Comparative Studies of Wild-Type and 
‘Cold-Mutant’ (Temperature Sensitive) Influenza Viruses: 
Genealogy of the Matrix (M) and Non-structural (NS) Proteins 
in Recombinant Cold-Adapted H3N2 Viruses

By ALAN P. KENDAL,* NANCY J. COX,* BRIAN R. MURPHY,†
SUSAN B. SPRING† and HUNEIN F. MAASSAB‡

* WHO Collaborating Center for Influenza, Respiratory Virology Branch, Bureau of 
Laboratories, Center for Disease Control, Public Health Service, Atlanta, Georgia 30333,
† Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, 
National Institutes of Health, Bethesda, Maryland 20014 and ‡ Department of Epidemiology, 
School of Public Health, University of Michigan, Ann Arbor, Michigan 48104, U.S.A.

(Accepted 6 May 1977)

SUMMARY

The matrix (M) protein of the H2N2 virus A/Ann Arbor/6/60 may be dis-
tinguished from M protein of several H3N2 viruses and A/New Jersey/76 (Hsw1N1) 
by SDS acrylamide gel electrophoresis using a discontinuous buffer system. The 
smallest RNA (RNA 8) of the A/Ann Arbor/6/60 virus may be distinguished from 
RNA 8 of several H3N2 viruses by acrylamide gel electrophoresis in 3 % or 3.6 % 
gels in the absence of urea, if electrophoresis is done at 30 to 35 °C or 20 °C 
respectively. Ten clones of conditionally-lethal temperature-sensitive (ts) mutants 
were studied, which derived their cold-adaption and ts genes from mutant 
A/Ann Arbor/6/60, and their haemagglutinin from the H3N2 virus A/Scotland/
840/74. Each clone was found to derive its M protein from A/Ann Arbor/6/60 
mutant, and its RNA 8 from A/Scotland/840/74. The only assignment of genes 7 
and 8 consistent with these findings for the recombinants is that in each parent 
virus (and in the recombinants) gene 7 codes for M protein, and gene 8 for NS 
protein. Furthermore, it may be concluded from the results that the biologically 
important ts lesions in the A/Ann Arbor/6/60 mutant parent are not present in 
the NS gene. In addition to the recombinants of A/Ann Arbor/6/60 and A/Scotland/
840/74, five independent ts/cold-adapted recombinants of A/Ann Arbor/6/60 mutant 
with H3N2 and Hsw1N1 wild-type viruses were examined, and all were found to 
tain the M protein of the A/Ann Arbor/6/60 mutant parent. This is suggestive 
that M protein may be at least partially responsible for the cold-adaptation and/or 
its properties of the A/Ann Arbor/6/60 mutant and the recombinants.

INTRODUCTION

The concept of transferring growth properties between influenza strains by recombination 
(Kilbourne et al. 1971) has been examined as a means for rapid development of attenuated 
influenza viruses that may be suitable for use as live influenza vaccines. Thus, in recent years 
several potentially attenuated H3N2 influenza virus strains have been produced by re-
combination of wild-type virus with (1) cold-adapted temperature-sensitive (ts) Hsw1N1 or
H2N2 viruses (Maassab, Kendal & Davenport, 1972; Maassab, 1975), (2) viruses containing genes originally present in ts mutants of A/Great Lakes/65(H2N2) virus (Murphy et al. 1972; Richman et al. 1975), and (3) laboratory-adapted viruses which have been demonstrated to be attenuated for man (Beare & Hall, 1971; Beare, Schild & Craig, 1975; Morris et al. 1975).

Since influenza contains eight discrete RNA genes (Pons, 1976), recombination without selective pressures can theoretically result in the production of \((2^8 - 2)\) new genotypes, assuming there is a random assortment of genes during recombination. Although reduction in the number of new genotypes is possible by selecting for markers such as a haemagglutinin (HA) and neuraminidase (NA) antigens, and temperature-sensitivity, many different recombinants are often still possible. For example, if recombinants of TS-1(E) (Murphy et al. 1972) and a new strain are produced which meet the criteria of (1) containing both ts lesions of TS-1(E) (Spring et al. 1975) and (2) containing HA and NA of the new strain, then 16 \((2^3)\) different genotypes are still possible. Therefore, in order to comprehend all the factors responsible for the attenuation of any recombinant influenza virus, we must identify every one of its genes. For practical reasons, procedures for doing this should be simple enough to allow many clones of viruses to be rapidly compared.

