An amino acid change in the non-structural NS2 protein of an influenza A virus mutant is responsible for the generation of defective interfering (DI) particles by amplifying DI RNAs and suppressing complementary RNA synthesis

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The mutated non-structural NS2 protein of an influenza A virus mutant, Wa-182, has been shown to be responsible for the production of defective interfering (DI) particles lacking the PA gene after a single cycle high-multiplicity infection. Using a subclone of Wa-182, A3/e-3, that inherited the Wa-182 phenotype but contained only a marginal amount of DI RNAs derived from the PA gene, we showed that replication of the PA genome RNA was suppressed primarily at the step of complementary RNA (cRNA) synthesis. On the other hand, the small amounts of DI RNA species present in the stock of A3/e-3 were shown to be replicated efficiently. These findings suggested that the suppression of cRNA synthesis of the PA gene was caused by preferential amplification of the DI RNAs. The suppression of PA gene cRNA synthesis subsequently resulted in suppression of both virion RNA synthesis and secondary transcription of the PA gene. Such aberrant replication of the PA gene was found to be attributable to an amino acid change in the NS2 protein at position 32, from isoleucine to threonine. These results suggest that the NS2 protein plays a role in promoting normal replication of the genomic RNAs by preventing the replication of short-length RNA species.

Introduction

The genome of influenza A virus consists of eight RNA segments of negative polarity (Inglis et al., 1976; Palese & Schulman, 1976; Ritchey et al., 1976). RNA synthesis is divided into three categories. Transcription of mRNAs from virion RNA (vRNA) is primed by 5' capped RNA fragments derived from host-cell mRNAs (Bouloy et al., 1979; Plotch et al., 1981). Replication of vRNAs proceeds in two steps: production of full-length complementary copies of the vRNAs, namely cRNAs, and reverse synthesis of progeny vRNAs from these template RNAs. These RNA replication steps, unlike transcription, are initiated without exogenous primers (Hay et al., 1977, 1982) but require one or more newly synthesized viral protein(s), e.g. nucleoprotein (Beaton & Krug, 1986; Shapiro & Krug, 1988).

RNA segment 8 of influenza A virus encodes two non-structural proteins, NS1 and NS2 (Lamb et al., 1978; Inglis et al., 1979; Lamb & Choppin, 1979). The NS2 protein is translated from the NS2 mRNA, which is produced by splicing the NS1 mRNA in the nucleus of host cells (Lamb & Lai, 1980, 1984). The function of the NS2 protein in virus replication remains unknown. Previous reports have indicated that synthesis of viral protein(s) is required as a trans-acting factor for the splicing of NS1 mRNA, suggesting that the NS2 protein is not synthesized in primary transcription (Lamb et al., 1978; Smith & Inglis, 1985). However, we have recently found that for several influenza A viruses the NS2 mRNA is produced at this stage irrespective of viral protein synthesis (Odagiri et al., 1991). This suggests that at least for these viruses, the splicing of NS1 mRNA depends solely on a cis-acting sequence in the NS1 mRNA itself, consistent with the results obtained from in vitro splicing of NS1 mRNA (Nemeroff et al., 1992) and the NS2 protein functioning from the early phase of infection. The splicing of NS1 mRNA has also been shown to depend on the efficiency of nucleocytoplasmic transport of the unspliced NS1 mRNA (Alonso-Caplen & Krug, 1991).

When influenza virus is serially passaged at a high m.o.i., small RNA species, namely subgenomic or defective interfering RNAs (sg/DI RNAs), derived

