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# The influenza virus haemagglutinin gene: cloning and characterisation of a double-stranded DNA copy

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ABSTRACT

A protocol has been developed for the synthesis of a double-stranded DNA (dsDNA) copy of the influenza virus RNA genome segment which codes for the major surface antigen, haemagglutinin (HA). This dsDNA copy was inserted, after digestion with $S_1$ nuclease and poly (dC) tailing with terminal transferase, into poly(dG)-tailed, PstI-cut, pBR322 DNA, and used to transform E. coli RR1. Tetracycline-resistant bacterial colonies were screened for the presence of plasmid containing the copied HA gene by testing their ability to hybridise to a specific, $^{32}$P-labelled, single-stranded DNA probe.

Four cloned hybrid plasmids, containing DNA complementary to the HA gene of the influenza strain 29C (a laboratory derivative of influenza A/NT/60/68 (1)) were analysed by restriction enzyme mapping. Each contained a dsDNA insert equivalent to a full length copy of the HA gene. The nucleotide sequence of a selected restriction fragment from the DNA inserted in one of these cloned plasmids (C89) was determined. The amino acid sequence deduced from these data agreed with the amino acid sequence determined for the corresponding region of HA from the influenza strain A/Mem/102/72, another member of the Hong Kong subtype, identifying the inserted dsDNA of C89 as an authentic copy of the influenza HA gene.

## INTRODUCTION

Influenza continues to be a major cause of illness and death in the human population because of the ability of the virus to alter its antigenic properties. Two types of antigenic variation have been recognised. A radical change (antigenic shift) in the properties of the surface proteins of the virus results in the appearance of a new viral subtype, antigenically unrelated to previous strains. Within a particular subtype, smaller progressive changes in the properties of surface antigens, referred to as antigenic drift, are also observed (reviewed in ref. 2).

In order to relate changes in viral antigenicity to changes in the structure of the major antigenic protein, haemagglutinin, comparisons have been made of the peptide maps of proteins from different influenza strains (for example, references 3, 4). The amino acid sequences of haemagglutinins

 

from two different viral subtypes of influenza A are also being determined, but this is taking considerable time to complete (5, 6). The development of rapid nucleotide sequencing methods now means that it is often easier to sequence a gene than the protein for which it codes. Therefore, our approach to the problem of antigenic variation in influenza has been at the nucleic acid rather than the protein level.

Influenza virus has an RNA genome, composed of at least eight distinct single stranded segments of negative polarity. In order to take advantage of DNA sequencing methods to analyse the structure of the haemagglutinin gene, the fourth largest of the RNA segments (7), we have developed a procedure to make a double-stranded DNA copy of the gene. The dsDNA was then inserted into the plasmid pBR322 and the hybrid plasmid was amplified in E. coli RR1 to provide large amounts of pure material for subsequent nucleotide sequence analysis.

## MATERIALS AND METHODS

Growth and Purification of Virus and RNA. The influenza strain 29C is a laboratory mutant derived by growing the parent field strain A/NT60/68 in the presence of the most avid fraction of homologous antibody (8) in embryonated chicken eggs (1). Other virus strains used were the field strain A/Eng/42/72 and the laboratory strain A/Mem/102/72. The virus was supplied by Dr. C. Hannoun and purified by Drs. V. Bender and B. Moss, as previously described (9). Viral RNA was prepared as before (9).

Purification of a double-stranded DNA Copy of the Haemagglutinin RNA. Total virion RNA (approximately 50 µg) was polyadenylated at its 3' terminus using E.coli terminal riboadenylate.transferase (9). After concentration of the polyadenylated RNA by ethanol precipitation (3 vol), total virion DNA ($^{32}$P-labelled) was synthesised using AMV reverse transcriptase (400 units/ml, lot G-678, generously supplied by Dr. J.W. Beard, St. Petersburg, Fla.) in the presence of {$\alpha$-$^{32}$P}dATP (Amersham) (9). The mixture of single-stranded (ss) cDNA species was freed from RNA, denatured and separated on thin 2.6% polyacrylamide gel electrophoresis in 7 M urea as described previously (9). The band of $^{32}$P-labelled DNA corresponding in size to the HA gene was eluted from the gel (19). A double-stranded copy of this single-stranded species was synthesised by incubating the labelled band 4 DNA in a 50-100 µl reaction mixture containing the four unlabelled deoxynucleoside triphosphates (500 µM each) and AMV reverse transcriptase (800 units/ml) at 42° for 4-5 hours in a sealed glass capillary in the presence of Tris-HCl, pH 8.3 (50 mM) $MgCl_2$ (10

