Comparative Studies of Wild-type and Cold-mutant (Temperature-sensitive) Influenza Viruses: Independent Segregation of Temperature-sensitivity of Virus Replication from Temperature-sensitivity of Virion Transcriptase Activity during Recombination of Mutant A/Ann Arbor/6/60 with Wild-type H3N2 Strains

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SUMMARY

RNA 1* (see end of Summary) of a cold-adapted and temperature-sensitive (ts) influenza virus mutant A/Ann Arbor/6/60 has a different mobility from RNA 1 of wild-type (wt) A/Ann Arbor/6/60 when subjected to electrophoresis through acrylamide/agarose gels in the absence of denaturing agents. Detection of this lesion in RNA 1 of the mutant virus was dependent on the temperature of the gel during electrophoresis. Because RNA 1 is believed to code for a protein involved in virus-specific RNA synthesis we compared phenotypes of virion transcriptases in the wt and mutant viruses. The enzyme of the mutant virus was found to be about 40% less active at 40 °C than the enzyme of the wt virus when related to their activities at 31 °C. Two cold-adapted ts recombinants which derive their RNA 1 from the mutant A/Ann Arbor/6/60 have virion transcriptases with a phenotype similar to that of their mutant parent. Three different cold-adapted ts recombinants, however, which also derive their RNA 1 from the mutant A/Ann Arbor/6/60, have virion transcriptases with a phenotype similar to that of wt virus. We conclude, therefore, that the conditional-lethal ts property of A/Ann Arbor/6/60 mutant and its recombinants is independent of the phenotypic marker observed for the A/Ann Arbor/6/60 mutant virion transcriptase, and that the lesion in RNA 1 of the mutant may also be unrelated to the observed difference between virion transcriptases of the mutant and wt A/Ann Arbor/6/60 viruses. The phenotypes of the virion transcriptases in recombinants did, however, correlate with the derivation of their RNA 2. This suggests that the increased temperature-sensitivity of virion transcriptase of the A/Ann Arbor/6/60 mutant is caused by either (1) a lesion (not necessarily conditionally lethal) that occurred in its RNA 2 during the course of cold-adaptation, or (2) a lesion in another gene whose product is a component of the virion transcriptase complex, but which lesion is only expressed phenotypically when there is a synergistic interaction in the transcriptase complex with the product of A/Ann Arbor/6/60 RNA 2.
* Our nomenclature for RNAs 1, 2 and 3 relates to results of electrophoresis under reference conditions where urea is omitted from gels and water at a temperature of 38 to 39 °C is circulated through the heat exchanger of the electrophoresis apparatus. Application of this nomenclature to describe in the text results obtained with other conditions of electrophoresis has only been done where control experiments have been performed which verify the validity of such an extrapolation.

**Introduction**

Methods proposed for preparation of live vaccine candidates from new strains of influenza virus include recombination (or more properly, reassortment) of wt isolates with ts and/or cold-adapted viruses previously found to have desirable levels of immunogenicity and attenuation (McKenzie, 1969; Maassab et al. 1972; Murphy et al. 1976). One virus used to prepare such 'attenuants' (influenza viruses whose virulence for a specific host has been attenuated by reassortment of genes from two or more parental strains) is the A/Ann Arbor/6/60 strain which was adapted to replicate efficiently at the temperature of 25 °C (that is suboptimal for wt virus) and which at the same time acquired a conditional-lethal ts phenotype so that it fails to replicate at a temperature of 39 °C (that is permissive for the wt virus; Maassab, 1967). We have therefore undertaken studies to define the genes in cold-adapted mutant A/Ann Arbor/6/60 virus which possess biologically significant lesions, so that ultimately it may be possible to understand the mechanisms for attenuation of this mutant and its recombinants. As one phase of the investigation we have been determining which genes are transferred from mutant A/Ann Arbor/6/60 to its cold-adapted recombinants and in previous reports we described methods for identifying the NP, M and NS genes in such viruses (Kendal et al. 1977; Cox & Kendal, 1978). We have now developed procedures that allow us to analyse the large RNAs (segments 1 to 3) in our cold-adapted viruses and in the course of that work found that RNA 1 of cold-adapted A/Ann Arbor/6/60 contains a mutation that, under certain conditions of electrophoresis, causes the RNA to migrate with a different mobility from that of RNA 1 in wt A/Ann Arbor/6/60 virus.

