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Identification of Sequence Changes in the Cold-Adapted, Live Attenuated Influenza Vaccine Strain, A/Ann Arbor/6/60 (H2N2)

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Nucleotide sequences have been obtained for RNA segments encoding the PB2, PB1, PA, NP, M1, M2, NS1, and NS2 proteins of the influenza A/Ann Arbor/6/60 (H2N2) wild-type (wt) virus and its cold-adapted (ca) derivative that has been used for preparing investigational live attenuated vaccines. Twenty-four nucleotide differences between the ca and wt viruses were detected, of which 11 were deduced to code for amino acid substitutions in the ca virus proteins. One amino acid substitution each was predicted for the PB2, M2, and NS1 proteins. Two amino acid substitutions were predicted for the NP and the PA proteins. Four substitutions were predicted for the PB1 protein. The biological significance of mutations in the PB2, PB1, PA, and M2 genes of the ca virus is suggested by currently available genetic data, a comparison with other available influenza gene sequences, and the nature of the predicted amino acid changes. In addition, the sequence data confirm the close evolutionary relationship between the genomes of influenza A (H2N2) and influenza A (H3N2) viruses.

INTRODUCTION

One approach for producing live attenuated influenza A vaccines is attenuation of new epidemic variants by reassortment with a cold-adapted (ca) temperature-sensitive (ts) mutant donor strain, A/Ann Arbor/6/60 (H2N2) (Maassab *et al.*, 1972; Kendal *et al.*, 1981; Cox, 1986). This prototype ca virus was derived by serial passage of the wild-type (wt) A/Ann Arbor/6/60 virus at successively lower temperatures until virus emerged that could replicate efficiently at 25° (Maassab, 1967; Kendal *et al.*, 1981). Although the ca A/Ann Arbor/6/60 donor virus has not been thoroughly tested in human volunteers, it is attenuated in a ferret model system (Maassab *et al.*, 1982). Reassortant viruses that received the hemagglutinin (HA) and neuraminidase (NA) genes from a new H1N1 or H3N2 epidemic variant virus and five or six of their other genes from the ca A/Ann Arbor/6/60 parent have been examined extensively. These ca reassortants had similar ca and ts properties in cell culture, and were attenuated, immunogenic, and phenotypically stable when administered to ferrets (Maassab *et al.*, 1982). They also were attenuated, immunogenic, and protective in challenge and field studies in humans (Davenport *et al.*, 1977; Hrabar *et al.*, 1977; Murphy *et al.*, 1979, 1980, 1981, 1982; Lazar *et al.*, 1980; Reeve *et al.*, 1980b; Mortiz *et al.*, 1980; Van Voorthuizen *et al.*, 1981; Cate and Couch, 1982; Wright *et al.*, 1982; Clements *et al.*, 1983; Belshe and Van Voris, 1984; Couch *et al.*, 1986).

It has not yet been determined if live ca viruses provide the theoretical benefits proposed for attenuated influenza vaccine (Stuart-Harris, 1980) or can be applied for influenza control on a large scale. Promising vaccine trials, however, have justified parallel laboratory studies to examine the molecular basis for attenuation and the genetic stability of the ca A/Ann Arbor/6/60 mutant. It was proposed previously that the restricted gene composition of ca reassortant viruses was advantageous for producing live attenuated influenza vaccines because viruses with five or six internal genes of the ca donor strain would be expected to have more uniform biological and attenuation properties (Cox *et al.*, 1979). Genetic stability of several vaccine strains having five or six genes from the ca parent was examined before and after replication in humans using T1 oligonucleotide mapping, and relative genetic stability was demonstrated (Cox and Kendal, 1984). To directly identify genes of the ca donor strain A/Ann Arbor/6/60 that contained mutations, we used a variety of biochemical techniques to compare RNAs and proteins of the ca and wt viruses. These procedures detected differences in each of the eight RNA segments of the ca mutant compared with the wt parent (Cox *et al.*, 1986). Here we present the entire nucleotide sequences for the six genes of the wt and ca A/Ann Arbor/6/60 viruses that are relevant for producing reas-

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sortant candidate vaccine viruses. Knowledge of these nucleotide sequences will be useful for monitoring genetic stability of attenuated vaccines during production and use as well as for understanding the molecular mechanism(s) of viral attenuation.

MATERIALS AND METHODS

Viruses and viral RNA

The A/Ann Arbor/6/60 wt virus and the derivation of the ca A/Ann Arbor/6/60 mutant have been described (Maassab, 1967; Kendal *et al.*, 1981). No further plaque purifications or end-point dilutions were performed. Viruses were propagated at a low multiplicity of infection in the allantoic cavity of 10-day-old embryonated eggs at 34° and purified as described previously (Cox and Kendal, 1984). Ca mutant and wt virus preparations used throughout this study for sequencing varied by no more than three egg passages for the mutant (E_4 to E_6) and by five egg passages for the wt (E_{28} to E_{32}). Sequencing of RNA obtained from these different passage levels of the same virus revealed no sequence differences. Virion RNA was extracted as described previously (Palese and Schulman, 1976) and dissolved to yield approximately 1 mg/ml.

Nucleotide sequence analysis of viral RNA

Sequence analysis was accomplished using either of two variations of the dideoxy chain termination method (Sanger *et al.*, 1977). The incorporative method was performed essentially as described previously (Air, 1979; Cox *et al.*, 1986). Each 5.5-μl reaction mixture contained 45 m*M* of Tris–HCl (pH 8.3), 45 m*M* of KCl, 4.5 m*M* of $MgCl_2$, 9 m*M* of DTT, 1.4 μM of dATP, 12.5 μCi of [α-^{32}P]-dATP (NEN Research Products, Boston, MA),[2] 45.5 μM of dGTP (9.1 μM of dGTP for the G reaction), 4.5 μM of dCTP (9.1 μM of dCTP for the C reaction), 45.5 μM of dTTP (9.1 μM of dTTP for the T reaction), 50–100 ng of RNA, 1–5 ng of primer, 3 units of reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL), and 1–5 μM of ddATP, ddCTP, ddGTP, or ddTTP. Reaction mixtures were incubated at 37° for 30 min, after which 1 μl of a chase mixture containing 1 m*M* each of dATP, dCTP, dGTP, and dTTP was added, and the incubation was continued at 37° for 30 min. Terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, MD) was added to the chase mixture when necessary to resolve sequence abiguities (DeBorde *et al.*, 1986). The reaction was stopped by adding 1 μl of 0.1 *M* EDTA, and samples were dried and finally dissolved in 5 μl of a formamide dye mixture.