The sequence data reported in this paper have been deposited in the DDBJ, EMBL and GenBank DNA databases under the accession numbers M35094 (Wa-182 = A3/e-3), M34829 (Aichi/2/68) and D90526 (Aichi/2/68, Ao).
predominantly from the polymerase genes (PB1, PB2 and PA) may become detectable (Jennings et al., 1983; Nayak et al., 1985). On the other hand, genomic RNAs corresponding to progenitors of such sg/DI RNAs are reciprocally reduced (Janda et al., 1979; Nakajima et al., 1979; De & Nayak, 1980). These sg/DI RNAs, in most cases, contain single internal deletions, retaining the original 3'- and 5'-terminal regions. Comparative nucleotide sequence analyses of the sg/DI RNAs and the genomic RNAs have indicated that the deleted regions of the sg/DI RNAs do not correlate with any specific sequences of the progenitor RNAs (Davis et al., 1980; Nayak et al., 1982; Jennings et al., 1983) and that the size and number of sg/DI RNAs are variable among DI preparations (Jennings et al., 1983; Nayak et al., 1989). However, neither the mechanism of sg/DI RNA generation nor the mechanism of suppression of genomic RNA replication has been elucidated, although two models for the production of sg/DI RNAs have been proposed, i.e. the ‘jumping polymerase’ model (Jennings et al., 1983) and the ‘rolling polymerase’ model (Nayak et al., 1985). At least three or four consecutive undiluted passages of virus are usually required to yield substantial amounts of DI particles, even when sg/DI RNAs are already present in the virus stocks (Janda et al., 1979; De & Nayak, 1980). Such multiple passages make it difficult to reproduce identical sg/DI RNA species primarily derived from a particular progenitor. We have, however, isolated an influenza A virus mutant, Wa-182, with a unique phenotype. We have demonstrated that after a single cycle of replication of Wa-182 at a high m.o.i., DI particles lacking the PA gene were predominantly produced (Odagiri & Tobita, 1990). In contrast, when the mutant was grown at low m.o.i., infectious progeny virus that retained the PA gene was produced. The mutated NS2 protein of Wa-182 was shown to be responsible for the selective suppression of replication and expression of the PA gene (Odagiri & Tobita, 1990).

To determine when the PA genome RNA synthesis is suppressed and how the mutated NS2 protein affects this, we have extended our study using a subclone of Wa-182, A3/e-3, which contained only a small amount of sg/DI RNAs. Here we show that both the suppression of PA gene replication and the amplification of PA DI RNAs, processes which are mediated by the mutated NS2 protein, occur primarily during cRNA synthesis.

Methods

Viruses and cells. A subclone of the NS2 mutant of influenza A virus Wa-182, designated A3/e-3, was obtained by plaque-to-plaque passages of Wa-182 in MDCK cells. Wa-182 was a reassortant possessing the mutated NS gene derived from A/Aichi/2/68 and the other seven genes from A/WSN/33 (Odagiri & Tobita, 1990). The nucleotide sequence of the NS gene of A3/e-3 was identical to that of Wa-182, and A3/e-3 inherited the phenotype of Wa-182. A3/e-3 contained a negligible amount of sg/DI RNAs derived from the PA gene, which were detectable only after amplification by the PCR for 25 cycles. AwNS/2 was a reassortant containing the wild-type NS gene of Aichi virus and the other seven genes of WSN virus. This reassortant was used as a control virus containing the wild-type NS gene. MDCK and MDBK cells were grown in Eagle’s MEM supplemented with 10% fetal calf serum.

32P labelling of virion RNAs. Cells were infected with virus at either a high m.o.i. (10 p.f.u./cell) or a low m.o.i. (6 × 10^4 p.f.u./cell) and incubated for 15 h or 34 h, respectively, in a phosphate-free maintenance medium containing [32P]orthophosphate at 2.96 MBq/ml. 32P-labelled RNAs were extracted from purified virions as described (Palese & Schulman, 1976) and were analysed by electrophoresis on 2.5% or 30% polyacrylamide gels containing 7 M-urea as described previously (Odagiri & Tobita, 1990).

Quantitative analysis of viral RNAs in infected cells. At various time intervals after infection with virus at 10 p.f.u./cell, the PA gene-specific RNAs (PA-cRNA, PA-vRNA and PA-mRNA) were quantified by a ribonuclease protection assay (RPA) (Ambion). Total RNA was extracted with 6 M-guanidinium thiocyanate solution containing 0.5% sodium N-lauroylsarcosine. After purification by centrifugation on a 57 M-CsCl cushion for 20 h at 27000 r.p.m. in an SW28 rotor. RNA samples were fractionated into poly(A) and poly(A) RNAs by two cycles of adsorption onto and elution from latex beads conjugated with oligo(dT) (Oligotex-dT30, Takara). The poly(A) RNAs were then treated with RNase-free DNase I at 0.3 units/μg RNA for 15 min at 37°C.