Results of complementation assays have been interpreted as suggesting that the A/Ann Arbor/6/60 mutant possesses a ts lesion in the same gene as an NIH mutant R1 (Spring et al. 1977a, b) which is thought to possess a ts lesion in its RNA 1 that codes for the P3 protein (Palese & Ritchey, 1977). Furthermore, other mutants having a ts lesion in their RNA 1 and P3 proteins, and known to be defective in complementary RNA synthesis (Krug et al. 1975), have been found to possess ts virion transcriptases (Mowshowitz & Ueda, 1976). We have therefore studied the effect of temperature on the activity and stability of the virion transcriptase in mutant A/Ann Arbor/6/60 and in some of its cold-adapted ts recombinants with defined gene compositions.

We describe here evidence for a change in the phenotype of the virion transcriptase activity of mutant A/Ann Arbor/6/60 and the independent segregation during recombination of the conditionally lethal ts property of the virus from its virion transcriptase phenotype. We also found that when preliminary gene assignments reported for cold-adapted recombinants AA-CR19 and AA-CR22 (Maassab et al. 1977) were re-evaluated to allow for the newly recognized aberrant electrophoretic mobility of RNA 1 of mutant A/Ann Arbor/6/60, these recombinants were found to resemble other cold-adapted recombinants in possessing RNA 1 from their mutant parent rather than from wt virus. From the final results of gene analysis of the cold-adapted recombinants whose virion transcriptases have also been studied, it is possible to correct the suggestion made on the basis of our earlier results (Kendal et al. 1978) that the RNA 1 of cold-adapted A/Ann Arbor/6/60 contains a lesion affecting the virion transcriptase phenotype. The deduction may be made, however, that the altered phenotype of virion transcriptase in cold-adapted A/Ann Arbor/6/60 is...
dependent on the presence of its RNA 2, suggesting that this gene may have undergone mutation during the process of cold adaptation.

**METHODS**

**Viruses.** Wt virus strains used were A/Ann Arbor/6/60 (H2N2), A/Ann Arbor/9/73 (H3N2), A/Dunedin/4/73 (H3N2) and A/Victoria/3/75 (H3N2) that had been originally isolated at the University of Michigan, Ann Arbor, or were from the collection of the WHO influenza centre, CDC. Selection of a cold-adapted ts mutant from A/Ann Arbor/6/60 has been described (Maassab, 1967). Recombinants AA-CR12, AA-CR13, AA-CR19 and AA-CR22 were obtained by growing, at 25 °C in the presence of A/Ann Arbor/6/60 immune serum, the progeny derived from chick kidney (CK) cells mixedly infected at 25 °C with mutant A/Ann Arbor/6/60 and wt A/Ann Arbor/9/73 (for AA-CR12), wt A/Dunedin/4/73 (for AA-CR13) or A/Victoria/3/75 (for AA-CR19 and AA-CR22). The A/Victoria/3/75 parents used in recombination experiments 19 and 22 differ in that for the latter experiment the virus was re-isolated at CDC by inoculating the original throat swab (kindly provided by Dr Brian Feery) into SPAFAS eggs and then adapted to grow in bovine kidney cells by Dr Brian Murphy. Passage histories of the cold-adapted recombinants were similar to those previously described for AA-CR18 (Kendal *et al.* 1977) except that each recombinant received a minimum of two plaque purifications. The recombinant AA-CR13 clone 9 is a separate clone from recombinant AA-CR13 (now designated as clone 0) that has been found not to have a ts phenotype (Spring *et al.* 1977a). As shown below (see Results) all the cold-adapted recombinants used in this study are also conditionally lethal ts mutants, like their mutant parent, A/Ann Arbor/6/60. All recombinants were shown by haemagglutination inhibition tests to derive their haemagglutinin (HA) from their wt H3N2 parents. Results of neuraminidase inhibition tests showed that all recombinants also derived their neuraminidase (NA) from the wild-type parent with the exception of AA-CR22 clone 17, which possesses the NA of A/Ann Arbor/6/60.

**Infectivity titrations.** Plaque titrations of wt and ts viruses were done in MDCK cells as previously described (Cox *et al.* 1977). Cells were maintained at 34 or 39 °C for 2 days before the second overlay containing neutral red was added and then the cells were returned to 34 or 39 °C incubators for 1 day before plaques were counted. The temperature of the 39 °C incubator, which was monitored each morning and evening by inspection of a certified thermometer adjacent to the flasks, did not fluctuate by more than ±0.2 °C from the set temperature after equilibrium had been reached.

