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The sequence of RNA segment 1 of influenza virus A/NT/60/68 and its comparison with the corresponding segment of strains A/PR/8/34 and A/WSN/33

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ABSTRACT

The complete nucleotide sequence of RNA segment 1 of influenza virus A/NT/60/68, corresponding to the PB2 protein, has been determined. It is 2341 nucleotides long, encoding a predicted product of 759 amino acids with a net charge of +27½ at neutral pH. The predicted amino acid sequence has been compared to the equivalent sequences in influenza viruses A/PR/8/34 and A/WSN/33. Evolutionary divergence, assuming a direct lineage from A/PR/8/34 and allowing for "laboratory drift", is 0.08% per year. The alignment of RNA segment 10 of A/NT/60/68 with segments 1 and 3 is completed, confirming that it is a mosaic of regions from these two segments.

INTRODUCTION

Influenza A viruses have negative stranded, segmented RNA genomes consisting of 8 essential segments (1), which code for at least 10 proteins (2). The largest of these segments are over 2,200 nucleotides long and code for the 3 polymerase proteins (PB2, PB1, PA)(3,4). Together with the viral RNA and nucleocapsid protein (NP), they make up the active transcribing structures (5,6). RNA segment 1 of influenza virus A/NT/60/68 encodes the PB2 protein (the smaller of the 2 basic P proteins), found in other influenza strains, to recognise host cell cap 1 structures (7-11). The capped RNA is cleaved 10-14 nucleotides from its 5' end (12-14), preferentially after a purine residue (15), by a cap 1-dependent virion endonuclease (15), thus producing an RNA primer. The larger basic P protein (PB1) appears to catalyse the addition of the first ribonucleotide (7,10), which is complementary to the 2nd or 3rd residue of the viral RNA (14,16) to the free 3' OH group of this primer. Additional processes such as elongation and termination of the nascent RNA chain remain to be assigned to specific proteins, although the acidic P protein (PA) may be involved in at least one of these functions (10). The proposed function of the 3 polymerase proteins and cross reference to the older nomenclature is summarized in Table 1.

TABLE 1 Function and nomenclature of polymerase proteins

RNA segment	Previous nomenclature	New nomenclature	Proposed function
1	P3† or P2*	PB2	Cap recognition
2	P1	PB1	Initiation of transcription
3	P2† or P3*	PA	?Elongation

† in A/PR/8/34 (38) * in A/FPV/Rostock (37)

Influenza is still a cause of mortality among the elderly. Specific vaccines directed against the surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA) are in use and have a protective value but the high rate of evolution of these proteins reduces the potential lifetime of such agents (17). The P proteins represent an alternative target for anti-viral agents, and it is of great interest to elucidate their mode of action in an attempt to give both a deeper understanding of the virus and to design physiologically safe drugs.

In the present study, we present the nucleotide sequence of RNA segment 1 of influenza virus A/NT/60/68, which codes for the PB2 protein. Its predicted amino acid sequence is compared with the corresponding segments of influenza viruses A/PR/8/34 (18) and A/WSN/33 (19) in order to estimate its rate of evolution and to look for conserved regions of possible functional significance. This sequence also completes the comparison of RNA segment 10 (20), a small RNA isolated from a preparation of A/NT/60/68, with segments 1 and 3 of A/NT/60/68 (21). We confirm that segment 10 is indeed a mosaic of these 2 segments, formed by some form of intragenic recombination.

MATERIALS AND METHODS

Cloning of full length segment 1 RNA of A/NT/60/68 Double stranded DNA was synthesized from a mixture of full length cDNA of segments 1, 2 and 3 of A/NT/60/68 virion RNA (kindly provided by Dr B M Moss) and cloned in the PvuII site of pBR322 as previously described (22,23). This entailed the use of 2 oligonucleotide primers such that the first 12 and last 13 nucleotide residues of the cloned RNA segments were derived from these primers and could differ slightly from the RNA sequence. 240 potential recombinants were first screened (24) with $[^{32}P]$ labelled short-copy cDNA obtained from A/NT/60/68