The second method used 5′-γ-^{32}P-labeled synthetic oligodeoxynucleotides for priming the reaction. Each 5-μl reaction mixture contained 50 m*M* of Tris–HCl (pH 8.3), 50 m*M* of KCl, 5 m*M* of $MgCl_2$, 10 m*M* of DTT, 50 m*M* each of dATP, dCTP, dGTP, and dTTP, 3.75 units of human placenta RNase inhibitor (Promega Biotec, Madison, WI), 200 ng of RNA, 1–5 ng primer, 3 units of reverse transcriptase, and approximately 10 μM of ddATP, ddCTP, ddGTP, or ddTTP. After incubation at 42° for 30 min, reaction products were analyzed on 6 and 8% polyacrylamide gels containing 100 m*M* of Tris–borate (pH 8.3), 5 m*M* of EDTA, and 8.3 *M* of urea.

Oligonucleotide primers were synthesized either by C. W. Naeve (St. Jude Children's Hospital) or by B. Holloway (Centers for Disease Control) on an Applied Biosystems Model 380A DNA synthesizer using the solid-phase phosphoramidite method. Primers used were as follows: the universal primer d(AGCAAAAGCAGG), PB2-28 d(ATGGAAAGAATAAAA), PB2-102 d(GGACCATATGGC), PB2-225 d(GACAAGAGGATA), PB2-451 d(CGCCGAAGAGTT), PB2-698 d(CAAGCAGTGTAT), PB2-905 d(GGATGGTGGACA) PB2-946 d(GCTGTGGAAATATGC), PB2-1125 d(TGGGAAAAGGGC), PB2-1349 d(ATGCTAAAGTGC), PB2-1570 d(CCTGAGGAGGTC), PB2-1780 d(CCTAAGGCCATT), PB2-2085 d(GGAGTCAGCTGT), PB2-2190 d(GGCTAATGTACT), PB1-13 d(CAAACCATTTGAATGG), PB1-208 d(GGAGCGCCCCAACT), PB1-400 d(CGTCAGACCTATGA), PB1-601 d(GACAACATGACCAAGA), PB1-745 d(AATGCAACACCCGGGA), PB1-909 d(CACAGAGCTTTC), PB1-1000 d(ACAAGAAATCAACCT), PB1-1105 d(AGCATGAAGCTCCGAA), PB1-1375 d(GTGAATGCACCA), PB1-1600 d(GGAGTAACAGTG) PB1-1801 d(GATGGAGGACCAAACT), PB1-2046 d(CCTCAACACAAG), PB1-2209 d(GACTTCGAGTCTGGA), PA-158 d(GCTTCATGTATT), PA-226 d(CCAAATGCACTT), PA-452 d(CACACATCCACA), PA-681 d(CCCGCCGAACTT), PA-876 d(GATGGATGCTTT), PA-1112 d(CGAGTCAGCTAA), PA-1339 d(GCAAGCATGAGA), PA-1540 d(AAAGGAAGATCTCA), PA-1601 d(CTCTCACTGACC), PA-1789 d(CTCCAACAAATC), PA-1800 d(CGAGAGTATGAT), PA-1900 d(AAAGGAGTGGAAGAA), PA-2009 d(CAAGAAAACTGC), PA-2026 d(GTCGTTCAGGCT), PA-2080 d(CTATATGAAGCAATT), NP-160 d(TTCTACATCCAA), NP-202 d(GAGGGGCGACUG), NP-397 d(CGAATCTGGCGC), NP-643 d(GGGATCAATGAT), NP-888 d(TGGACCTGCCGT), NP-1015 d(CACAAGAGTCAG), NP-1285 d(GTGCAAAGAAACCTTC), M-239 d(CGAGGACTGCAG), M-438 d(GCTGACTCCCAG), M-719 d(GATCTTCTTGAAAATTTG), M-895 d(GAGGGCCTTCTA), NS-207 d(G-

[2] Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Public Health Service or the Department of Health and Human Services.

GAAAGCAGATAGTG), NS-384 d(GACCAGGCAATC), NS-597 d(ACAGTTCGAGTC) and NS-742 d(GAAGTGAGACAC). Primer numbers correspond to the position (in the mRNA sense sequence) of the first nucleotide in the primer.

Nucleotide sequence analysis of mRNA

The nucleotide sequence of the first 25–40 nucleotides of the 3′ region of each gene was obtained using synthetic primers complementary to viral mRNA. Confluent MDCK cells were infected at a multiplicity of approximately 2 PFU/cell and harvested by scraping at 6 or 8 hr postinfection for the wt and mutant viruses, respectively. Cells were washed once in ice-cold phosphate-buffered saline and lysed in a buffer containing 5.8 *M* of guanidinium–hydrochloride, 50 m*M* of Tris–HCl (pH 7.6), 10 m*M* of EDTA, 2% sodium lauryl sarkosinate, and 1% β-mercaptoethanol prepared as described previously (Maniatis *et al.*, 1982). RNA isolated by centrifugation through a CsCl cushion (Maniatis *et al.*, 1982) was suspended in 20 m*M* Tris–HCl (pH 7.4), 1 m*M* EDTA and 0.1% sodium dodecyl sulfate (SDS), ethanol precipitated, and stored at −70° in sterile distilled water. Approximately 50 μg of total RNA was used in each incorporative sequencing reaction carried out as described above. Primers used were as follows: mNS-771 d(ATGGGGCATCACCTAGTT), mM-911 d(ACATCTTCAAGTCTCTGT), mNP-1433 d(CGACGGATGCTCTGATTT), mPA-2151 d(GCTTTTTCAGCAAGCTCGACAATC), mPB1-2233 d(GAGGATCTCCAGTATAAG), and mPB2-2253 d(GTTAGTATCTCGCGAGTG). Primer numbers correspond to the position (in the virion sense RNA sequence) of the first nucleotide in the primer.

Computer analysis

Nucleotide sequences were stored and manipulated on a Digital Equipment Corp. VAX computer using version 5 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Devereaux *et al.*, 1984).

RESULTS

Nucleotide sequences of the NS1, NS2, M1, M2, NP, PA, PB1, and PB2 genes of the A/Ann Arbor/6/60 mutant and wt viruses were determined directly using the dideoxy chain termination method (Sanger *et al.*, 1977) modified for sequencing from virion RNA (Air, 1979). Bands in two lanes of the reaction were observed occasionally at a single nucleotide position. (These postions are indicated by asterisks in the figures). The darkest band was read as the correct nucleotide. In addition, the NS and M genes of the wt virus were cloned into pBR322 and sequenced using the chemical sequencing method of Maxam and Gilbert (1980). Results using the chemical method were identical to those using the dideoxy chain termination method (data not shown). The sequences of the HA and NA genes of the A/Ann Arbor/6/60 viruses were not determined because they are irrelevant for producing reassortant candidate vaccine strains. Sequences of H2N2 virus genes have been published previously for only the NS, NA, and HA genes. Therefore, each gene sequence of the wt A/Ann Arbor/6/60 virus was also compared with influenza A (H3N2) gene sequences available in the Gen Bank data base at the time of analysis.