**Analysis of virion RNA.** Procedures for growth of virus in CK cells in the presence of $^3$H-uridine and for purification of virus were similar to those used in previous studies (Kendal *et al.* 1977). To compare the electrophoretic mobilities of RNAs of different viruses and recombinants, we used slab gels 28 cm long × 14 cm wide × 3 mm thick, poured between glass plates. One plate was in close contact with a lucite heat exchanger through which temperature-controlled water was circulated and the other glass plate was in contact only with air over most of its outer surface. The apparatus was purchased from Bio-Rad Laboratories, Richmond, Calif., and was modified to permit continuous re-circulation of buffer between the upper and lower buffer reservoirs during electrophoresis. Gels lacking urea contained 3% acrylamide cross-linked with 0.15% bis-acrylamide and 0.5% agarose; buffers were similar to those used previously in tube gels lacking urea (Kendal *et al.* 1977; Cox & Kendal, 1978). Gels containing 6 M-urea were prepared with 2.8% acrylamide, 0.14% bis-acrylamide and 0.5% agarose (Floyd *et al.* 1974). All slab gels were allowed to set at room temperature for 2 h and then at 4 °C overnight. Before samples were applied, the gels were equilibrated to the temperature of the heat exchanger in the electrophoresis apparatus for 4 to 6 h, and pre-electrophoresed for 2 h at 125 V. Virion RNAs were subjected
to electrophoresis at 150 V for about 20 h on gels without urea and for about 40 h on gels containing urea. The gels were processed for fluorographic detection of isotope by the method of Bonner & Laskey (1974) using pre-flashed X-ray films.

**RNA polymerase assays.** Viruses for RNA polymerase assays were grown in eggs at 33 °C for 2 days and, with the exception of wild-type A/Victoria/3/75, concentrated by centrifuging allantoic fluid harvests in a Beckman type 35 rotor at 30000 rev/min for 1 h. Virus-containing pellets were resuspended in a small vol of 0.1 M-tris-HCl buffer, pH 7.5 and disaggregated by a short (usually 30 s) period of sonication in plastic tubes placed in a Raytheon sonicator. The preparations were then centrifuged for 1 h at 25000 rev/min through 12 to 60% linear sucrose gradients in a Beckman SW 27.1 rotor and the opalescent band of virus midway down the gradient was recovered through a needle inserted in the side of the centrifuge tube. Virus was freed from sucrose by dilution with tris-HCl buffer and by centrifugation for 1 h at 35000 rev/min in a Beckman type 40 rotor. After the pellet had been resuspended in a small vol. of 0.1 M-tris-HCl buffer, pH 7.5, we further purified each virus by centrifugation through a second 12 to 60% linear sucrose gradient for 1 h at 40000 rev/min in a Beckman SW 50 rotor, or alternatively for 1.5 h at 33000 rev/min in a Beckman SW 36 rotor. The sharp band of virus near the bottom of the gradient was recovered and freed from sucrose as described above and then resuspended at a concentration of about 10 to 20 mg protein/ml in 0.1 M-tris-HCl buffer, pH 7.5, containing 1% glycerol. Virus was usually used in polymerase assays immediately after purification, or after storage for 1 or 2 days at +4 °C, which did not cause any appreciable loss in enzyme activity except for A/Victoria/3/75 wild-type virus. Occasionally virus was used after being stored at −70 °C for several weeks without any intervening thawing. Because it was found that A/Victoria/3/75 virus purified by the above method had very low or undetectable levels of virion transcriptase activity, this virus was purified by an alternative procedure. Chilled, clarified allantoic fluid was mixed with washed chicken red blood cells to a 1% final concentration of cells and placed in the cold for 1 h. Agglutinated cells were recovered by centrifugation in a refrigerated instrument for 10 min at about 1000 g and the supernatant carefully discarded. Cells were resuspended to 10% of the original vol. of allantoic fluid and incubated in a 37 °C water bath for 1 h, with occasional gentle swirling. The mixture was then centrifuged for 10 min at 1000 g and the supernatant recovered. Samples of 20 ml of the virus-containing supernatant were layered over discontinuous gradients prepared in Beckman SW 27 cellulose nitrate tubes with 5 ml each of 60%, 30% and 15% sucrose (w/v) in tris-HCl buffer, and centrifuged for 1 h at 25000 rev/min. The opalescent virus band at the upper surface of the 60% sucrose solution was recovered through the side of the tube with a syringe and needle, freed from sucrose and resuspended in glycerol-containing tris-HCl buffer similarly to other viruses. All assays of A/Victoria/3/75 virus polymerase were done in the afternoons with virus purified during the mornings of the same days.

