A/WSN wt	—	1.8×10^6	1.8×10^5

At a low moi, MDCK cells were infected with 6×10^{-4} pfu per cell and incubated for 26 hr. At a high moi, MDCK cells were infected with 10 pfu per cell and incubated for 9 hr.

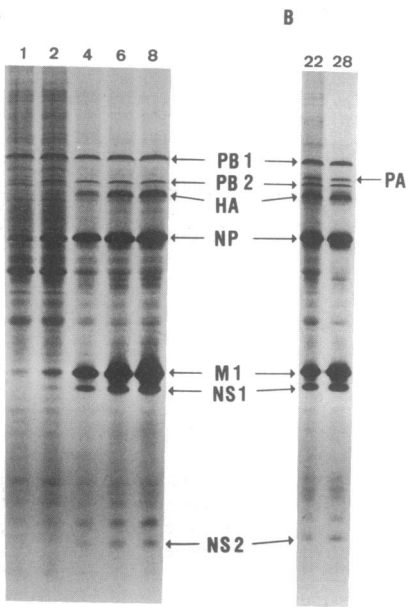

FIG. 3. Viral protein synthesis of Wa-182 in infected cells at a high moi (*A*) and a low moi (*B*). The numbers at the top of lanes indicate time after infection in hr.

did not contain the RNA segment 3 (*PA* gene) but did contain a newly formed subgenomic RNA (sgRNA) between the *M* and *NS* genes (lane DI). The other RNA segments were comparable in quantity between the N and DI virions. Thus, loss of the *PA* gene correlated with the formation of a large number of DI particles and the sgRNA. When RNA samples were electrophoresed longer on a high-concentration polyacrylamide gel (Fig. 4*B*), both N and DI virions were shown to have at least 10 sgRNAs ranging from ≈940 to 300 bases

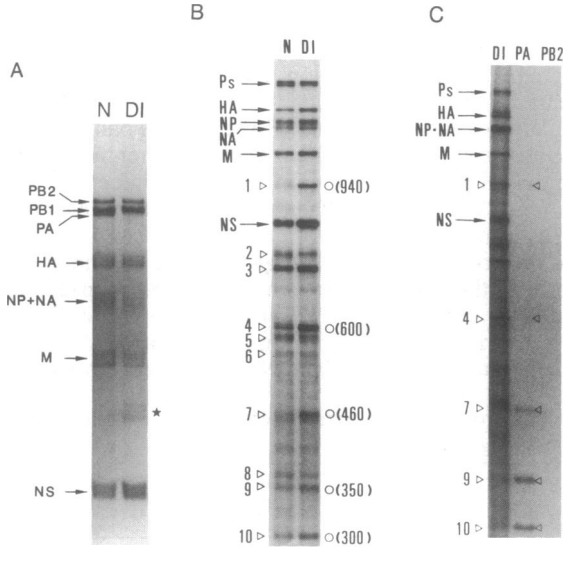

FIG. 4. PAGE of vRNAs of Wa-182. Lanes: N, normal infectious vRNA; DI, DI vRNA. (*A*) [^{32}P]vRNA samples were electrophoresed on a 2.5% polyacrylamide gel containing 6 M urea. The star indicates sgRNA. (*B*) RNA samples were 5'-end-labeled with [γ-^{32}P]-ATP (20) and electrophoresed on a 3.0% polyacrylamide gel containing 6 M urea. The numbered arrowheads show sgRNAs, and the numbers in parentheses show approximate length (bases) of the sgRNAs. Open circles indicate the sgRNAs that greatly increased in quantity in the DI virion. (*C*) sgRNAs selected by hybridization with Wa-182 PA cDNA (lane PA) and Wa-182 PB2 cDNA (lane PB2) probes. Lengths of the numbered sgRNAs correspond to those shown in *B*.

long (numbered 1–10). The content of sgRNAs 1, 4, 7, 9, and 10 was greatly increased in the DI virions compared to the N virions (marked by open circles), and sgRNAs 2, 3, 5, 6, and 8 were present in almost comparable amounts in the N and DI virions.