mM), KCl (70 mM) and dithiothreitol (10 mM). An aliquot of the reaction mixture was made 0.5% with respect to sodium dodecyl sulphate and analysed by 2.6% polyacrylamide thin gel electrophoresis without urea (2 hr at 25 mA) to determine the extent of conversion to double-stranded DNA. The bulk of the DNA was purified by adjusting the reaction mixture to 10 mM EDTA, 0.5% SDS, and extracting with an equal volume of a water-saturated phenol-chloroform (1:1)mixture. The organic phase was re-extracted with 25 µl aliquots of 50 mM Tris-HCl buffer pH 7.6, containing 1 mM EDTA, until all radioactive material was recovered. The dsDNA in the combined aqueous layers was purified by filtration through a micro-column of Sephadex G100.

Digestion of dsDNA with $S_1$ Nuclease. The dsDNA was digested with $S_1$ nuclease from Aspergillus oryzae for 30 min at 37°C in a 200 µl reaction mixture containing 30 mM sodium acetate (pH 4.5), 0.3 M NaCl, 3 mM $ZnCl_2$, 50 µg/ml of E.coli tRNA and 1000 units/ml of $S_1$ nuclease (Miles). The reaction was assayed either by electrophoresis on 4% polyacrylamide thin gels in 95% formamide in Tris-borate-EDTA buffer at half the usual (18) concentration or (more routinely) by electrophoresis on 2.6% acrylamide gels. The $S_1$-digested dsDNA was purified by adding 50 µl of 0.1 M Tris base, 0.5% SDS, 10 mM EDTA and an equal volume of phenol-chloroform (1:1) mixture. The phenol layer was re-extracted with 25 µl aliquots of 50 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, and the dsDNA was recovered from the combined aqueous layers by precipitation with ethanol. Chromatography on Sephadex G100 was again used to purify the dsDNA.

Tailing of dsDNA and pBR322. Approximately 30 dCMP residues were added to the 3' termini of the $S_1$-cleaved, dsDNA using terminal transferase (minimal nuclease P.-L. Biochemicals). DNA samples (5-20 ng) were incubated at 37°C for 30-45 min with 2 µl enzyme in potassium cacodylate (0.15 M), Tris-HCl pH 7.6 (30 mM), dithiothreitol (10 mM), dGTP (100 µM) and $CoCl_2$ (1 mM) in a 10 µl reaction volume. Since dsDNA was $^{32}$P-labelled, the time required for the poly(dC) addition was checked in a parallel experiment by following the incorporation of {$^3$H}dCTP into acid-insoluble material, using as a substrate pBR322 DNA fragments generated by digestion with AluI. After tailing, the dsDNA was precipitated with ethanol and separated from material of lower molecular weight by electrophoresis on a thin 2.6% polyacrylamide gel. The full length dsDNA band, representing 5-10% of the total $^{32}$P-labelled material, was eluted from the gel (19). Supercoiled pBR322 DNA (15) was converted to the linear form by cleavage with the restriction enzyme PstI (New England Biolabs). After fraction of the reaction mixture by electro-

phoresis on a thin 2.6% polyacrylamide gel, the band of linear DNA was eluted (19). Approximately 30 dGMP residues were added to the 3' termini of the linear plasmid as estimated from the incorporation of {$^3$H}dGTP into acid-insoluble DNA and using the conditions described above. The tailed plasmid was extracted with phenol and separated from low molecular weight material by passage through a microcolumn of Sephadex G100.