To measure the amount of PA-specific RNA molecules, riboprobes were transcribed in vitro in the presence of [α-32P]UTP (30 TBq/mmol) from a cDNA copy of the PA RNA of Wa-182 which was inserted into the Small site of plasmid Bluescript (pPA/2) (see Fig. 5a). To assay vRNA species, a positive-sense probe, corresponding to nucleotides 8 to 581 of PA mRNA, was transcribed by T7 RNA polymerase from pPA/2 linearized with StuI, and was hybridized with the poly(A) RNA fraction for 16 h at 50°C. At the end of the incubation period the hybrid RNAs were digested with a mixture of 0.5 units/ml of RNase A and 10 units/ml of RNase T1 followed by electrophoresis on a 5% polyacrylamide gel containing 8 M-urea. To quantify viral cRNAs and mRNAs, a negative-sense probe that corresponds to nucleotides 1799 to 2232 of PA-vRNA was transcribed by T3 RNA polymerase from pPA/2 linearized with TaqI, and was hybridized with either poly(A) RNAs (for cRNA) or poly(A) RNAs (for mRNA) (see Fig. 5a). After digestion with RNases the hybrids were distinguishable on 5% polyacrylamide gels containing 8 M-urea because the mRNAs were 15 nucleotides shorter than the cRNAs. The radioactivities of undigested bands in the gel were measured using a BAS 2000 Bioimaging Analyser after exposure for 12 h on an imaging plate (Fuji Film). The data were expressed as the arbitrary copy number of RNA species, calculated from the radioactivity/nucleotide length of the undigested band in 1 μg of total RNA. In control experiments the linearity of the RPA response was shown to be ensured when the 32P-labelled riboprobe (1 × 10^5 c.p.m.) was hybridized with probe-specific RNAs over a range from 50 to 5000 pg, with an average error of 14.6% in three experiments. This range was found to be sufficient when 0.3 μg of poly(A) RNA or 1 or 3 μg of poly(A) RNA were subjected to RPA to measure PA-mRNA, PA-vRNA and PA-cRNA, respectively. Since both positive- and negative-sense RNAs (cRNA and vRNA) are present in the poly(A) RNA fraction, these RNA species would hybridize to each other and thereby reduce hybridization between cRNA and the negative-sense probe during the RPA. To examine this, the efficiency of hybridization between cRNA and the 32P-labelled negative-sense probe was analysed in the presence of various amounts of negative-sense RNA. The hybridization between cRNA and the 32P
Influenza virus cRNA synthesis suppression

100

60

20

0

Input competitor RNA (pg)

Fig. 1. Inhibition of the hybridization between PA-cRNA and the \(^{32}\)P probe by adding competitor RNAs. PA-cRNA (500 pg) synthesized \textit{in vitro} from a cDNA copy of the A3/e-3 PA gene, corresponding to nucleotides 1701 to 2232, was hybridized with \(1 \times 10^5\) c.p.m. of \(^{32}\)P-labelled negative-sense probe in the presence of various amounts of unlabelled probe RNA as a competitor. The hybrid RNAs were analysed by RPA as described in Methods. The ratio of the radioactivity of hybrid RNA formed in the presence of competitor RNA to that of hybrid RNA formed in the absence of competitor RNA is shown as the efficiency of hybridization.

probe was decreased markedly when the ratio of the competitor RNA to cRNA became greater than 0.6 (Fig. 1).

Quantification of PA RNAs by filter hybridization. To quantify the PA-specific RNA of A3/e-3 by hybridization on nitrocellulose filters, MDBK cells infected with A3/e-3 were labelled with 9.25 MBq/ml of \([\text{\textsuperscript{3}H}]\)uridine for 3 or 4 h. After extraction and fractionation into poly(A) RNA, the labelled RNAs were hybridized with positive- or negative-sense M13 DNA containing the Wa-182 PA gene, corresponding to nucleotides 8 to 2002, bound to nitrocellulose filters, as described previously (Shapiro et al., 1987). After heating of the filters for 5 min at 90 °C, the radioactivity of PA RNAs eluted from the filters was counted and the amount was calculated on the basis of the incorporation of \([\text{\textsuperscript{3}H}]\)uridine.

Results

Reduction in the yield of infectious particles after consecutive undiluted passages

Our initial goal using the NS2 mutant, Wa-182, was to determine which RNA replication process of the PA gene is suppressed by the mutated NS2 protein. The stock of Wa-182 has been found to contain several sg/DI RNA species derived from the PA gene (Odagiri & Tobita, 1990). Such pre-existing sg/DI RNAs in the virus stock might interfere with normal replication of the genomic RNAs from the beginning of infection, and thereby make it difficult to elucidate the stage at which replication of the PA gene is primarily suppressed. Therefore, we attempted to obtain a Wa-182 stock free from sg/DI RNAs and isolated a subclone (A3/e-3) with a negligible amount of sg/DI RNAs which were detectable only after amplification by PCR.