virion RNA allowing us to isolate full length segment 2 and segment 3 clones as previously described (22). Surprisingly, segment 1 clones were absent in the initial screening but were subsequently detected by screening (24) with $[^{32}P]$ labelled short-copy cDNA derived from the heterologous virion RNA, A/USSR/90/77, also provided by Dr B M Moss. Two independent segment 1 clones, referred to as A/NT/60/68/1 and 2, gave a distinctive HinfI restriction enzyme profile when analysed by 5% polyacrylamide gel electrophoresis versus marker segment 2 and segment 3 clones (results not shown). A/NT/60/68/1 and 2 differed from one another in a total of 7 of their HinfI bands (results not shown), suggesting they were cloned in opposite orientations in the PvuII site of pBR322 and that, in addition, clone 2 lacked an internal HinfI site. Sequencing of residues 1174 to 1428 confirmed this hypothesis (36). Subsequent cloning of material from the same full length double-stranded DNA preparation into a modified pBR322 vector gave 2 clones with an internal HinfI restriction pattern identical to that of clone 1. Thus 3 out of 4 RNA segment 1 clones are taken to be identical to that sequenced.

Subcloning of sonicated fragments in M13mp9 and sequencing 15 μgs of plasmid DNA (25) derived from clone A/NT/60/68/1 was sonicated (26) to an average size of 400 base pairs and the ends repaired by incubation with the Klenow subfragment of E.coli DNA polymerase I in the presence of all 4 deoxynucleoside triphosphates. After heating the reaction mixture at 70° for 10 mins, a one-tenth aliquot was blunt-end ligated (27,9) using T4 DNA ligase (from N. Gascoyne) to approximately 5 μg of SmaI cut and calf intestinal phosphatase treated (23) M13mp9 (28). The ligation mixture (10 μl) was used to transform competent E.coli JM103 (29) and recombinants selected as clear plaques by insertional inactivation of β-galactosidase (29). 200 recombinants were transferred as an ordered array onto a lawn of E.coli JM103 on a fresh plate and phage was transferred by blotting in duplicate onto nitrocellulose filters for screening (30) for specific influenza segment 1 containing sequences. Two probes were used: firstly a $[^{32}P]$ labelled cDNA prepared against an A/NT/60/68 vRNA template (23) in the presence of all 4 deoxynucleoside triphosphates, and secondly 1 μg of A/NT/60/68 vRNA was partially degraded by boiling for 15 mins in 2 μl of deionised formamide in 1mM $MgCl_2$ and labelled with $[\gamma\text{-}^{32}P]$ ATP using T4 phosphokinase (31). Free deoxynucleoside triphosphates were separated on a 1ml Sephadex G100 column. Single stranded DNA from the 60 specific influenzal clones was prepared by standard procedures (32). The phage inserts were sequenced using the Sanger dideoxy sequencing technique (33) in conjunction with a 'universal' 17 nucleotide long primer (34). 95%

of the sequence was obtained in this manner. A consensus of the sequence data was prepared using the Staden programs (35). Two contigs were constructed, with an intervening unknown region, estimated to be 10-20 nucleotides (corresponding to nucleotides 689-703) in length by comparison with the known sequence of segment 1 of A/PR/8/34 (18). To complete this unknown region, a BstN1 restriction fragment was labelled and sequenced by standard Maxam and Gilbert sequencing procedures (36). Further ambiguous regions were sequenced in this manner. Nucleotides 2165-2314, 490-658 and 863-921 were obtained by a common DdeI digestion followed by digestion of the specific bands with AccI, RsaI and RsaI respectively. The sequence shown in figure 1 represents a consensus of 75% of viral (negative) strand and 93% of the positive strand with 3% of this sequence, (nucleotides 1323-1333, 1371-1378 and 1851-1878 inclusive), present in a single copy. A consensus of the above information is available upon request.

RESULTS

Figure 1 shows the sequence of RNA segment 1 of influenza virus A/NT/60/68 as deduced from clone A/NT/60/68/1, written in the mRNA (positive) sense. It is 2341 nucleotides in length with a viral RNA base composition of 23% A, 24.6% C, 17.9% G and 34.5% U. There is one long open reading frame from nucleotide numbers 28-2304 coding for a predicted product of 759 amino acids. No other open reading frames longer than 250 nucleotides exist in the mRNA sense. Since the longest two other open reading frames contain neither a methionine residue nor a splicing acceptor site close to the 5' ends, and alternative forms of segment 1 mRNAs have not been demonstrated, it is unlikely they are utilized. The second clone, A/NT/60/68/2, with the altered HinfI profile (see Methods) has a G residue at nucleotide 1312, causing a change in the predicted amino acid sequence from Asn $\longrightarrow$ Asp at residue 429.