The findings of Palese & Schulman (1976a), suggesting that some RNAs of different influenza strains have distinctive electrophoretic mobilities under certain conditions, encouraged us to determine whether this approach could be used to study recombinant influenza viruses prepared as candidate live vaccine strains. Additionally, we have observed that the matrix (M) protein of an attenuated A/Ann Arbor/6/60(H2N2) strain can be distinguished from the M protein of several other strains, by analysing the viruses in a high-resolution electrophoresis system (Laemmli, 1970). We describe in this report how concurrent comparisons of the RNA and proteins of cold-adapted temperature-sensitive influenza viruses prepared by recombination of wild-type A/Ann Arbor/6/60 mutant enabled us to identify the genealogy of their non-structural (NS) and/or M proteins. It is anticipated that this type of mapping study will complement genetic analysis (Richman et al. 1975; Spring et al. 1975, 1977), and biochemical analysis of mutant virus replication (Kendal, Kiley & Maassab, 1973) in determining the nature of the genes responsible for attenuation in cold-adapted and/or ts influenza viruses.

**METHODS**

*Viruses.* Five wild-type viruses used were A/Ann Arbor/6/60(H2N2), A/Queensland/6/72(H3N2), A/Scotland/840/740(H3N2), A/Victoria/3/75(H3N2) and A/New Jersey/8/76(H5N1). Derivation of a cold-adapted, temperature-sensitive mutant from A/Ann Arbor/6/60 virus has been previously described in detail (Maassab, 1967). Derivation of a cold-adapted, temperature-sensitive virus AA-CR6 by recombination of wild-type A/Queensland/6/72 and the cold-adapted mutant A/Ann Arbor/6/60 has also been described (Maassab, 1975). The recombinant (AA-CR18) of wild type A/Scotland/840/74 and cold-adapted mutant A/Ann Arbor/6/60 was obtained in a manner similar to that used for obtaining AA-CR6, by infecting chick kidney (CK) cells with a mixture containing \(10^5\) EID\(_{50}\) of each of the wild-type and mutant viruses, which were then grown at \(25^\circ C\) until cytopathic effects were observed (about 3 days). Progeny virus was passaged twice more at \(25^\circ C\) in CK cells in the presence of immune serum to A/Ann Arbor/6/60, and then at \(25^\circ C\) in CK cells without immune serum. After this fourth CK passage, the virus was grown under agar overlay at \(25^\circ C\) in CK cells, and ten distinct plaques were picked as a source of the AA-CR18.
recombinant clones. Stocks of each clone were prepared by inoculating embryonated eggs at 33 °C with undiluted material recovered from each plaque. This harvest was passaged once more in eggs at 33 °C to provide stocks used for genetic, biological and biochemical analysis. Recombinant AA-CR19 was prepared similarly, but using A/Victoria/3/75(H3N2) as wild-type parent. Recombinant AA-CR22 was also prepared using A/Victoria/3/75 as a wild-type parent. The recombination experiment was performed independently from that above using as parent A/Victoria/3/75 virus that had been re-isolated from original throat washings (supplied by Dr Brian Feery, Commonwealth Serum Institute, Melbourne) by inoculating directly into specific pathogen-free eggs (SPAFAS, Storrs, CT). During the cloning procedure, plaques were picked, and each passaged directly by plaquing in CK cells under agar overlay. After antigenic analysis two clones with A/Victoria/3/75 haemagglutinin were selected for study, one having the neuraminidase of A/Victoria/3/75 and the other having the neuraminidase of A/Ann Arbor/6/60. Recombinant AA CR24 was prepared similarly to AA-CR22, but using a SPAFAS E2 passage of A/New Jersey/8/76(Hsw1N1) as wild-type parent virus. Only a single clone of recombinant has been studied so far, having the antigenic composition Hsw1N2. All clones of recombinants had the following properties, to be reported elsewhere: (1) cold-adaptation (ability to produce plaques in CK cells at 25 °C), (2) temperature-sensitivity (failure to produce plaques in CK cells at 39 °C). Recombinants AA-CR6 and AA-CRI8 have also been examined by complementation analysis and shown to possess a conditional-lethal ts lesion mapping in the same gene as a ts lesion of the A/Ann Arbor/6/60 parent.