Reaction mixtures for RNA polymerase assays included GpG to stimulate virion transcriptase as described by McGeoch & Kitron (1975). Concentrations of reagents were (after addition of virus as described below): 50 mm-tris-HCl, pH 8.2, 150 mm-KCl, 5 mm-dithiothreitol, 4 mm-MgCl₂, 2 mm-ATP, 0.4 mm-CTP, 0.4 mm-UTP, 0.1 mm-GTP including ³H-GTP at a sp.act. of 80 mCi/mmol, and 6.25 mm-GpG. To initiate RNA polymerase reactions we treated purified concentrated virus with 0.5% Triton N101 for 1 min at 40 °C and then 40 μl samples of treated virus containing 400 to 800 μg of protein were rapidly mixed with 560 μl samples of reaction mixtures that had been pre-warmed to the required temperatures. Pre-incubation of virus with detergent was done at 40 °C to ensure that the virus enzyme would, from the first moments of contact with substrates, be equilibrated at the elevated temperature that might be restrictive for RNA synthesis. Identical samples of the same Triton-treated virus preparations were always used to initiate reactions in parallel
assays at different temperatures. To measure incorporation of $^3$H-nucleotide into acid-insoluble products we removed duplicate samples of 40 μl from the reaction mixtures at appropriate intervals and pipetted them into 5 ml samples of cold 5% trichloracetic acid. After standing for 30 min in melting ice, the precipitated RNA was poured on to glass fibre filters (Whatman GF/C) which were then washed successively five times with 10 ml vol of cold 5% trichloracetic acid and five times with cold ethanol. Filters were dried in a hot-air oven and radioactivity was counted after toluene-based scintillation fluid was added. All values were corrected for a background (about 100 ct/min) determined with duplicate samples taken from reaction mixtures immediately after viruses were added.

Chemicals. Acrylamide, bis-acrylamide and agarose were purchased from Bio-Rad Laboratories, Richmond, Calif. Unlabelled nucleotides were purchased from PL Biochemicals Inc., Milwaukee, Wis., and GpG from Sigma Chemical Co., St Louis, Mo.; $^3$H-GTP was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Evidence for the existence of a lesion in RNA 1 of mutant influenza A/Ann Arbor/6/60

The electrophoretic mobilities of double-stranded hybrid RNAs or single-stranded virion RNAs have been observed to differ between some wt and mutant reoviruses or influenza viruses respectively (Ito & Joklik, 1972; Palese et al. 1977). Presumably this is a result of conformational changes in the RNAs which are due to alterations in base-pairing possibilities caused by the presence of new base sequences in the mutant virus RNAs. We have therefore compared virion RNAs of mutant and wt A/Ann Arbor/6/60 by acrylamide gel electrophoresis under non-denaturing conditions, where it might be expected that maximum conformation-dependent differences in RNA migration rates would be observed between mutant and wt RNAs. Electrophoresis was done in 28 cm long slab gels to obtain better resolution of large RNA species than we have previously obtained with 10 to 12 cm tube gels (Cox & Kendal, 1978). Because the relative migration rates of influenza RNAs in this non-denaturing gel system are highly temperature-dependent (Cox & Kendal, 1978; Kendal et al. 1977), we compared A/Ann Arbor/6/60 mutant and wt RNAs in experiments performed at different temperatures.

When the slab gel electrophoresis apparatus was run with 38 °C water circulated through its heat exchanger, the migration order of HA, NA and NP genes was similar to that previously observed when electrophoresis was done in a tube gel apparatus at 46 °C and a total of eight RNA species were resolved reproducibly (see Fig. 4). No differences were seen between migration rates of RNAs in wt and mutant A/Ann Arbor/6/60 viruses (not shown). When the temperature of circulating water in the heat exchanger of the slab gel apparatus was maintained at 32 °C, RNAs 2 and 3, and RNAs 5 and 6 of each virus did not clearly resolve from each other, but RNAs 1, 4, 7 and 8 gave distinct bands (Fig. 1). At this lower temperature of electrophoresis a difference between migration rates of RNA 1 of the mutant and wt A/Ann Arbor/6/60 was reproducibly observed.