The predicted protein of 759 amino acids (Fig. 1) is basic, with a net charge of +27½ at pH 7.0, assuming glutamic and aspartic acid take the value -1, arginine and lysine the value +1, and histidine the value +½ and that no post-translational modification has occurred. The calculated molecular weight of 85,947 daltons, is consistent with the predicted weight of 80-100 daltons for the protein from SDS gels.

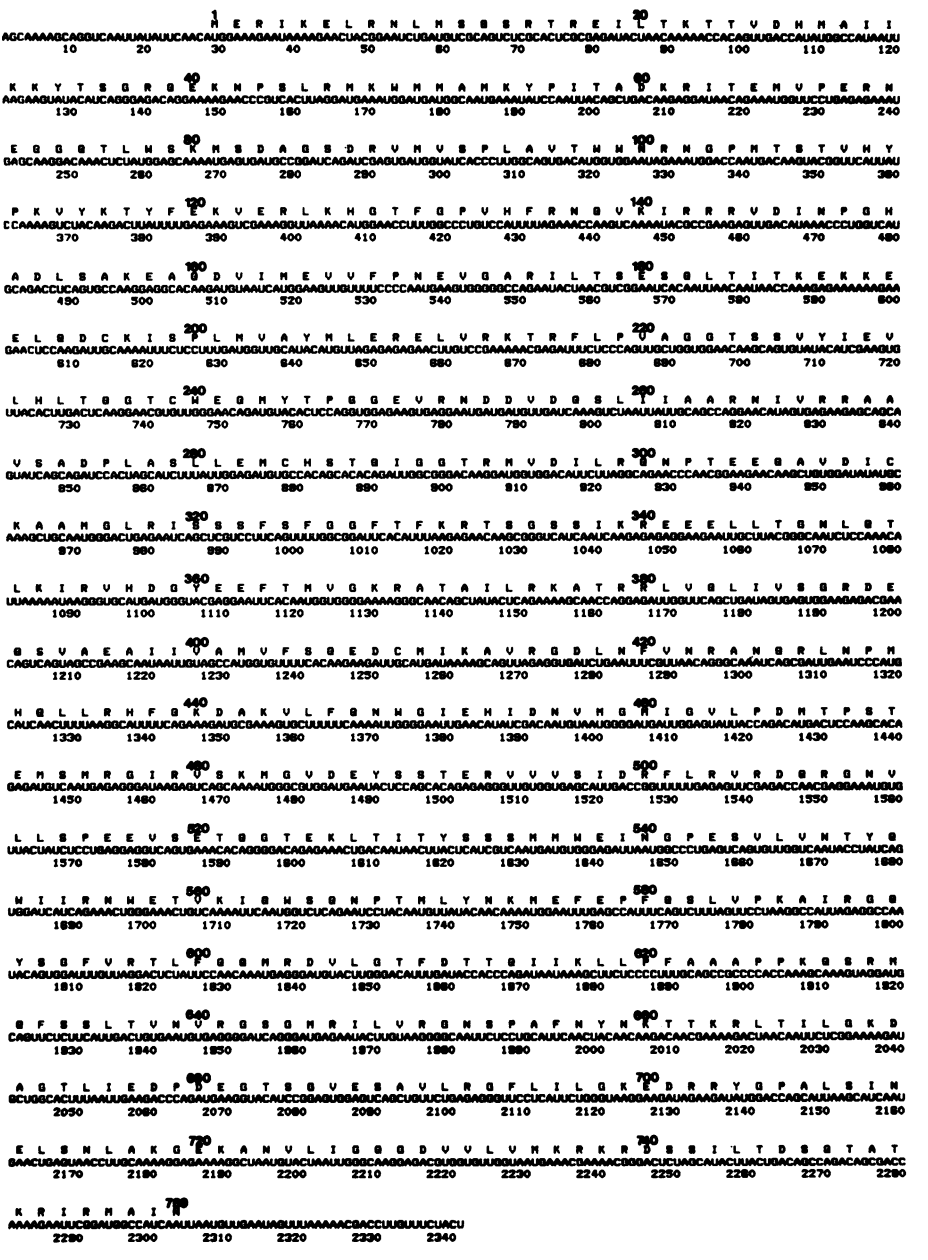

Figure 1. Nucleotide sequence of segment 1 of A/NT/60/68 and the predicted amino acid sequence of the PB2 protein.