Isotope-labelling of viruses. Viruses were grown in CK cell monolayers infected with an m.o.i. of about 10 to 50 EID50/cell. After a 30- to 45-min period for virus adsorption, residual inoculum was removed and monolayers were washed once with balanced salt solution. To label virus RNA with 3H-uridine we added Eagle’s minimum essential medium containing 100 to 200 µCi of 3H-uridine/ml. To label virus RNA with 32P we used a medium containing 1 mM-sodium citrate in place of sodium or potassium phosphate, to which we added 0.5 to 1 mCi of carrier-free 32P/ml. Virus polypeptides were labelled by using medium containing 10% of the standard leucine concentration and either 5 µCi of 14C-leucine or 25 µCi of 3H-leucine/ml. Virus was harvested after 18 to 20 h growth at 34 °C and a small amount of purified unlabelled A/Ann Arbor/6/60 virus was added as carrier. In those experiments requiring comparison of 3H- and 32P-labelled RNA of different viruses, fluids containing the two viruses were mixed before purification. 3H-leucine and 14C-leucine labelled viruses were purified separately. In all cases purification was done by ultracentrifugal pelleting, velocity sedimentation for 45 min at 25000 rev/min through a 12% to 60%, w/v, sucrose gradient in a Beckman SW 27.1 rotor, and final pelleting of the virus present in a visible band. Purified virus was resuspended in 0.1 M-tris-HCl buffer, pH 7.5, and was used within a few hours for RNA analysis.

RNA electrophoresis procedures. Gels for RNA analysis contained 0.5% agarose, electrophoresis buffer [30 mM-sodium dihydrogen phosphate 30 mM-tris base, 0.9 mM-EDTA, 0.2% sodium dodecyl sulphate (SDS), pH 7.8], the stated concentration of acrylamide, and, in addition, bis-acrylamide at 1/20 the concentration of acrylamide. Polymerization of gels was initiated at room temperature by adding 10^-3 (v/v) N,N',N'-tetramethyl ethylene diamine (TEMED) and 0.1% of fresh ammonium persulphate. After 2 h the gels were placed in a refrigerator for at least 2 h before use. Gels were pre-run at 50 V for 1 h before samples were loaded. Virus samples (40 µl) were mixed with 10 µl of sample buffer (60% sucrose, 5% SDS, 6 mM-EDTA, 5 mM-tris base, pH 7.5) and heated to 56 °C for 2 min to solubilize RNA for electrophoresis. During electrophoresis, temperature was controlled
by using a water-jacketed glass electrophoresis chamber through which was circulated the output from a refrigerated/heated bath maintained at a constant temperature \( \pm 0.1 \) °C.

**Protein electrophoresis procedures.** The discontinuous electrophoresis system used was similar to that described by Laemmli (1970). Ten cm resolving gels containing 11% total acrylamide (0.3% bis-acrylamide), 0.375 M-tris-HCl, pH 8.9, buffer, and 0.1% SDS were polymerized at room temperatures by adding 0.3 \( \times \) \( 10^{-3} \) (v/v) TEMED and 0.032% of ammonium persulphate. After the resolving gels had set, they were overlaid with 3 cm spacer gels containing 3.6% acrylamide (including 0.18% bis-acrylamide), 0.125 M-tris-HCl, pH 6.7, and 0.1% SDS. Spacer gels were polymerized similarly to the resolving gels. The electrode buffer, pH 8.9, contained 0.05 M-tris base, 0.38 M-glycine and 0.1% SDS. Virus samples were prepared by boiling them for 2 min in dissociation buffer containing 10 mM-disodium monohydrogen phosphate, 2.5% SDS, 5% (v/v) 2-mercaptoethanol, and 10% glycerol. Gels were electrophoresed with a constant current of 0.9 mA/gel for 16 h.

**Isotope counting.** All gels were frozen to \(-60\) °C, partially thawed and then cut with a Mickle gel slicer into 1 mm segments. Protein or RNA was extracted from the gel slices by incubation for 5 h at 35 °C in 4 ml of fluid containing 15 parts of scintillation mixture ‘320’ and one part of solubilizer ‘TS1’ (Research Products International). Samples containing \(^3\)H and \(^32\)P were counted in a scintillation counter with windows set for a 3% spillover of counts into the \(^3\)H channel, and samples containing \(^3\)H and \(^14\)C were counted with windows set for a 20% spillover of \(^14\)C counts into the \(^3\)H channel. Corrections for spillover were made when necessary.

**Materials.** All isotopes were obtained from New England Nuclear, Massachussetts, and reagents for acrylamide gel electrophoresis from Bio-Rad, California.