To determine whether the *PA* gene is the progenitor of sgRNAs, Northern blot analysis using pBSM13+ DNA containing full-length PA, PB2, and PB1 cDNAs derived from Wa-182 was carried out. sgRNAs in DI virions were detected when probed with the PA cDNA but not when probed with the PB2 and PB1 cDNAs (data not shown), demonstrating that the sgRNAs in the DI virion, if not all sgRNAs, originated predominantly from the *PA* gene, and that the *PB1* and *PB2* genes did not produce a detectable amount of sgRNA. On the basis of these results, we examined which species of sgRNA is derived from the *PA* gene. Fig. 4*C* shows sgRNA patterns selected by hybridization with the PA and PB2 cDNA probes of Wa-182. As shown in the lane PA, the PA cDNA probe selectively hybridized with sgRNAs 1, 4, 7, 9, and 10, whereas the PB2 cDNA probe did not hybridize with any sgRNAs, in agreement with the Northern blot analysis (lane PB2). However, the intensity of the hybridized sgRNAs did not correlate with that of sgRNAs contained in the DI particles, implying that the reactivity with the PA cDNA may vary depending on the species of sgRNA. From these results, it was apparent that at least sgRNAs 1, 4, 7, 9, and 10, which were greatly increased in quantity in the DI virion, were derived from the *PA* gene and that the DI particles of Wa-182 were formed by the deletion of the *PA* gene.

Determination of the Gene Inducing the Deletion of *PA* Gene. Our experiment has shown that the *PA* genomic RNA of Wa-182 and A/WSN were identical (21). This result strongly suggests that the aberrant replication of the *PA* gene of Wa-182 is caused extragenically by a Wa-182 gene other than the *PA* gene. We examined the *NS* gene of Wa-182, because it was a heterologous gene of Aichi origin. We isolated two single-gene reassortants that possessed the *PA* or *NS* gene derived from Wa-182 with seven other genes derived from A/Ann Arbor wt virus and characterized their growth patterns at high moi and low moi (Table 2). The single-gene reassortant of *PA* (182AA/23-2) produced amounts of infectious progeny comparable to Ann Arbor wt parent at both infection conditions. On the other hand, the single-gene reassortant of *NS* (182AA/30) yielded a greater than normal amount of DI particles at a high moi but produced a normal amount of infectious particles at a low moi. These growth patterns imply that the unusual characteristics of Wa-182 are due to the *NS* gene. This finding was verified with vRNA profiles of the single-gene reassortants prepared at high moi (Fig. 5*A*). 182AA/23-2 and its wt parent contained all eight RNA segments (lanes 1 and 3), but 182AA/30 did not contain a detectable amount of the *PA* gene, as was observed with Wa-182 (lane 2). 182AA/30 possessed two sgRNAs that differed from the sgRNA of the wt parent, although the progenitor gene of these sgRNAs has not been identified. In addition, the suppression of the PA protein synthesis was characteristically observed only when 182AA/30 was grown at a high moi (Fig. 5*B*). Consequently, the defect in the normal replication of the *PA* gene and the suppression of PA protein synthesis are extragenically regulated by the *NS* gene of Wa-182.

Nucleotide Sequence of the *NS* Gene. Fig. 6 shows the nucleotide sequences of the *NS* genomic RNAs of Wa-182 and A/Aichi wt. The *NS* genes were 890 nucleotides long and code for 237 amino acids in the *NS1* region (positions 27–737) and for 121 amino acids in the *NS2* region (positions 27–56 and 529–861). The *NS* gene of Wa-182 contained three mutations at positions 389, 593, and 698. The latter two mutations were involved in reading frames of both NS1 and NS2 proteins. In the *NS1* reading frame, the three mutations