Effect of temperature on virion transcriptase activity in wild-type and mutant A/Ann Arbor/6/60 viruses

In preliminary studies we determined that optimal conditions for the virion transcriptase assay included addition of GpG to the reaction mixtures as described by McGeoch & Kitron (1975) and use of Mg$^{2+}$ as divalent cation, similarly to results of Plotch & Krug (1977). Optimum pH and temperature for the in vitro assay were 8.0 to 8.4 and 31 to 34 °C respectively (not shown).

The phenotypes of the wt and mutant virus transcriptases were compared by examining
Fig. 1. Polyacrylamide gel electrophoresis of virion RNAs in wt and mutant (mt) A/Ann Arbor/6/60 (A/AA/6/60) viruses. Purified viruses grown in the presence of \( ^3 \)H-uridine were disrupted by incubation at 56 °C for 5 min in buffer containing 30 mM-sodium dihydrogen phosphate, 30 mM-tris base, 0.9 mM-EDTA and 1% sodium dodecyl sulphate. Disrupted viruses were applied to the top of a vertical slab gel 28 cm × 14 cm × 3 cm containing 3% acrylamide, 0.15% bisacrylamide and 0.5% agarose, in a Bio-Rad apparatus. Water at a temperature of 32 °C was circulated through the heat exchanger of the apparatus, which was also modified to permit continuous re-circulation of buffer between the anode and cathode reservoirs. Electrophoresis was carried out at 150 V (95 mA) for 20 h and RNA bands were detected by fluorography. Control experiments with several recombinants of known RNA composition verified that the same RNA species migrated as band 1 at this temperature of electrophoresis and at the higher temperature when re-circulating water in the heat exchanger was maintained at 38 °C.
Fig. 2. Thermal inactivation at 40 °C of virion transcriptase in wild-type (●—●) and mutant (▲—▲) A/Ann Arbor/6/60 disrupted with 0.5% Triton N101. Purified viruses pre-warmed at 30 °C were disrupted by the addition of pre-warmed Triton N101 to a concentration of 0.5% and were incubated at 40 °C for 60 min. At selected time points duplicate samples of 20 μl were removed and added to 230 μl vol. of RNA polymerase reaction mixture pre-warmed to 31 °C. Duplicate 40 μl samples (20 to 30 μg virus protein) were removed from the RNA polymerase reaction after 0 to 30 min, and the amount of 3H radioactivity incorporated from 3H-GTP into TCA-precipitable product was determined, with the counts recorded for the 0 min sample used as a background correction. Composition of the enzyme reaction mixture is described in the text. Results are the mean of determinations from three experiments.

The kinetics of thermal inactivation of enzyme activity at 40 °C. Rates of inactivation of transcriptase activity of detergent-disrupted virions were similar for both viruses, in that about one-third of the activity was lost by 7.5 min, two-thirds by 15 min and after 30 min only 15 to 20% of the activity remained (Fig. 2).

Virion transcriptase activities of the mutant and wt A/Ann Arbor/6/60 viruses at 25, 31 and 40 °C were also compared (Fig. 3). For both viruses there was a temperature-dependent time lag before the nucleotide precursor was incorporated into TCA-precipitable RNA. The lag was approximately 5 min at 25 °C and 2.5 min at 31 °C. There was no lag at the higher temperature of 40 °C, but at this higher temperature net synthesis of RNA continued at a maximum rate for only about 5 to 7.5 min and little net RNA synthesis was observed after about 15 min, presumably as a result of thermal inactivation of transcriptase. This contrasted with results at 25 and 31 °C, at which temperatures, maximum net synthesis of RNA was more prolonged. Analogous results to these have been observed with the cytoplasmic RNA polymerase from cells infected with one strain of fowl plague virus (Paffenholz & Scholtissek, 1973). Although our experiments demonstrated a considerable reduction in total RNA synthesis by wt virus when the temperature of the in vitro reaction was raised from 31 to 40 °C, a greater temperature-dependent reduction in RNA synthesis was observed for the mutant virus. Thus in the experiment shown in Fig. 3, at 40 °C the wt virus synthesized approx. 55% of the RNA made at 31 °C measured after a 15 min reaction, whereas at 40 °C the mutant virus synthesized only about 30% of the RNA made at 31 °C. Similar differences between the two A/Ann Arbor/6/60 viruses were observed at any time point in the first 30 min of the virion transcriptase reaction where significant
levels of \(^3\)H-nucleotide incorporation had occurred. In 10 repetitive determinations of \(^3\)H-nucleotide incorporation after a 15 min reaction, the mean ratio of virion transcriptase activity at 40 °C to that at 31 °C was 0.38 for wt A/Ann Arbor/6/60 and 0.24 for the mutant virion transcriptase. The finding of an approx. 40% lower transcriptase activity for the mutant virus at 40 °C, compared to wt virus when activities were normalized against those for each virus at 31 °C, was significant at the level \(P < 0.05\) for these ten determinations. We did not observe, however, a significantly increased efficiency of RNA synthesis at 25 °C as compared to 31 °C for the cold-adapted mutant viruses.