**RESULTS**

**Comparison of migration rates of H2N2 and H3N2 structural polypeptides in a discontinuous electrophoresis system**

To determine whether differences could be detected between the migration rates of A/Ann Arbor/6/60(H2N2) and H3N2 virus structural polypeptides, we grew virus A/Ann Arbor/6/60(H2N2) in the presence of \(^14\)C-leucine, and H3N2 viruses in the presence of \(^3\)H-leucine. After purification, mixtures of \(^14\)C-labelled and \(^3\)H-labelled viruses were co-electrophoresed on 11% acrylamide gels. A discontinuous buffer system was used which affords higher resolution than most continuous buffer systems (Laemmli, 1970). In the experiment illustrated, four polypeptides were identified in each virus (Fig. 1). Although occasionally a small peak corresponding to P protein was seen in some virus preparations, the largest sized peak reproducibly seen (peak 1) represents the virion HA, which has previously been shown to be predominantly in the uncleaved form for influenza viruses grown in the chick kidney cell system in the absence of trypsin (Kendal et al. 1973). For the H3N2 viruses A/Queensland/6/72 and A/Scotland/840/74, the HA peak migrated slightly slower than the HA peak of the H2N2 A/Ann Arbor/6/60 virus. The second polypeptide (peak 2) corresponds to the virus nucleoprotein, NP and its migration rate was identical for each virus. Each virus also contained small amounts of a polypeptide (peak 3) migrating slightly faster than the nucleoprotein. This polypeptide had a different migration rate for each virus, and is therefore believed to represent a small amount of the haemagglutinin cleavage product HA\(_1\), rather than a host cell contaminant or neuraminidase which cannot normally be identified. The fourth polypeptide (peak 4) present in each virion was the M protein. This component of both the H3N2 viruses examined migrated slightly more
Influenza M and NS genes

Fig. 1. Comparison of structural polypeptides of two H3N2 wild-type viruses and A/Ann Arbor/6/60(H2N2) strain. Purified $^3$H-leucine labelled H3N2 viruses were mixed with $^{14}$C-leucine labelled A/Ann Arbor/6/60 virus and co-electrophoresed in 11% acrylamide gels with a discontinuous buffer system containing SDS. (a) A/Scotland/840/74 and A/Ann Arbor/6/60; (b) A/Queensland/6/72 and A/Ann Arbor/6/60. Peaks 1, 2, 3 and 4 correspond to HA, NP, HA1, and M respectively. HA2 has been run off the bottom of the gel in this experiment. (a) ---, $^3$H-A/Scotland/840/74; --, $^{14}$C-A/Ann Arbor/6/60. (b) ---, $^3$H-A/Queensland/6/72; --, $^{14}$C-A/Ann Arbor/6/60.

Fig. 2. Comparison of structural polypeptides of the H3N2 recombinant virus AA-CR6 and its H2N2 parent A/Ann Arbor/6/60. $^3$H-leucine labelled AA-CR6 recombinant and $^{14}$C-leucine labelled A/Ann Arbor/6/60 were co-electrophoresed on an 11% acrylamide gel with a discontinuous buffer system containing SDS. Peaks are numbered as in Fig. 1. ---, $^3$H-AA-CR6; --, $^{14}$C-A/Ann Arbor/6/60.
slowly than the M protein of the H2N2 virus A/Ann Arbor/6/60. Although the difference was small, it was reproducibly observed in this electrophoresis system, when the H2N2 and the H3N2 viruses labelled with different isotopes were co-electrophoresed in a single gel. It was similarly shown that the M protein of A/Victoria/3/75 migrated more slowly than that of A/Ann Arbor/6/60, and that the M protein of A/New Jersey/76(HSw1NI) migrated faster than that of A/Ann Arbor/6/60 (results not shown).

Several recombinant viruses derived by genetic interaction of the A/Ann Arbor/6/60 virus with H3N2 or HSw1NI viruses were therefore examined by the same procedures, to see whether the genealogy of their M proteins could be determined by comparative electrophoresis. For recombinant AA-CR6 peaks 1 and 3, corresponding to the HA and HA1, migrated differently from the corresponding peaks of A/Ann Arbor/6/60 (Fig. 2) and had migration rates similar to those of peaks 1 and 3 of the parent A/Queensland/6/72 from which AA-CR6 derived its HA antigen. By contrast, the M protein (peak 4) of AA-CR6
co-migrated with the M protein of the A/Ann Arbor/6/60 parent, which, as shown above, is different from that of A/Queenland/6/72.