DISCUSSION

1. Comparison of the predicted amino acid sequence of the PB2 protein among differing influenza A virus strains

Figure 2 shows the differences in the amino acid sequence between that reported here and the corresponding segments of A/PR/8/34 (18) and A/WSN/33 (38). Of the 30 amino acid differences (Fig. 2) between A/NT/60/68 and A/PR/8/34, 22 are due to single nucleotide mutations. The remainder show 2 substitutions within the codon, but, in each case, one of these could be a silent change. A similar comparison between the amino acid sequences of A/NT/60/68 and A/WSN/33 indicates 35 differences, 32 of which are single site mutations. 2 changes are due to 2 substitutions within a single codon and one nucleotide change is probably silent. In the single case of amino acid residue 453, all 3 nucleotides differ. Most amino acid changes between the three strains are conservative in nature (e.g. R → L) or semi-conservative (S → A, N → S). But 2 of the differences between A/PR/8/34 and A/NT/60/68 are non-conserved, at residues 309 (G → D) and 453 (P → H). Between A/WSN/33 and A/NT/60/68, 5 non-conserved changes occur at residues 195 (G → D), 296 (N → D), 630 (G → R), 740 (N → D), and 453 (S → H). These alterations give a slightly increased overall charge of +28 for the PB2 protein of A/PR/8/34 and +29 for A/WSN/33. We consider it unlikely that these non-conserved residues play an important functional role in Cap recognition because of their charge variability between different strains of influenza. The marked change in second-

Influenza strain	43	67	82	103	105	107	114	121	185	195	250	251	271	292	296
A/WSN/33	A	I	N		V		I		T	G	A		T	I	N
A/NT/60/68	S	V	S	G	M	S	V	K	I	D	V	R	A	T	D
A/PR/8/34	A	I	N	A		N	I	R				K		I	

Influenza strain	299	309	338	344	358	368	382	394	444	451	453	463	471	478	480
A/WSN/33			V	V	E	R	I	I	A		S	I		V	I
A/NT/60/68	K	D	I	L	D	K	V	V	V	I	H	V	T	I	V
A/PR/8/34	R	G	V	V	E	R	I	I		V	P	I	I	V	I

Influenza strain	491	493	494	588	613	630	636	638	655	661	667	674	676	684	740
A/WSN/33	A	K	I	V	A	G		I	I		V	P	T	A	N
A/NT/60/68	T	R	V	I	T	R	L	V	A	T	I	T	I	S	D
A/PR/8/34					A		F		V	A	V		T	A	

Figure 2. Amino acid differences between the PB2 proteins of A/NT/60/68, A/WSN/33 and A/PR/8/34.

ary structure which can occur upon substitution of a proline residue by any other amino acid suggests that residue 453 has limited structural significance.

If a similar comparison is carried out between the influenza viruses A/PR/8/34 and A/WSN/33, 27 differences are seen (Fig. 2). Since the initial isolates were obtained just a year apart, few changes might be expected between them. However, both viruses have been passaged within the laboratory, and in particular, A/WSN/33 has undergone repeated passage to select a neurotropic variant. We know from a comparison of the nucleotide sequences encoding the mature HA1 polypeptide (39,40), and the MS_2 (41,32) and NS (42, 27) proteins, that the predicted amino acid sequences can differ between different laboratory isolates of A/PR/8/34. The variation is 7 out of 325, 2 out of 96, 1 out of 230, and 2 out of 121 residues in the polypeptides HA1, MS_2, NS_1 and NS_2, respectively, or a mean average of 1.5% per protein. Hence, we might expect that "laboratory drift" accounts for some of the observed differences between the three strains, particularly in relation to A/WSN/33. A/NT/60/68 has been passaged infrequently in the laboratory; hence we think it reasonable to assume that little laboratory drift has occurred since its isolation in 1968.

