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Analysis of an Influenza A Virus Mutant with a Deletion in the NS Segment

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The influenza virus host range mutant CR43-3, derived by recombination from the A/Alaska/6/77 and the cold-adapted and temperature-sensitive A/Ann Arbor/6/60 viruses, has previously been shown to possess a defect in the NS gene. To characterize this defect, nucleotide sequence data were obtained from cloned cDNAs. The CR43-3 NS gene was found to be 854 nucleotides long and to derive from the NS gene of the A/Alaska/6/77 parent virus by an internal deletion of 36 nucleotides. Direct sequencing of RNA 8 of CR43-3 virus confirmed that the deletion in the NS1-coding region was not an artifact that was generated during the cloning procedure. Protein analysis indicated that the NS1 protein of CR43-3 virus was synthesized in equal amounts in the restrictive (MDCK) cells as well as in the permissive (PCK) host cells. Also, indirect immunofluorescence studies of virus-infected cells showed that the NS1 protein of CR43-3 virus, like that of the parent viruses, accumulates in the nuclei of both cell systems. Although no differences in synthesis or localization of the NS1 protein could be detected, a consistent reduction in M1 protein was noted in CR43-3 virus-infected, nonpermissive cells as compared with that of the permissive host. Since analysis of the CR43-3 virus required us to obtain the NS nucleotide sequence of the 1977 isolate A/Alaska/6/77, we were able to compare this sequence with those of corresponding genes of earlier strains. The result of this analysis supports the idea of a common lineage of human influenza A viruses isolated over a 43-year period.

The influenza A virus genome is composed of eight single-stranded RNA segments of negative polarity that code for at least 10 polypeptides (for review, see references 17 and 28). The smallest of the gene segments codes for two overlapping polypeptides, NS1 and NS2 (12, 18) which are only found in the infected cell (8, 9, 14, 20). The function of these gene products in the influenza virus life cycle is not well characterized, although several NS gene mutants have been isolated (1, 30, 32, 34–36, 42). Most of the NS mutants that have been described in detail are temperature dependent and are derived from the influenza A fowl plague virus (1, 13, 30, 32, 42). In this paper we report on the defect of an NS mutant virus that exhibits a restricted host range phenotype that is not temperature dependent. The mutant virus, CR43-3, was accidentally generated in a recombination experiment performed in primary chicken kidney (PCK) cells at the nonpermissive temperature of 38°C with A/Alaska/6/77 and the cold-adapted and temperature-sensitive (*ts*) A/Ann Arbor/6/60 (A/AA/6/60) viruses as parents (22). CR43-3 virus displays a host range phenotype different from that of either parent. The mutant virus forms plaques in PCK cells, but not in Madin-Darby canine kidney (MDCK) cells, whereas the parent viruses and an isogenic recombinant virus (CR43-7) plaque equally well in both cell systems (21a). Previous analysis of the RNA gene constellation of CR43-3 virus by electrophoresis in polyacrylamide gels showed that CR43-3 possesses an NS segment with an aberrant gel migration. In this paper we show that the CR43-3 NS gene is derived from the A/Alaska/6/77 NS gene by an internal deletion of 36 nucleotides. This deletion occurs in the NS1-coding region of RNA 8, resulting in an NS1 polypeptide that is 12 amino acids shorter than that of the A/Alaska/6/77 virus. This is the first report of a deletion mutation in an influenza virus gene that appears to be associated with a restricted host range phenotype.

In the course of sequence analysis of the NS gene of CR43-3 virus, we also obtained the complete sequence of the NS gene of A/Alaska/6/77 virus. The sequence of the NS gene of this field isolate permitted comparison with known NS sequences of earlier strains (2, 15, 19, 41). Analysis of the sequences of six human influenza A virus NS genes indicates a sequential evolutionary relationship among these strains isolated over a 43-year period.

MATERIALS AND METHODS

Viruses and cells. Influenza viruses A/Alaska/6/77 (H3N2), A/AA/6/60 (H2N2) (21), and the mutant CR43-3 (H3N2) were grown in the allantoic cavity of 10-day-old embryonated eggs. The A/AA/6/60 and CR43-3 viruses are cold adapted and temperature sensitive and therefore were grown at 33°C for 3 days, whereas A/Alaska/6/77 virus was grown at 35°C for 2 days. Viruses were purified using sucrose gradient centrifugation, and the RNA was extracted as reported previously (29).

PCK cell cultures were prepared as described previously (27). Both PCK and MDCK cell cultures were used for viral protein analysis and indirect immunofluorescence studies of virus-infected cells.

Cloning and sequencing. The purified RNAs of A/Alaska/6/77, A/AA/6/60, and CR43-3 viruses were transcribed into double-stranded cDNAs by using reverse transcriptase and synthetic dodecamer oligonucleotide primers (2). *Eco*RI linkers were blunt end ligated to the cDNAs to facilitate insertion into the unique *Eco*RI site of pBR322. The DNA, containing virus-specific inserts, was used to transform competent *Escherichia coli* C600 cells. The ampicillin-resistant transformants obtained were initially screened for

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NS gene-specific sequences by the in situ colony hybridization method of Grunstein and Hogness (11). The probe used was a nick-translated cDNA copy of the previously cloned A/FM/1/47 virus NS gene (15). The plasmid DNAs of the NS-positive colonies were further analyzed for the presence of full-length NS gene inserts (4).

