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Norton, Gerard Patrick

I. ESTABLISHMENT OF A SENSITIVE ASSAY SYSTEM FOR THE
DETECTION OF NONSENSE SUPPRESSOR TRANSFER-RNA ACTIVITY IN
HIGHER EUKARYOTIC CELLS AND II. ANALYSIS OF INFECTIOUS
INFLUENZA A AND B VIRUS VARIANTS WITH LONG CARBOXYL TERMINAL
DELETIONS IN THE NS1 POLYPEPTIDES AND III. EXAMINATION OF
INFLUENZA VIRUS GENE PRODUCTS EXPRESSED BY VACCINIA VIRUS
RECOMBINANTS

City University of New York

Ph.D. 1987

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POLYPEPTIDES
AND
EXAMINATION OF INFLUENZA VIRUS GENE PRODUCTS EXPRESSED BY
VACCINIA VIRUS RECOMBINANTS

by

GERARD P. NORTON

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Biomedical Sciences in partial fulfillment of the
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1987 .

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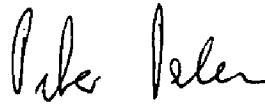
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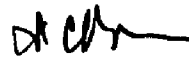
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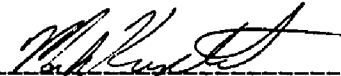


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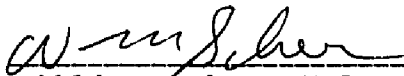
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Abstract

Establishment of a sensitive assay system for the detection of nonsense suppressor tRNA activity in higher eukaryotic cells,

and

Analysis of infectious influenza A and B virus variants with long carboxyl terminal deletions in the NS1 polypeptides

and

Examination of influenza virus gene products expressed by vaccinia virus recombinants.

by

Gerard P. Norton

Adviser: Professor Peter Palese

Establishment of a sensitive assay system for the detection of nonsense suppressor tRNA activity in higher eukaryotic cells.

The assay used for the measurement of suppressor tRNA activity in eukaryotic cells is based on the tRNA-mediated readthrough of either amber or ochre stop codons in influenza A virus NS1 cistrons (Young et al., 1983b). The suppressed NS1 readthrough product can be quantitated following electrophoresis of the cell extract on SDS polyacrylamide gel electrophoresis. Suppressor tRNA genes were cloned into the late region of SV40 DNA and the recombinant vectors were used to express suppressor tRNA

activity in infected CV-1 cells (Laski et al., 1982, 1984; Ho et al., 1986). The amount of readthrough NS1 gene product after superinfection of these cells with influenza viruses indicated that the efficiency of suppression was 31-49%. Permanent CHO cell lines expressing suppressor tRNA activity were also established (Ho et al., 1986). Cells containing an average of 200 copies of the amber suppressor tRNA gene showed a 3% level of suppression. An improved detection of the readthrough NS1 protein by Western blot analysis allowed identification of suppressor tRNA activity of only 1% in cells containing an average of 20 copies of the suppressor tRNA gene. Thus the suppressor tRNA activity in permanently expressing cells correlates with the number of copies of suppressor tRNA genes contained in the cells. However, the level of suppressor tRNA activity that can be achieved in cells permanently expressing tRNA genes is much lower than that of cells lytically infected with SV40 tRNA gene recombinant vectors.

Analysis of infectious influenza A and B virus variants with long carboxyl terminal deletions in the NS1 polypeptides.

An influenza A virus, A/turkey/Oregon/71, was shown by protein gel analysis to code for an NS1 protein approximately half the size of those of other influenza A viruses. Sequence analysis of the NS gene of this virus

revealed a 10 nucleotide deletion resulting in an NS1 reading frame of only 124 amino acids. This truncated NS1 polypeptide retained its karyophilic pattern as detected by immunofluorescence analysis of virus infected cells. Also, the A/turkey/Oregon/71 virus grew to high titer in embryonated chicken eggs in comparison to other influenza A viruses.

A laboratory variant of an influenza B virus, clone 201, was identified which codes for a truncated NS1 protein. Sequence analysis of the NS gene revealed a 13 nucleotide deletion resulting in a shortened NS1 protein of only 127 amino acids. Two other influenza B virus NS genes which have been sequenced possess NS1 proteins of 281 amino acids. As shown for the NS1 proteins of other influenza B viruses, the truncated NS1 polypeptide of B virus clone 201 was found to localize in the nucleus of infected cells. It appears that large deletions in the carboxyl terminus of the NS1 proteins of influenza A and B viruses can be tolerated without affecting the functional integrity of the NS1 polypeptide.

Analysis of influenza virus gene products expressed by vaccinia virus recombinants.

Vaccinia virus recombinants containing the cloned hemagglutinin (HA) genes of influenza viruses, A/PR/8/34

(H1 subtype) and A/Japan/305/57 (H2 subtype), directed the expression of influenza viral HA proteins in infected CV-1 cells. Western blot analysis revealed that these vaccinia-HA recombinant vectors produced levels of HA proteins approximately 1/2-1/5 of those seen in cells infected with the appropriate influenza viruses. Migration of the vector-produced HA protein on SDS gels was noticeably slower than that of HA protein induced by influenza virus infection. However, the apparent molecular weight of the HA protein expressed by the vaccinia-HA vector decreased after neuraminidase treatment. This result suggests that the vector-produced HA protein contained neuraminic acid residues.

The PB1, PB2, PA, NP and NS genes of influenza A/PR/8/34 virus were cloned into vaccinia virus. The expression of these influenza virus gene products from the respective vaccinia recombinant virus-infected cell extract were analyzed by immunoblot techniques.

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Abbreviations

BAP	bacterial alkaline phosphatase
BPB	bromophenol blue
CEF	chicken embryo fibroblast
CHO	Chinese hamster ovary
CV-1	African green monkey kidney
DDW	double distilled water
DMTr	dimethoxytrityl
DTT	dithiothreitol
EtBr	ethidium bromide
IPTG	isopropyl-B-D-thiogalactopyranoside
MDCK	Madin Darby canine kidney
MOI	multiplicity of infection
MTX	methotrexate
m. u.	map unit
NaOAc	Na Acetate
NC	nitrocellulose
NTE	NaCl, Tris-HCl, EDTA buffer
PCK	primary chicken kidney
PFU	plaque forming units
PNK	polynucleotide kinase
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SSC	NaCl, Na Citrate
TCM	Tris-HCl, MgCl ₂ , CaCl ₂ buffer
TE	Tris-HCl EDTA buffer

TY **tryptone yeast**

X-gal **5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside**

Specific Aims

A. Recently developed genetic engineering techniques have made it possible to introduce nonsense suppressor (Su^+) tRNA genes into higher eukaryotic cells. My objective was to further develop a system to study the expression of Su^+ tRNA genes using a suppressor tRNA assay based on tRNA-mediated readthrough of an influenza virus NS1 protein (Young et al., 1983b). This assay was successfully used by me to determine the level of suppression in CV-1 cells infected with SV40 recombinant viruses containing (Su^+)tRNA^{Tyr}, (Su^+)tRNA^{Gln} or (Su^+)tRNA^{Lys} genes (Laski et al., 1984; Ho et al., 1986). Furthermore, it has been used to measure suppression in CHO cells permanently expressing a human lysine suppressor tRNA (Ho et al., 1986). It was also my aim to determine if natural suppressor tRNA activity exists in higher eukaryotic cells. In order to realize this goal, I devised a more sensitive suppressor tRNA assay using Western blotting to quantitate the amount of NS1 readthrough product. Although this assay reliably detected a level of suppression between 0.1 to 0.5%, natural suppressor tRNA activity was not detected in CHO cells.

B. Another aspect of my thesis was to determine if the overlap that exists between the carboxyl terminal coding region of the NS1 polypeptide and the main body of the NS2 coding region was essential for the infectivity of

influenza A and B viruses. Two influenza viruses, (A/turkey/Oregon/71 and clone 201) were found to have deletions in the NS1 coding region of the NS gene such that the sequences coding for the carboxyl terminal region of the NS1 polypeptide and the main body of the NS2 polypeptide do not overlap (Norton et al., 1986). Despite these deletions these viruses behaved like wild-type influenza A and B viruses.

C. A number of vaccinia virus recombinants containing cloned cDNA copies of influenza virus genes were constructed by Drs. G.L. Smith and B. Moss at the National Institutes of Health for possible use in vaccine production. My goal was to examine, by Western blot analysis, these vaccinia virus recombinants for their ability to express these cloned influenza virus genes. The hemmagglutinin, nucleoprotein and NS1 protein were all found to be expressed to the same level in these vaccinia recombinant viruses as in influenza A/PR/8/34 virus-infected cells.

The following publications were published during my graduate studies at the Mount Sinai School of Medicine and represent a collaboration with Dr. Costantin A. Bona.

Mond, J.J., G. Norton, W. E. Paul, I. Scher, F.D. Finkleman, S. House, M. Schaefer, P.K.A. Mongini, C. Hansen and C. Bona. 1983. Establishment of an inbred line of mice that express a synergistic immune defect precluding in vitro responses to type 1 and type 2 antigens, B cell mitogens and a number of T cell-derived helper factors. J. Exp. Med. 158:1401-1414.

Waters, S.J., S.D. Waksal, G.P. Norton and C. Bona. 1984. Antigen recognition by a T cell clone outside the context of the major histocompatibility complex. J. Exp. Med. 159:305-312.

Moran, T.M., M. Monestier, A.C.K. Lai, G. Norton, M.A. Reale, M.A. Thompson, J.L. Schulman R. Riblet and C. Bona. 1986. Characterization of variable region genes and shared crossreactive idiotypes of antibodies specific for antigens of various influenza viruses. Viral Immunol. (in press).

Reale, M.A., A.J. Manheimer, T. Moran, G. Norton, C.A. Bona and J.L. Schulman. 1986. Characterization of monoclonal antibodies specific for sequential influenza A/PR/8/34 virus variants. J. Immunol. 137:1352-1358.

Introduction

Influenza virus

Influenza viruses are the only members of the orthomyxoviridae family, and are classified into three distinct types (A, B, and C), based on antigenic differences between their nucleoprotein (NP) and matrix (M) protein (Pereira, 1969). The orthomyxoviruses are enveloped animal viruses of approximately 100 nm in diameter, with a segmented genome of 8 single-stranded RNAs of negative polarity (Palese, 1977a). Influenza A viruses have been isolated from humans, mammals and birds, and are classified into subtypes according to their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), (Table 1). Influenza B and C viruses have not been divided into subtypes.