In order to attempt to correct for the laboratory drift factor when estimating the rate of evolution of segment 1 from A/PR/8/34 or A/WSN/33 to A/NT/60/68, we have set up the hypothesis that the two earlier strains were identical when isolated. Alterations which occurred in the field are taken to be those where there is a common amino acid for the earlier strains, which differs from that found in A/NT/60/68, e.g. residues 43, 67, 82, etc. (a total of 20). All other alterations, e.g. 103, 105, 107, bar residues 453 and 655, are accredited to laboratory drift alone of either A/PR/8/34 or A/WSN/33. Positions 453 and 655, at which all 3 strains differ, are classed as field mutations for A/NT/60/68 and laboratory drift for A/WSN/33, since, if the A/PR/8/34 amino acid is taken as the ancestral residue, just a single amino acid change gives both the A/WSN/33 and A/NT/60/68 residues. There are 30 amino acid changes between A/PR/8/34 and A/NT/60/68 giving a value of 30/759 or 3.9% observed evolutionary drift. Assuming that 20 residues are actual field changes and the remainder laboratory drift we calculate a corrected evolutionary drift of 20/30 x 3.9% = 2.6%, or 0.08% per year. Furthermore, we calculate the laboratory drift of A/PR/8/34 to be 1.4% and that for A/WSN/33 to be 2.1%. These values are similar to the known laboratory drift of various proteins of A/PR/8/34 (see above).

Selection for the PB2 protein occurs regardless of whether drift

happens in the laboratory or the field. Thus comparisons of the amino acid sequence may still give an insight into functionally important regions. Amino acid alterations (Fig. 2) are randomly spaced throughout the molecule but tend to conserve its basic nature. This is compatible with its proposed function of recognising the negatively charged host cell cap 1 structures (7-11). The specific residues which bind the "cap" have not been identified to date. There are no obvious regions of high basicity in the primary sequence (Fig. 1) which may carry out this function, but this does not exclude their possible existence in the 3 dimensional structure. It would be of interest to determine the residues involved and to see if they are conserved between strains.

2. "Evolution" of influenza proteins

Possible amino acid sequence divergence from initial isolates during laboratory passage may prevent us determining the absolute rates of evolution of A/NT/60/68 proteins. Nevertheless, if the rate estimations are restricted to those between genes of two strains, the comparative rates of evolution of the genes can be studied. It is assumed that there is equivalent drift of the genes, that direct lineage exists and that no major reassortment occurred between the strains. Figure 3 shows the evolution of the proteins encoded by segments 1 (the sequence determined in this study), 2, 3, 4, 5 and 6 of A/NT/60/68 (21-23, 52, 53) and segments 7 (2) and 8 (54) of A/Udorn/72 with respect to the prototype virus A/PR/8/34 (18,27,32,39,55,56,51). Segments 7 and 8 of A/NT/60/68 have not yet been sequenced. Thus those of A/Udorn/72 were chosen to complete the comparison. The resulting percentages fall into two main groups, those less than 0.3% amino acid changes per year and those greater than 0.9%. All internal proteins are found in the first class of highly conserved molecules. The surface glycoproteins, the haemagglutinin (HA1 subunit) and neuraminidase (NA) show a marked percentage increase, indicating a larger variability. It should be noted that the figure of 1% for the HA1 domain and NA represents point alterations and does not include spaces or deletions included to allow a colinear comparison of the two sequences. It is therefore an artificially low value. Thus internal proteins are conserved because of selection to perform specific functions within the virion and the external proteins are variable because of antigenic selection by the host immune systems.

3. The Origin of RNA segment 10

One of the aims of this study was to confirm previous work defining the origin of a small RNA (segment 10) found in a preparation of A/NT/60/68 virus