Complete nucleotide sequences of the cloned A/Alaska/6/77 and CR43-3 virus NS genes were determined by the chemical method of Maxam and Gilbert (24). Since a series of NS genes have already been sequenced (2, 3, 15, 19, 41), most of the sequence information was obtained from one strand of the cDNA fragments. To confirm that the sequence of RNA 8 of CR43-3 was identical around the deletion points to that of the cloned cDNA, the RNA was directly sequenced by the Sanger chain termination method (31). The sequencing reactions were performed as previously described (16). The primer used for dideoxy sequencing was an *Eco*RI-*Dde*I restriction fragment corresponding to nucleotide positions 1 through 153 of the cloned NS gene of A/Alaska/6/77 virus. The complete nucleotide and deduced amino acid sequences were stored, edited, and compared in an IBM 370 computer at the University Computing Center of the City University of New York by using published programs (37–39).

Analysis of viral polypeptides. Confluent monolayers of MDCK and PCK cells in 35-mm dishes were infected with approximately 10^8 PFU of virus (A/Alaska/6/77, A/AA/6/60, and CR43-3) per ml for 1 h at 34°C. At various times postinfection (p.i.), the cells were pulse-labeled for 1 h with [^{35}S]methionine (200 μCi/ml) as described previously (29). Cell lysates were prepared and infected cell proteins were separated by electrophoresis on a 7 to 14% linear gradient sodium dodecyl sulfate-polyacrylamide gel (43).

Indirect immunofluorescence of virus-infected cells. MDCK and PCK cells were seeded onto 13-mm glass cover slips and grown to 60 to 100% confluency. Monolayers of both cell types were infected with approximately 10^8 PFU of virus per ml for 1 h at 34°C. At various times after infection, the cells were acetone fixed. A monospecific rabbit antiserum to *E. coli*-produced NS1 protein followed by a fluorescein-conjugated goat antibody to rabbit 7S immunoglobulin G was used for indirect immunofluorescence analysis of virus-infected cells as described elsewhere (42a).

RESULTS

cDNA analysis of NS segments. Polyacrylamide-urea gel electrophoresis of the glyoxalated RNAs of many influenza A virus strains has shown identical gel migration for the NS segments (7). These data indicate a size conservation among NS genes which is supported by nucleotide sequence data (2, 3, 15, 19, 41). In contrast, the NS segment of CR43-3 virus migrates faster under these gel conditions (data not shown). Similar results were obtained when the cDNAs of A/Alaska/6/77, A/AA/6/60, and CR43-3 viruses were compared on polyacrylamide gels. Figure 1 shows the analysis of both the single- and double-stranded cDNA copies of the A/AA/6/60 and CR43-3 viral genomes. The cDNA products of the first and second strand reverse transcriptase reactions were treated with S1-nuclease and electrophoresed in parallel on the gel. From the relative mobilities and known sizes of the double-stranded cDNAs on the gel, we calculated that the CR43-3 NS segment is approximately 40 bases shorter than the NS segment of A/AA/6/60 virus. This suggested that the CR43-3 NS gene has a deletion. It should also be noted that the CR43-3 NS cDNA consistently showed a decreased

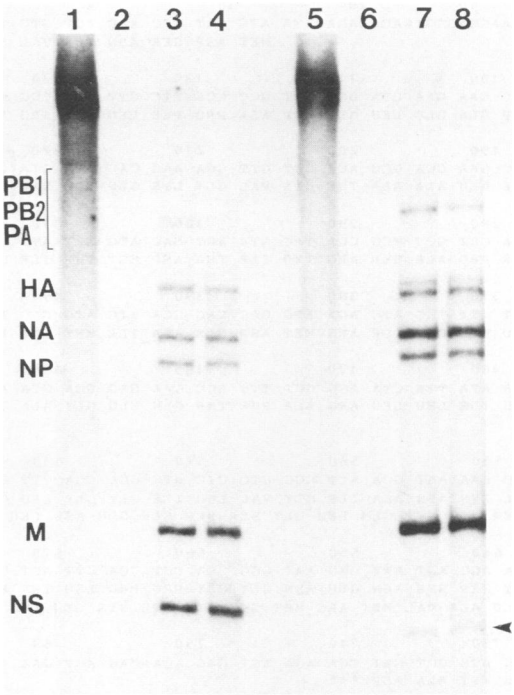

FIG. 1. Electrophoresis of the reverse-transcribed cDNA copies of A/AA/6/60 and CR43-3 viral genes in a 5% polyacrylamide gel. Lanes: 1, A/AA/6/60 single-stranded cDNA; 2, A/AA/6/60 single-stranded cDNA after S1 nuclease treatment; 3, A/AA/6/60 double-stranded cDNA; 4, A/AA/6/60 double-stranded cDNA after S1 nuclease treatment; 5, CR43-3 single-stranded cDNA; 6, CR43-3 single-stranded cDNA after S1 nuclease treatment; 7, CR43-3 double-stranded cDNA; 8, CR43-3 double-stranded cDNA after S1 nuclease treatment. The proteins coded by the double-stranded cDNA transcripts are indicated. The faster migrating, NS-specific cDNA of CR43-3 virus is indicated by the arrow.

intensity relative to the other viral gene segments. This could indicate that either the amount of RNA 8 in the viral preparation is low or that cDNA transcription of this segment is not efficient. The migration of the NS cDNA of the A/Alaska/6/77 virus was identical to that of the corresponding segment of the A/AA/6/60 virus (data not shown).