Studies were also made of the M protein in viruses derived by recombination of A/Ann Arbor/6/60 and the H3N2 virus A/Scotland/840/74. As described in Methods, ten clones were obtained from the recombination. Each clone possessed the cold-adaptation and ts properties of the cold-adapted A/Ann Arbor/6/60 parent, but the HA of A/Scotland/840/74. Four of the AA-CR18 clones (1, 2, 6, and 7) also contained the NA of A/Scotland/840/74, whereas the other six clones contained the NA of A/Ann Arbor/6/60 (result not shown). Each of the ten recombinant virus clones was labelled with \(^3\)H-leucine and co-electrophoresed with \(^{14}\)C-leucine-labelled A/Ann Arbor/6/60 and \(^{14}\)C-leucine-labelled A/Scotland/840/74 (H3N2). The profile of polypeptides for AA-CR18 recombinants demonstrated the presence of HA, HA\(_1\), and HA\(_S\) (peaks 1, 3 and 5 respectively) with electrophoretic mobilities similar to those of the corresponding peaks of the H3N2 parent virus. The M protein peak of each AA-CR18 recombinant, however, had an identical electrophoretic mobility to that of the A/Ann Arbor/6/60 parent and differed from the M protein of A/Scotland/840/74. This is shown for clone 6 in Fig. 3, and similar results (not shown) were obtained for the other nine AA-CR18 clones. Similar experiments have also been performed with recombinant AA-CR19, with the two clones of AA-CR22, and with AA-CR24, which have HA antigens from H3 or HswI strains but ts and cold adaptation properties of A/Ann Arbor/6/60 mutant. In each case, the M protein in the recombinants has reproducibly been shown to be derived from the mutant A/Ann Arbor/6/60 parent, and to differ from the M protein in the relevant wild-type parent. Thus, including AA-CR6, a total of 15 clones of cold-adapted ts recombinants have been examined which contain HA antigens of wild-type H3N2 or HswI strains and growth characteristics of the mutant parent A/Ann Arbor/6/60. Each of the recombinants contained the M protein of the A/Ann Arbor/6/60 parent. This contrasts with the presence in recombinants of NA derived from either parent (described above) or NS derived from either parent (see below).

Comparison of the small RNA genes 7 and 8 in H2N2 and H3N2 viruses and recombinants AA-CR6 and AA-CR18

To obtain further information about the relationship between the A/Ann Arbor/6/60 and H3N2 parent viruses and their recombinants, we compared virion RNAs by gel electrophoresis techniques. We tried many conditions of electrophoresis to determine those which were optimum for differentiating between the RNA species of the influenza strains we wished to compare. The usefulness of 2%, 2.5%, 3% and 3.6% acrylamide concentrations, with or without 6 M-urea, and the effect of electrophoresis at temperatures of 5, 20 and 35 °C were all examined. Differences between the H2N2 and H3N2 strains we have studied were usually greatest when RNA was electrophoresed in 3% acrylamide gels at a temperature about 10 °C above ambient without incorporation of urea in the gels.

For the studies described in detail below, RNA profiles were always compared by co-electrophoresis of two viruses, one labelled with \(^3\)H-uridine and the other with \(^3\)P.

RNA from virus A/Ann Arbor/6/60 consistently resolved into seven RNA species when electrophoresed through 3% acrylamide gels at 30 to 35 °C in the absence of urea. We believe that one large RNA species was inadequately resolved, and RNA species are therefore labelled 1 to 8 as shown in Fig. 4. In the same electrophoresis system, the largest RNA species of A/Scotland/840/74 virus also incompletely resolved, although their pattern of migration was clearly dissimilar to that of the largest RNA species in A/Ann Arbor/6/60.
Fig. 4. Comparison of A/Ann Arbor/6/60(H2N2) and A/Scotland/840/74 (H3N2) virion RNAs by co-electrophoresis in a 3% acrylamide gel at 35 °C. 3H-uridine labelled A/Ann Arbor/6/60 and 32P-labeled A/Scotland/840/74 viruses were co-electrophoresed in a 15 cm gel (3% acrylamide concentration) at 10 mA/gel for 6 h. For clarity the A/Ann Arbor/6/60 RNA profile (bottom) is shown separately from the A/Scotland/840/74 RNA profile (top). Vertical lines are used to indicate those A/Ann Arbor/6/60 RNA segments migrating with different mobilities from RNA segments of A/Scotland/840/74 virus.