Influenza virus causes a highly contagious acute respiratory illness known as influenza. The 1918-1919 pandemic of the "Spanish influenza" was estimated to cause about 500 million cases and 20 million deaths worldwide (Robins, 1986). This pandemic generated much interest in determining the causative agent of influenza. It was subsequently found that swine influenza could be transmitted to ferrets by filtered mucus, indicating that the causative agent was a virus (Shope et al., 1931). The

TABLE 1. Hemagglutinin and neuraminidase subtypes of influenza A viruses isolated from humans, swine, horses and birds.

Subtypes	Species of origin ^c			
	Humans	Swine	Horses	Birds
Hemagglutinin:				
H1 ^a (H0, H1, Hw) ^b	PR/8/34	Sw/Ia/15/30	-	Dk/Alb/35/76
H2 (H2)	Sing/1/68	-	-	Dk/Ger/1215/73
H3 (H3, Hw7, Hwq2)	HK/1/68	Sw/Taiwan/70	Eq/Miami/1/63	Dk/Ukr/1/63
H4 (Hw4)	-	-	-	Dk/Cz/56
H5 (Hw5)	-	-	-	Tern/S.A./61
H6 (Hw6)	-	-	-	Ty/Mass/3740/65
H7 (Hwq1, Hwv1)	-	-	Eq/Prague/1/56	FPV/Dutch/27
H8 (Hw8)	-	-	-	Ty/Ont/6118/68
H9 (Hw9)	-	-	-	Ty/Wis/1/66
H10 (Hw10)	-	-	-	Ck/Ger/N/49
H11 (Hw3)	-	-	-	Dk/Eng/56
H12 (Hw10)	-	-	-	Dk/Alb/60/76
H13	-	-	-	Gull/Md/704/77
Neuraminidase:				
N1 (N1)	PR/8/34	Sw/Ia/15/30	-	Ck/Scot/59
N2 (N2)	Sing/1/57	Sw/Taiwan/70	-	Ty/Mass/3740/65
N3 (Nav2-3)	-	-	-	Tern/S.A./61
N4 (Nav4)	-	-	-	Ty/Ont/6118/68
N5 (Nav5)	-	-	-	Sh/Austral/1/72
N6 (Nav1)	-	-	-	Dk/Cz/56
N7 (Neq1)	-	-	Eq/Prague/1/56	FPV/Dutch/27
N8 (Neq2)	-	-	Eq/Miami/1/63	Dk/Ukr/1/63
N9 (Nav6)	-	-	-	Dk/Mem/546/74

^a Current subtype designation.

^b Previous subtype designation.

^c The reference strains of influenza viruses, or the first isolates from that species are presented.

^d Adapted from Murphy and Webster (1986).

first human influenza A virus was isolated in 1933 (Smith et al., 1933).

Influenza A virus undergoes continual antigenic change resulting in the isolation of new strains each year. Antigenic drift occurs in the HA and NA proteins of the virus via point mutations in these genes, which result in amino acid substitutions that alter the antigenic sites. Antigenic change can be reproduced in the laboratory by growing influenza viruses in the presence of monoclonal antibodies to HA protein. Antibodies to HA neutralize virus infectivity by binding at or near the antigenic regions of this protein. Antigenic variants that survive this antibody selection against the HA protein are isolated at high frequencies (Webster and Laver, 1980; Lubeck et al., 1980; Yewdell et al., 1979). Occasionally major antigenic variation is observed with influenza A viruses. This antigenic shift is associated with the emergence of pandemic viruses in the human population. Antigenic shift is thought to occur via a recombinational event between a human and animal virus that possesses different surface glycoproteins (Webster and Laver, 1975). Since 1918, major antigenic shifts occurred with the H1N1 subtype being replaced by the H2N2 subtype in 1957 which in turn was replaced by the H3N2 subtype in 1968. In 1977 the H1N1 subtype reappeared and continues to cocirculate with H3N2 viruses.

Structure of influenza virus

The structures of influenza viruses of type A and B are similar. The virion consists of two surface glycoproteins HA and NA for A (Fig.1) and B viruses. The HA and NA polypeptides are attached via a short stretch of hydrophobic amino acids to a lipid envelope which is derived from the membrane of the host cell. Beneath the lipid envelope lies the matrix protein which surrounds the ribonuclear core. This core consists of eight single-stranded RNA molecules, the nucleocapsid protein (NP) and the three P proteins (PB1, PB2, and PA). In addition, the virus codes for three nonstructural proteins (NS1, NS2, and M2) which are only in infected cells (Lamb, 1983). The eight gene segments of an influenza A virus and the proteins they encodes are described in Table 2.

The three largest proteins of the virus are PB1, PB2, and PA with apparent molecular weights of 96,000, 87,000 and 85,000 daltons and calculated molecular weights of 85,900, 86,500 and 82,400 daltons. Recombinant analysis of influenza A/PR/8/34 and A/HK/68 viruses revealed that RNA segment 1 (identified by migration on polyacrylamide gels after electrophoresis) codes for P3 (PB2), RNA segment 2 for P1 (PB1) and RNA segment 3 for P2 (PA) (Palese et al., 1977b; Ritchey et al., 1977). Using in vitro transcription experiments, PB2 was implicated in binding to

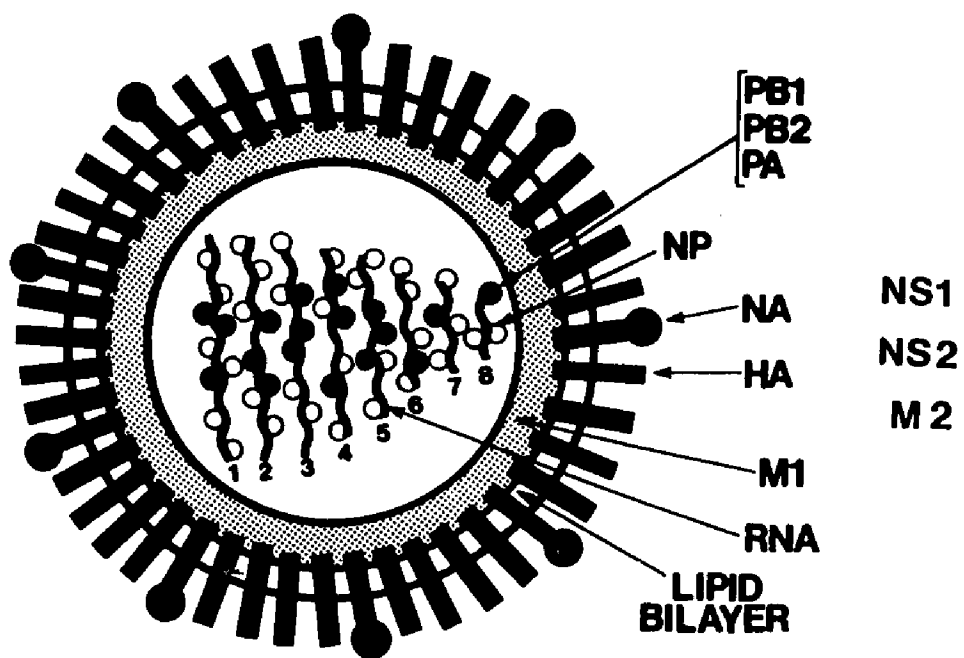


Figure 1. Schematic diagram of the structure of an influenza virus particle. Proteins PB1, PB2, PA and NP comprise the virion core and are associated with the genome RNAs. M1 is in close contact with the lipid bilayer. The HA and NA spikes are surface glycoproteins. The 8 single-stranded RNA segments are indicated by numbers 1 to 8. The proteins M2, NS1 and NS2 are only found in infected cells and are nonstructural proteins of the virus.

TABLE 2. INFLUENZA A VIRUS RNA AND PROTEINS^a

Segment	Length ^b (Nucleotides)	Gene Name	Encoded Protein	Amino Acid ^c Length (Nascent Protein)	Calculated Mol. Wt.	Approx. No. Molecules per Virion
1	2,341	PB2	PB2	759	86,500	30-60
2	2,341	PB1	PB1	757	85,900	30-60
3	2,233	PA	PA	716	82,400	30-60
4	1,778	HA	HA	566	64,000	500
5	1,565	NP	NP	498	56,300	1,000
6	1,413	NA	NA	454	51,300	100
7 ^d	1,027	M1	M1	252	28,500	3,000
		M2	M2	97	11,000	
8 ^e	890	NS1	NS1	230	26,000	
		NS2	NS2	121	13,700	
	13,588					

^aModified from Lamb (1983)

^bValues for A/PR/8/34 strain

^cDeduced from nucleotide sequence

^dRNA Segment 7 encodes 2 proteins

^eRNA Segment 8 encodes 2 proteins

the cap structure of the host cell mRNA primer (Ulmanen et al., 1981, 1983; Blaas et al., 1982a, 1982b; Penn et al., 1982) and PB1 was found to be associated with the initiation of transcription and elongation (Ulmanen et al., 1981; Plotch et al., 1981; Braam et al., 1983). Temperature-sensitive (ts) mutants defective in RNA synthesis suggested that PB1 and PB2 are involved in messenger RNA synthesis and PA and NP in virion RNA synthesis (Krug et al., 1975; Palese et al., 1977a; Scholtissek and Bowles, 1975).

The fourth largest RNA segment codes for the HA protein. The HA is responsible for attachment of the virus to cell surfaces via neuraminic acid molecules as well as for penetration of the virus into the cell during the early stages of infection (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Antibodies to the HA protein may neutralize the infectivity of influenza virus. The HA protein is synthesized as a single polypeptide that undergoes post-translational processing. An amino-terminal signal sequence is removed and further cleavage results in two disulfide-linked polypeptide chains, HA1 and HA2, with apparent molecular weights of 36,000 and 27,000, respectively. The HA on the surface of the virion exists as a trimer of non-covalently linked HA monomers (Roth et al., 1983). Cleavage of the HA into HA1 and HA2 is required for virion infectivity (Lazarowitz and Choppin,

1975; Klenk et al., 1975; Rott, 1979). The HA molecule is anchored to the viral envelope by the carboxyl terminal region of the HA2 polypeptide (Gething and Sambrook, 1982; Sveda et al., 1982).

The nucleoprotein is encoded by RNA segment 5 and is part of the internal complex that is associated with the RNA segments and the three P proteins (Compans et al., 1972). The NP protein is a type specific antigen of influenza viruses, because antibody to the nucleoprotein of A viruses is cross-reactive with the NP proteins of all A viruses, but not those of either B or C viruses. The NP protein of the A viruses possesses at least three nonoverlapping antigenic areas with one domain being identical in all strains tested. Monoclonal antibodies to this conserved domain inhibit in vitro transcription of viral RNA, suggesting that this region of NP protein is involved in RNA transcription (van Wyke et al., 1981). Recent data suggests that the NP protein is involved in influenza A virus transcription-antitermination of template RNA synthesis (Beaton and Krüg, 1986).