Nucleotide sequences of cDNAs. To confirm that the CR43-3 virus NS gene contained a deletion mutation and to localize the exact nucleotide position of this defect, the complete nucleotide sequence of a full-length NS gene of CR43-3 virus, cloned into pBR322, was determined (Fig. 2). This NS gene was found to be 854 nucleotides long, which is 36 bases shorter than that reported for NS genes of all other influenza A viruses (2, 3, 15, 19, 41). Comparison of the CR43-3 NS sequence with the other known NS gene sequences indicated that the deletion occurred around nucleotide position 220. To precisely define the deletion junctions and to determine the parental origin of the CR43-3 NS gene, it was necessary to obtain sequence information of the NS genes of the parent viruses. We compared the CR43-3 NS sequence to the previously obtained partial sequence of the NS gene of the wild-type A/AA/6/60 virus (28a). In the region sequenced, 6 out of 55 nucleotides differed from those in the CR43-3 NS sequence. These base differences made it very unlikely that CR43-3 derived its NS gene from the A/AA/6/60 parent. Therefore, we proceeded to clone and sequence the NS gene

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        10         20         30         40         50         60         70         80         90
AGCAAAAGCAGGGTGACAAAGACATA ATG GAT TCC AAC ACT GTG TCA AGT TTC CAG GTA GAT TGC TTC CTT TGG CAT GTC CGA AAA CAA GTT
                           MET ASP SER ASN THR VAL SER SER PHE GLN VAL ASP CYS PHE LEU TRP HIS VAL ARG LYS GLN VAL

       100        110        120        130        140        150        160        170        180
GTA GAC CAA GAA CTA GGT GAT GCC CCA TTC CTT GAT CGG CTT CGC CGA GAT CAG AGG TCC CTA AGG GGA AGA GGC AGC ACT CTC GGT CTA
VAL ASP GLN GLU LEU GLY ASP ALA PRO PHE LEU ASP ARG LEU ARG ARG ASP GLN ARG SER LEU ARG GLY ARG GLY SER THR LEU GLY LEU

       190        200        210        220        230        240        250        260        270
GAC ATC GAA GCA GCC ACC CAT GTT GGA AAG CAG ATA GTA [GAG AAG ATT CTG AAG GAA GAA TCT GAT GAG GCA CTT] AAA ATG ACC ATG GCC
ASP ILE GLU ALA ALA THR HIS VAL GLY LYS GLN ILE VAL [GLU LYS ILE LEU LYS GLU GLU SER ASP GLU ALA LEU] LYS MET THR MET ALA

       280        290        300        310        320        330        340        350        360
TCC ACA CCT GCT TCG CGA TAC ATA ACT GAC ATG ACT ATT GAG GAA TTG TCA AGA GAC TGG TTC ATG CTA ATG CCC AAG CAG AAA GTG GAA
SER THR PRO ALA SER ARG TYR ILE THR ASP MET THR ILE GLU GLU LEU SER ARG ASP TRP PHE MET LEU MET PRO LYS GLN LYS VAL GLU

       370        380        390        400        410        420        430        440        450
GGA CCT CTT TGC ATC AGA ATG GAC CAG GCA ATC ATG GAG AAG AAC ATC ATG TTG AAA GCG AAT TTC AGT GTG ATT TTT GAC CGA CTG GAG
GLY PRO LEU CYS ILE ARG MET ASP GLN ALA ILE MET GLU LYS ASN ILE MET LEU LYS ALA ASN PHE SER VAL ILE PHE ASP ARG LEU GLU

       460        470        480        490        500        510        520        530        540
ACC CTA ATA TTA CTA AGG GCT TTC ACC GAA GAG GGA GCA ATT GTT GGC GAA ATC TCA CCA TTG CTT TCT TTT CCA GGA CAT ACT ATT GAG
THR LEU ILE LEU LEU ARG ALA PHE THR GLU GLU GLY ALA ILE VAL GLY GLU ILE SER PRO LEU LEU SER PHE PRO GLY HIS THR ILE GLU
                                                                                                  ASP ILE LEU LEU ARG