Comparison of the RNA of recombinant AA-CR18 (clone 6) with the RNAs of the two parent viruses A/Ann Arbor/6/60 and A/Scotland/840/74 indicated that RNA 8 of the recombinant virus was identical in migration rate to RNA 8 of A/Scotland/840/74 virus and different from RNA 8 of A/Ann Arbor/6/60 virus (Fig. 5). Comparison of the migration rates of other RNA species consistently indicated that (1) the large RNA species 1 to 3 of CR18 probably were derived partially from A/Ann Arbor/6/60 and partially from A/Scotland/840/74 parents and (2) RNA 4 of AA-CR18 was derived from A/Scotland/840/74 parent, which is consistent with the belief that RNA 4 codes for influenza HA (Palese & Schulman, 1976a). Resolution of genes 5 and 6 in the AA-CR18 recombinant was insufficient to conclusively determine their derivation although it appears probable that gene 6 was derived from the A/Scotland/840/74 parent. The results shown also suggest that RNA 7 of AA-CR18 recombinant is identical in migration to RNA 7 of A/Ann Arbor/6/60 parent and distinct from RNA 7 of the A/Scotland/840/74 parent. However, it was not always possible to observe this with the conditions of electrophoresis used in this study.

To verify that RNA 8 of the recombinant AA-CR18 was not derived from the A/Ann
Fig. 5. Comparison of RNAs in A/Ann Arbor/6/60 (H3N2), A/Scotland/840/74 (H3N2),\textsuperscript{a} and their H3N2 recombinant AA-CRI8-clone 6. Conditions of electrophoresis were as described for Fig. 4. Gel 1 (a, b) the profile of \textsuperscript{3}H-uridine A/Ann Arbor/6/60 RNA is plotted in (b), and the co-electrophoresed \textsuperscript{32}P-AA-CRI8 clone 6 RNA is plotted separately in (a). Gel 2 (c, d): the profile of \textsuperscript{3}H-uridine A/Scotland/840/74 is plotted in (d) and the co-electrophoresed \textsuperscript{32}P AA-CRI8 clone 6 RNA is plotted separately in (c). Arbor/6/60 parent the virus RNAs were also examined by electrophoresis in 3-6% acrylamide gels. When electrophoresis was conducted at 35 °C RNA 8 of the two viruses migrated similarly (not shown). However, when the electrophoresis was conducted at 20 °C, then RNA 8 of AA-CRI8 clone 6 migrated more rapidly than RNA 8 of A/Ann Arbor/6/60.
Fig. 6. Comparison of A/Ann Arbor/6/60 (H2N2) and recombinant AA-CR18 clone 6 virion RNAs by co-electrophoresis in a 3.6% acrylamide gel at 20 °C. 3H-uridine A/Ann Arbor/6/60 and 3P-labelled AA-CR18 clone 6 viruses were co-electrophoresed in a 10 cm gel at 4 mA/gel for 16 h. For clarity the A/Ann Arbor/6/60 RNA profile (bottom) is shown separately from the A/Scotland/840/74 RNA profile (top).

(Fig. 6). In this respect RNA 8 of AA-CR18 clone 6 resembled RNA 8 of A/Scotland/840/74 virus (results not shown). With the different electrophoresis conditions it was also verified that gene 4 of the AA-CR18 (H3N2) recombinant was not derived from the H2N2 parent A/Ann Arbor/6/60. Studies similar to those described above were performed for the nine additional clones of AA-CR18, which all contain the H3 antigen of A/Scotland/840/74 and the ts and cold-adaptation genes of A/Ann Arbor/6/60. Each clone contained an RNA species 8 that in its electrophoretic migration behaviour was similar to the RNA 8 of its H3N2 parent, A/Scotland/840/74, and distinct from that of A/Ann Arbor/6/60. By contrast, analysis of the recombinant AA-CR6 consistently demonstrated it to contain RNA species 8 that was similar to RNA 8 of A/Ann Arbor/6/60 and different from RNA 8 of the H3N2 parent, A/Queensland/6/72 (Fig. 7). It was also reproducibly shown that RNA 4 of the AA-CR6 (H3N2) recombinant was derived from its H3N2 parent, and not from the A/Ann Arbor/6/60 parent. In some experiments RNA 7 of the AA-CR6 recombinant was found to be similar to RNA 7 of the A/Ann Arbor/6/60 parent, which is also shown in Fig. 7.