**Phenotype of virion transcriptase in cold-adapted, ts recombinant viruses deriving RNA 1 from mutant A/Ann Arbor/6/60**

To determine whether the altered phenotype of the virion transcriptase activity of mutant A/Ann Arbor/6/60 might be caused by the observed lesion in RNA 1 of this mutant, and to determine whether the altered characteristic of the virion transcriptase was related to the conditionally lethal ts phenotype of the mutant, we examined the properties of the virion transcriptase in recombinant ts viruses deriving their RNA 1 from mutant A/Ann Arbor/6/60. Recombinants AA-CR12 clone 0 and AA-CR22 clone 17 had virion transcriptases whose net RNA synthesis at 40 °C was only about 24 to 25% of that at 31 °C, similar to their mutant parent A/Ann Arbor/6/60 and less than their wt parents A/Ann Arbor/9/73 and A/Victoria/3/75 (Table 1). The recombinants AA-CR13 clone 9, AA-CR19 clone 0 and AA-CR22 clone 1, however, had virion transcriptases whose net RNA synthesis at 40 °C...
Table 1. In vitro virion transcriptase activity* of wild-type (wt) mutant, and recombinant influenza viruses, at two temperatures

<table>
<thead>
<tr>
<th>Viruses</th>
<th>3H-GMP incorporation at 40 °C</th>
<th>3H-GMP incorporation at 31 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant A/Ann Arbor/6/60</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Wt A/Ann Arbor/9/73</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Wt A/Dunedin/4/73</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Wt A/Victoria/3/75</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Recombinant AA-CR12 clone 0†</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Recombinant AA-CR13 clone 9‡</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Recombinant AA-CR19 clone 0§</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Recombinant AA-CR22 clone 1§</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Recombinant AA-CR22 clone 17§</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction time was 15 min. Each recombinant was assayed in parallel with its mutant and wt parents, and each result is the mean of at least three determinations.

† Parental viruses of this recombinant were mutant A/Ann Arbor/6/60 and wt A/Ann Arbor/9/73.
‡ Parental viruses of this recombinant were mutant A/Ann Arbor/6/60 and wt A/Dunedin/4/73.
§ Parental viruses of this recombinant were mutant A/Ann Arbor/6/60 and wt A/Victoria/3/75.

Table 2. Plaque titrations of parental and recombinant influenza A viruses in MDCK cells at two temperatures

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Infectivity titre* at 34 °C</th>
<th>Infectivity titre* at 39 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant A/Ann Arbor/6/60</td>
<td>8.9</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Wt A/Ann Arbor/9/73</td>
<td>8.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Wt A/Dunedin/4/73</td>
<td>8.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Wt A/Victoria/3/75</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Recombinant AA-CR12 clone 0</td>
<td>8.0</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Recombinant AA-CR13 clone 9</td>
<td>8.0</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Recombinant AA-CR19 clone 0</td>
<td>8.1</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Recombinant AA-CR22 clone 1</td>
<td>8.2</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Recombinant AA-CR22 clone 17</td>
<td>8.4</td>
<td>&lt; 3</td>
</tr>
</tbody>
</table>

* Titre expressed as log_{10} (p.f.u./ml). All results are the mean of two determinations.

was about 60% of that at 31 °C, similar to their wt parents A/Dunedin/4/73 and A/Victoria/3/75, but more than twice as great as their mutant parent A/Ann Arbor/6/60 (Table 1). All the recombinant viruses were conditionally lethal ts mutants with an efficiency of plaquing at 39 °C which was less than 0.001% of that at 34 °C (Table 2). Identification of genes in the recombinants demonstrated them all to contain RNA 1, 3, 6 (RNP) and 8 (NS) from A/Ann Arbor/6/60 mutant (Fig. 4 and 5), although their HA and usually NA were derived from their wt parents. The M protein of the recombinants is also derived from mutant A/Ann Arbor/6/60 as determined by polyacrylamide gel electrophoresis of virion proteins (Kendal et al. 1977 and unpublished results). The two sets of viruses differed, however, in that RNA 2 of AA-CR12 clone 0 and AA-CR22 clone 17 was derived from the mutant parent, whereas RNA 2 of AA-CR13 clone 9, AA-CR19 clone 0 and AA-CR22 clone 1 was derived from the wt parents (Fig. 4 and 5).