       550        560        570        580        590        600        610        620        630
GAT GTC AAA AAT GCA ATT GGG GTC CTC ATC GGA GGA CTT GAA TGG AAT GAT AAC ACA GTT CGA GTC TCT AAA ACT CTA CAG AGA TTC GCT
ASP VAL LYS ASN ALA ILE GLY VAL LEU ILE GLY GLY LEU GLU TRP ASN ASP ASN THR VAL ARG VAL SER LYS THR LEU GLN ARG PHE ALA
 MET SER LYS MET GLN LEU GLY SER SER SER GLU ASP LEU ASN GLY MET ILE THR GLN PHE GLU SER LEU LYS LEU TYR ARG ASP SER LEU

       640        650        660        670        680        690        700        710        720
TGG GGA AGC AGT AAT GAG AAT GGG GGA CCT CCA CTT ACT CCA AAA CAG AAA CGG AAA ATG GCG AGA ACA GCT AGG TCA AAA GTT CGA AGA
TRP GLY SER SER ASN GLU ASN GLY GLY PRO PRO LEU THR PRO LYS GLN LYS ARG LYS MET ALA ARG THR ALA ARG SER LYS VAL ARG ARG
 GLY GLU ALA VAL MET ARG MET GLY ASP LEU HIS LEU LEU GLN ASN ARG ASN GLY LYS TRP ARG GLU GLN LEU GLY GLN LYS PHE GLU GLU

       730        740        750        760        770        780        790        800        810
GAT AAG ATG GCT GAT TGA AGA AGT GAG ACA CAG ACT GAA AAC AAC TGA GAA TAG TTT TGA GCA AAT AAC ATT CAT GCA AGC CTT ACA GCT
ASP LYS MET ALA ASP ***
 ILE ARG TRP LEU ILE GLU GLU VAL ARG HIS ARG LEU LYS THR THR GLU ASN SER PHE GLU GLN ILE THR PHE MET GLN ALA LEU GLN LEU

       820        830        840        850        860        870        880        890
ACT ATT TGA AGT GGA ACA GGA GAT AAG AAC TTT CTC ATT TCA GCT TAT TTA A TGATAAAAAACACCCTTGTTTCTACT

LEU PHE GLU VAL GLU GLN GLU ILE ARG THR PHE SER PHE GLN LEU ILE ***
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FIG. 2. Nucleotide sequence of the cloned NS genes of influenza A viruses A/Alaska/6/77 and CR43-3. The deduced amino acid sequences of the NS1 and NS2 polypeptides, respectively, are shown below the nucleotide sequence. The region of the A/Alaska/6/77 NS gene which is deleted in the CR43-3 NS gene is denoted by brackets. The arrows indicate the nucleotide positions where the NS1 mRNA is spliced to generate the NS2 mRNA.

of A/Alaska/6/77 virus. Excluding the area of the deletion (positions 222 through 257), the NS gene sequences of A/Alaska/6/77 and CR43-3 viruses were identical, indicating that CR43-3 derived its NS gene from the A/Alaska/6/77 parent (Fig. 2). However, it should be noted that base positions 400, 403, and 415 in the CR43-3 NS gene and position 521 in the A/Alaska/6/77 NS gene were not unambiguously identified.

Direct RNA sequencing. To eliminate the possibility that the deletion in the NS-specific cDNA of CR43-3 virus was an artifact generated in the cloning procedure, RNA 8 of CR43-3 was directly sequenced by the dideoxy method in the region corresponding to nucleotides 166-290 of the A/Alaska/6/77 NS gene (Fig. 3).

Amino acid sequences. The deduced NS1 and NS2 amino acid sequences of the A/Alaska/6/77 and CR43-3 NS genes are also presented in Fig. 2. The NS1 protein of A/Alaska/6/77 virus consists of 237 amino acids, whereas the NS1 of CR43-3 virus consists of only 225 amino acids. The deletion in the NS1 protein of CR43-3 virus corresponds to positions 66 through 77 of the A/Alaska/6/77 NS1 protein. The deduced length of the NS2 polypeptides of both A/Alaska/6/77 and CR43-3 viruses was found to be 121 amino acids, which is identical to that reported for all other NS2 proteins of influenza A viruses (2, 3, 15, 19, 41).

Viral protein analysis. Since CR43-3 virus shows a restricted host range phenotype, [^{35}S]methionine-labeled proteins of MDCK and PCK cells infected with CR43-3 and its parent viruses were analyzed on sodium dodecyl sulfate-polyacryl-

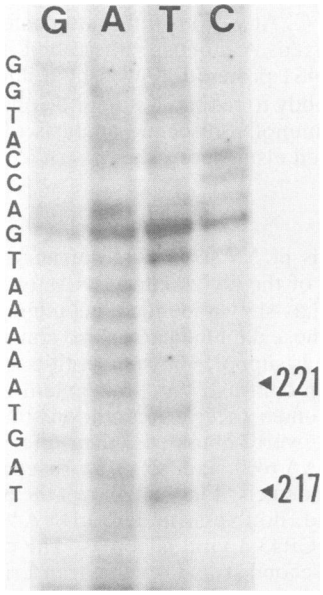

FIG. 3. Dideoxy sequencing of RNA 8 of CR43-3 virus. The primer used to generate this sequence was as described in the text. The bases indicated extend from nucleotide position 217 of the CR43-3 NS segment. Beyond nucleotide position 221, the CR43-3 virus NS sequence is different from that of A/Alaska/6/77 virus. The dideoxy nucleotide-terminated DNA chains were fractionated on an 8% polyacrylamide–7 M urea sequencing gel.