The neuraminidase (NA) surface glycoprotein is the second subtype-specific molecule on the virion and it is coded for by RNA segment 6. NA exists as a tetramer on the surface of the virus particle with the individual polypeptide chains (MW 56,000) held together by disulfide bonds. The NA protein has a neuraminidase activity which

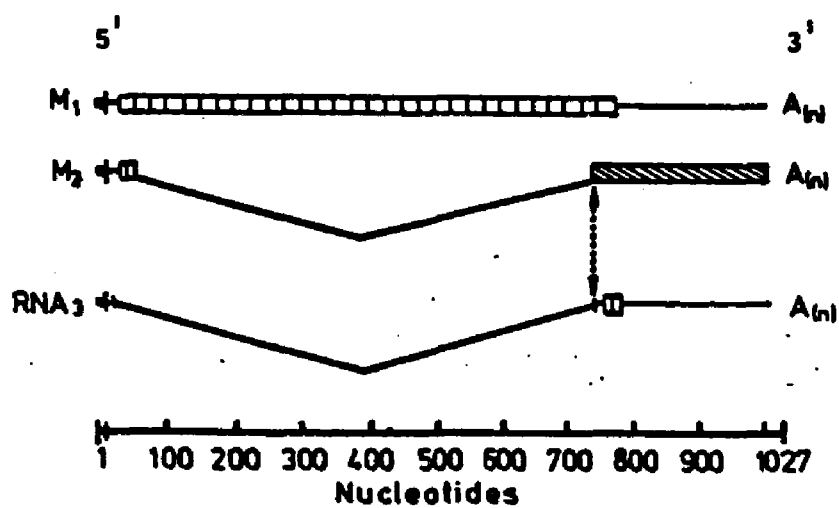
can hydrolytically cleave the ketosidic linkage between sialic acid and an adjacent sugar (Gottschalk, 1957). Mutants of influenza virus defective in neuraminidase form aggregates at the surface of infected cells, indicating that this enzyme functions to remove neuraminic acid residues from cell surfaces as well as from the hemagglutinin of virus particles (Palese and Schulman, 1974).

The influenza B virus NA gene codes for two proteins, NA and NB, from a bicistronic mRNA. NB has no homology with any of the influenza A virus proteins and its function is unknown (Lamb, 1982).

Unlike the HA protein, the signal sequence of the NA protein is not proteolytically removed after the protein is translocated across the ER membrane (Blok et al., 1982). Also in contrast to the HA protein, the NA protein is anchored in the viral lipid bilayer via its amino terminal sequence which is hydrophobic.

RNA segment 7 encodes two proteins, M1 and M2. Three mRNAs transcribed from RNA 7 have been isolated in virus infected cells (Fig.2). One mRNA is a colinear transcript of the genome segment and it is responsible for production of the M1 protein (matrix protein MW 27,500) which is a type-specific antigen. The M2 mRNA is generated from the M1 mRNA by a splicing event (Fig.2). The M2 protein (MW 15,000) has been identified only in infected cells and not

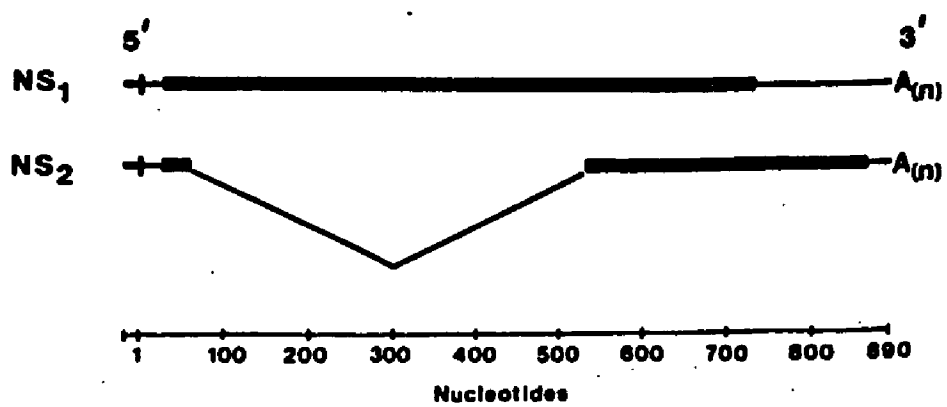
Figure 2. Structure of the mRNAs derived from RNA 7 (M gene) of influenza A virus. The filled-in boxes at the 5' ends of the mRNAs represent nucleotides derived from host cell mRNAs. The thin lines at the 5' and 3' termini of the mRNAs represent noncoding regions. The boxes designate coding nucleotides. The M2 mRNA is translated in a different reading frame from that used for the M1 in the region 740-1004. No evidence has been obtained that mRNA₃ is translated, but its coding potential (which corresponds to the COOH-terminal region of the M1 protein) is indicated by the dashed arrow. The V-shaped lines represent nucleotides removed by splicing in the M2 and mRNA₃ transcripts (from Lamb et al., 1981). The A(n) at the 3' end of mRNAs represent poly A tails. The M1-specific mRNA can code for a 248 amino acid-long polypeptide (box with verticle lines). The spliced M2-specific mRNA encodes a polypeptide which shares the first 14 amino acids with that of M1 (box with verticle lines).



in the virus and is therefore a nonstructural viral protein (Lamb and Choppin, 1981). Recently, it has been determined that M2 is an integral membrane protein that is expressed at the cell surface (Lamb et al., 1985; Zebedee et al., 1985). In addition, a third mRNA has been found that could encode an 8 amino acid-long peptide identical to the carboxyl terminus of M1 (Lamb and Choppin, 1981). However, such a peptide has not been shown to be synthesized in virus-infected cells.

The smallest RNA segment (NS) (890 nucleotides) of the influenza A virus genome encodes two nonstructural proteins, NS1 and NS2. (Lamb, 1983). The NS1 mRNA is a colinear transcript of the virion RNA, and the NS2 mRNA appears to derive from the NS1 mRNA through a splicing mechanism which deletes 473 nucleotides (positions 56 to 529) from the body of the message (Lamb and Lai, 1980) (Fig.3). This results in an open reading frame for the NS2 mRNA of 56 nucleotides that are shared with the NS1 protein. The remainder of the 121 amino acid-long NS2 protein is translated in a +1 reading frame (Lamb, 1983). The NS1 protein (MW 26,000) is synthesized early in infection and accumulates in the cell nucleus (Briedis et al., 1981), whereas the NS2 protein (MW 14,000) is made late in infection (Lamb et al., 1978). Influenza A virus NS1 and NS2 proteins have been localized in the nucleus of infected cells by immunofluorescence experiments (Young et

Figure 3. Structure of the mRNAs derived from RNA 8 (NS gene) of influenza A virus. The thin line at the 5' and 3' termini of the NS1 and NS2 mRNAs represent noncoding regions. The thick lines represent the coding regions of the two mRNAs. In the region 529-861 the NS2 mRNA is translated in a reading frame different from that used for NS1. The V-shaped thin line in the NS2 mRNA represents the interrupted sequence in the translated region of the NS2 mRNA. The medium line and bar before nucleotide 1 at the 5' termini represent the heterogeneous nucleotides derived from cellular mRNAs that are donated to the NS1 and NS2 mRNAs (from Lamb and Lai, 1980).



al., 1983a; Greenspan et al., 1985). The function of these two polypeptides in the virus replication cycle remains unclear.

The structure of the NS gene of influenza B viruses is similar to that of the A viruses (for review, Lamb, 1983; Air and Compans, 1983). The NS1 and NS2 proteins contain 281 and 122 amino acids, respectively. Eleven amino acids at the N-terminus of NS1 and NS2 are shared.

Suppressor tRNA mediated readthrough of influenza A viruses NS1 genes

Three of the sixty four possible triplet codons do not code for any of the twenty amino acids, but, rather signal polypeptide chain termination when encountered in the reading frame of a messenger RNA sequence. These three codons, UAG, UAA, and UGA, are termed nonsense or termination codons. When a termination codon is introduced via mutation into the reading frame of a messenger RNA sequence premature termination takes place at this site. Peptide chain termination occurs on the ribosome and requires soluble protein factor(s) (release factors RF) in both prokaryotes and eukaryotes (Caskey and Campbell, 1979). The effect of termination codons can be reversed by suppressor tRNA molecules: termed amber, ochre and opal. Amber, ochre, and opal suppressor tRNAs read the UAG, UAA, and UGA codons, respectively.

Transfer RNA suppression of nonsense mutations is a well-studied phenomenon which has been used successfully to analyze the genetics of prokaryotic and lower eukaryotic systems (Steege and Soll, 1979; Gorini and Beckwith, 1966; Capecchi et al., 1975; Sherman, 1982). Specifically, nonsense suppression by suppressor tRNAs was instrumental in studying T4 phage and lambda phage genes as well as RNA phages (Epstein et al., 1963; Miller et al., 1977; Horiuchi, 1975). In contrast, nonsense suppression has not been well studied in higher eukaryotes.

With the advent of recombinant DNA technology, it has now become feasible to take a second look at tRNA suppression in higher eukaryotic cells. Specifically, the availability of SV40 recombinant vectors containing amber and ochre Su⁺ tRNA genes (Laski et al., 1982; 1984; Ho et al., 1986) and the establishment and isolation of viral (Summers et al., 1975; Cremer et al., 1979; Summers et al., 1983; Rawlins and Muzyczka, 1980) and *Drosophila* (Kubli et al., 1982) nonsense mutants hold the promise of allowing the study of genomes of cellular and viral origin via this approach. Development of this technology, required a rapid and quantitative assay to determine the level of suppressor activity in a specific cell. One such assay for the measurement of amber suppressor activity has been developed. It is based on the tRNA-mediated readthrough of the amber stop codon in the NS1 polypeptide of influenza

Figure 4. Carboxyl terminus of the NS1 protein of influenza virus A/Cam/46 (H1N1). Su⁺ tRNA - mediated readthrough results in a product which is twenty amino acids longer than the amber codon-terminated polypeptide.

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A/Cam/46 virus (Young et al., 1983b) (Fig.4). Experiments described in this thesis rely on the utilization of this assay.