NS1 and NS2 sequences

The smallest RNA segment of influenza virus is 890 nucleotides long and codes for the NS1 and NS2 polypeptides that are found only in infected cells (Inglis *et al.*, 1979; Lamb and Choppin, 1979; Lamb *et al.*, 1980). The functions of NS1 and NS2 are unknown. The complete nucleotide sequences for the NS genes of the A/Ann Arbor/6/60 mutant and wt viruses are shown in Fig. 1. Two nucleotide differences were detected between the ca mutant and wt viruses with a coding change at nucleotide 483 in the portion of the gene that encodes NS1 and a noncoding change at nucleotide 813 in the portion of the gene that encodes NS2. The former nucleotide change encodes an alanine to threonine substitution in the mutant NS1 protein at amino acid 153. Comparison of the sequence we obtained for the A/Ann Arbor/6/60 wt NS gene with the sequence for this gene published previously (Buonagurio *et al.*, 1986) revealed complete agreement, with only one additional change compared to the sequence of the A/WSN/33 virus in a region not sequenced previously (T at nucleotide 35 in both ca and wt A/Ann Arbor/6/60 viruses). Volunteer studies with reassortant candidate vaccine strains having an RNA 8 from the H1N1 or H3N2 wild-type parent indicated that this gene is not required for attenuation (La Montagne *et al.*, 1983). Studies with single gene reassortants are also consistent with the conclusion that RNA 8 does not contribute significantly to attenuation (Snyder *et al.*, 1987).

M1 and M2 sequence

RNA segment 7 is 1027 nucleotides long and codes for at least two proteins, M1 and M2 (Allen *et al.*, 1980; Winter and Fields, 1980; Lamb *et al.*, 1981). The M1 protein is a viral structural protein of 252 amino acids. The M2 protein is 97 amino acids in length and, al-

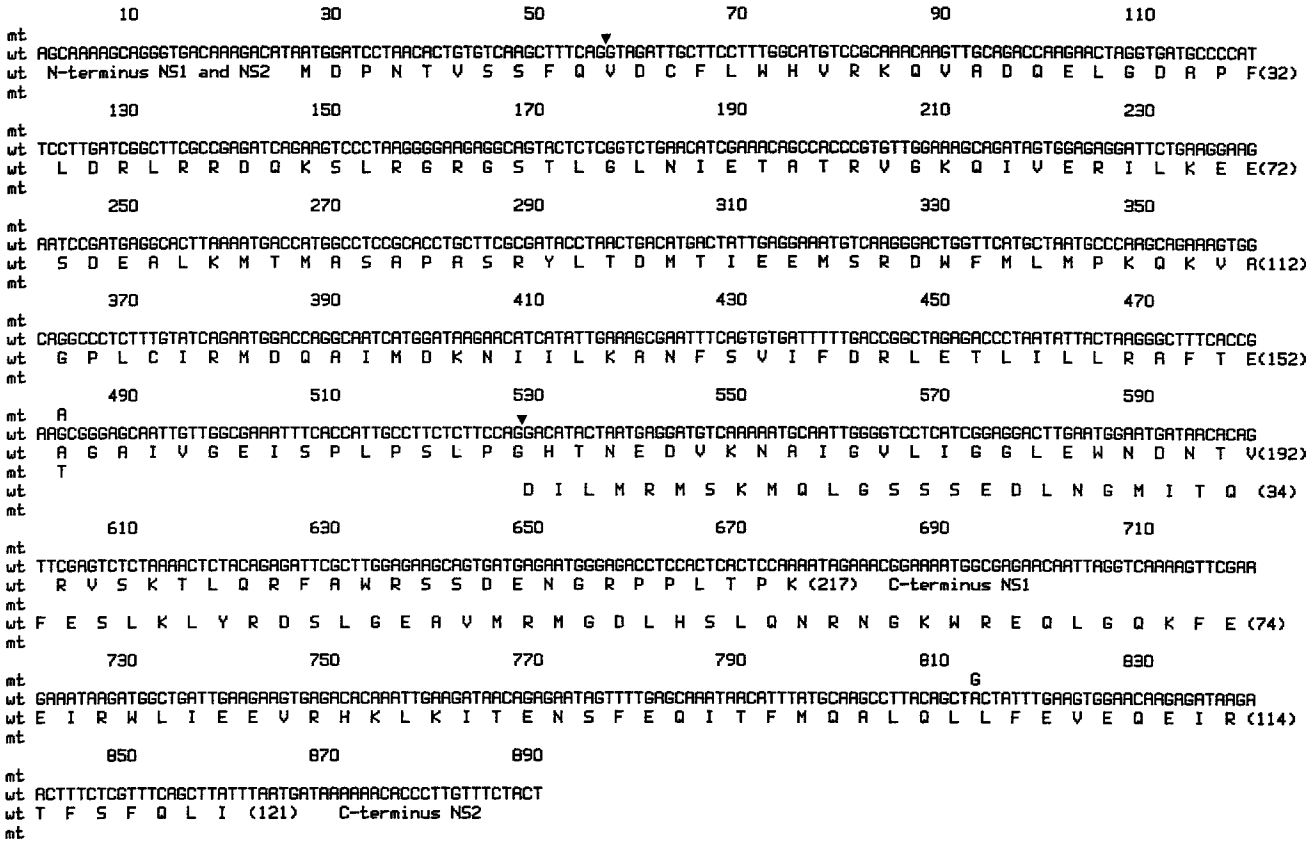

FIG. 1. Nucleotide and protein sequences of RNA segment 8 of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence. Splice junctions for NS2 are indicated by arrowheads.

though its function is unknown, this protein is known to be inserted into plasma membranes of infected cells (Zebedee *et al.,* 1985). Complete nucleotide sequence data for RNA 7 of A/Ann Arbor/6/60 ca mutant and wt viruses (Fig. 2) demonstrate that there was a single difference (G to T) at nucleotide 969. This nucleotide substitution encodes a change from alanine in the wt to serine in the mutant at amino acid 86 of the M2 protein. Comparison of the Ann Arbor/6/60 M2 sequences with those of other strains indicated that the M2 genes of the three other wt human strains sequenced to date have alanine at amino acid 86 of M2. This amino acid has been identified as one that varies between human and avian influenza strains (Buchler-White *et al.,* 1986). Amino acid 86 is a part of the trypsin-sensitive carboxy-terminal tail (Zebedee *et al.,* 1985). One ca candidate vaccine strain, CR 31 Clone 3, that received the segment 7 from its wt parent in addition to the HA and NA genes (Cox *et al.,* 1979) was attenuated in seronegative adult volunteers (La Montagne *et al.,* 1983), but was less restricted in its replication in humans than a similar reassortant with segment 7 from the ca parent (Murphy *et al.,* 1981). In addition, a reassortant with only the genes encoding the PA, M1, and M2 proteins from the ca A/Ann Arbor/6/60 virus was as attenuated in human volunteers as another reassortant virus having all but the HA and NA genes from the ca donor virus (Snyder *et al.,* 1985). Further studies with single segment reassortant viruses also suggest that either the M1 or M2 proteins may play a role in attenuation (Snyder *et al.,* 1987). Sequence data presented here suggest that the M2 protein may be responsible for the observed biological findings.