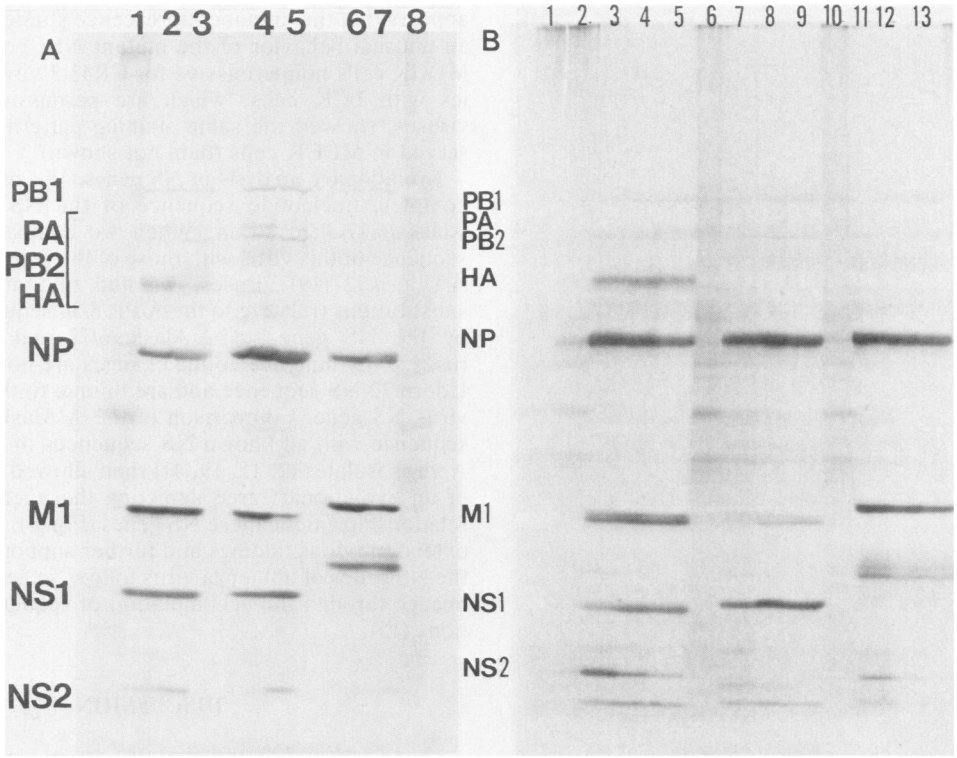

FIG. 4. Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis of viral proteins synthesized in PCK and MDCK cells. (A) Cells were infected with virus and pulse-labeled for 1 h at 8 h p.i. with [^{35}S]methionine as described in the text. Lanes: 1, mock-infected PCK cells; 2, A/AA/6/60 virus-infected PCK cells; 3, A/AA/6/60 virus-infected MDCK cells; 4, CR43-3 virus-infected PCK cells; 5, CR43-3 virus-infected MDCK cells; 6, A/Alaska/6/77 virus-infected PCK cells; lane 7, A/Alaska/6/77 virus-infected MDCK cells; 8, mock-infected MDCK cells. Virus-specific proteins are indicated. (B) MDCK cells were infected with virus and pulse-labeled for 1 h at the indicated times. Lanes 2, 6, and 10 were infected for 3 h; lanes 3, 7 and 11 were infected for 6 h; lanes 4, 8, and 12 were infected for 9 h, and lanes 5, 9, and 13 were infected for 12 h. Lanes: 1, mock-infected MDCK cells; 2 through 5, A/AA/6/60-infected MDCK cells; 6 through 9, CR43-3 virus-infected MDCK cells; 10 through 13, A/Alaska/6/77 virus-infected MDCK cells. Virus-specific proteins are indicated.

amide gels (Fig. 4A). No major differences in protein synthesis patterns could be detected in A/AA/6/60 virus-infected MDCK and PCK cells at 8 h p.i. (Fig. 4A, lanes 2 and 3). Similarly, cell extracts from A/Alaska/6/77 virus-infected MDCK and PCK cells showed comparable protein patterns after polyacrylamide gel electrophoresis (Fig. 4A, lanes 6 and 7). This result was not unexpected, since neither virus displays a growth restriction in the two host cells. Analysis of MDCK and PCK cells infected with CR43-3 virus (8 h p.i.) revealed that the NS1 protein is synthesized in comparable amounts in both cell systems (Fig. 4A, lanes 4 and 5). However, from this and other gels (data not shown) it was found that the M1 protein is reduced by approximately 50% relative to the NS1 polypeptide in the nonpermissive MDCK cells as compared with the permissive PCK cells infected with CR43-3 virus. This finding was obtained by quantitating densitometer tracings of different autoradiographs and was confirmed by time course experiments.