Natural suppression by suppressor tRNAs is best known in prokaryotic systems. For example, QB phage and bacteriophage lambda appear to rely on opal (UGA) suppression for replication to occur (Hofstetter et al., 1974; Engelberg-Kulka et al., 1979). In eukaryotic systems it appears that "natural" suppressor tRNAs are used for the synthesis of tobacco mosaic virus (Pelham, 1978) and possibly for that of Moloney leukemia virus (Philipson et al., 1978; Yoshinaka et al., 1985). Indirect evidence based on sequencing studies also suggests that translation of mRNAs of Sindbis and Middleberg viruses relies on the presence of natural suppressor tRNAs (Strauss et al., 1983). However, it has not been proven that the presence of suppressor tRNAs is required in any of these systems since in all cases alternative mechanisms could be responsible for the control of translation. Additional evidence pointing to the existence of tRNA suppression in higher eukaryotes derives from the following experiments. Geller and Rich (1980) and Diamond and Dudock (1981) reported the isolation of Su+ tRNAs from rabbit reticulocytes and bovine liver. In each case these tRNAs were shown to possess suppressor activity only in cell-free systems of reticulocyte origin. Furthermore, Hatfield et

al.(1983)and O'Neill et al.(1985) isolated clones containing genes coding for opal suppressor tRNAs from chicken liver and a human DNA library. Although these results suggest that tRNA-mediated suppression exists in higher eukaryotic cells, direct evidence for this does not exist.

During preparation of this thesis Capone et al. (1986) reported on a sensitive assay for detection of amber, ochre and opal suppressor tRNA activity in mammalian cells. The genes for suppressor tRNAs as well the bacterial chloramphenicol acetyltransferase (CAT) gene containing nonsense mutations were cotransfected into mammalian cells. They provided the first evidence that an opal suppressor tRNA gene is functional in mammalian cells. They also showed that different amino acids can be inserted at the same site of mutation in the CAT gene without loss of enzymatic activity.

Analysis of influenza virus gene products expressed by vaccinia virus recombinants

Recombinant DNA technology has made it possible to insert heterologous genes into a variety of different viruses. The development of such vectors has proven useful for the study of viral protein expression. Several viruses, including SV40 virus, bovine papillomavirus and retroviruses, have been used as expression vectors.

Influenza viral proteins have been successfully expressed in mammalian cells by these viral vectors containing cloned cDNA copies derived from influenza virus genes (Sveda and Lai, 1981; Gething and Sambrook, 1981; Hartman et al., 1982; Lin and Lai, 1983; Davis et al., 1983; Lamb and Lai, 1982; Krystal et al., 1984, 1986; Lamb and Lai, 1984; Penn and Mahy, 1984; Townsend et al., 1984; Lin et al., 1984; Sambrook et al., 1985; Braam-Markson et al., 1985).

In addition, Panicalli et al. (1983) and Smith et al. (1983) have reported that vaccinia recombinants containing the cloned hemagglutinin (HA) genes from influenza viruses expressed HA proteins in infected cells. The HA protein synthesized in cells infected with the vaccinia-HA vector was glycosylated and transported to the cell surface (Smith et al., 1983). Furthermore, the HA protein expressed in infected cells could be cleaved with trypsin into HA1 and HA2 subunits and reacted with specific anti-HA antibody (Smith et al., 1983). A segment of this thesis deals with the characterization of different influenza virus proteins (PB1, PB2, PA, HA, NP and NS1) expressed in vaccinia recombinant virus infected cells.

Materials and Methods

Influenza A and B virus growth and purification

The influenza A and B viruses used in this thesis are; A/Cam/46, A/Tex/1/68, A/turkey/Oregon/71, B/Lee/40, B/Yamagata/1/73 and clone 201. All influenza viruses were propagated in embryonated chicken eggs according to the following protocol. A 10^{-3} dilution of virus seed was made in phosphate-buffered saline (PBS), (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) containing 0.9 mM CaCl_2 , 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 U/ml penicillin G, 100 ug/ml streptomycin sulfate and 0.2% bovine albumin (Miles Scientific Laboratories, Inc.; Elkhart, IN). Virus (100 ul) was inoculated into the allantoic cavity of 10 day old embryonated eggs. Eggs were then incubated for 48 hours at 37°C or 35°C for A and B viruses, respectively. Following refrigeration of the eggs for 1 hour at -20°C, allantoic fluid was harvested, pooled and clarified by centrifugation at 10,000 rpm for 15 minutes. The supernatant fluid was removed and the virus titer was determined by the hemagglutination assay described below. Virus was concentrated by centrifugation at 4°C in a Ti45 rotor at 35,000 rpm for 1 hour. The supernatant fluid was decanted and the virus pellet was resuspended in NTE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The virus suspension was layered onto 30 ml continuous gradients of

30 to 60% (w/v) sucrose in NTE buffer. Gradients were formed in 25 x 89 mm ultra-clear SW27 tubes (Beckman Instruments, Inc.; Palo Alto, CA). Sucrose gradients were centrifuged in an SW27 rotor at 4°C for 3 hours at 25,000 rpm. After centrifugation, the bluish virus band was observed in the dark using a high intensity lamp and aspirated with a 10 ml syringe after puncturing the tubes. Virus was concentrated by first diluting the suspension 1:8 in NTE and pelleting it in an Ti45 rotor at 35,000 rpm for 45 minutes. Virus pellets were resuspended in NTE buffer for subsequent RNA extraction.

Quantitation of virus by hemagglutination assay

Virus was quantitated by the hemagglutination (HA) assay (Sever, 1962). A virus sample (25 ul) was added to the first well of a row in a 96 well Linbro microtiter plate (Flow Laboratories, Inc.; McLean, VA). Virus diluent (25 ul) was added to each well in the appropriate rows of the plate. Two-fold serial dilutions of the sample were made using 25 ul stainless steel diluters. An additional 25 ul of diluent was added to all wells followed by 50 ul of 0.5 % human type O or chicken red blood cells in PBS. The microtiter plate was then mixed on a Cooke microtiter mixer for 5 seconds followed by incubation on ice for 1 hour to allow for hemagglutination of red blood cells by the virus. The hemagglutination titer, expressed as

hemagglutinating units (HA U) per ml is the reciprocal of the highest dilution exhibiting hemagglutination.

Quantitation of influenza A and B viruses by plaque assay

Influenza A and B viruses were plaqued in Madin Darby canine kidney (MDCK) cells as reported by Tobita et al. (1975). Confluent monolayers of MDCK cells (10^6) in 35 mm dishes were infected with 0.1 ml of serial 10-fold dilutions of virus sample. Virus was allowed to adsorb to cells for 1 hour at 37°C and 35°C for A and B viruses, respectively. After adsorption, 2.5 ml of agar overlay medium consisting of REM medium (M.A. Bioproducts; Walkersville, MD) supplemented with 100 U/ml penicillin G, 100 ug/ml streptomycin sulfate, 0.2% bovine albumin, 2 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM HEPES, Earle's balanced salt solution, 0.1% (w/v) DEAE dextran, 0.6% (w/v) Oxoid agar (Oxoid Ltd; Hampshire, England), 0.1% (w/v) NaHCO_3 , and 1 ug/ml 1:250 trypsin (Difco Laboratories; Detroit MI) was added to each dish. The agar was allowed to solidify, then the dishes were inverted and incubated for 2 to 4 days at 37°C or 35°C. When plaques became visible, the agar overlay was removed with a spatula and the infected-cell monolayers were stained with a solution of 0.1% crystal violet (Fisher Scientific; Springfield, N.J.) and 20% methanol for 10 minutes. The stain was removed and the monolayers were washed with tap water.

Growth of SV40 recombinant virus stocks containing amber suppressor (Su^+) tRNA genes

Confluent monolayers of African green monkey kidney (CV-1) cells (2×10^7) in 75 cm^2 plastic flasks were infected with 0.5 ml of SV40 Su^+ tRNA recombinant virus stocks (Laski et al. 1982b; 1984; Ho et al., 1986). Virus was allowed to adsorb to the cells for 1 hour at 37°C . After adsorption 15 ml of 3T3 medium containing DMEM (Gibco Laboratories; Grand Island, NY) supplemented with 2% heat-inactivated fetal bovine serum (56°C for 1 hour), 100 U/ml penicillin G, 100 ug/ml streptomycin sulfate, and 0.15% (w/v) NaHCO_3 was added per flask and allowed to incubate for 4 to 5 days at 37°C . When 80% complete cytopathic effect (CPE) was observed, cells were freeze-thawed by placing the flasks at -70°C for 5 minutes followed by room temperature incubation for 10 minutes. This procedure was repeated 2 more times. The entire contents of the flasks were then centrifuged at 2,500 rpm for 10 minutes and the supernatant fluid was aliquoted and stored at -70°C .

Maintenance of MDCK, C127I and CV-1 cells

MDCK, C127I and CV-1 cells were propagated in 75 cm^2 plastic flasks. Cell growth medium for MDCK cells was minimal essential medium (MEM) with Earle's salts (Gibco Laboratories; Grand Island, NY) supplemented with 100 U/ml

penicillin G, 100 ug/ml streptomycin sulfate, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine (M.A. Bioproducts; Walkersville, MD) and 0.15% (w/v) NaHCO_3 . CV-1 and C127I cells were grown in 3T3 medium as described above. Confluent cell monolayers were split by removing the growth medium and washing with 10 ml of PBS. Three ml of trypsin-EDTA solution (0.1% 1:250 trypsin (Difco Laboratories), 0.1% (w/v) EDTA, 0.025% (w/v) NaHCO_3) in PBS was added to release the cells from the plastic solid support. After 5 minutes incubation at 37°C the cells were dissociated from the solid support by gentle tapping of the flask. Eight ml of growth medium was added and the cells were mixed by aspiration using a 10 ml pipet. The cell suspension was transferred to a 50 ml plastic Falcon conical tube (Becton Dickenson; Oxnard, CA) and centrifuged for 10 minutes at 1,500 rpm. The cell pellet was resuspended in 4 ml of growth medium. A 1:10 dilution of the cell suspension was made and the cells were counted with a hemocytometer under an inverted microscope. After an appropriate dilution of cells in growth medium, the cells were seeded into 75 cm^2 flasks (4×10^6 cells/flask), 35 mm dishes (2.5×10^5 cells/dish) or 13 mm glass cover slips (10 cover slips in a 60 mm dish/ 5×10^5 cells). Confluent monolayers were obtained following incubation at 37°C in a humidified CO_2 incubator.