NP sequence

The nucleoprotein gene [RNA segment 6 of the A/Ann Arbor/6/60 viruses under electrophoretic conditions used to separate RNA (Cox *et al.,* 1985)] is 1565 nucleotides in length and encodes a basic structural protein of 498 amino acid residues that specifically interacts with RNA molecules to form ribonucleoprotein complexes (Winter and Fields, 1981; Huddelston and Brownlee, 1982) and has sequences that direct its migration into the nuclei of infected cells (Davey *et al.,* 1985). Three nucleotide changes were detected in the

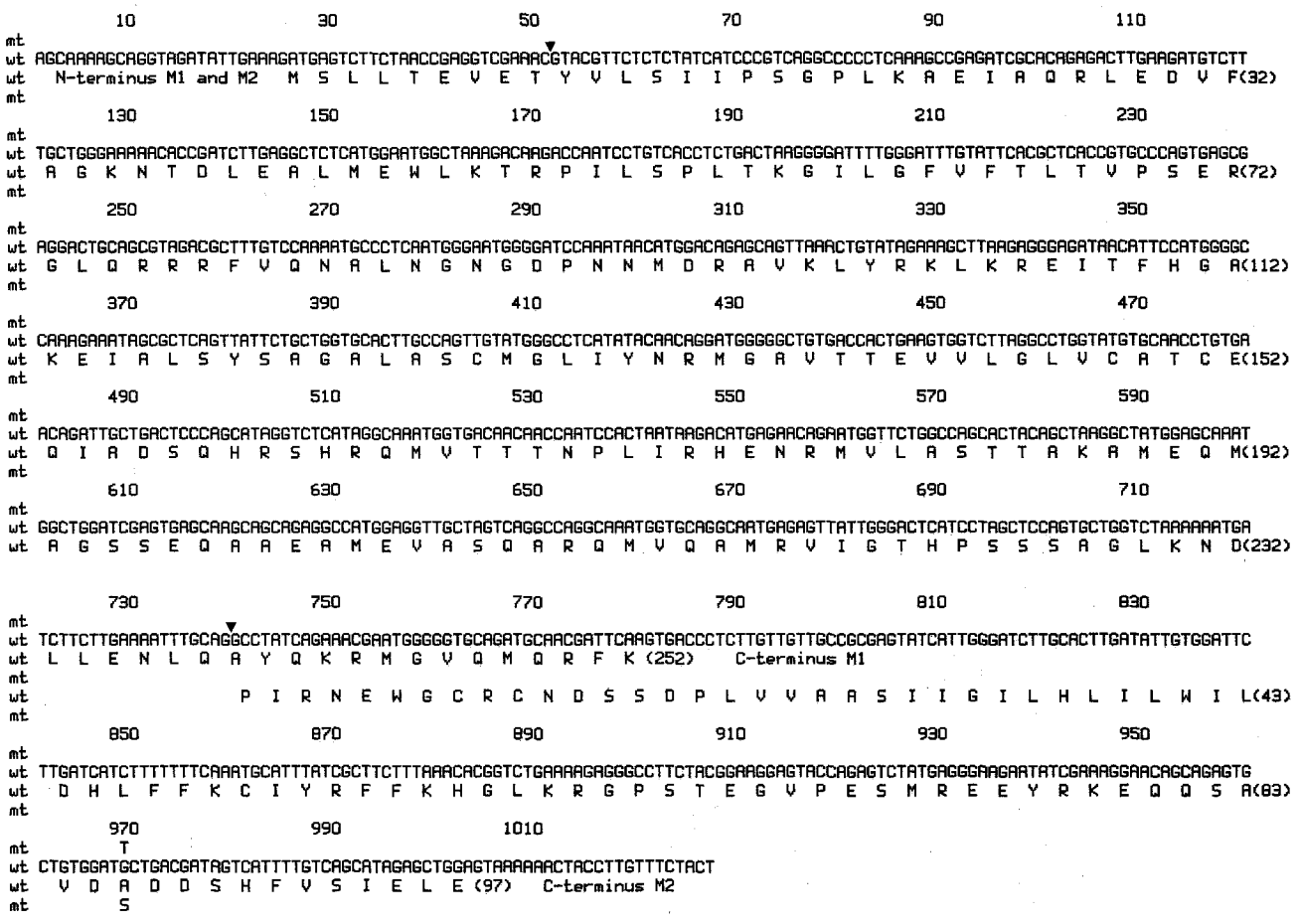

FIG. 2. Nucleotide and protein sequences of RNA segment 7 of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence. Splice junctions for M2 are indicated by arrowheads.

mutant NP gene sequence (Fig. 3) A change at nucleotide 113 encodes an amino acid substitution from threonine in the wt to asparagine in the ca mutant at amino acid 23, and a change at nucleotide 146 encodes an amino acid substitution from aspartic acid in the wt to glycine in the ca mutant at amino acid 34. The change at nucleotide 113 is not present in NP genes of other influenza viruses sequenced, while the change at nucleotide 146 is also present in the highly passaged laboratory strain, A/PR/8/34. The other change in the NP gene of the mutant is the insertion of a single A nucleotide in an A-rich region (1545–1550) that is near the putative polyadenylation signal (Robertson *et al.*, 1981). This insertion may not be important because other influenza virion RNA segments have poly(U) tracts of varying lengths in corresponding positions. Although a ca NP gene was present in all the candidate vaccine strains we examined (Cox *et al.*, 1986), no compelling genetic evidence exists to show that the NP genes of the ca mutant and wt A/Ann Arbor/6/60 viruses are associated with different phenotypic properties (Snyder *et al.*, 1987); therefore, the significance of the mutations in RNA 6 is unknown.

PA sequences

RNA segment 3 is 2233 nucleotides in length and encodes an acidic polymerase protein (PA) 716 amino acids in length (Fields and Winter, 1982; Bishop *et al.*, 1982b; Robertson *et al.*, 1984). The complete nucleotide sequences for the PA genes of A/Ann Arbor/6/60 mutant and wt viruses are presented in Fig. 4. Four nucleotide changes were detected in the mutant gene, with two of these changes located in the same codon. A change at nucleotide 20 (U to C) is in the noncoding region. Comparison of the A/Ann Arbor/6/60 sequences with published sequences revealed that A/PR/8/34 and the Rostock strain of fowl plague virus have C at nucleotide 20 (Fields and Winter, 1982; Robertson *et al.*, 1984), while A/NT/60/68, like the A/Ann