The reduction in the yield of infectious particles after consecutive undiluted passages of A3/e-3 and wild-type virus AwNS/2 were compared (Fig. 2). When MDCK cells were infected with A3/e-3 at low m.o.i., infectious virus was produced normally, i.e. like wild-type virus (passage 0). On the other hand, when A3/e-3 was grown at a high m.o.i. the yield of infectious virus was drastically decreased from the initial passage (passage 1), although the haemagglutinin titre was not reduced until the second undiluted passage. The reduction in the virus yield was enhanced during the subsequent undiluted passages. In contrast, the yield of infectious particles was not diminished during the first cycle of wild-type virus infection at a high m.o.i. (passage 1) and was reduced only during the second and subsequent undiluted passages. These results demonstrated that the subclone A3/e-3 retained the phenotypic characteristic of the NS2 mutant Wa-182 which is readily converted from normal infectious virus to DI virus by a single cycle of infection at a high m.o.i. (Odagiri & Tobita, 1990).

Viral RNAs in particles grown at low and high m.o.i.s

To demonstrate that the A3/e-3 virions grown at a high m.o.i., like those of Wa-182, lacked the PA gene and...
Fig. 3. Virion RNA of A3/e-3 (a and c) and AwNS/2 (b and d). $^{32}$P-labelled virion RNA extracted from the virions was analysed on a 30% polyacrylamide gel containing 7 M-urea (a and b) and 2.5% polyacrylamide gel containing 7 M-urea (c and d). Lane L indicates virus stock grown at a low m.o.i. Lanes 1 and 2 represent the viruses obtained after the first and second passages at high m.o.i., respectively, as shown in Fig. 2. Open triangles indicate sg/DI RNA species and dots indicate contaminating host cellular RNA.

Fig. 4. Virion RNA of single NS gene reassortants, 29 (lane 2), 31 (lane 3) and 39 (lane 4), and the parent wild-type virus A/Ann Arbor/6/60 (lane 1) grown at a high m.o.i. The reassortants contained the NS gene of A3/e-3 and the other seven genes of Ann Arbor virus. $^{32}$P-labelled virion RNA was analysed on a 2.5% polyacrylamide gel containing 7 M-urea. Open triangles a, b and c indicate sg/DI RNAs derived from the PB1, PB2 and PA genes, respectively, of the Ann Arbor virus.

contained sg/DI RNA species derived from the PA gene, vRNAs of A3/e-3 and wild-type virus were compared with respect to particles produced at a low m.o.i. (corresponding to Fig. 2, passage 0) and at a high m.o.i. (corresponding to Fig. 2, passage 1 and 2). At a low m.o.i. all eight RNA segments of A3/e-3 were present in normal amounts like those of the wild-type virus. No sg/DI RNA species were detected (Fig. 3a, lane L), although by 25 cycles of PCR two sg/DI RNA species...
were detected in the virus stock (data not shown). On the other hand, when A3/e-3 was grown at a high m.o.i., two sg/DI RNA species were detectable (Fig. 3a, lane 1). By nucleotide sequencing the sg/DI RNAs 1 and 2 were shown to correspond to the PA gene with internal deletions of residues 205 to 1876 and 208 to 1977, respectively (see Fig. 5a). Furthermore, these sg/DI RNA species were also shown to be identical to those present in the stock of A3/e-3, suggesting that the small amount of sg/DI RNAs in the A3/e-3 stock were efficiently amplified by a single cycle of infection at a high m.o.i. These sg/DI RNAs were inherited by descendant DI viruses produced by the second undiluted passage without changes in size or nucleotide sequence (Fig. 3a, lane 2).

The amplification of the sg/DI RNA species coincided with a drastic decrease in the amount of the PA gene of A3/e-3 (Fig. 3c, lane 1). The decrease was more striking after the second undiluted passage (Fig. 3c, lane 2). It should be noted that after the first undiluted passage of A3/e-3 an sg/DI RNA, derived from the PB2 gene, with a deletion of nucleotides 295 to 2029 also became detectable by PCR. The appearance of this sg/DI RNA was accompanied by a reduction in the PB2 gene in addition to that of the PA gene at the second undiluted passage (Fig. 3c, lane 2). These results suggested that the generation of sg/DI RNAs always preceded reduction in their progenitor polymerase genes and that the target polymerase gene was not confined to the PA gene.