Annealing of dsDNA to pBR322 and Transformation of E.coli BR1. Annealing mixtures contained 4 ng poly(dC)-tailed dsDNA and 150 ng poly(dG)-tailed pBR322 DNA in 20 µl of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.6, and 0.2 mM EDTA. After 3 min at 65°, the mixture was held at 42° for 2 hr and then cooled to 15° over a 4 hr period. Transformation of the EK1 host, E.coli RR1, under CII containment conditions (Australian Academy of Science Recombinant DNA Committee) was by standard methods (15). Cells were plated on L-agar containing tetracycline (12.5 µg/ml) and incubated at 37°.

Identification of Transformants Containing Hybrid Plasmids and Screening of their Size. Tetracycline-resistant colonies were dotted onto nitrocellulose filters on agar (as above) and grown for 24 hr. Filters were treated as described (16) except for the omission of the proteinase K digestion step. After baking, filter preparation and hybridisation were as described by Botchan et al. (17) except that the concentrations of Ficoll, bovine serum albumin and polyvinylpyrrolidone during hybridisation were increased 5-fold. The probe for hybridisation was a $^{32}$P-labelled single-stranded DNA copy of the influenza HA gene, synthesised by reverse transcriptase in the absence of added primer (Both, Brownlee and Sleigh, manuscript in preparation). The size of the DNA inserted into positive clones was assayed (J. Shine, personal comm.) by transferring individual colonies to 20 µl water. Lysozyme (0.5 mg/ml), EDTA (25 mM) and pancreatic RNase (100 µg/ml) were added, and lysis was carried out at 4° for 30 min. Then 5 µl of a solution of SDS (5%), sucrose (20%) and bromophenol blue (0.2 w/v) was added and tubes were heated at 70° for 5 min. After vigorous mixing (Vortex) samples were loaded onto a 1.0% agarose gel in Tris/borate/EDTA (18). Electrophoresis was carried out at 4 V/cm for 4 hr. DNA bands were located by ethidium bromide staining.

Terminal Labelling of Restriction Fragments and Nucleic Acid Sequencing. Terminal labelling of restriction fragments and procedures for nucleotide sequencing were according to published procedures (9, 19).

## RESULTS

The reaction conditions described below for the preparation of dsDNA from

the HA RNA gene are, in our experience so far, applicable to many, if not all influenza strains from the Hong Kong sub-type.

Synthesis of Single Stranded cDNA from Total Influenza Virion RNA. Although we were specifically interested in copying the HA gene, we chose to separate its ss cDNA product rather than the RNA gene itself, since the former was more stable and $^{32}P$-labelled. Single stranded cDNA was transcribed from total polyadenylated virion RNA by AMV reverse transcriptase primed with $p(dT)_{12-18}$ as described in Methods and the products were resolved on a polyacrylamide gel as shown in Fig. 1. The pattern of the dominant cDNA products mimics very closely the pattern of the template RNA, suggesting that these cDNA species are full length copies of the RNA genome segments (9).

Synthesis of Double Stranded DNA. Band 4 ss cDNA, cut out and eluted from the gel and purified as in Methods, was converted to the ds form by reverse transcriptase, using the inherent ability of the cDNA to "self prime". Initially, various incubation conditions were tried, e.g. varying time, temperature and deoxynucleoside triphosphate concentrations. With the conditions described in Methods used on an analytical scale, we could obtain essentially complete conversion of single to dsDNA (Fig. 2a). In preparative experiments we observed approximately 50% conversion to double stranded material (Fig. 2b).

Digestion of dsDNA with $S_1$ Nuclease. The terminal hairpin loop in dsDNA synthesised by reverse transcriptase was nicked with $S_1$ nuclease. Optimum reaction conditions (enzyme and NaCl concentration, time and temperature) for conversion of DNA from the closed to the open ended form were determined by assaying the reaction products on a polyacrylamide gel containing 95% formamide (see Methods - results not shown). Double stranded DNA samples being prepared for cloning using the optimum $S_1$ digestion conditions were routinely analysed by electrophoresis on a non-denaturing gel to check that the $S_1$ digestion was satisfactory (see Methods). Such an analysis is shown in Fig. 3. Some lower molecular weight material resistant to $S_1$ nuclease was present in the sample, and is presumably short double-stranded material.