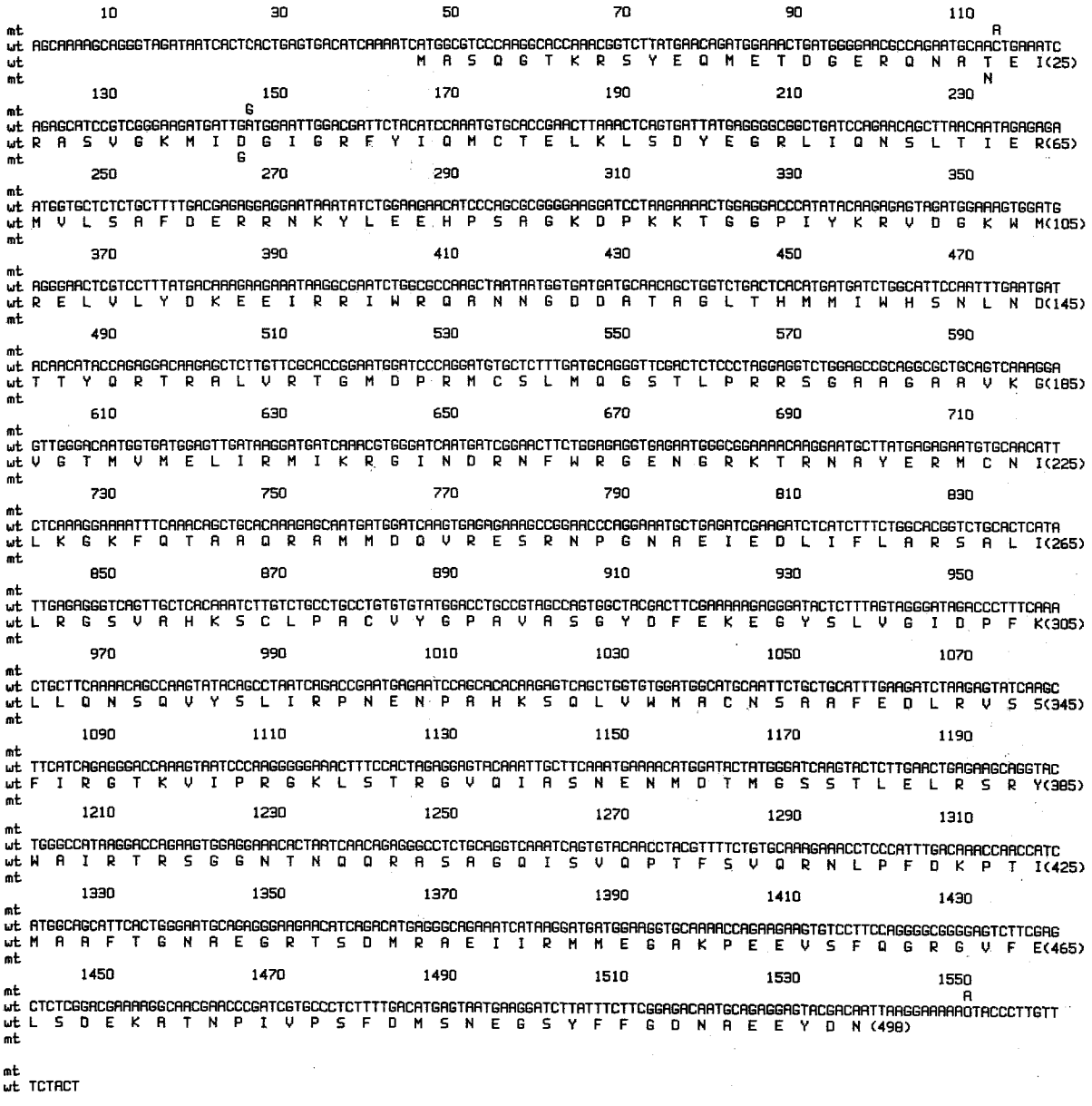

FIG. 3. Nucleotide and protein sequences of RNA segment 6 (NP) of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence. The single nucleotide insertion is indicated by a 0 in the wt sequence at position 1550.

Arbor/6/60 wt, has U (Bishop *et al.*, 1982b). Thus it seems unlikely that this U to C change is important in attenuation. It is possible, however, that this change could alter base pairing of the ends of the RNA segment or polymerase recognition (Skehel and Hay, 1978; Robertson, 1979; Stoeckle *et al.*, 1987). A single base change in the noncoding region of the poliovirus 3 genome has been shown to affect attenuation (Evans *et al.*, 1985). A change from A to G at nucleotide 1861 encodes a lysine to glutamic acid substitution at amino acid 613. While this is a change from a basic to an acidic residue, it is not likely by itself to be involved in attenuation because comparison with other sequences revealed that A/PR/8/34, A/FPV/Rostock/34, and A/NT/60/68, like the A/Ann Arbor/6/60 mutant virus, all have glutamic acid at residue 613. The two other changes are in the same codon (TTA to CCA at nucleotides 2167 and 2168), resulting in a predicted amino acid substitution at residue 715 (the penultimate amino acid in the PA protein) from leucine in the wt to