On the other hand, when virion RNAs of wild-type virus grown at a low m.o.i. were amplified by PCR, several species of sg/DI RNA derived from the PA gene were detected (data not shown). However, there was neither an amplification of these sg/DI RNAs nor a significant decrease in the progenitor PA gene during the initial undiluted passage (Fig. 3b and d, lanes 1). These results suggest that in the case of wild-type virus the amplification of pre-existing sg/DI RNAs and the selective decrease in their progenitor gene were prevented during the initial cycle of infection at a high m.o.i.

Table 1. Nucleotide and amino acid differences in NS2

<table>
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<tr>
<th>Virus</th>
<th>Nucleotide (amino acid) positions</th>
<th>Phenotypes*</th>
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<tr>
<td></td>
<td>593 (32)</td>
<td>PA gene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>suppression</td>
</tr>
<tr>
<td></td>
<td>698 (67)</td>
<td>sg/DI RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>amplification</td>
</tr>
<tr>
<td>Wa-182</td>
<td>C Thr A Glu</td>
<td>+</td>
</tr>
<tr>
<td>A3/e-3</td>
<td>C Thr A Glu</td>
<td>+</td>
</tr>
<tr>
<td>AwNS/2</td>
<td>U Ile A Glu</td>
<td>-</td>
</tr>
<tr>
<td>Aichi/2/68</td>
<td>U Ile G Gly</td>
<td>-</td>
</tr>
<tr>
<td>Aichi/2/68 (Ao)</td>
<td>U Ile A Glu</td>
<td>-</td>
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* The phenotypes found in one-step growth at a high m.o.i. are shown.

Identification of the mutation responsible for the phenotype of A3/e-3

To determine whether the mutated NS gene of A3/e-3 is solely responsible for the preferential amplification of the sg/DI RNAs and the selective decrease in their progenitor genes, the NS gene was singly transferred to another wild-type virus, A/Ann Arbor/6/60. The virion RNAs of the NS single-gene reassortants were likewise examined after a single passage at a high m.o.i. (Fig. 4). In the case of wild-type Ann Arbor virus, the stock of which contained substantial amounts of sg/DI RNAs derived from the PB1 and PA genes, no polymerase genes were decreased even after growth at a high m.o.i. (Fig. 4, lane 1). On the other hand, the PA gene of all of the reassortants examined was decreased by the acquisition of the NS gene from A3/e-3 virus (Fig. 4, lanes 2, 3 and 4). Furthermore, the PB1 and/or PB2 genes were also decreased. These reassortants were shown to contain several sg/DI RNA species. Nucleotide sequencing of these sg/DI RNAs (Fig. 4, open triangles) showed that they were derived from the PA, PB1 and/or PB2 genes of the Ann Arbor virus, but not from the A3/e-3 polymerase genes. We have not determined whether these sg/DI RNA species were newly derived from the polymerase genes of Ann Arbor virus during reassortment or were secondary derivatives from pre-existing sg/DI RNAs in the Ann Arbor virus stock that underwent additional deletions. From these results, it was concluded that the A3/e-3 NS gene was solely responsible for both the amplification of the sg/DI RNAs and the selective decrease in their progenitor polymerase genes, excluding the possibility that the suppression of certain polymerase genes was caused by the sg/DI RNAs of A3/e-3 which might have been transferred to the reassortants along with the mutant NS gene.