DISCUSSION

Comparison of matrix protein in parent and recombinant viruses

Although Laver & Downie (1976) observed minor differences in the peptide compositions of M proteins of influenza A strains it was nevertheless surprising to observe that the electrophoretic migration rate of the M protein in an H2N2 virus was different from the migration rate of M protein in several H3N2 viruses and in A/New Jersey/8/76 (Hsw1N1).
Fig. 7. Comparison of RNAs in A/Ann Arbor/6/60 (H2N2), A/Queensland/6/72 (H3N2) and their H3N2 recombinant AA-CR6. Conditions of electrophoresis were as described for Fig. 5. Gel 1 (a, b): the profile of $^3$H-uridine A/Ann Arbor/6/60 RNA is plotted in (b), and the co-electrophoresed $^{32}$P AA-CR6 RNA is plotted in (a). Gel 2 (c, d): the profile of $^3$H-uridine A/Queensland/6/72 RNA is plotted in (d), and the co-electrophoresed $^{32}$P-AA-CR6 RNA is plotted in (c).
In the absence of additional data, it is not possible to conclude whether the small but reproducible differences in migration rates of the M proteins of the H2N2 and H3N2 strains studied resulted from differences in sizes of the proteins, or resulted from a failure of the electrophoresis system to eliminate differences between their electrical charges and/or conformations. Regardless of the explanation, the simple procedure employed provided a rapid method of determining the genealogy of the M proteins in the recombinant viruses examined, without recourse to the more complex procedures of isolating the M proteins and mapping their tryptic peptides.

Each one of the 15 cold-adapted recombinant viruses examined was found to contain the M protein of the cold-adapted parent A/Ann Arbor/6/60. It cannot, however, be assumed that the ten clones of AA-CR18 are totally independent viruses. The clones may in fact be multiple isolations of only two viruses (see below) present in the mixture obtained from the fourth chick kidney passage step of the procedure used to isolate recombinants. Since four of the AA-CR18 clones contained neuraminidase of A/Scotland/840/74, and six of the AA-CR18 clones contained neuraminidase of A/Ann Arbor/6/60, it is apparent that we have examined a minimum of two independent AA-CR18 recombinants deriving cold-adaptation and ts properties from A/Ann Arbor/6/60 mutant, and HA from wild-type A/Scotland/840/74.

No selective pressure other than the ability to replicate at 25 °C was applied that might have affected segregation of the M protein genes during active production of the recombinants AA-CR6, AA-CR18, AA-CR19, AA-CR22 or AA-CR24. The observation that the M protein found in 15 cold-adapted viruses (including at least seven independent clones) recovered from five recombination experiments is exclusively that of the cold-adapted parent, A/Ann Arbor/6/60 (in contrast to the reassortment of NA and NS genes that we have found) is therefore suggestive that influenza M protein may be involved in cold-adaptation. Additional independent recombinant virus clones will continue to be studied so that further evidence may be obtained about this hypothesis.

Comparison of RNAs in parent and recombinant viruses

In previous reports where analysis of RNA migration patterns has been used to identify the genealogy of RNA species in recombinant influenza viruses (Palese & Schulman 1967a, b; Ritchey, Palese & Schulman, 1976), differences between the migration rates of corresponding genes in different virus strains have often been small. For our study, therefore, we used gels cast in tubes, rather than in slabs, and detected radioactivity by slicing gels, rather than by autoradiography, so that for comparative studies we could electrophorese in a single gel $^3$H- and $^{32}$P-labelled RNAs of two viruses. In this way we could reliably detect differences of as little as about 5% in the migration rates of corresponding RNA species of different strains, which is an equivalent sensitivity to that found when urea is incorporated into the electrophoresis gels so as to sharpen bands of RNA (Floyd, Stone & Joklik, 1974). We found, however, that the presence of urea during electrophoresis of RNAs of the H2N2 strains we studied reduced the differences in migration patterns so that they could not be reliably observed when radioactivity in the virion RNAs was detected by the gel slicing technique.

Ongoing studies comparing influenza virus RNAs by slab gel electrophoresis also showed that differences in migration rates of RNA species of H2N2 and H3N2 strains are often seen best when urea is omitted from the gel, confirming our observations made using tube gels. Our studies have also demonstrated that influenza strains may vary in the way their RNA migration patterns are affected by temperature when electrophoresed in the
Influenza M and NS genes

Influenza M and NS genes

Fig. 8. Possible gene assignments for RNA 7 and 8 in A/Ann Arbor/6/60 and A/Scotland/840/74 viruses, and in recombinants that could theoretically be derived from these parents in each instance. In each case, the upper and lower band represent the positions of RNAs 7 and 8 after electrophoresis on a 3-6% acrylamide gel at 20 °C. Subscripts ‘60’ and ‘74’ for M and NS genes designate RNA from A/Ann Arbor/6/60 and A/Scotland/840/74 respectively. Results for AA-CRI8 recombinant are consistent only with those predicted for recombinant 1 in case 1.

absence of urea. This has previously been observed to occur even when urea is incorporated into the electrophoresis gels used for studying RNAs of influenza viruses (M. B. Ritchie and P. Palese, personal communication), or cellular messenger RNAs (Gross et al. 1976). Optimal detection of differences between the RNA migration patterns of influenza strains may therefore require the use of different temperatures, gel concentrations, and denaturing agents depending on the viruses being compared. This may particularly apply to live virus vaccine development since the choice of viruses to be studied is dictated by their relevance, and not by the ease of differentiating between their structural components.