Figure 4B shows that M1 protein synthesis in CR43-3 virus-infected MDCK cells was reduced throughout the infection cycle relative to that in the parental virus-infected MDCK cells. Time points were taken at 3, 6, 9, and 12 h p.i. (Fig. 4B, lanes 2 through 13). This time course experiment further suggests that the NS1 defect in CR43-3 virus causes a reduction in M1 protein synthesis. It should be noted that the A/Alaska/6/77 HA (H3 subtype) produced in MDCK or in PCK cells does not form a sharp band under the gel conditions used and thus cannot be identified on the autoradiogram. In contrast, the A/Ann Arbor HA, belonging to the H2 subtype, forms visible bands on gels obtained with extracts from infected PCK and MDCK cells (Fig. 4A and B).

Indirect immunofluorescence analysis of virus-infected cells. MDCK cells infected with CR43-3 and its parent viruses were acetone fixed 3 h p.i. and stained by using monospecific rabbit antiserum against NS1 and fluorescein-conjugated goat anti-rabbit antibody. Both parents, as well as CR43-3 virus, induced the synthesis of NS1, which was found localized in the nucleoplasm of MDCK cells (Fig. 5). This indicates that the mutant protein accumulates in the same cellular compartment in which the wild-type protein is found during the course of infection. At later time points (6, 9, and 12 h p.i.), CR43-3 and A/AA/6/60 virus-infected MDCK cells continued to display intense nucleoplasmic staining. Uninfected cells treated with the anti-NS1 rabbit antiserum and fluorescein-conjugated goat anti-rabbit antibody did not exhibit any positive immunofluorescence (data not shown). It should be noted, however, that the A/Alaska/6/77 virus-infected MDCK cells exhibited NS1-specific nucleolar staining at later time points (6, 9, and 12 h p.i.). Previously, it had been observed that some influenza virus strains, but not others, include nucleolar staining specific for NS1 late in infection. This observation, however, could not be correlated with any biological activity of these strains (42a). It thus