Transformation of *E. coli* RR1 by the HA Gene Copy Annealed to Plasmid. Approximately 4 ng of poly(dC)-tailed dsDNA, prepared from the HA gene of influenza strain 29C, was annealed to an excess (approximately 150 ng) of poly(dG)-tailed pBR322 DNA. Terminal transferase reaction conditions were adjusted to produce polynucleotide tails of approximately 30 residues in each case. When the mixture, after annealing, was used to transform *E. coli*


Fig.1

FIGURE 1. Polyacrylamide gel electrophoresis of ss cDNA synthesised from the total RNA extracted from influenza A/NT60/68. $^{32}$P-labelled ss cDNA species were separated on a 2.6% polyacrylamide gel containing 7M urea. The cDNA bands are numbered according to the RNA gene segment to which they correspond in size (9).

RR1, 118 tetracycline-resistant bacterial colonies were obtained. Of these, 30 were transformed by plasmid with no detectable DNA inserted, although the background rate of transformation obtained with the poly (dG)-tailed plasmid itself was very low - approximately 2 colonies under these conditions. From the total, 34 colonies immobilised on nitrocellulose filters hybridised to a $^{32}$P-labelled, single-stranded HA gene-specific DNA probe, indicating that

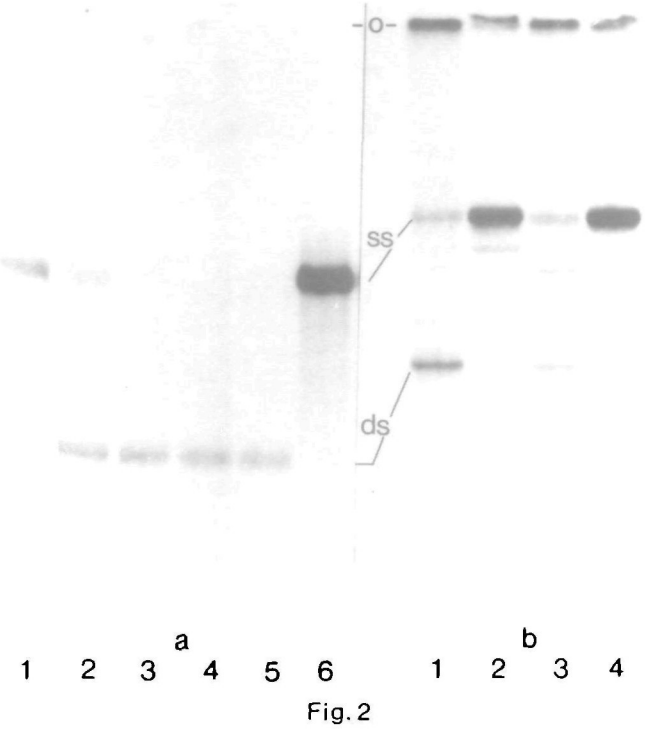


Fig. 2

FIGURE 2. *Conversion of ss cDNA corresponding to HA gene RNA to dsDNA by reverse transcriptase.* Reaction products were separated by electrophoresis on a 2.6% polyacrylamide gel. Results for influenza strain A/Mem/102/72 are shown in (a) and for A/NT60/68/29C and A/Eng/42/72 in (b). In (a) slots 1-5 show the progress of conversion of ss to dsDNA hourly over the 5 hour incubation period. Slot 6 shows the original ss cDNA. In (b), slots 2 and 4 show ss cDNA for strains 29C and A/Eng/102/72 respectively, while slots 1 and 3 show the conversion of the ssDNA to dsDNA under the standard preparative reaction conditions.

these bacteria were transformed by hybrid plasmids containing HA-specific DNA inserts (Fig. 4). The sizes of these inserts were of the size expected for a full length HA gene copy (approx. 1.8 kb) (results not shown). In the only case examined where a smaller hybrid plasmid was observed, it was found that the inserted DNA was still 1.8 kb long, but that part of the plasmid had been deleted, perhaps due to tailing at a nick within the plasmid.