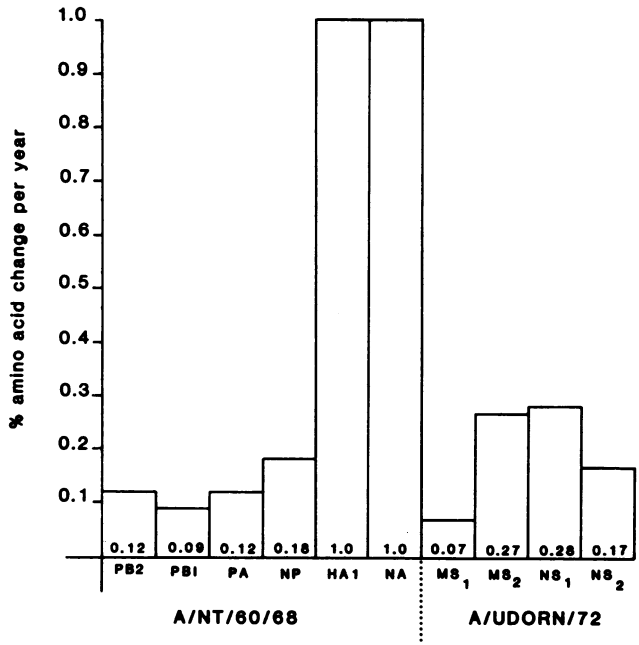

Figure 3. Histogram showing the different rates of evolution of viral proteins. Rates are calculated as % amino acid change of A/NT/60/68 for PB2, PB1, PA, NP, HA1, NA and for A/Udorn/72 for the MS and NS proteins with reference to A/PR/8/34. Data are calculated from references 22, 21, 23, 52, 53, 2, 54, 18, 55, 56, 39, 51, 32, and 27.

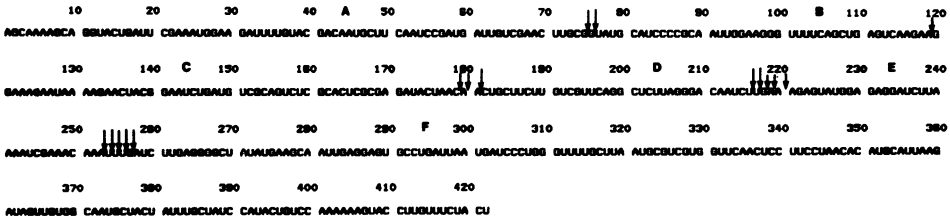

Figure 4. The nucleotide sequence of segment 10 of A/NT/60/68 and its alignment with segments 1 and 3 from A/NT/60/68. Arrows indicate alternative junctions between the sections.

Region A nucleotides 1 to (75 → 76) of segment 3
Region B " (1971 → 1972) to 2014 of segment 3
Region C " 30 to (89 → 91) of segment 1
Region D " (2015 → 2017) to (2051 → 2055) of segment 3
Region E " (86 → 89) to (126 → 130) of segment 3
Region F " (2065 → 2069) to 2233 of segment 3

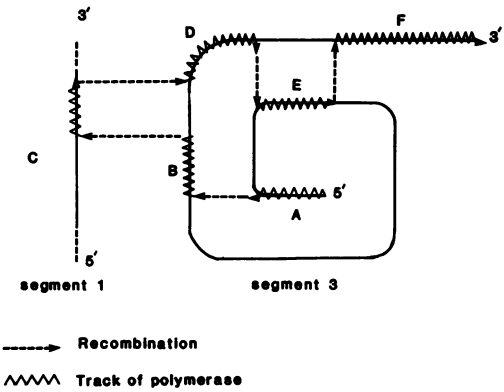

Figure 5. A hypothetical arrangement for intragenic recombination between RNA segments 1 and 3.

(20). Sequence analysis of this RNA and its comparison to segments 1 and 3 of A/PR/8/34 indicated a mosaic structure for segment 10 (18). Intragenic recombination had not previously been seen in influenza viruses. Figure 4 shows the alignment of segment 10 to specified regions of 1 and 3, found using a computer program to scan for homology (43). No mismatches within the regions were found, although ambiguities in the positioning of junctions remain. Figure 5 illustrates a hypothetical arrangement of segments 1 and 3 allowing such a recombination event by "polymerase jumping". The two ends of segment 3 are exactly reproduced in band 10.

4. General discussion

Control of influenza infection by the conventional methods of vaccination has achieved little success. A cap-recognising protein, such as PB2, presents a potential target for specific anti-influenza virus drugs. A 24K cap binding protein (CBP), found within the host cell, has also been described (44,45). It would be of interest to compare the amino acid sequence and active residues of this protein to those of PB2 to study any functional similarities and to determine whether agents against PB2 might not also affect CBP. It has been shown that the RNA segment coding for the PB2 protein of A/PR/8/34 is involved in the recognition of target cells by an anti-influenza cytotoxic T cell line (46). This may be due to the presence of a small amount of PB2 on the surface of an infected cell or an antigen-presenting cell or may be an indirect effect of PB2 on some other viral product.

Studies on functional P proteins have been limited by their scarcity within the virion (approximately 8% of total protein in the viral core (38)).

The development of both eukaryotic (47,48) and prokaryotic expression systems of cloned genes (49,50) should allow increased quantities of these proteins to be made available for research.

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