Preparation of primary chicken kidney (PCK) cells

Embryonated chicken eggs were incubated until they hatched on day 21. Chickens were used for primary kidney culture preparation when 1 day old as described by Odagiri et al. (1982). Chickens were sacrificed and the kidneys were removed and transferred to a 60 mm petri dish containing 5 ml of sterile PBS. The kidneys were washed 3 times with PBS before being minced with scissors and transferred to a 50 ml Falcon tube. The minced tissue was washed 3 times in PBS and 2.5 ml of 0.25% trypsin in PBS was added per kidney. The tube was vortexed and incubated at 37°C for 10 minutes and this step was repeated again. The tube was then mixed at room temperature for 5 minutes using a stirring bar. This was followed by the addition of 5 ml of 10% fetal bovine serum to inactivate the trypsin. Cells were centrifuged at 1,500 rpm for 10 minutes and the pellet was resuspended in 10 ml of 199 medium (Gibco Laboratories; Grand Island, NY) containing Earle's salts 10% fetal bovine serum, 200 U/ml penicillin G, 200 ug/ml streptomycin sulfate, 2 mM L-glutamine, MEM nonessential amino acids, 0.1% NaHCO₃, and fungizone (Flow Laboratories Inc.; McLean, VA). The chick cell suspension was filtered through sterile gauze and more medium was added to a volume of 6.25 ml/chick kidney. Cells were seeded onto 35 mm dishes (5 dishes/chick) and then incubated for 2 days at 37°C.

Preparation of chicken embryo fibroblast (CEF) cells

Eleven day old embryonated chicken eggs (5) were washed in alcohol and embryos were liberated and placed in a petri dish containing 10 ml of PBS. The internal organs, head, wings and feet were removed and discarded. The bodies were transferred to a 60 mm petri dish and washed 3 times with PBS before being transferred to another petri dish. Twenty ml of 0.25% trypsin/PBS were added and the bodies were minced with scissors. The minced tissue and 40 ml of 0.25% trypsin/PBS were added to a sterile 250 ml Erlenmeyer flask. The contents of the flask was then mixed using a stirring bar for 10 minutes at 37°C. The supernatant fluid was decanted and 160 ml of 0.25% trypsin/PBS was added to the flask and incubated for an additional 50 minutes at 37°C. Ten ml of 10% fetal bovine serum was added to inactivate the trypsin. The cell suspension was filtered through sterile gauze and transferred to 50 ml Falcon tubes. Cells were centrifuged at 1,500 rpm for 10 minutes and the pellets were resuspended in 10 ml of minimal essential medium (MEM) with Earle's salts (Gibco Laboratories; Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 ug/ml streptomycin sulfate, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine (M.A. Bioproducts; Walkersville, MD) and 0.15% (w/v) NaHCO_3 . Cells were counted and seeded onto 35 mm dishes 3×10^6

cells/dish and incubated overnight at 37°C.

Maintenance of Chinese hamster ovary (CHO) cell lines transformed with the gene for amber suppressor tRNA lysine

The CHO-K1 cell transformants were grown in MDCK growth medium containing different concentrations of MTX (0.1, 1, 10, 100 uM) Ho et al., 1986).

Extraction of viral RNA

Virion RNA was extracted according to Ritchey et al. (1976a) in a 30 ml glass Corex tube fitted with a rubber stopper. All glassware was baked at 250°C to inactivate ribonucleases and sterilize the glassware. To the virus solution, 10% sodium dodecyl sulfate (SDS) (Pierce Chemical Co.; Rockford, IL) was added to a concentration of 0.33% and vigorously mixed. Then, 1/10 volume of 10X RSB buffer (0.1 M Tris-HCl, pH 7.4, 0.1 M KCl, 0.015 M $MgCl_2 \cdot 6H_2O$) and 5 mg/ml proteinase K (Beckman Instrument, Inc.; Palo Alto, CA) in RSB were added. The tube was vortexed and incubated in a 56°C water bath for 15 minutes. One-tenth volume of LiCl buffer (5% SDS 0.1 M acetate buffer pH 4.9, 1.4 M LiCl) and 1 volume of phenol (redistilled and saturated with RSB buffer) were added and the tube was mixed for 20 minutes on a wrist-action shaker (Burrell Corporation; Pittsburg, PA). The tube was centrifuged for 10 minutes

at 3,000 rpm to separate the two phases. The top aqueous layer was removed and transferred to a new Corex tube and phenol chloroform extracted (1:1 ratio of phenol to chloroform). The tube was centrifuged for 10 minutes at 3,000 rpm to separate the two phases and the upper phase was phenol/chloroform extracted again until the interface formed after centrifugation was clear. The RNA was precipitated by the addition of 2.5 volumes of 100% ethanol. After incubation at -70°C for 30 minutes, the RNA was pelleted by centrifugation at 4°C for 20 minutes at 11,000 rpm (Sorvall SS-34 rotor). The supernatant fluid was removed and the RNA pellet was lyophilized in a Speed Vac Concentrator (Savant Instruments, Inc.; Hicksville, NY). The RNA was resuspended in 0.4 ml of 0.3 M NaOAc with a sterile transfer pipet and placed in a 1.5 ml Eppendorf polypropylene tube. The RNA solution was extracted twice with two volumes of ethyl ether and residual ether was removed by evaporation with nitrogen gas. The RNA, was precipitated with 2.5 volumes of 100% ethanol and collected by centrifugation at $12,000\times g$ for 15 minutes in an Eppendorf model 5412 tabletop microfuge. The RNA pellet was rinsed with 70% ethanol and centrifuged for 5 minutes. The RNA was lyophilized and resuspended in 100 μl of double distilled water (DDW). A dilution of the sample was made and the RNA concentration and purity was determined by spectrophotometric analysis by scanning 230-280 nm

wavelengths.

Gel electrophoresis of viral RNAs

Influenza virus RNAs were electrophoresed in polyacrylamide vertical slab gels using a Tris-borate-EDTA (TBE) buffer system modified from Peacock and Dingman (1967). The 10X TBE buffer of pH 8.3 consisted of 0.89 M Trizma base, 0.89 M boric acid, and 0.025 M disodium EDTA. A 40% stock of polyacrylamide was prepared in sterile DDW with acrylamide (>99.9% pure, Bio Rad Laboratories; Rockville Centre, NY) and N'N'-methylenebisacrylamide (ultra pure, Bethesda Research Laboratories; Gaithersburg, MD) in a ratio of 38:2. The stock solution was filtered using a Nalgene filtration device (Nalge Co.; Rochester, NY). A stock of 10 M urea (ultra pure, Schwarz/Mann Biotech; Cleveland, OH) was prepared in sterile DDW that was gravity filtered through Whatman qualitative paper. Seventy five ml of gel solution consisting of 1X TBE, 3% polyacrylamide, and 6 M urea was used. Gel polymerization was initiated by the addition of 750 ul of 10% (w/v) ammonium persulfate (AP) (Fisher Scientific) and 75 ul of N,N,N'N' tetramethylethylenediamine (TEMED) (Bio Rad Laboratories). The gel was cast between autoclaved glass plates, 26 x 16 x 0.5. One plate was notched on both edges of one end and the other was sandblasted on one side. The sandblasted side of the plate faced the gel surface. The

plates were separated by plexiglass spacers (Aquebogue; Aquebogue, NY) of 1.5 mm thickness. A 15 tooth (6 mm wide separated by 1.6 mm) 1.5 mm thick plexiglass comb was used to form the sample wells.

Preparation of RNA for electrophoresis

One ug of influenza virus RNA was added to a 1.5 ml Eppendorf tube for subsequent gel electrophoresis analysis. An equal volume of 2X running mix (40% (w/v) glycerol, 2X TBE, 0.05% (w/v) XC, and 0.05% (w/v) BPB) was added. The sample was heated at 56°C for 5 minutes and loaded onto the gel with a Hamilton syringe. The gel was run at room temperature for 12 hours at a constant voltage of 175 Volts.

³⁵S-Methionine labeling of viral proteins

Influenza virus proteins were pulse-labeled as described by Ritchey et al. (1976b). Confluent monolayers of MDCK, CV-1, CHO-K1 and PCK cells in 35 mm dishes were infected with 0.1 ml of undiluted virus seed. Virus was adsorbed to the cells for 1 hour at the appropriate temperature. After adsorption, 2 ml per dish of maintenance medium was added which consisted of MEM with Earle's salts supplemented with 0.2% bovine albumin, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 1000 ug/ml streptomycin sulfate and 0.12% NaHCO₃. The infected-cell

monolayers were incubated at the appropriate temperature for the desired length of time post infection before viral proteins were labeled. Maintenance medium then was removed and monolayers were washed with PBS. One ml/dish of protein labeling medium was added which consisted of Hanks' balanced salt solution, 0.5% NaHCO_3 , 0.004% (w/v) phenol red, 100 U/ml penicillin G, 100 ug/ml streptomycin sulfate, 0.2% (w/v) glucose supplemented with 200 uCi/ml L-[^{35}S] methionine (specific activity 1000 Ci/mmol; New England Nuclear; Boston, MA). The cells were pulse-labeled for 1 hour. The labeling medium was removed and the monolayers were washed with PBS. Two hundred ul of protein-loading buffer containing 30% (v/v) glycerol, 3% 2-mercaptoethanol, 2% SDS, 0.025% BPB and 0.01X buffer 11B (5.7 g Trizma base dissolved in 100 ml of DDW and was adjusted to pH 7.1) was added per dish. The cells were scraped off the dish with a rubber policeman and transferred to a 1.5 ml Eppendorf tube.

Viral protein gel electrophoresis

^{35}S -Methionine-labeled virus-infected cell extracts were electrophoresed on 7-14% linear gradient SDS-polyacrylamide gels (Young and Palese, 1979).

DDW was used to make up stock solutions of polyacrylamide (30:0.8 ratio of acrylamide to bis-acrylamide), 10% SDS, 60% (v/v) glycerol and 10% AP. Buffer

11A (20X resolving gel buffer) contained 36.3 g of Trizma base dissolved in 100 ml of DDW and was adjusted to pH 8.9. The 7.0% gel solution (24 ml) was prepared containing 7.0% polyacrylamide, 5.2% (v/v) buffer 11A, and 0.1% SDS. The 14.0% gel solution (24 ml) consisted of 14% polyacrylamide, 5.2% buffer 11A, 0.1% SDS, and 20% (v/v) glycerol. To start polymerization 75 μ l of 10% AP and 18 μ l of TEMED were added to the 7% and 14% gel solutions. The gradient gel was cast using a gradient formed between glass plates, 26 x 16 x 0.5 cm, separated by 1.5 mm spacers. The gradient gel was filled to within 4 cm of the top of the glass plates and 2 ml of isobutanol was layered on top of the gel solution to ensure a smooth edge on the top of the gel. Polymerization was complete approximately 45 minutes later, then the isobutanol was decanted and the top of the gel mold was washed 3X with DDW and excess water was removed with 3MM chromatography paper. The stacking gel solution was prepared and consisted of 4.6% polyacrylamide, 12.5% buffer 11B, 0.2% SDS 0.15% AP and 0.15% TEMED. The stacking gel was poured directly on top of the resolving gel and a 15 tooth (6 mm wide separated by 1.6 mm) comb was inserted to form the sample wells. The electrophoresis running buffer contained 0.025 M Trizma, 0.19 M glycine (Sigma Chemical Co.; St. Louis, MO), pH 8.25 and 0.1% SDS.