DISCUSSION

Results of biological, biochemical and gene mapping studies with five clones of cold-adapted viruses derived from four recombination experiments are summarized in Table 3. It is apparent that the conditional-lethal ts phenotype of the recombinants is independent of the in vitro phenotype of the virion transcriptase since three recombinants
Fig. 4. Polyacrylamide gel electrophoresis of virion RNAs in recombinant and parental viruses. Electrophoresis was carried out as described for Fig. 1, except that the water circulated through the heat exchanger of the electrophoresis apparatus was maintained at 38 °C. (a) Mutant parent A/Ann Arbor/6/60, recombinant AA-CR 12 and wt parent A/Ann Arbor/9/73; (b) mutant parent A/Ann Arbor/6/60, recombinant AA-CR 13 clone 9 and wt parent A/Dunedin/4/73 detected by fluorography for 3 days or 1 day (right hand lane of A/Dunedin/4/73). Comparison of RNAs 4, 5 and 6 of recombinant and parent viruses shows that the recombinants derive RNA 4 from their wt parents, but RNA 6 from mutant A/Ann Arbor/6/60. Since RNAs 4, 5 and 6 between them code for HA, NA and NP proteins and since antigenic analysis showed that each recombinant derived its HA and NA from its wt parent, it follows that RNA 6 codes for the NP gene of A/Ann Arbor/6/60 mutant. This result following slab gel electrophoresis is similar to that observed in tube gels when electrophoresis is performed at 46 °C (Cox & Kendal, 1978). Verification that RNA 1 of AA-CR 13 clone 9 was derived from its mutant parent was obtained from results obtained when electrophoresis was carried out at 35 and 31 °C (not shown).
Fig. 5. Polyacrylamide gel electrophoresis of virion RNAs in wt A/Victoria/3/75, mutant A/Ann Arbor/6/60 viruses and their cold-adapted recombinants. (a) Electrophoresis was carried out as described for Fig. 1 except that water at a temperature of 38.5 °C was circulated through the heat exchanger of the apparatus; (b) electrophoresis was carried out on a slab gel containing 6 M-urea. For this gel, water at 30.5 °C was circulated through the heat exchanger. Although it is not possible from these results to deduce whether RNAs banding in positions 1 and 3 are comparable genes under the different conditions of electrophoresis, analysis of the results shown does permit the conclusion that the RNA banding in position 2 is the same gene under both sets of conditions. The gene derivations shown in Table 3 for the recombinants AACR19 and CR22 clones 1 and 17 are the only derivations consistent with the results in Fig. 5(a) and (b), and with previous findings (Cox & Kendal, 1978; Kendal et al. 1977).
Table 3. Comparison of properties of five clones of cold-adapted recombinant viruses and of their A/Ann Arbor/6/60 mutant parent

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Cold-adaptation</th>
<th>Temperature-sensitivity</th>
<th>Virion transcriptase</th>
<th>RNA1</th>
<th>RNA2</th>
<th>RNA3</th>
<th>HA</th>
<th>NA</th>
<th>NP</th>
<th>M</th>
<th>NS</th>
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</thead>
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<td>AA-CR12 clone 0</td>
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<td>×</td>
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</tr>
<tr>
<td>AA-CR13 clone 9</td>
<td>×</td>
<td>×</td>
<td>o</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
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<td>×</td>
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<tr>
<td>AA-CR19 clone 6</td>
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<td>×</td>
<td>o</td>
<td>×</td>
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<td>×</td>
<td>×</td>
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<tr>
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<td>×</td>
<td>×</td>
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</table>

* Stated phenotypic properties of the recombinant (ability to replicate at 25 °C; reduced replication at 39 °C; relative in vitro activity of virion transcriptase at 40 °C compared to 31 °C) either resemble those of the mutant parent A/Ann Arbor/6/60 (×) or of wt parental virus (o).