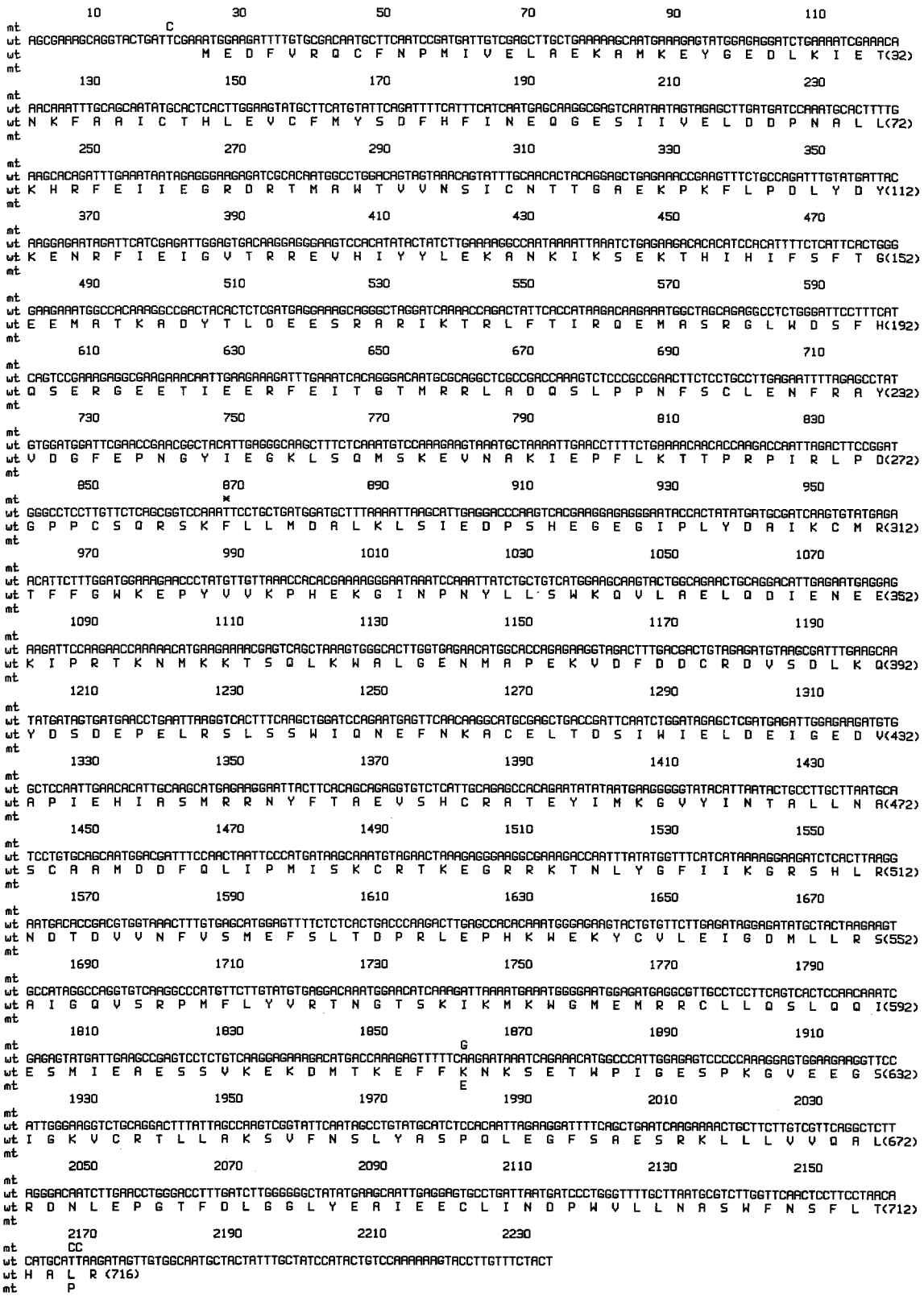

FIG. 4. Nucleotide and protein sequences of RNA segment 3 of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence.

proline in the ca mutant. Although two nucleotide changes were found in the same codon, only a single nucleotide change from CCA to CTA is required for reversion back to leucine by the mutant. The other three influenza viruses sequenced to date have leucine at residue 715 of their PA proteins. The PA gene has been demonstrated to be involved in the cold-adaptation and attenuation properties of the mutant (Odagiri *et al.*, 1986; Snyder *et al.*, 1985, 1987), so this leucine to proline change may be relevant to virus phenotype.

PB1 sequence

RNA segment 2 is 2341 nucleotides in length and encodes a basic polymerase protein (PB1) 757 amino acids in length (Winter and Fields, 1982; Bishop *et al.*, 1982a; Sivasubramanian and Nayak, 1982). Seven nucleotide differences were detected between the A/Ann Arbor/6/60 mutant and wt PB1 gene sequences (Fig. 5). Changes at nucleotides 123 and 486 were noncoding changes. Four coding changes were found at nucleotides 1195, 1395, 1766, and 2005 followed by a noncoding change at nucleotide 2019. The four coding changes result in amino acid substitutions of lysine to glutamic acid at amino acid 391, glutamic acid to aspartic acid at amino acid 457, glutamic acid to glycine at amino acid 581, and alanine to threonine at amino acid 661. None of these four changes exists in the three other wild-type PB1 genes sequenced to date, so any or all of them may be involved in the biological properties of the virus. Because RNA 2 of the mutant was absent in several ca reassortant vaccines that were fully attenuated (La Montagne *et al.*, 1983), its role in attenuation has been questioned. Earlier genetic evidence (Cox *et al.*, 1981) and more recent evidence using single gene reassortants in several animal models, however, suggest that mutations in RNA 2 may contribute to the temperature-sensitive and attenuation phenotypes of the ca A/Ann Arbor/6/60 virus (Snyder *et al.*, 1987).

PB2 sequence

RNA segment 1 is 2341 nucleotides in length and encodes a basic polymerase polypeptide (PB2) of 759 amino acids (Fields and Winter, 1982; Kaptein and Nayak, 1982; Jones *et al.*, 1983; Roditi and Robertson, 1984). The complete nucleotide sequences of the A/Ann Arbor/6/60 mutant and wt PB2 genes are shown in Fig. 6. Seven nucleotide differences were detected between mutant and wild-type sequences, at nucleotides 141, 426, 821, 1182, 1212, 1353, and 1923. Only the A to G change at nucleotide 821 encodes an amino acid change, from asparagine in the wt to serine in the mutant at amino acid 265. Examination of the other PB2 sequences available for other wt influenza A viruses revealed that asparagine is present at amino acid 265 in each case. However, a lower passage (E_3) A/Ann Arbor/6/60 wild-type virus sequenced in another laboratory, like the ca mutant, had serine at amino acid 265 (Herlocher, personal communication). The other six changes, A to G, U to C, A to U, C to U, U to G, and A to G at nucleotides 141, 426, 1182, 1212, 1353, and 1923, respectively, are all silent mutations within the coding region. All A/Ann Arbor/6/60 ca reassortant vaccines tested in clinical trials thus far (except single segment reassortants) derive their PB2 gene from the ca parent (Cox *et al.*, 1986; Maassab, personal communication), and early genetic data indicated that RNA 1 may be involved in the temperature sensitivity of the mutant (Spring *et al.*, 1977). More recent results using single segment reassortant viruses also indicate that RNA 1 contributes to the temperature-sensitive phenotype of the ca mutant (Snyder *et al.*, 1987).

DISCUSSION

Here we describe the complete nucleotide sequences for the genes encoding the NS1, NS2, M1, M2, NP, PA, PB1, and PB2 proteins of the cold-adapted and wild-type A/Ann Arbor/6/60 (H2N2) viruses. Few sequences are published for genes of H2N2 viruses. Such data are of evolutionary and epidemiologic significance because H2N2 viruses are proposed to be the immediate ancestors of the H3N2 subtype (Scholtissek *et al.*, 1978; Nakajima *et al.*, 1982). Comparisons between the wt A/Ann Arbor/6/60 gene sequences and those published previously for the H3N2 viruses A/NT/60/68 or A/Udorn/72 indicate that yearly rates of mutation of 0.36, 0.24, 0.28, 0.40, 0.68, and 0.49% occurred for the NS, M, NP, PA, PB1, and PB2 gene segments, respectively. These observations are consistent with the derivation of these genes in the H3N2 viruses from previously circulating H2N2 viruses.