The protein samples in loading buffer were boiled for 4

minutes to denature the proteins and immediately loaded onto the 7-14% gradient gel. The proteins were electrophoresed for a total of 1600 voltage hours which allowed the BPB tracking dye to run off the gel. After electrophoresis, the stacking gel was removed and the resolving gel was fixed in 25% methanol, 8% glacial acetic acid for 30 minutes with slow shaking at room temperature. The fixed gel was then dried on a Bio Rad slab gel dryer and exposed to Cronex X-ray film (Dupont; Wilmington, DE) at -70°C .

Western blot assay

Confluent monolayers of CHO-K1 Su^+ tRNA, CV-1, or C127I cells in 35 mm dishes were infected with influenza or vaccinia recombinant viruses at 37°C . At 24 hours after infection, the cell lysate was separated by electrophoresis on 7-14% gradient polyacrylamide gels as described in the previous section. The separated proteins were transferred to DEAE-membrane (NA45, Schleicher and Schuell; Keene, NH) by the Bio Rad transblot system (0.2 Amps 2 hours) and the DEAE membrane was incubated in blocking solution containing 3% bovine albumin (fraction V, Sigma), 1% heat-inactivated goat serum (Gibco) and 0.05% tween 40 (Sigma) in Tris basic salt (TBS) buffer (50 mM Tris-HCl, pH 7.5 and 0.2 M NaCl) for 1 hour at room temperature (Hawkes et al., 1982). The membrane was then treated with a 1:50-1:200

dilution of primary antibody (polyclonal rabbit anti-A/PR/8/34, anti-A/Japan/305/57 virus antibody, anti-A/NS1 antibody [Young et al., 1983a], mouse monoclonal A/PR/8/34 virus HA specific antibody PY202, [Moran et al., 1984]) for 16 hours at room temperature. After washing at room temperature with TBS buffer 3X for 15 minutes each, the DEAE-membrane was treated with either ^{125}I -labeled goat anti-rabbit IgG or ^{125}I -labeled goat anti-mouse Ig. (Specific activity 100 uCi/ml, New England Nuclear, Boston, MA) for 2 hours at room temperature. The membrane was washed 3X with TBS buffer and exposed to Cronex X-ray film (Dupont, Wilmington, DE) at -70°C .

Production of influenza B virus anti-NS1 antiserum

Confluent monolayers of MDCK cells in 35 mm dishes were infected for 16 hours with B/Lee/40 virus at a multiplicity of infection (MOI) of 10 at 33.5°C . Whole cell lysates were prepared and electrophoresed on SDS-polyacrylamide gels as described above. Gel slices containing the NS1 protein band were excised from the gel and placed in dialysis tubing (MW cut off 12K, Spectrapor, Spectrum Medical Industries Inc., Los Angeles, CA) and electroeluted overnight (100 Volts 8 milliamps) at 4°C . The amount of NS1 was quantitated using low MW protein standards (Bio Rad Laboratories, Rockville Center, NY) run on the same gel. Approximately 10 ug of NS1 protein in suspension was

combined with an equal volume of Freund complete adjuvant (Difco Laboratories; Detroit, MI) and was used for subcutaneous injection into New Zealand White rabbits at 4 sites along the back. Three weeks later this procedure was repeated except that incomplete adjuvant (Difco Laboratories; Detroit, MI) was used. The animals were boosted at 2 week intervals for an additional month and bled 1 week after the final boost.

Immunofluorescence of virus infected cells

MDCK or CV-1 cells were grown to confluency on 13 mm cover slips at ca. 10^5 cells per cover slip. The next day these cells were infected for 8 and 20 hours with influenza A and B viruses, respectively, at an MOI of 10. Immunofluorescence experiments of influenza B viruses were carried out in CV-1 cells in order to reduce possible cross-reactivity of host cell proteins with the anti-B/NS1 antiserum made from the NS1 protein expressed in B/Lee/40 virus-infected MDCK cells. Influenza A virus-infected cells were fixed with 1% formaldehyde for 1 hour at room temperature followed by incubation with 0.1% Triton X-100 for 5 minutes, and treated with acetone at -20°C for 5 minutes. Influenza B virus-infected cells were fixed with acetone for 10 minutes at -20°C . The infected cells were incubated with an anti-A/NS1 antiserum at a 1:5 dilution or with an anti-B/NS1 antiserum at a 1:40 dilution

for 30 minutes at 37°C. Cells were then washed and stained with fluoresceinated affinity purified goat anti-rabbit IgG (Cappel, Cooper Biomedical, Scientific Division, West Chester, PA) at a 1:100 dilution for 30 minutes at 37°C. Cells were viewed on a Leitz fluorescence microscope.

Immunoprecipitation

Confluent monolayers of MDCK cells in 35 mm dishes were infected with approximately 10 PFU/cell of influenza virus (A/turkey/Oregon/71, A/PR/8/34, B/Lee/40, clone 201) for 1 hour at 37°C (A viruses) or 33.5°C (B viruses). At six hours post infection cells were pulse-labeled with [³⁵S] methionine (200 uCi/ml) for 1 hour as reported previously (Ritchey et al., 1976). After the labeling period, cells were lysed in 200 ul of RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% (v,v) Triton X-100, 0.1% SDS, 0.01 M Tris-HCl, pH 7.4) and 1 mM phenylmethylsulfonylfluoride). Samples (25 ul) of labeled cell extracts were incubated overnight at 4°C with rabbit sera (25 ul). Formalin-fixed Staphylococcus aureus cells (100 ul; Bethesda Research Laboratories, Rockville, MD) were added and the mixture was incubated for 30 minutes at 4°C. Samples were pelleted (2 minutes at 15,000 x g) at 4°C and suspended in 200 ul of RIPA buffer. The samples were pelleted again and washed once with DDW. Finally, samples were resuspended in loading buffer, boiled for 3 minutes, centrifuged for 10 seconds and the supernatant fluid was subjected to

electrophoresis on 7-14% linear gradient SDS-polyacrylamide gels as described previously (Young and Palese, 1979).

Neuraminidase treatment of virus-infected cells.

Confluent monolayers of CV-1 or mouse C127I cells in 35 mm dishes were infected with vaccinia HA recombinant viruses or influenza viruses. At 24 hours post infection cells were lysed in 200 ul of RIPA buffer/dish and 50 ul aliquots were treated with 50 ul of neuraminidase (NA) from Clostridium perfringens (500 U/ml) (Gibco) in 50 mM sodium acetate buffer pH 5.5, 9 mg/ml NaCl, and 1 mg/ml CaCl_2 for 1 hour at 37°C. The HA protein samples treated with or without NA were analyzed by the Western blot assay described above.

Cloning of the influenza A/turkey/Oregon/71 virus NS gene

Double-stranded (ds) cDNA was synthesized from influenza A/turkey/Ore/71 virus RNA by using reverse transcriptase and synthetic oligonucleotide primers as described by Baez et al. (1980). Since the 3' and 5' ends of all eight influenza virus RNAs are conserved, primers complementary to these 3' and 5' terminal nucleotides will prime first and second strand cDNA synthesis, respectively.

The reaction for first strand cDNA synthesis consisted of 200 ug of purified vRNA, reverse transcriptase (RT) buffer [50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 20 mM

dithiothreitol (DTT), 12 mM $MgCl_2$, 0.5 U/ul RNasin (Promega Biotec; Madison, WI), 0.5 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia P-L Biochemicals Inc.; Milwaukee, WI), 10 ug 3' end primer d(AGCAAAAGCAG)rG (Collaborative Research, Inc.; Waltham, MA), 100 uCi [α - ^{32}P]-dATP in Tricine (specific activity 3,000 Ci/mole; New England Nuclear; Boston, MA) and 500 U/ml avian myeloblastosis virus (AMV) RT (Molecular Genetic Resources, Inc.; Tampa, FL) in a volume of 300 ul. The mixture was incubated at 42°C for 1.5 hours and made 0.2% SDS, 0.02 M EDTA and 0.3 M NaOAc to terminate the reaction. The mixture was twice phenol/chloroform (1:1) and ether extracted. The RNA/DNA hybrids were ethanol precipitated by the addition of 2.5 volumes of 100% ethanol and incubated at -70°C for 20 minutes. The ds hybrids were collected by centrifugation for 15 minutes at 12,000 x g and the pellet was washed with 70% ethanol and lyophilized. The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) and the RNA strands of the hybrids were removed by pancreatic RNase A (25 ug/ml) digestion at 37°C for 30 minutes. NaOAc was added to the mixture at a final concentration of 0.3 M, and the solution was extracted twice with phenol/chloroform (1:1) and ether. The aqueous phase was passed through a Sephadex G-75 (Pharmacia Fine Chemicals; Piscataway, NJ) gel filtration column (20 x 0.8 cm, polypropylene; Kontes; Vineland, NJ) that was equilibrated with NTE. The first

radioactive peak eluted from the column contained the ss cDNA and the fractions containing this peak were counted by measuring Cerenkov radiation. The ss cDNA was ethanol-precipitated, lyophilized and resuspended in 20 μ l of DDW.

5' end primer d(AGTAGAAACAAG) (1 μ g), complementary to the 3' end of the first strand cDNA, was mixed with first strand cDNA for 2 minutes at 90°C and cooled to room temperature for 5 minutes. Reverse transcriptase buffer, 0.5 mM each of dATP, dGTP, dTTP, dCTP and 500 U/ml of RT were added to the reaction to give a final volume of 100 μ l. The reaction was incubated at 37°C for 1.5 hours and made 0.2% SDS, 0.02 M EDTA and 0.03 M NaOAc. Following phenol/chloroform (1:1) and ether extraction the ds cDNA was collected by gel filtration on a Sephadex G-75 column (20 x 0.8 cm) in NTE buffer.