† Stated genes are either derived from the mutant parent A/Ann Arbor/6/60 (×) or from wt parental virus (o). Relative migration positions for RNAs 1, 2 and 3 are given for the reference conditions where electrophoresis is carried out in slab gels lacking urea, as described in the Methods, with water at a temperature of 38 to 39 °C circulated through the heat exchanger of the electrophoresis apparatus.

(AA-CR13 clone 9, AA-CR19 clone 6 and AA-CR22 clone 17) are restricted in replication at 39 °C but possess virion transcriptase with wt phenotype in our assay. Furthermore, the particular transcriptase phenotypic marker we have studied is unlikely to be specified by RNA 1 of mutant A/Ann Arbor/6/60 since that gene is present in all the recombinants. Accordingly we are unable, on the basis of the present studies, to ascribe any biological significance to the existence of the lesion in RNA 1 of mutant A/Ann Arbor/6/60 (demonstrated by electrophoretic techniques, Fig. 1) which might support the view that a lesion in this gene is responsible for the conditionally-lethal ts phenotype of this virus and its recombinants (Palese & Ritchey, 1977; Spring et al. 1977a, b) although our results do not exclude this possibility.

Our findings do, however, suggest that during the process of cold-adaptation a mutation occurred in RNA 2 of A/Ann Arbor/6/60 which affects the phenotype of the virion transcriptase in our in vitro assay. This conclusion is predicated on two observations. (i) All three recombinants possessing wt RNA 2 have a wt virion transcriptase phenotype whereas the recombinants possessing mutant RNA 2 have a mutant virion transcriptase phenotype in our assay. The correlation between transcriptase phenotype and RNA 2 derivation was valid not only for clones from different recombination experiments (e.g. AA-CR12 and AA-CR13) but also for clones from the same recombination experiment (e.g. AA-CR22 clones 1 and 17). (ii) The derivation of other genes coding for proteins implicated in RNA synthesis (RNAs 1 and 3 and the nucleoprotein gene) is invariant in the recombinants studied. Although the simplest explanation for these results is the existence of a lesion in RNA 2 directly affecting the transcriptase phenotype, an alternative explanation is possible. Thus, the transcriptase phenotype of the A/Ann Arbor/6/60 virus might have been altered during cold-adaptation by mutation in any or all of the RNAs 1, 3 and the NP gene, but the mutation is only expressed in conjunction with RNA 2 of the A/Ann Arbor/6/60 virus and is suppressed on replacement of this gene by RNA 2 of another virus. Due to the restricted numbers of different gene constellations found so far for cold-adapted recombinant viruses (unpublished results) these alternative hypotheses cannot yet be directly evaluated. Any lesions in the RNA polymerase genes of the mutant are in addition to the previously described lesion in the gene coding for the haemagglutinin (Kendal et al. 1973).
Since recombinants AA-CR19 clone o and AA-CR22 clone 1 have proved to be attenuated in human volunteer studies (Maassab et al. 1977; personal communication from Drs R. Couch and T. Cates), it is unlikely that the lesion in the haemagglutinin gene or that suggested for RNA 2 of mutant A/Ann Arbor/6/60 are essential for attenuation, as both these genes in AA-CR19 clone o and AA-CR22 clone 1 are derived from the wt parent. Additional studies are in progress to attempt to identify which genes, or constellations of genes, are associated with the various biological properties of cold-adapted A/Ann Arbor/6/60 and its recombinants, and evidence has been obtained that the lesions in the A/Ann Arbor/6/60 mutant contribute to its temperature-sensitive behaviour in recombination assays with group III mutants of WSN virus (Kendal et al. 1978; personal communication from Dr S. Spring), as well as in plate-complementation assays with the NIH mutant R1 (Spring et al. 1977a, b).

Detection of multiple lesions in the A/Ann Arbor/6/60 virus increases the problems of genetic analysis. The compensation for this is the possibility that recombinants deriving most of their genes from this mutant which has multi-step lesions are less likely to regain their virulence by reversion when administered as vaccines to man than if they contained genes from a mutant virus having a small number of single-step lesions. Results from human volunteer and animal model studies are in agreement with this view, since reversions in ts or cold-adapted markers have usually been independent in viruses recovered from hamsters or human volunteers infected with cold-adapted recombinants (Spring et al. 1977b; B. R. Murphy & H. F. Maassab, unpublished results).

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REFERENCES


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