We have identified 24 nucleotide differences between the A/Ann Arbor/6/60 ca mutant donor strain and its wt parent (Table 1). These 24 nucleotide changes constitute 0.2% of the total number of nucleotides sequenced. Our previous estimate (based on T_1 oligonucleotide mapping studies) that as many as 40–60 nucleotide changes might have occurred in the total genome of the ca mutant (Cox *et al.*, 1986) was inaccurate. Comparison of deduced amino acid sequences for the parental and mutant viruses revealed 11 amino acid differences or 0.26% of the total number of amino acids (Table 1). Sequences for four other pairs of wt and attenuated viruses have been compared, and the

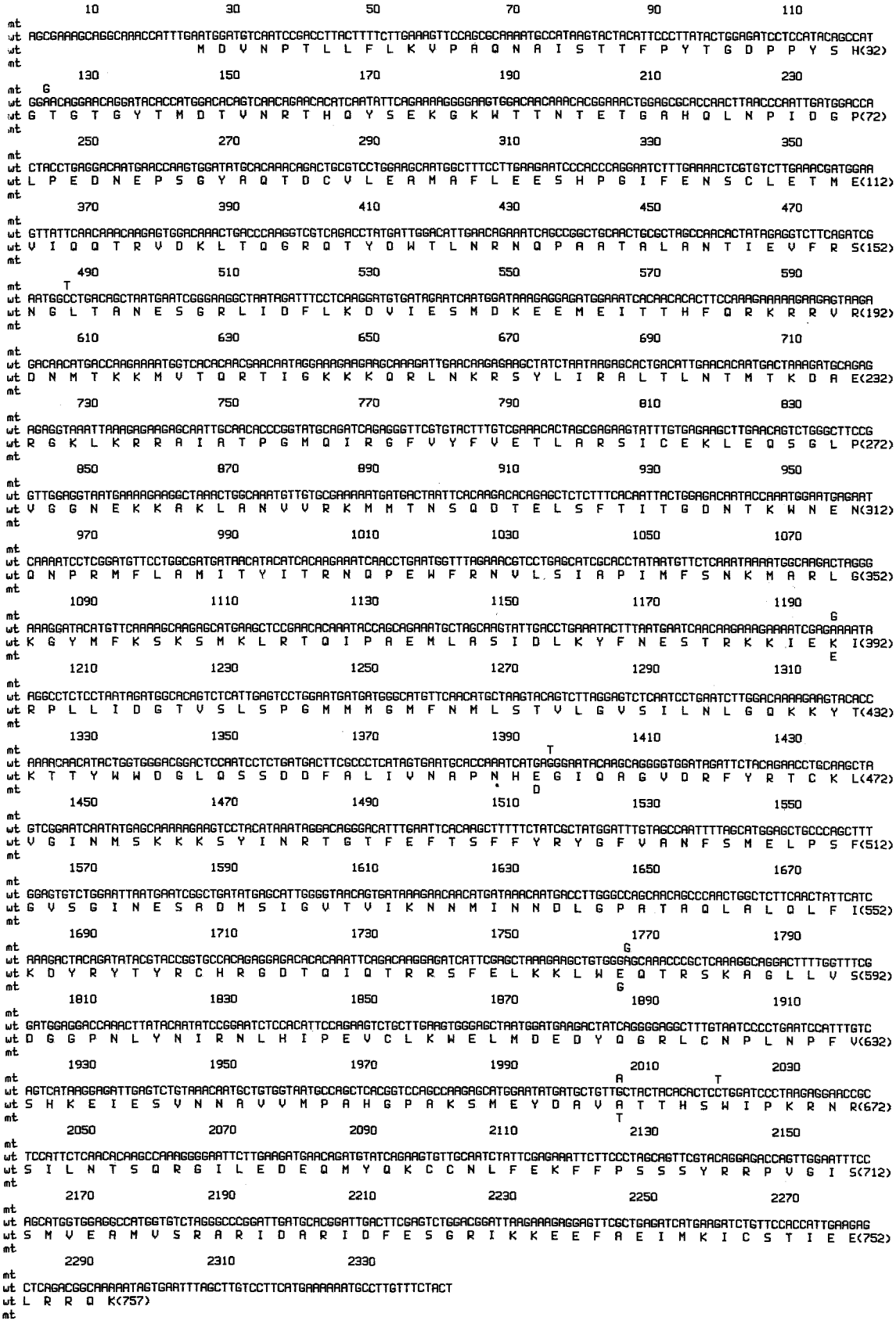

FIG. 5. Nucleotide and protein sequences of RNA segment 2 of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence.

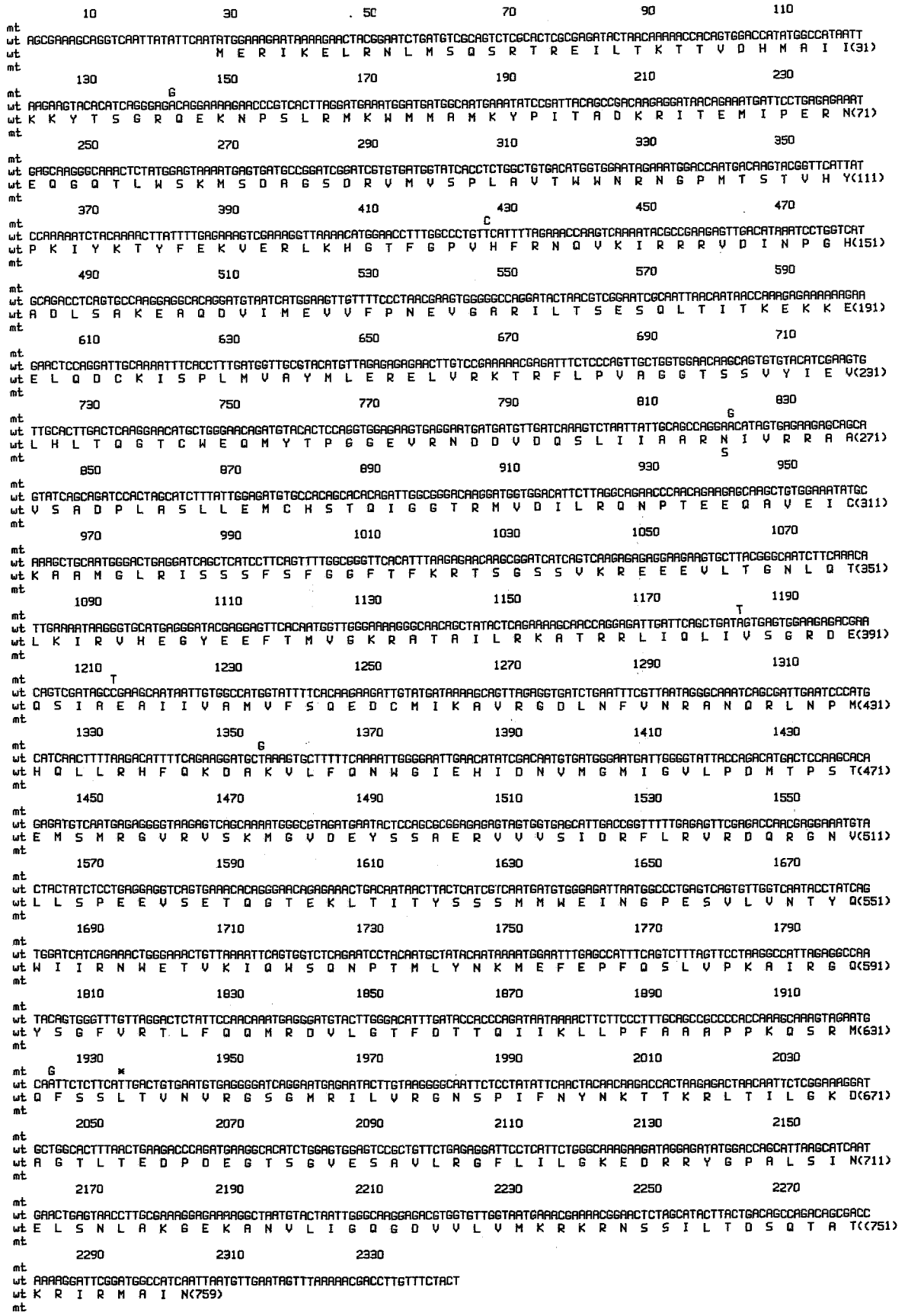

FIG. 6. Nucleotide and protein sequences of RNA segment 1 of the A/Ann/Arbor/6/60 wt and ca mutant viruses. Complete sequences are shown for the wt viruses. Nucleotide changes detected in the mutant RNA are shown above the wt sequence, while predicted amino acid changes in the mutant are shown below the wt sequence.