The nucleotide sequence of the NS gene of A3/e-3 was shown to be identical to that of Wa-182 (Table 1), i.e. there are two mutations in the NS2 reading frame, at
positions 593 and 698 relative to the wild-type NS gene of the Aichi virus (Odagiri & Tobita, 1990). To identify which mutation is responsible for the A3/e-3 characteristic, the nucleotide sequence of the NS gene of A3/e-3 was compared with those of different wild-type viruses: AwNS/2, Aichi and another stock of Aichi (Aichi, Ao). As shown in Table 1, guanine at residue 698 of Aichi virus had been changed to adenine in AwNS/2 as well as in A3/e-3. Furthermore, Aichi Ao virus was also shown to have adenine at this position. Consequently, the nucleotide difference at position 698 between A3/e-3 and Aichi virus should not be crucial in the phenotype of
Fig. 5. Quantification of the PA gene-specific RNAs synthesized in MDCK cells infected with viruses at a high m.o.i. (a) Plasmid DNA construct containing the PA cDNA of Wa-182 (pPA/2); two riboprobes in the positive- and negative-senses that were transcribed from pPA/2 by T7 and T3 RNA polymerases, respectively (grey column in upper panel). By using each riboprobe, RNA species corresponding to full-length RNA (1) and DI RNAs (2) shown by grey columns were detected. The expected number of nucleotide of each RNA molecule is shown in parentheses. (b, c and d) cRNA, mRNA and vRNA species, respectively, detected by the RPA in infected cells at the indicated time (h) p.i. lanes P, Probe; lanes U, RNAs from mock-infected cells; lanes A(+) and A(−), markers for mRNA and cRNA, respectively. Arrowheads and open triangles indicate PA gene-specific RNAs and DI RNAs, respectively. (e) Amounts of the cRNA species (upper panel) and the mRNA species (lower panel); (f) amount of the vRNA species, determined as described in Methods. The closed and open circles represent PA gene-specific RNAs and the sum of the DI RNA species, respectively.
A3/e-3. Cytosine at position 593 was found to occur only in A3/e-3 and Wa-182, whereas in wild-type viruses the nucleotide residue at this position was uracil. This mutation in the A3/e-3 and Wa-182 NS genes would cause an amino acid change from isoleucine to threonine at position 32 in the NS2 protein and this reduced its electrophoretic mobility (data not shown). The results indicate that this amino acid substitution was responsible for the altered structure of the NS2 protein that determined the phenotype of A3/e-3. Since isoleucine at position 32 in the protein is well conserved among most influenza A viruses including avian strains (data not shown), this residue may be crucial for the function of the NS2 protein.

Suppression of PA gene RNA synthesis

To determine at which RNA replication stage the production of the A3/e-3 PA gene is suppressed in the initial undiluted passage, synthesis of PA RNAs (PA-cRNA, PA-vRNA and PA-mRNA) as well as synthesis of the DI RNA molecules derived from the PA gene (DI-cRNA, DI-vRNA and DI-mRNA) were analysed quantitatively by the RPA as described in Methods. Using the negative-sense PA probe, both cRNAs and mRNAs specific for the PA gene were detected. These RNA species were distinguishable from each other by fractionation of the samples into poly(A)⁺ RNAs and poly(A)⁻ RNAs, and by a difference in electrophoretic migration rate owing to the presence or absence of the region downstream from the poly(A) signal, i.e. mRNA was 15 nucleotides shorter than cRNA (see Fig. 5c, lane 4). In wild-type virus-infected cells, the synthesis of PA-cRNA was enhanced dramatically between 3 and 4 h post-infection (p.i.) (Fig. 5b, panel AwNS/2) with the maximum amount 4-2 times greater than that of the PA-cRNAs of A3/e-3 (compare Fig. 5e, upper panels A3/e-3 and AwNS/2). Although DI-cRNA species of wild-type virus were synthesized together with the PA-cRNA, their synthesis never predominated over that of PA-cRNA synthesis, unlike that of A3/e-3 virus. In A3/e-3-infected cells, PA-cRNA and the smaller DI-cRNAs were first detected at 1-5 h p.i. and their syntheses increased in parallel until 3 h p.i. (Fig. 5b, panel A3/e-3). PA-cRNA synthesis, however, did not increase thereafter, whereas the DI-cRNA synthesis was enhanced greatly between 3 and 3-5 h p.i. and the amount of PA-cRNA remained lower than that of DI-cRNAs (Fig. 5e, upper panel of A3/e-3). Since both cRNA and vRNA species are present in the poly(A)⁺ RNA fraction, the lower synthesis of the A3/e-3 PA-cRNA may be attributable to inhibition of the hybridization between PA-cRNA and the ³²P probe by PA-vRNA as a competitor. To examine this, the PA-cRNA and PA-vRNA of A3/e-3 labelled with [³H]Jurdine were also quantified by filter hybridization as described in Methods. As shown in Table 2, the amount of PA-vRNA synthesized by 3 h p.i. was less than 60 % of that of PA-cRNA, excluding the above possibility (see Fig. 1) and supporting the reliability of quantification by the RPA. From these results, it was concluded that replication of the PA gene of A3/e-3 was suppressed primarily at the cRNA synthesis stage. This suppression was probably caused by a reciprocal amplification of the DI-cRNA species. It is noteworthy that the synthesis of both PA-cRNA and DI-cRNA molecules of A3/e-3 was found to occur 30 min earlier than that of wild-type virus (Fig. 5b).