Table 2. Growth characteristics of Wa-182 × A/AA/60 wt reassortants at a high moi and a low moi

Virus	Genome derived from Wa-182	Virus yield at a high moi			Virus yield at a low moi		
		pfu/ml	HAU/ml	pfu/HA	pfu/ml	HAU/ml	pfu/HA
182AA/23-2	*PA*	6.6×10^7	128	5.1×10^5	6.8×10^8	256	2.7×10^6
182AA/30	*NS*	1.6×10^5	256	6.3×10^2	5.1×10^7	128	4.0×10^5
A/AA 60 wt	None	3.9×10^7	256	1.5×10^5	3.4×10^8	256	1.3×10^6

Amount of the input virus at a high moi and a low moi was 10 pfu per cell and 1×10^{-4} pfu per cell, respectively. The progeny viruses were harvested 13 hr after infection at a high moi and 26 hr after infection at a low moi. All genes of the reassortants were originated from the A/AA/60 wt except as indicated. HAU, hemagglutinin units.

were silent, whereas in the *NS2* reading frame, the mutations at positions 593 (T → C) and 698 (G → A) gave rise to two amino acid substitutions from isoleucine to threonine and from glycine to glutamic acid, respectively. These results suggest that the anomalous replication of the *PA* gene of Wa-182 was mainly caused by the mutated NS2 protein.

DISCUSSION

We have described the generation of influenza DI particles by extragenic intervention of the *NS* gene. Reassortant Wa-182 possesses properties that may elucidate the generation of DI RNAs. Wa-182 can be readily converted from a standard infectious virus to a DI virus by a single high-multiplicity infection (Fig. 2), in sharp contrast to the observation that a comparable amount of DI particles of A/WSN was obtained after at least three consecutive high-multiplicity passages of the standard virus (ref. 22; unpublished data). The conversion of Wa-182 from a standard infectious virus to a DI virus is not due to preexisting DI particles in the stock virus, since all subclones isolated from the stock virus also converted to DI viruses when grown at high moi, and is inherited by progeny subclones as shown when Wa-182 was plaque-purified five times (Table 1).

DI virions of Wa-182 completely lacked the *PA* gene but contained at least five sgRNAs originating from the *PA* gene (Fig. 4), indicating that the DI particles of Wa-182 were formed by deletion of the *PA* gene. However, this deletion was not due to mutations in the *PA* gene, since the *PA* gene of Wa-182 was shown to have no mutation when compared with the progenitor gene of A/WSN (21). These results suggest that a mutation or a unique stretch of the nucleotide sequence, such as a uracil-rich region (5), on the polymerase genes is not a prerequisite for the generation of DI RNAs. These results are consistent with previous studies on DI RNAs in which no consensus sequences that may favor the generation of DI RNAs were found (6) and the sequences at