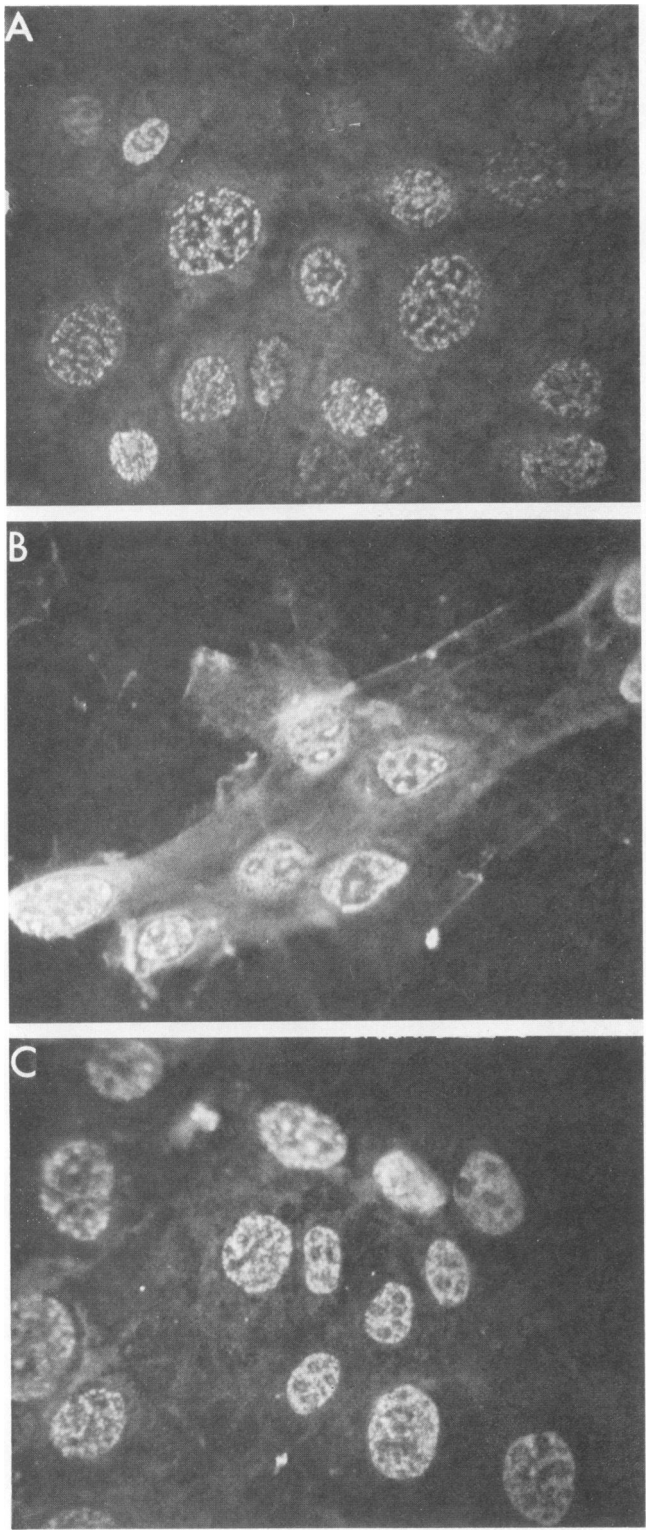

FIG. 5. Indirect immunofluorescent staining of NS1 protein in influenza virus-infected MDCK cells. MDCK cell monlayers were adsorbed with virus for 1 h at 34°C. At 3 h p.i., the cells were acetone fixed and incubated with a monospecific rabbit anti-NS1 antibody followed by an anti-rabbit fluorescein-conjugated antibody. Shown are MDCK cells infected with (A) CR43-3 virus, (B) A/Alaska/6/77 virus, and (C) A/AA/6/60 virus. The nucleoplasm of cells infected with all three viruses shows intense staining at this early time point.

appears that the immunofluorescence studies do not point to an unusual behavior of the mutant NS1 polypeptide in the MDCK cells nonpermissive for CR43-3 virus. Control studies with PCK cells, which are permissive for all three viruses, showed the same staining pattern for NS1 as observed in MDCK cells (data not shown).

Evolutionary analysis of NS genes. We have obtained the complete nucleotide sequence of the NS gene of the A/Alaska/6/77 field strain. When we compare the NS gene sequence of this virus with those of the A/PR/8/34 (2) and the A/Udorn/72 (19) viruses, we find that most of the base substitutions (relative to the A/PR/8/34 sequence) are shared by the NS genes of A/Alaska/6/77 and A/Udorn/72 viruses. Only nine nucleotide changes are not found in the A/Udorn/72 NS sequence and are unique to the A/Alaska/6/77 virus NS gene. Comparison of the A/Alaska/6/77 virus NS sequence with all known NS sequences of human influenza A virus isolates (2, 15, 19, 41) then allowed the construction of an evolutionary tree depicting the nucleotide sequence relationship among these NS genes (Fig. 6). The present data extend previous findings and further support the notion that the NS genes of influenza virus follow, in general, a common lineage through the accumulation of sequential point mutations (15).

DISCUSSION

In this study, we have analyzed the defect in the NS segment of the influenza virus host range mutant CR43-3. Sequence data obtained from cloned cDNAs revealed that

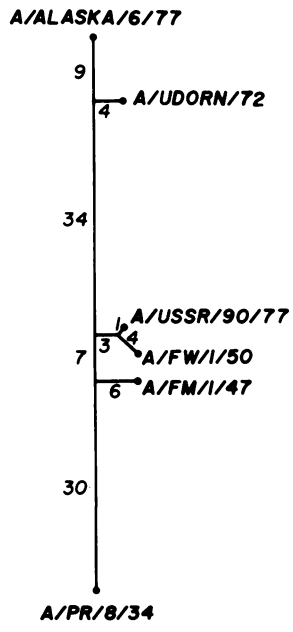

FIG. 6. Evolutionary tree depicting the nucleotide relationship among NS genes of human influenza A viruses. The NS gene of the oldest isolate examined, A/PR/8/34, is used as a baseline. The numbers indicate single base changes relative to the A/PR/8/34 NS sequence. Nucleotide positions 183, 290, 538, 757, and 762 were omitted from the analysis because a second point mutation might have occurred at these positions. Inclusion of these hypothetical nucleotide changes would not permit construction of an evolutionary tree with additive genetic distances.

the CR43-3 NS gene derived from the NS gene of the A/Alaska/6/77 parent by a deletion of 36 nucleotides. Dideoxy sequencing of RNA 8 confirmed the sequence data obtained from the cloned cDNA, indicating that the deletion of the NS clone was not an artifact. The CR43-3 virus NS deletion is in the NS1-coding region. Consequently, the CR43-3 NS1 protein is 12 amino acids shorter than that of the A/Alaska/6/77 parent. The stretch of 12 amino acids not found in the CR43-3 NS1 protein is conserved among NS1 proteins of six human influenza A virus isolates (2, 15, 19, 41). Only in position 67 of the NS1 of A/Udorn/72 and A/Alaska/6/77 viruses was an amino acid change noted which resulted in a conservative arginine to lysine substitution. However, in the NS genes of A/FPV/Rostock/34 (2) and A/duck/Alberta/60/76 (3) viruses, two and four amino acids changes, respectively, are observed, indicating that functional NS proteins may not require a high degree of amino acid conservation in this region.

To determine a possible mechanism for the genesis of the CR43-3 virus NS gene, the sequences flanking the deletion region of the progenitor A/Alaska/6/77 NS gene were compared with the consensus splice junction sequences for mRNA processing (25). No sequence homologies were observed. The A/Alaska/6/77 deletion junctions were also compared with the sequences reported for the sites of RNA polymerase detachment and reinitiation in influenza virus defective interfering RNAs (26). The A/Alaska/6/77 NS sequence at the deletion junctions does not appear to show any nucleotide homologies with reported defective interfering RNA sequences. However, it should be noted that the junction regions of the influenza virus defective interfering RNAs sequenced to date also fail to show a consensus sequence (26).