The results described above for the HA gene copy from influenza strain 29C show a high incidence of apparently full-length dsDNA inserted into the plasmid. The material used in this experiment was of approximately uniform

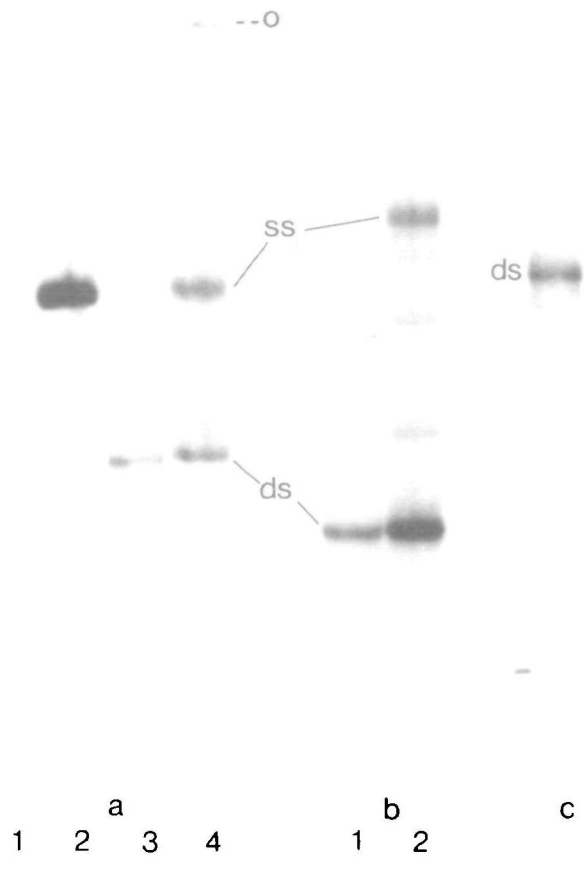


FIGURE 3. Digestion of ds cDNA with $S_1$ nuclease. Reaction products were separated by electrophoresis on a 2.6% polyacrylamide gel. (a) The conditions for digestion were determined using influenza A/Mem/102/72 cDNA. Slots 1 and 2 show ssDNA after and before $S_1$ nuclease treatment. Slots 3 and 4 show the corresponding samples of dsDNA. In (b), dsDNA from influenza A/NT60/68/29C is shown before and after $S_1$ digestion (slots 2 and 1 respectively) and (c) shows the material from (b), slot 1, after the addition of 20-30 dC residues with terminal transferase.

size, since the band of poly(dC)-tailed dsDNA was separated from lower molecular weight material by elution from the gel shown in Fig. 3(c). In contrast, 8-10 ng of dsDNA prepared from the HA gene of the influenza strain A/NT60/68 was annealed to poly(dG)-tailed pBR322 without the final polyacrylamide gel sizing step. This material gave approximately 800 tetracycline-resistant bacterial colonies, when the transformation was carried out as

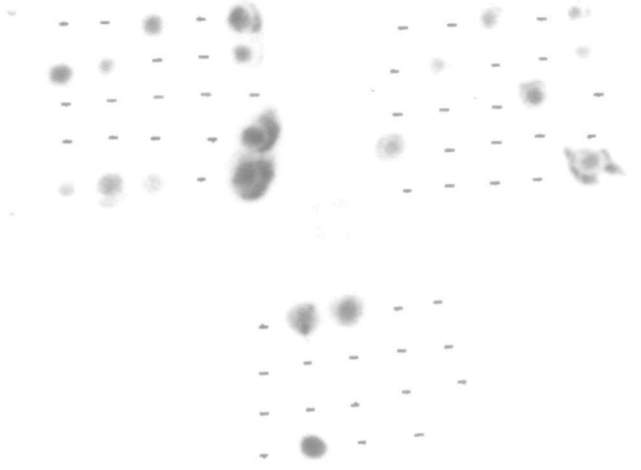

FIGURE 4. Identification of some of the E. coli RR1 colonies, grown on nitrocellulose filters, transformed by plasmids containing inserts of dsDNA prepared from gene 4 of strain 29C. Dashes indicate the position on the filters of colonies that failed to hybridise to the radiolabelled band 4-specific ssDNA probe, which was prepared from A/Mem/102/72 RNA. Filter 10 contained 25 colonies, none of which annealed to the DNA probe.

described above. The colonies, immobilised on nitrocellulose filters, hybridised to varying degrees with a ssDNA probe copied from the HA gene of influenza A/Mem/102/72. The amount of DNA inserted into the plasmid varied widely, but even among the most strongly hybridising transformants, no DNA insert equivalent to a full-length copy of the HA gene was identified. Since the dsDNA used for this transformation contained a prominent band of apparently full-length material similar to that shown in Fig. 3(c), the advantage of separating the largest dsDNA from lower molecular weight material before annealing to the plasmid is obvious.