Approximately 5000 cpm aliquots of the ss and ds cDNA products were treated with S1 nuclease (Sigma Chemical Co.; St. Louis, MO) and analyzed by electrophoresis on a 4% polyacrylamide gel (stock acrylamide:bisacrylamide ratio 29:1)/TBE gel (26 x 16 cm, 1.5 mm thick).

Modification of ds cDNA for insertion into pBR322

Since the NS gene-specific ds cDNA of the A/turkey/Ore/71 virus was not digested by the restriction enzyme Bam HI, Bam HI synthetic linkers, d(CGCGGATCCGCG) (New England Biolabs; Beverly, MA) were added. In order to

phosphorylate the Bam HI linkers, 50 uCi of [gamma-³²P]-ATP (specific activity 3,000 Ci/mole; New England Nuclear; Boston, MA) was lyophilized in an 1.5 ml Eppendorf tube. Then 2 ug of Bam HI linkers and 10 U of T4 polynucleotide kinase (PNK) in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) were added in a final volume of 20 ul. The reaction was incubated for 30 minutes at 37°C. Ten U of T4 PNK along with a final concentration of 1 mM ATP were added and the reaction was incubated for an additional 60 minutes. The ds cDNA was phosphorylated at 37°C for 60 minutes in a 20 ul reaction volume containing 1 mM ATP, 10 U T4 (PNK) (Bethesda Research Laboratories; Gaithersburg, MD) in Wu buffer (66 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 15 mM DTT, 0.1 mM ATP). An additional 10 U of T4 PNK was added and the reaction was incubated for another 60 minutes.

The kinase reactions were terminated by incubation at 65°C for 10 minutes. The ds cDNA and linkers were pooled, phenol/chloroform (1:1) and ether-extracted and ethanol-precipitated. The ligation reaction (100 ul of Wu buffer) containing the kinased cDNA and linkers, 0.5 mM ATP and 400 U T4 DNA ligase (New England Biolabs, Beverly, MA) was incubated for 15 hours at 14°C followed by 5 minutes at 70°C. An aliquot was stored for future analysis and the bulk of the reaction was digested with an excess of 100 U

of Bam HI according to the manufacturer's specifications to convert polymerized linkers to monomers. The reactions were monitored by electrophoresis on a 4% polyacrylamide/TBE gel (26 x 16 x 0.5 cm, 1.5 mm thick). In order to separate the cDNA linker molecules, the reaction was loaded onto a sepharose CL-6B (Pharmacia Fine Chemicals; Piscataway, NJ) column (30 x 1.8 cm) which was equilibrated with NTE buffer. The cDNA eluting in the first radioactive peak was ethanol-precipitated, lyophilized and resuspended in 200 ul DDW.

Preparation of cloning vector and ligation of cDNA

The vector pBR322 was linearized by complete digestion with Bam HI and the 5' ends of the vector were dephosphorylated. The reaction mixture (100 ul) consisted of 25 ug of linearized pBR322 (18 pM 5' termini) and 256 U of bacterial alkaline phosphatase (BAP) (Bethesda Research Laboratories) in BAP buffer (10 mM Tris-HCl, pH 8.0, 120 mM NaCl). The mixture was incubated at 65°C for 60 minutes and then cooled to room temperature. The reaction was extracted twice with phenol/chloroform (1:1) and ether and the DNA was ethanol-precipitated, lyophilized and resuspended in 200 ul DDW.

The ligation reaction contained 100 ul of the cDNA reaction, 400 U T4 DNA ligase (New England Biolabs) in Wu buffer, 0.5 mM ATP and 200 ng BAP-treated pBR322. The reaction was at 14°C for 15 hours.

Preparation of competent bacterial cells and transformation

An overnight culture of E. coli C600 cells (20 ml) was grown in TY medium (10 g Difco Bacto-tryptone, 10 g Difco bacto-yeast extract, 5 g NaCl per liter). An aliquot of the culture was inoculated into 50 ml of TY medium and the cells were grown at 37°C to logarithmic phase (O.D.590=0.6). The bacterial cells were made competent according to the method reported by Dagert and Ehrlich (1979). The cells were centrifuged at 3,000 rpm for 10 minutes and resuspended in 10 ml of cold, sterile 100 mM CaCl₂, 10 mM Tris-HCl, pH 8.0 and incubated on ice for 60 minutes. Following centrifugation at 4°C at 3,000 rpm the pellet was resuspended in 2 ml of 100 mM CaCl₂, 10 mM Tris-HCl, pH 8.0.

The 100 ul ligation product was added to 100 ul of competent cells and 100 ul of TCM buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 10 mM CaCl₂). This mixture was placed on ice for 30 minutes followed by a heat-shock at 42°C for 2 minutes. Then 500 ul of TY medium plus ampicillin (50 mg/liter) was added to the mixture followed by incubation at 37°C for 45 minutes. Aliquots of 20, 50 and 100 ul of the transformation reaction were plated onto TY agar plates (20 g Difco-agar/liter) containing ampicillin (50 mg/liter). The agar plates were incubated at 37°C for approximately 16 hours.

Identification of recombinant clones

E. coli colonies containing plasmids with influenza virus-specific genes were identified by the in situ colony hybridization method of Grunstein and Hogness (1975). Bacterial colonies were streaked onto TY plus ampicillin agar plates and incubated at 37°C for 15 hours. The bacterial streaks were replica-plated onto several nitrocellulose (NC) membranes (0.45 μ m, 82.5 mm circles; Schleicher & Schuell; Keene, NH) that were on top of the agar plates. Bacteria on these NC filters were propagated at 37°C for 15 hours and the replica plates were refrigerated at 4°C until needed.

Four large trays containing two sheets of Whatman 3MM chromatography paper were saturated with one of the following solutions: I) 0.5 N NaOH, 1 M Tris-HCl, pH 8.0; II) 1 M Tris-HCl, pH 8.0, 10X SSC (1.5 M NaCl, 0.15 M $\text{NaC}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$); III) chloroform; and IV) 10X SSC. In order to lyse the bacterial cells and denature the DNA the nitrocellulose membranes were transferred to the first tray for 10 minutes. Following neutralization in the second tray (10 minutes) the NC filters were air-dried on 3MM paper and then transferred to the tray containing chloroform. The tray was shaken for 3 minutes during which time the bacterial streaks were no longer visible. The filters were air-dried again on 2 sheets of 3 MM paper and

placed in the tray with 10X SSC. The filters were then air-dried and baked in a vacuum oven at 80°C for 2 hours in order to fix the DNA to the filters.

Nick translation of NS gene-specific hybridization probe

The nick translation reaction was carried out using a ³²P nick translation system (New England Nuclear; Boston, MA). The reaction volume (25 ul) contained 100 uCi of [alpha-³²]-dATP (specific activity 3,000 Ci/mmole), 0.5 ug of NS gene DNA (in TE buffer). 20 uM each of cold dGTP, dCTP, dTTP, 48 U/ml E. coli DNA polymerase I and 0.02 U/ml pancreatic DNase I in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂. The reaction was incubated at 14°C for 2 hours and terminated with 100 ul of nick translation stop buffer containing 0.5 M EDTA, 0.2% SDS and 0.3 M NaCl. A Sephadex G-75 chromatography column (20 x 0.8 cm, Kontes; Vineland, NJ) in NTE buffer was used to separate the labeled DNA from the unincorporated ³²P. The first peak eluted from the column contained the labeled DNA.

Hybridization of probe to bacterial clones

The baked NC filters were prehybridized in hybridization buffer [1X Denhardt's: 0.02% Ficoll-70, 0.02% polyvinyl-pyrrolidone-40, 0.02% BSA-fraction V; (Sigma Chemical Co.; St. Louis, MO), 3X SSC, 0.1% SDS] by incubation at 65° for 2 hours. Following denaturation by

boiling for 10 minutes the hybridization probe was added to a plastic bag containing the filters in 15 ml hybridization buffer. Following hybridization for 12 hours at 65°C in a shaking water bath the probe was removed from the bag and stored at -20°C for future use and the filters were washed 2 times at 65°C for 30 minutes in prewarmed 3X SSC, 0.1% SDS with gentle agitation. Two ml of methyl green (0.01%) was added during an additional wash with 0.1% SDS and 0.1% SSC in order to locate the positions of the bacterial colonies. The filters were air dried, mounted on a rigid support and exposed at -70°C to Cronex X-ray film with an intensifying screen (Dupont; Wilmington, DE).

Rapid screen-analysis of bacterial colonies

The NS-positive colonies were selected from the master plate and inoculated into 3 ml of TY medium. The cultures were grown at 37°C for 15 hours and screened by the rapid alkaline method (Birnboim and Doly, 1979) for the presence of plasmids with full-length inserts. Half of the culture was transferred to another Eppendorf tube and the cells were centrifuged at 12,000 x g for 2 minutes. The cells were resuspended in 100 ul of solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA) containing 2 mg/ml lysozyme (Sigma Chemical Co.; St. Louis, MO). Cells were kept on ice for 30 minutes, then 200 ul of solution II (0.2 N NaOH, 1.0% SDS) was added and the solution was mixed until it became clear. The tube was kept on ice for an

additional 5 minutes then 150 ul of solution III (3 M NaOAc, pH 4.8) was added and the contents were mixed and incubated for 60 minutes. The samples were centrifuged at 12,000 x g for 10 minutes and plasmid DNA was precipitated from the supernatant fluids with isopropanol at -70°C for 15 minutes. Following centrifugation pellets were resuspended in 0.1 M NaOAc (100 ul). The DNA was reprecipitated with ethanol, lyophilized and resuspended in DDW (50 ul).

Half of the DNA preparation was digested with Bam HI at 65°C for 60 minutes in a 150 ul reaction volume consisting of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT and 0.1 ug/ul RNase A. The DNA was ethanol precipitated, lyophilized and resuspended in 10 ul DDW. An equal volume of 2X sample loading buffer (0.1% BPB, 0.1% XC, 20% (v,v) glycerol in 2X TBE) was added to the DNA and the sample was loaded onto a 6 x 1.5 mm well formed in a 3 mm thick horizontal 1.0% agarose (ultra pure; Bethesda Research Laboratories; Gaithersburg, MD) gel containing 0.5 ug/ml ethidium bromide (EtBr). Electrophoresis buffer was TBE containing 0.5 ug/ml EtBr. The samples, along with molecular weight markers, were electrophoresed at 4°C for 2 hours at 75 mA (constant current). After electrophoresis, the agarose gel was photographed under short wave 254 nm UV illumination with a Tiffen (Hauppauge, NY) dark red filter #29. The film used was Polaroid 3000

speed, black and white land pack film, type 107C and the exposure was for 1/4 second at f 4.5.