Fields and Winter have reported the sequence of a small cloned DNA derived from regions of RNA 1 and 3 of A/NT/60/68 virus (10). The model proposed for the generation of this mosaic segment postulates that the viral polymerase can switch templates during the synthesis of either positive or negative RNA strands. The signal for the detachment of the polymerase on the RNA template may be a cluster of uridine residues (three to seven bases). The polymerase then resumes transcription with a different template molecule. At all points of polymerase reinitiation, the dinucleotide 3′-AC-5′ is found at or within one to three nucleotides of the reinitiation points. In the case of the CR43-3 NS gene, uridine-rich sequences were not found at the polymerase detachment site with either the positive or negative strand as a potential template. At the reinitiation point, with the negative strand as possible template, a 3′-AC-5′ dinucleotide was found four bases away from the reinitiation junction, but the presence of a dinucleotide in this position may not, in itself, be significant. In summary, the mechanism responsible for the generation of the CR43-3 virus NS gene does not appear to be related to the mechanisms proposed for either mRNA splicing or for the formation of the mosaic RNA. (In this context, it should be noted that the existence of the mosaic segment has not been demonstrated with direct RNA sequencing. It is thus possible that these mosaic sequences are reverse transcriptase artifacts and do not occur under natural conditions). Although we have no evidence to suggest that the CR43-3 gene has defective interfering properties, it is possible that the CR43-3 NS gene originated by the same (unknown) mechanism that leads to the generation of defective interfering RNAs.

The mutant NS1 protein of CR43-3 virus appeared to be synthesized in approximately equal amounts in the nonpermissive (MDCK) and in the permissive (PCK) cell system. In the restrictive MDCK cells, the NS1 protein of CR43-3 virus accumulates in the nuclei as shown by specific NS1 immunofluorescence of acetone-fixed MDCK cells. The NS1 nucleoplasmic staining pattern displayed by CR43-3 virus-infected MDCK cells was similar to that observed for A/AA/6/60 virus-infected MDCK cells. An identical NS1 staining pattern has previously been noted for several wt influenza virus strains (44). It should also be noted that a similar pattern of nucleoplasmic staining was exhibited in permissive PCK cells infected with CR43-3 virus.

Although the NS mutant CR43-3 did not show aberrant NS1 synthesis in the nonpermissive MDCK cells, a reduction in M1 protein of approximately 50% was observed in MDCK cells as compared with that in PCK cells infected with CR43-3 virus. Reduced synthesis of M1 protein has been reported for three NS mutants of fowl plague virus: *ts*47 (1, 30, 42), *ts*mNS (42), and *ts*412 (13). Since M1 protein is synthesized late in infection, NS1 may be required for efficient expression of late viral functions (for review, see reference 23). Although *ts*47, *ts*mN3, and *ts*412 are all defective in the NS segment, they do not exhibit identical phenotypes at the nonpermissive temperature. In addition to a common defect in M1 synthesis, *ts*47 and *ts*mN3 show defects in viral RNA synthesis, whereas *ts*412 displays reduced hemagglutinin production at the nonpermissive temperature. The variable phenotypic characteristics exhibited by these NS mutants could be attributed to the fact that it has not yet been determined whether the temperature-sensitive lesion in RNA 8 of *ts*47, *ts*mN3, and *ts*412 affects NS1, NS2, or both polypeptides (1, 13, 30, 42). Thus it will be interesting to study the RNA phenotype of CR43-3 virus, which appears to have a defect in the NS1 only. At this time, however, no data are available regarding the synthesis of virus-specific viral RNA and cRNA in CR43-3-infected permissive or nonpermissive cells.

In the process of characterizing the NS defect of the CR43-3 virus, the complete nucleotide sequence of the A/Alaska/6/77 virus NS gene was also determined. Comparison of previously determined NS sequences of human influenza A viruses (2, 15, 19, 41) enabled us to calculate a rate of variation in the NS segment of approximately two nucleotide changes per year (15). The A/Alaska/6/77 virus was isolated 5 years later than the A/Udorn/72 virus. The nucleotide sequence of the A/Alaska/6/77 virus NS gene reveals nine unique base changes that are not found in the NS gene of A/Udorn/72 virus. This value is thus consistent with the previous estimate of the annual variation rate for the NS gene. The NS sequence data presented here also support our earlier finding that the major process by which NS genes of influenza A viruses evolve is through the accumulation of sequential point mutations (15).

The study of temperature-sensitive mutants of influenza virus has greatly aided in the assignment of functional roles to specific viral proteins. Through nucleotide sequence analysis we have now determined that the defect of the CR43-3 virus mutant is a deletion mutation in the NS gene. It appears likely that this deletion in the NS1 polypeptide is associated with the restricted host range of the virus. With the possible exception of variants with deletions or insertions in the hemagglutinin and neuraminidase genes (5, 6, 16), there is no precedent for a conditional lethal mutant of influenza virus with a defined deletion. Further analysis of the CR43-3 mutant may be valuable for determining the function of the NS gene products in influenza virus replication.

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