Restriction Enzyme Mapping of Hybrid Plasmids. Preliminary experiments, in which $^{32}$P-labelled dsDNA was digested with selected restriction enzymes, gave some information on the restriction patterns expected for the copied HA gene. Similar patterns were observed when four of the cloned plasmids (C44, C38,

C74 and C89) containing the largest DNA inserts, were digested with an array of restriction enzymes and compared with fragments from plasmid pBR322. The four hybrid plasmids contained inserts of approximately 1850 bases. Allowing 40-80 bases for the poly(dC)-poly(dG) links, and 20-50 bases for the priming by $p(dT)_{12-18}$ of synthesis of the first DNA strand, these inserts are close to full-length copies of the HA gene, which has been estimated at 1760 (±) 40 bases (9). Restriction patterns obtained for C89 are shown in Fig. 5.

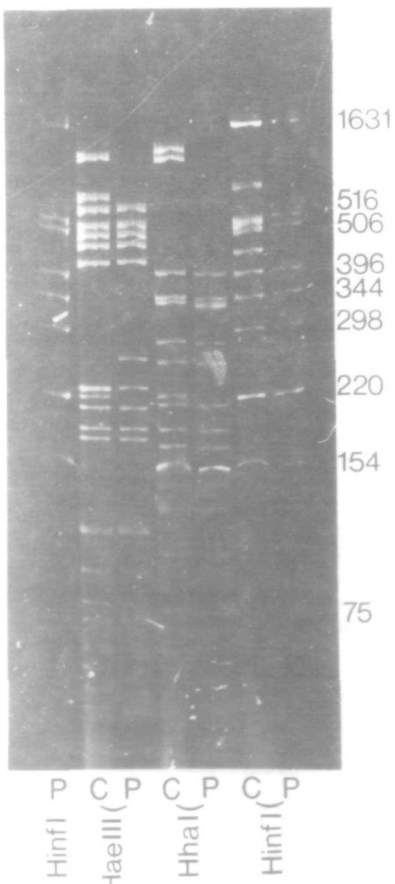


FIGURE 5. Restriction endonuclease digestion patterns for DNA from the plasmid pBR322 (P) and from cloned hybrid plasmid C89 (C), which contained DNA prepared from the HA gene of strain 29C. DNA fragments prepared by digestion with restriction enzymes HhaI, HaeIII and HinfI, were separated by electrophoresis on a 4% polyacrylamide gel. Figures on the right hand side show the sizes (in base pairs) of the HinfI digestion products of pBR322 (20).

Restriction enzyme digestion products of dsDNA before and after $S_1$ treatment were compared by electrophoresis on a denaturing (98% formamide) gel (21). The fragment which appeared as double length in the DNA sample not treated with $S_1$ was thus identified as the one containing the terminal hairpin loop. From this information it was possible to determine the orientation of the DNA inserted into the plasmid; in C89 and C78 the orientation was opposite to that in C38 and C44. Some minor variations in restriction patterns were observed even among cloned plasmids with similarly oriented inserts, probably attributable to slight differences in the lengths of the poly(dC)-poly(dG) joints.

A series of single and double restriction enzyme digests of C89 DNA provided the data for the preliminary restriction enzyme cleavage map shown in Fig. 6. DNA was separated by electrophoresis on 4-6% polyacrylamide gels or on 1.2% agarose gels, so that any very small fragments, resulting from cuts at adjacent sites by the same enzyme, would not have been detected. The restriction enzyme cleavage map was the basis for identifying and isolating suitable DNA fragments for nucleotide sequence analysis.