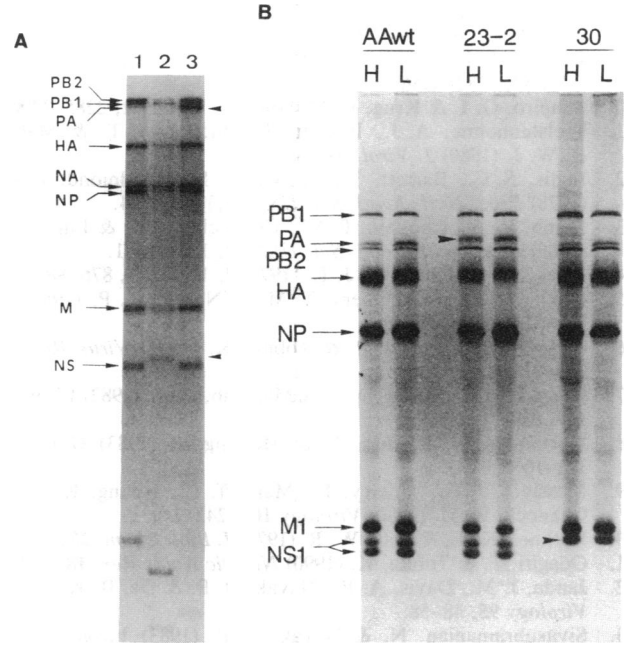

FIG. 5. (*A*) [^{32}P]vRNAs of single-gene reassortants prepared at a high moi. RNAs were electrophoresed on a 2.8% polyacrylamide gel containing 7 M urea for 22 hr at 35°C. Lanes: 1, A/Ann Arbor wt; 2, 182AA/30; 3, 182AA/23-2. Arrowheads show genes originating from Wa-182. (*B*) Viral protein patterns of the single-gene reassortants in infected cells. Virus-infected cells at a high moi (lanes H) and a low moi (lanes L) were pulse-labeled for 30 min with [^{35}S]methionine at 6 hr and 25 hr after infection, respectively. Lanes: AAwt, A/Ann Arbor wt; 23-2, *PA* single-gene reassortant 182AA/23-2; 30, *NS* single gene reassortant 182AA/30. Arrowheads indicate the proteins derived from Wa-182.

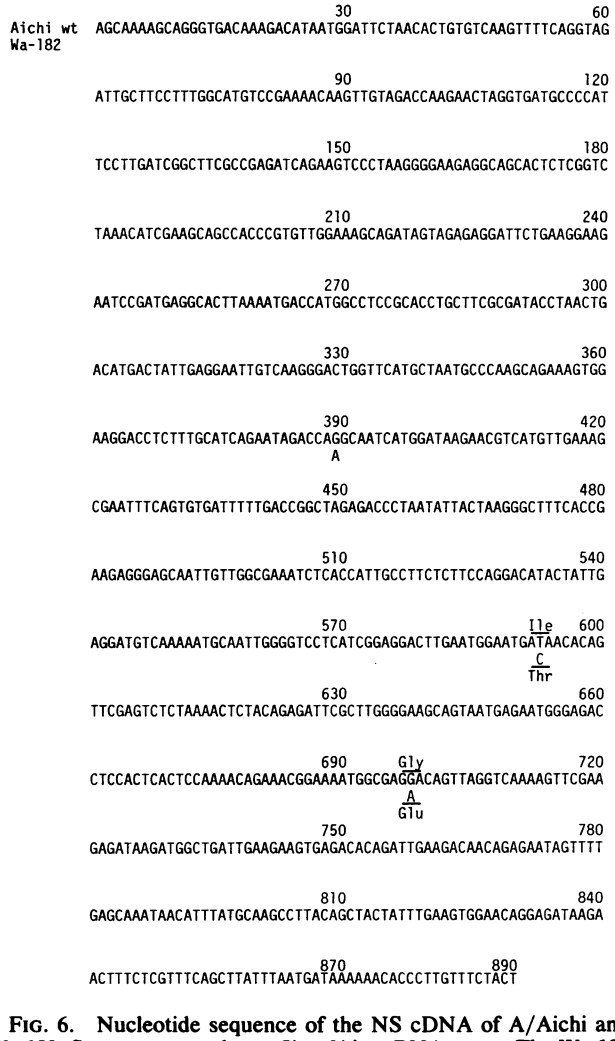

FIG. 6. Nucleotide sequence of the NS cDNA of A/Aichi and Wa-182. Sequences are shown 5′ to 3′ in mRNA sense. The Wa-182 sequence identical to that of Aichi is not reproduced. Amino acids indicated above (for Aichi) and below (for Wa-182) are deduced from the NS2 reading frame.

the flanking regions of progenitor RNAs giving rise to different DI RNAs did not resemble the consensus cellular RNA splicing sequences (5, 7, 23).