Both PA-mRNA and DI-mRNA species of A3/e-3 became detectable from 1 h p.i. in similar amounts and they were not amplified until 2 h p.i. (Fig. 5c, panel A3/e-3), implying that these RNA species were primary transcripts. We also observed that the amount of PA-mRNA synthesized in the presence of cycloheximide was essentially identical between A3/e-3 and wild-type virus (data not shown). These results suggest that PA-mRNA synthesis was not suppressed for A3/e-3 during the primary transcription phase, nor was synthesis greatly enhanced even in the late phase of infection, in contrast to the DI-mRNA species that were synthesized markedly between 3-5 h and 4 h p.i. (Fig. 5c and e, panel A3/e-3). In contrast to the mutant, the PA-mRNA of wild-type virus was synthesized further between 3 h and 4 h p.i. (Fig. 5c and e, panel AwNS/2). These results suggest that PA-mRNA synthesis of A3/e-3 was suppressed at the stage of secondary transcription. This resulted in a reduction in the PA protein synthesis (Odagiri & Tobita, 1990).

For A3/e-3, PA-vRNA synthesis was also shown to be less efficient than the DI-vRNA synthesis, whereas the PA-vRNA synthesis of wild-type virus was greatly enhanced between 5 h and 6-5 h p.i. (Fig. 5d and f). Using the appropriate riboprobe we failed to find a DI-vRNA species for the wild-type virus. The kinetics of the PA-vRNA synthesis of A3/e-3 were similar to those of the PA-cRNA synthesis. Because cRNA synthesis precedes vRNA synthesis and the cRNA molecules serve as

<table>
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<th>Time p.i. (h)</th>
<th>vRNA (pg/µg total RNA)</th>
<th>cRNA (pg/µg total RNA)</th>
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<tr>
<td>3</td>
<td>259.5</td>
<td>465.5</td>
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<td>4</td>
<td>510.0</td>
<td>1038.1</td>
<td>2.04</td>
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*The amount of PA RNAs was the sum of the number of full-length RNA and DI RNAs.
the templates for vRNA replication, the restricted synthesis of the PA RNA species of A3/e-3 is suggested to be primarily attributable to the suppression of cRNA synthesis.

Discussion
In the present study, we have shown that suppression of the PA gene replication of the NS2 mutant, A3/e-3, occurred primarily at the cRNA synthesis stage. The mutated NS2 protein was demonstrated to be responsible for this phenomenon, since the suppression phenotype was transferred together with the mutated NS gene to reassortant viruses (Fig. 4). Comparative nucleotide sequencing of the NS genes revealed that an amino acid change from isoleucine to threonine at position 32 in the NS2 protein was responsible. The suppression was shown to coincide with the preferential amplification of DI-cRNA species derived from the PA gene. In fact, the PA-cRNA synthesis was strongly suppressed when the DI-cRNA synthesis was enhanced (Fig. 5).

It has been shown that viral proteins synthesized through the primary transcription of viral mRNAs are required for cRNA synthesis (Hay et al., 1977; Barrett et al., 1979). However, the primary mRNAs of the mutant were shown to be synthesized in the same amount as those of wild-type virus, and they were able to be translated in vitro into the three polymerase, NP, M1 and NS1 proteins (data not shown). It is therefore concluded that the suppression of PA-cRNA synthesis was not due to the failure of primary mRNA transcription.

Reduction in PA-vRNA replication of the mutant can be considered to be attributable to the suppression of the PA-cRNA synthesis, since cRNA synthesis precedes vRNA synthesis and cRNAs serve as the templates for replication of progeny vRNAs (Barrett et al., 1979; Shapiro et al., 1987). In the present study, PA-vRNA synthesis of A3/e-3 was shown to begin 2 h later than PA-cRNA synthesis, when PA-cRNA synthesis was already suppressed (Fig. 5). Furthermore, the restricted synthesis of the PA-cRNA should result in less efficient PA-mRNA synthesis in secondary transcription (Fig. 5), for which the amplification of vRNA replication supplies the templates (Smith & Hay, 1982; McCauley & Mahy, 1983; Shapiro et al., 1987). From these results, it is concluded that the impaired synthesis of the PA-cRNA caused a corresponding reduction in PA-vRNA replication and subsequently in secondary PA-mRNA synthesis.