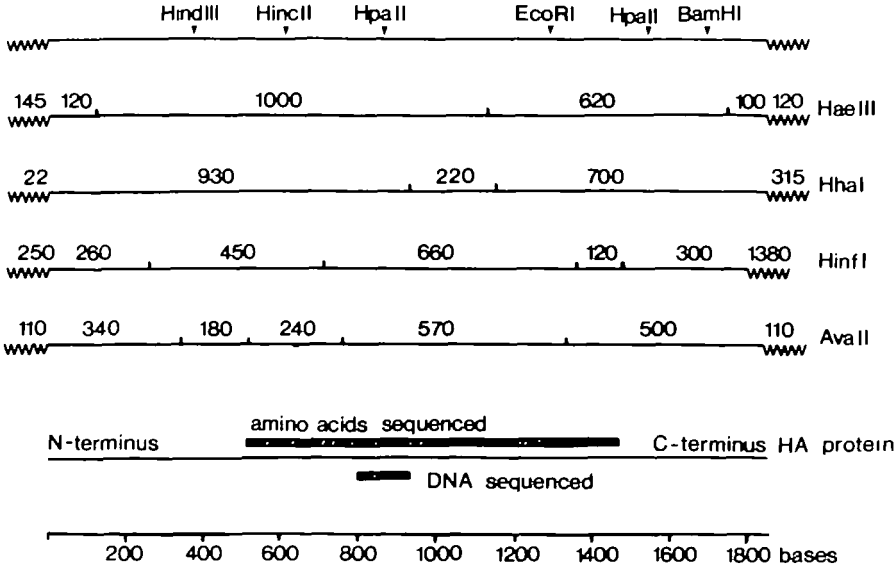


FIGURE 6. Preliminary restriction enzyme cleavage map of the cloned dsDNA copy of the HA gene from strain 29C. Wavy lines are plasmid DNA attached to the ends of the insert. The map also shows the corresponding sections of HA whose amino acid sequence has been published (22) and the DNA fragment which was prepared for nucleotide sequence analysis.

Nucleotide Sequencing of the C89 Insert. Although hybridisation with the HA gene-specific DNA probe, and the similarity of restriction enzyme digestion products suggested that the DNA inserted into plasmid C89 was an HA gene copy, conclusive proof could only be obtained by correlating a part of the nucleotide sequence with the known amino sequence for HA. Thus, plasmid C89 was digested with AvaII and the fragment ends were radioactively labelled by filling them in with reverse transcriptase in the presence of $\alpha^{32}$P-dATP. The AvaII fragments were re-cut with HhaI, and separated by gel electrophoresis. Part of the sequence of a fragment of approximately 130 bases eluted from the gel is shown in Fig. 7. It begins 7 bases from the AvaII cut, and represents the 3'→5' sequence of the non-coding strand. Conversion to the 5'→3' polarity of the complementary strand shows that the base sequence predicts an amino acid sequence which agrees with that determined for amino acids 53-77 of the CNBR-2 fragment of HA from the influenza strain A/Mem/102/72 (22) (Fig. 8). Exceptions occur at residues 74, where Ile in Memphis appears to be a Val in strain 29C, and at residue 58 where there is a Leu → Gln change. This latter change has also been observed in comparisons of peptides from these strains (23). Thus, the comparison of nucleic acid and amino acid sequence data shows conclusively that the DNA inserted in plasmid C89 is a copy of the HA gene.

## DISCUSSION

We have described procedures for the preparation of a dsDNA copy of the RNA gene coding for HA from the strain 29C. Plasmid pBR322 DNA annealed to the ds cDNA was then used to transform E. coli RRl in order to provide large amounts of cloned DNA for subsequent analysis. Proof that the DNA inserted into cloned hybrid plasmid was an authentic HA gene copy was obtained by comparing the nucleotide sequence of a fragment of the inserted DNA with the amino acid sequence from the corresponding region of the protein.

A variety of RNA molecules has now been copied and cloned, using the same series of basic steps followed in this work. However, we found it necessary to optimise conditions at each stage, since even under the most favourable conditions, approx. 50 μg of total influenza RNA yielded only ~2 ng of the full length dsDNA copy. The procedure we have described seems to be suitable for copying and cloning the HA gene from several different influenza strains, and may be applicable to the other genome segments as well. A similar protocol for the synthesis of dsDNA copies of the genome segments from Fowl Plague Virus has recently been described (24).