TABLE 1

SUMMARY OF COMPARATIVE SEQUENCE DATA FOR A/Ann Arbor/6/60 WILD-TYPE AND MUTANT VIRUSES

RNA segment	No. of changes	Nucleotide change		Position changed		Amino acid change	
		wt	MT	Nucleotide	a.a.	wt	MT
1 (PB2)	1 Coding change	AAC	AGC	821	265	Asn	Ser
	4 Noncoding changes	AGA	AGG	141	—	—	—
		GTT	GTC	426	—	—	—
		ATA	ATT	1182	—	—	—
		GCC	GCT	1212	—	—	—
		GCT	GCG	1353	—	—	—
		CAA	CAG	1923	—	—	—
2 (PB1)	4 Coding changes	AAA	GAA	1195	391	Lys	Glu
		GAG	GAT	1395	457	Glu	Asp
		GAG	GGG	1766	581	Glu	Gly
		GCT	ACT	2005	661	Ala	Thr
	3 Noncoding changes	GGA	GGG	123	—	—	—
		GGC	GGT	486	—	—	—
		TCC	TCT	2019	—	—	—
3 (PA)	2 Coding changes	AAG	GAG	1861	613	Lys	Glu
		TTA	CCA	2167-8	715	Leu	Pro
	1 Noncoding change	T	C	20	—	Noncoding	
Region 6 (NP)	2 Coding changes	ACT	AAT	113	23	Thr	Asn
		GAT	GGT	146	34	Asp	Gly
	Single nucleotide insertion	GAAAAAT	GAAAAAAT	1550	—	Noncoding	
Region 7 (M1)	—	—	—	—	—	—	—
(M2)	1 Coding change	GCT	TCT	969	86	Ala	Ser
8 (NS1)	1 Coding change	GCG	ACG	483	153	Ala	Thr
(NS2)	1 Noncoding change	CTA	CTG	813	—	—	—

total number of nucleotide or amino acid changes acquired during attenuation represents less than 1% of the genome or protein in each case (reviewed by Cohen *et al.*, 1987). There are more differences between wt and ca A/Ann/Arbor/6/60 viruses than between wt and vaccine strains of poliovirus 3 but fewer differences than between wt and vaccine strains of poliovirus 1, yellow fever virus, hepatitis A virus HM-175 (Cohen *et al.*, 1987), and influenza B viruses (DeBorde *et al.*, 1988). It seems clear that relatively few changes are present in a variety of attenuated RNA viruses. Because viral RNA genomes are extremely heterogeneous (Domingo *et al.*, 1985), sequencing of a viral RNA template results in a consensus sequence consisting of the base present at the greatest frequency at each nucleotide position. Sequence variations present in minor subpopulations remain undetected. These low frequency sequence variations, however, would not be expected to contribute significantly to the phenotypic properties of the virus.

During the many clinical studies conducted with investigational ca vaccines derived from the ca A/Ann Arbor/6/60 virus, over 900 isolates from human volunteers have been tested for the phenotypic properties of cold-adaptation and temperature-sensitivity, and no true revertants have been detected (Murphy *et al.*, 1980; Wright *et al.*, 1982; Maassab, personal communication). Similarly, reversion has not been demonstrated in ferrets (Maassab *et al.*, 1982). This is in contrast to results with investigational ts influenza live attenuated vaccines (Murphy *et al.*, 1980; Tolpin *et al.*, 1981). It is likely, therefore, that more than one of the mutations identified here are biologically important, so that if reversion or suppression of one mutation occurs, attenuation conferred by other independent mutation(s) remains. This is supported by data obtained with single gene reassortants (Snyder *et al.*, 1987). Thus, all available data support the previous suggestion that candidate vaccine strains be selected on the basis of having six internal genes of the mutant donor parent since multiple mutations throughout the genome might confer increased stability (Cox *et al.*, 1979, 1986).

Now that the precise nature of the mutations in the A/Ann Arbor/6/60 ca donor strain has been deter-

mined, it would be relatively simple to sequence rapidly the relevant areas of each gene in viruses prepared for human use or in viruses recovered from infected volunteers to monitor the stability of these mutations during production and use of ca reassortant candidate vaccines. This approach would not detect suppressor mutations (Scholtissek, 1984), however, and monitoring of phenotypic properties would also be important.

Because it would be expected that a combination of nonspecific mutations and mutations specific for the phenotypic properties of the ca A/Ann Arbor/6/60 virus might occur during the process of cold-adaptation, we have attempted to deduce the likelihood that the mutations detected are involved in the phenotypic properties of the virus. After taking into consideration the available data, we suggest that the changes in the ca A/Ann Arbor/6/60 virus that are of particular interest for future study are as follows: (1) the alanine to serine change at amino acid 86 in the M2 protein, (2) the leucine to proline change at amino acid 715 in the PA protein, (3) the asparagine to serine change at amino acid 265 in PB2, and (4) any or all of the four changes in the PB1 protein. While it is useful to attempt to identify those changes most likely to be involved in the phenotypic properties of the ca virus, conclusions made at this time must be viewed as preliminary for the following reasons: (1) Some contradictions appear in results from genetic complementation or recombination experiments that used different ts mutant sets (Spring *et al.,* 1977; Reeve *et al.,* 1980a; Cox *et al.,* 1981). (2) Conclusions about the genetic basis of attenuation made with single segment reassortants may not apply for different wild-type parents. (3) Mixing polymerase genes of two human influenza viruses may in itself attenuate a reassortant (Florent, 1980). (4) Some of the mutations in the A/Ann Arbor/6/60 ca mutant that are also present in other wt sequences may be biologically significant in the context of other sequence changes. Experiments that use site-specific mutagenesis and DNA expression systems to demonstrate the effect of a particular mutation in a functional assay as well as experiments to obtain and sequence phenotypic revertants will be necessary to further define the biological importance of the mutations reported here. Until definitive studies are complete, it is advisable that, to minimize the risk of reversion to virulence, vaccines used in open populations contain all genes except those coding for HA and NA from the ca parent.

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