With the conditions we adopted, comparison of the RNA migration profiles of recombinant viruses AA-CRI8 clones 1 to 10 and parent viruses A/Ann Arbor/6/60 and A/Scotland/840/74 demonstrated that all AA-CRI8 clones possessed an RNA species 8 derived from the A/Scotland/840/74 parent. Furthermore, the AA-CRI8 recombinants were shown to derive their M protein from A/Ann Arbor/6/60, and not from A/Scotland/840/74. On the basis of their sizes, RNA species 7 and 8 were believed to code for the M and non-structural (NS) proteins (Pons, 1976). A firm assignment could not justifiably be made on
the basis of size determinations for the RNA species, however, since RNA migration can be affected by conformation, and it is unknown whether processing of mRNA might occur after transcription from virion RNA templates. Results we obtained for AA-CRI18 (clear resolutions of RNA 7 and 8 on electrophoresis, derivation of M protein from A/Ann Arbor/6/60, and derivation of RNA 8 from A/Scotland/840/74) correspond to those predicted for one of two recombinants that might be produced in the case where RNA 7 and RNA 8 of both parents code for M and NS proteins respectively (Fig. 8). Only if RNA 7 and 8 are assigned in this way can we account for the production of a recombinant with the properties of AA-CRI18. Thus, even though we have been unable to reproducibly identify the genealogy of RNA 7 in AA-CRI18 virus by electrophoresis procedures, nevertheless we are able to deduce that RNA 7 is derived from the A/Ann Arbor/6/60 mutant virus, and codes for M protein, and that RNA 8, which is derived from the A/Scotland/840/74 wild-type virus, codes for the NS protein. Ritchey et al. (1976) recently concluded that RNA 7 and 8 in HoN1 and H3N2 influenza viruses coded for M and NS proteins respectively. We could not, however, extrapolate that result to the virus we needed to study since we have had to perform electrophoresis in the absence of urea in order to detect differences between H2N2 and H3N2 strains, and since the order of migration of influenza genes may not always be the same in different virus strains (Ritchey et al. 1976).

**Significance of results for the development of live attenuated influenza vaccines**

Our finding that RNA 8 in the AA-CRI18 cold-adapted ts recombinant viruses was not derived from the cold-adapted ts A/Ann Arbor/6/60 parent eliminated the NS gene as being responsible for the cold-adaptation and ts properties of the mutant parent which were transferred to the AA-CRI18 recombinants. Spring et al. (1977a, b) have shown that the ts lesion of the cold-adapted parent and recombinant viruses detected by complementation analysis with a set of NIH ts mutants maps in group I, similarly to one ts lesion of TS-1(E) attenuation virus (Richman et al. 1975). This complementation group I ts lesion of TS-1(E) is probably present in the largest RNA segment of that virus (Palese & Ritchey, 1977). Recently we have obtained evidence consistent with the above findings, by showing that mutant A/Ann Arbor/6/60 probably has a lesion in its RNA 1 which causes temperature-sensitivity of the virion polymerase (Kendal et al. 1977). Additional genetic analysis of mutant A/Ann Arbor/6/60, and identification of the genealogy of all RNAs in the recombinant live virus vaccine strain AA-CRI19, also showed, however, that a ts lesion exists in RNA 3 of the mutant (Kendal et al. 1977). The existence of multiple ts lesions in the mutant A/Ann/Arbor 6/60 virus, as well as the possibility that additional lesions responsible for cold-adaptation might be present in other genes, such as RNA 7, makes it desirable to determine the genetic composition of cold-adapted recombinants produced with new epidemic strains before selecting clones for evaluation as candidate live virus vaccines.

Expert technical assistance was provided by Ms Judy Galphin and Ms Eve Bingham. Studies at the University of Michigan were supported by U.S. Army Medical Research and Development Command, contract no. DADA 17-73-C-3060 and National Institutes of Health, contract no. I-A172521.
Influenza M and NS genes

REFERENCES


(Received 23 February 1977)