The genetic studies clearly demonstrated that characteristics of Wa-182 were transferred to another wt virus A/Ann Arbor by the *NS* gene of Wa-182, whereas transfer of the Wa-182 *PA* gene did not affect the wt phenotype of A/Ann Arbor (Table 2 and Fig. 5). Thus the *NS* gene of Wa-182 extragenically affects the replication of the *PA* gene, resulting in the defect of the *PA* gene and the generation of sgRNAs from the *PA* gene. This phenomenon could be "true" extragenic suppression by the *NS* gene rather than the "extragenic suppression" that has been used for pseudorevertants of temperature-sensitive influenza virus mutants (24–26) or of reovirus (27) that arise as the result of a second mutation or exchange of a gene other than the temperature-sensitive gene. It is noteworthy that the anomalous replication of the genomic RNA induced by the extragenic suppression of Wa-182 *NS* gene was found not only in the *PA* gene but also in the *PB2* gene. In fact, the amount of the *PB2* gene in the *NS* single-gene reassortant (182AA/30) was also slightly decreased (Fig. 5). This result suggests that all three polymerase genes may be affected by the *NS* gene of Wa-182, although for Wa-182 the magnitude of suppression to the *PA* gene might have been more striking than the other polymerase genes by some undetermined interactions.

The *NS* gene of Wa-182 contained three mutations relative to the wt *NS* gene of A/Aichi, which resulted in two amino acid substitutions from isoleucine to threonine and from glycine to glutamic acid, in the NS2 protein (Fig. 6). These changes should affect the secondary structure of the protein. In addition, another reassortant that possessed wt *NS* gene of Aichi and other genes identical to those of Wa-182 failed to exhibit any characteristics of Wa-182 (data not shown). Therefore, it is likely that the mutations in the NS2 reading frame result in the mutated NS2 protein that causes aberrant replication of the polymerase genes.

When the cells were infected with Wa-182 at a high moi, the PA protein synthesis was greatly reduced (Fig. 3). This suppression occurred in an association with the Wa-182 *NS* gene (Fig. 5). If the mutated NS2 protein is a main cause of the suppression, it would be interesting to know whether or not the PA protein synthesis of Wa-182 is suppressed from the early phase of infection cycle, since the NS2 protein has been shown to be synthesized in the late phase (28). We have observed that, in primary transcription in the cells infected with Wa-182 at a high moi or with A/WSN wt all mRNA species including the PA mRNA were synthesized in comparable amounts and that these transcripts were translatable into the proteins *in vitro*. However, after the primary transcription, the amount of the PA-specific mRNA did not increase with subsequent incubation, in contrast to that of wt virus (unpublished results). Consequently, the synthesis of the PA protein is likely not to be suppressed at least during the early phase in infection, although the product may remain undetectable due to a lack of subsequent accumulation.

Deletion of the normal replication of the *PA* gene of Wa-182 and defective *PA* gene expression were observed only after infection at a high moi (Figs. 3 and 4). The question as to the difference between low moi and high moi remains to be answered.

The *PA* gene of A/WSN wt contains two internal complementation regions at positions 2221–2232 vs. 1501–1490 and positions 2220–2226 vs. 2051–2056 (21), which are expected to make loop structures. This wt virus does not efficiently generate sgRNAs even when infected at a high moi (Fig. 1). These results suggest that the generation mechanism of sgRNAs (or DI RNAs) could not be explained only by interactions of viral polymerases with certain RNA templates that form many loops in the tertiary structure of ribonucleoproteins (6, 8)—the "jumping polymerase" (6) and "rolling polymerase" (29) models. The present observations would propose the possibility that NS2 protein might be a cofactor for the normal movement of the viral polymerase on the RNA template in the tertiary structure of the ribonucleoproteins. For Wa-182, the NS2 protein might have lost such a regulatory function by the mutations of the *NS* gene.

We have shown above that the mutated NS2 protein may suppress the normal replication of *PA* gene and lead to DI virus formation. The reassortant Wa-182, therefore, should be a suitable tool to explore not only the generation mechanism of DI virus but also the role of NS2 protein in influenza vRNA replication.

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