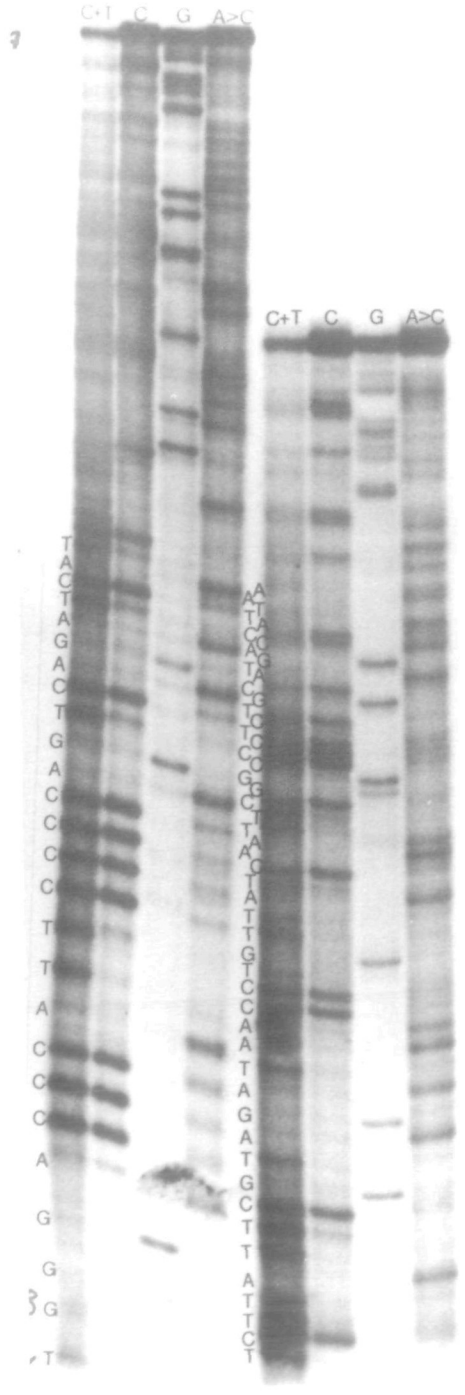


FIGURE 7. Sequencing of a 130 base pair fragment of inserted dsDNA from the cloned hybrid plasmid C89. The AvaII/HhaI restriction fragment, labelled at the AvaII-cut end, was partially degraded according to the procedure of Maxam and Gilbert (19). Reaction products were separated by electrophoresis on a 20% polyacrylamide gel containing 7M urea.

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      5'  A CCC UGG GUA AGG GGU CAG UCU AGU AGA AUA AGC AUC
29C       PRO TRP VAL ARG GLY GLN SER SER ARG ILE SER ILE
Mem       PRO TRP VAL ARG GLY LEU SER SER ARG ILE SER ILE
                  55

          UAU UGG ACA AUA GUU AAG CCG GGA GAC GUA CUG GUA AUU  3'
29C       TYR TRP THR ILE VAL LYS PRO GLY ASP VAL LEU VAL ILE
Mem       TYR TRP THR ILE VAL LYS PRO GLY ASP ILE LEU VAL ILE
          65                                  75
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FIGURE 8. The base sequence for C89 DNA determined from Fig. 7 (top line) and the amino acid sequence it predicts (middle line) are compared with the amino acid sequence determined for the corresponding region of HA from A/Mem/102/72 (bottom line) (22). The two amino acids not conserved between the two strains are enclosed in boxes; the altered nucleotides responsible for the changes are marked (•). The numbers refer to the amino acid residues of the CNBr-2 fragment of Memphis HA (22).

With the detailed restriction enzyme cleavage map prepared for C89, it will now be possible to select suitable restriction fragments to use as probes in cloning HA gene copies from other influenza strains, and also to determine the complete nucleotide sequence of the gene copy inserted in C89. In this way we should be able to study antigenic variation in HA much more readily at the genetic level than has been possible at the protein level. Already our nucleotide sequencing data have detected changes in amino acid sequence between the HA of A/Mem/102/72 and 29C, at least one of these changes being confirmed by peptide mapping data (23). The changes can be attributed to single base substitutions in the RNA, but whether the amino acid changes contribute to antigenic variation will not become apparent until many more influenza strains can be compared in the same way.

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