It is likely that DI RNAs are replicated more efficiently than their full-length progenitor RNAs because of their smaller size. If the preferential amplification of the DI-cRNA species of A3/e-3 could be interpreted on this basis, the DI-cRNA species of wild-type virus would be expected to be synthesized as efficiently as those of the NS2 mutant. However, in wild-type virus-infected cells neither preferential amplification of the DI-cRNA species nor suppression of the PA-cRNA synthesis occurred, although by using PCR the stock of wild-type virus was shown to contain several sg/DI RNA species derived from the PA gene (data not shown). Similarly, for another wild-type virus, A/Ann Arbor/6/60, replication of the polymerase genes was not suppressed, although the virus stock contained considerable amounts of sg/DI RNA species derived from the PB1 and PA genes (Fig. 4). In addition, we have observed that in the mutant-infected cells the ratio of the PA genomic RNA to PA DI-vRNAs was 50.5%, whereas in the progeny viral particles the ratio was 9.6%, indicating that the short-length DI-vRNA species were preferentially incorporated into progeny virions. This finding implies that the selective packaging of DI-vRNAs may be an additional mechanism for producing DI particles lacking the PA gene, as shown in Fig. 3.

On the other hand, the NS2 protein of the mutant was found to be responsible for both enhanced amplification of the DI-cRNA synthesis and suppression of the PA-cRNA synthesis. Because only the NS2 protein differed between the mutant and wild-type virus, AWNS/2 (Table 1), the preferential synthesis of the DI-cRNA species and the suppression of cRNA synthesis of their progenitor may be caused by a trans-acting mechanism mediated by the mutant NS2 protein. This postulate is supported by the results of two independent experiments in which the suppression of the PA gene was transferred together with the mutant NS gene to reassortant viruses (Fig. 4 and Odagiri & Tobita, 1990). The NS2 protein of the mutant as well as those of several wild-type viruses was found to be synthesized in the early phase of infection (Odagiri et al., 1991), consistent with the mutated NS2 protein adversely affecting the process of cRNA synthesis.

For DI viruses, the affected polymerase genes have been shown to be the progenitors of the sg/DI RNAs (Davis & Nayak, 1979; Nakajima et al., 1979; Akkina et al., 1984). In the case of the NS2 mutant, the PA gene was affected at the first undiluted passage. However, when the mutant was further passaged without dilution, another polymerase gene, PB2, was also decreased (Fig. 3). Similarly, in the case of the single NS gene reassortants, A3AA/29 and A3AA/31, the decrease in both the PA and PB1 genes accompanied the acquisition of the mutant NS gene (Fig. 4). These results indicate that the target area suppressed by the mutated NS2 protein is not confined to the PA gene. By PCR analyses, we observed that the apparent generation of sg/DI RNA species always preceded suppression of RNA replication of their progenitor (T. Odagiri & K. Tobita, unpublished data). Furthermore, in the single NS gene reassortants,
progenitors of the sg/DI RNA species were always those genes whose replication was greatly suppressed (Fig. 4). These observations suggest that the presence of certain sg/DI RNA species is a prerequisite for the selective suppression of its progenitor RNA replication and that the origin of pre-existing sg/DI RNA in the virus stock might determine the target gene of the suppression.

Since in an artificial vaccinia virus vector-driven replication system influenza virus RNA has been shown to be replicated by the three polymerase proteins and the nucleoprotein alone (Huang et al., 1990), NS2 as well as NS1 protein may not be essential for viral RNA synthesis. The mutated NS2 protein of A3/e-3, however, was shown to suppress cRNA synthesis of the PA gene and to enhance the amplification of PA sg/DI RNA species. To account for the selective suppression of PA cRNA synthesis it is speculated that PA DI-cRNAs amplified by the mutated NS2 protein might be hybridized with the 5'- and/or 3'-terminal regions of the progenitor PA vRNA, since DI-cRNAs retain both 5' and 3' termini. Such specific hybridization might interfere with the normal movement of viral polymerase and suppress subsequent cRNA synthesis from genome RNA. The mutated NS2 protein may facilitate such specific hybridization, although both cRNA and vRNA are coated with nucleoproteins. Consequently, the NS2 protein of the wild-type virus may play a role in promoting normal replication of the genomic RNAs by preventing aberrant replication of sg/DI RNAs and hybridization between genomic RNAs and their complementary sg/DI RNAs.

We thank Dr M. Tashiro (Department of Virology, Jichi Medical School, Japan) for many helpful discussions and N. Ogawa for technical assistance. This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Science, Education and Culture, Japan, the Mochida Memorial Foundation, and the Naito Foundation.

References


(Received 7 June 1993; Accepted 10 September 1993)