Large scale purification of plasmid DNA

From 1 liter of bacterial cell culture grown to O.D. 590 nm = 0.6 plasmid DNA was obtained using the previously described method of Birnboim and Doly (1979). Cells were centrifuged at 8000 rpm, at 4°C for 15 minutes and the bacteria were resuspended in 40 ml of NTE and pelleted again. The bacteria were resuspended in 14 ml of solution I plus 4 mg/ml lysozyme and transferred into two Beckman Type Ti45 tubes. The samples were mixed and kept on ice for 30 minutes, then 28 ml of solution II was added. The tubes were vortexed and kept on ice for 5 minutes until the contents were clear, then 21 ml of solution III was added to each tube. The samples were mixed and kept on ice for another 60 minutes. Chromosomal DNA, high molecular weight RNA and protein were pelleted by centrifugation in a Ti45 rotor at 30,000 rpm for 20 minutes at 4°C. The plasmid DNA in the supernatant was precipitated twice with an equal volume of isopropanol and centrifuged at 12,000 rpm for 20 minutes in Ti45 tubes. The DNA was then lyophilized, resuspended in 4 ml of 10 mM Tris-HCl, pH 7.5 plus RNase A (100 ug/ml) and incubated at 37°C for 30 minutes in order to digest the RNA. The solution was made 0.3 M NaOAc and extracted with phenol/chloroform (1:1), ethanol-

precipitated and resuspended in 1 ml of NTE containing 10% (v/v) glycerol. The nucleic acid sample was applied to a Bio-Gel A-50 agarose (Bio Rad Laboratories; Rockville Center, NY) column (30 x 1.8 cm) in NTE buffer, in order to separate the DNA from the RNA. Elution of the DNA peak was monitored by UV absorbance at 254 nm. The DNA was ethanol-precipitated and centrifuged at 11,000 rpm (Sorvall SS-34 rotor) for 30 minutes at 4°C. The pellet was resuspended in 400 ul of 0.3 M NaOAc and placed in an Eppendorf tube. The DNA was phenol/chloroform (1:1)-extracted, ethanol-precipitated and centrifuged at 12,000 x g for 15 minutes. The DNA pellet was lyophilized and resuspended in 200 ul TE buffer. The resultant plasmid DNA was quantitated by UV absorbance at 260 nm and the DNA concentration was adjusted to 1 ug/ul.

Isolation of NS gene insert

One hundred and eighty ug of plasmid DNA was digested at 37°C for 2 hours with 13 ul BamHI (16 U/ul) in medium salt buffer (100 mM NaCl 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) in a final volume of 100 ul. To one half of the BamHI-digested plasmid containing the NS insert was added 350 ul of sterile DDW and 400 ul of 2X loading buffer. The sample was loaded into a 5 cm x 2 mm well formed in a 1% agarose (ultra pure; Bethesda Research Laboratories;

Gaithersburg, MD) 13 x 20 cm x 1 cm thick gel containing 0.5 ug/ml EtBr. The DNA was electrophoresed at 4°C for 2 hours at 75 mA (constant current). When the NS insert had separated from the remainder of the plasmid DNA by about 8 cm the current was shut off and a cut was introduced into the gel below the insert band. DEAE cellulose membrane NA45 (Schleicher & Schuell, Inc.; Keene, NH) was cut to a size approximately 1 mm larger in height and width than the insert DNA band. The membrane was placed in 10 mM EDTA, pH 7.5 for 10 minutes, 0.5 M NaOH for 5 minutes and washed 3 times with DDW before inserting into the gel slit. Electrophoresis was continued until all the insert migrated onto the DEAE membrane, as confirmed by UV light visualization. After elution the excess membrane was trimmed and discarded and the remainder was cut into several pieces and placed in an Eppendorf tube in which low salt buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0) had been added. The paper was transferred to another Eppendorf tube containing high salt buffer (300-700 ul) (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8.0) and heated at 65°C for 45 minutes. The DNA was phenol/chloroform 1:1-extracted, ethanol-precipitated, lyophilized and resuspended in 100 ul DDW. The DNA concentration was determined by spotting 1 ul of sample and comparing the intensity of fluorescence with known quantities of DNA on a 1% agarose plate containing 0.5 ug/ml of EtBr.

M13 Cloning

M13mp18 and M13mp19 are filamentous DNA phage that differ from wild-type phage in that lac DNA was inserted into an intergenic region (Messing et al., 1981). In addition several restriction sites were cloned into the lac DNA (Messing et al., 1981). Therefore, intact M13mp18 and M13mp19 produce a functional B-galactosidase enzyme forming blue plaques under conditions of IPTG induction using X-gal as the enzyme substrate. When DNA is inserted into any of these restriction sites it interrupts production of the functional B galactosidase gene and white plaques are formed.

Before the full length NS gene and its Tag I-digested fragments could be cloned, the vectors (M13mp18 and M13mp19) were digested with Bam HI and Acc I restriction enzymes, respectively. Five ug of M13mp18 DNA (5 ul) (New England Biolabs; Beverly, MA) was digested at 37°C for 2 hours with 16 U (1 ul) of Bam HI in 2 ul of medium salt buffer and 15 ul of sterile DDW. Five ug of M13mp19 DNA (New England Biolabs; Beverly, MA) was digested at 37°C for 2 hours with 25 U of Acc I (2.5 ul) in 2 ul of medium salt buffer plus 15 ul of sterile DDW. The plasmid digestions were verified by electrophoresis of 1 ug of each plasmid (digested and undigested) and molecular weight size markers on 1% agarose gels containing EtBr (0.5 ug). After the

electrophoresis run the DNA was visualized by UV light and photographed as described above.

Tag I restriction fragments of the NS gene were generated in order to allow rapid sequencing of the entire NS gene. This was done by digesting 100 ng of NS insert (20 ul) with 2 ul (12 U) Tag I restriction enzyme in 2.2 ul 10X low salt buffer 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ at 65°C for 2 hours.

Twenty ng of linearized M13 phage DNA (mp18 and mp19), 200 ng of NS insert (full length or Tag I-digested), 2 ul of T4 DNA ligase (400 U/ul) and 10 ul of ligation buffer (660 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 150 mM DTT, 0.1 mM ATP) were brought up to a volume of 100 ul with DDW, and incubated for 14 hours at 15°C.

In order to prepare transformation-competent cells, the stock of E. coli JM109 (Messing et al., 1981) was streaked onto a minimal agar plate (Bacto-agar, 15 g/l; K₂HPO₄, 10.5 g/l; KH₂O₄, 4.5 g/l; (NH₄)₂SO₄, 1 g/l; sodium citrate 2H₂O, 0.5 g/l; MgSO₄·7H₂O, 0.2 g/l; thiamine-HCl, 5 ug/l; glucose, 2 g/l). The plate was incubated overnight at 37°C and stored at 4°C and used as a stock of single colonies. A single colony of JM109 cells was transferred to 2 ml of TY medium (Bacto tryptone 10 g/l; Bacto yeast extract 5 g/l; NaCl 5 g/l) and grown for 12 hours at 37°C. This freshly expanding population of cells was diluted 1:100 into 50 ml of TY medium and propagated until a density of OD 590 nm =

0.6 was reached. Another colony was propagated to obtain exponentially growing cells upon which the transformation mixture was to be plated). The bacteria were then centrifuged at 2500 RPM for 5 minutes and resuspended in 25 ml of sterile 100 mM CaCl_2 and kept on ice until the transformants were ready for plating.

Fifty μl of each ligation reaction was added to 100 μl of competent cells and incubated on ice for 30 minutes. The cells were subjected to heat-shock for 2 minutes at 42°C , 400 μl of TY medium was added and incubation was continued at 37°C for 30 minutes. Three 13 x 100 mm sterile glass tubes containing 3 ml of molten (45°) plating agar (6 g Bacto agar per liter of TY medium) were prepared. Four μl of the transformation reaction was added to the first tube, 40 μl to the second and 400 μl to the third and 10 μl of 100 mM IPTG, 50 μl of 2% X-gal and 0.2 ml of exponentially growing JM109 cells were added to each tube. The contents of each tube were poured onto TY media plates (15 g Bacto agar per liter of TY medium, autoclaved and poured onto petri dishes). Undigested M13mp18 and M13mp19 phage DNA and linearized M13 phage DNA ligated in the absence of insert DNA were included as controls. The plates were incubated at 37°C for 12 hours and observed for the appearance of clear plaques.

Preparation of template DNA for dideoxy sequencing

A single colony of JM109 was grown in TY media (5 ml) for 12 hours at 37°C. Two ml of TY media containing 100 ul of exponentially growing JM109 were inoculated with a single colorless plaque. The sample was incubated for 3 hours in a 37°C incubator shaker then 50 ml of fresh TY media was added to the sample and incubation continued for an additional 4 hours. The sample was pelleted at 10,000 rpm for 10 minutes at 4°C. The supernatant fluid (50 ml) was collected and 10 ml of 20% (w/v) polyethelene glycol (6000 molecular weight) in 2.5 M NaCl was added and the reaction was incubated on ice with occasional agitation for 2 hours. The mixture was centrifuged again at 10,000 rpm for 10 minutes and the supernatant fluid was discarded. To the pellet, 0.1 ml of 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA was added and the sample was vortexed. Fifty ul of phenol saturated with 0.5 M Tris-HCl, pH 7.5 was added in order to extract the M13 DNA. The sample was centrifuged at 3000 rpm for 10 minutes and the aqueous phase was saved. To the aqueous phase 100 ul of 3 M Na Acetate, pH 6.0 and 2.5 ml of 100% ethanol were added at -70°C for 45 minutes. Following incubation the DNA was centrifuged at 12,000 rpm for 15 minutes at 4°C, lyophilized and resuspended in 100 ul 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The concentration of DNA was verified by electrophoresing 1 ul of the sample on a 1% agarose gel containing 0.5 ug/ml of EtBr. The desired concentration of template DNA was 0.1 ug/ul.

Dideoxy sequencing of M13 NS recombinant DNA

The complete sequence of the NS gene of the A/turkey/Ore/71 virus was obtained using the modified dideoxy chain termination method of Sanger et al. (1977).

Two μCi of [α - ^{35}S]-dATP (specific activity 1000 Ci/mmole; New England Nuclear; Boston, MA) was aliquoted to the A, C, G and T reaction tubes. The radioactive solutions were lyophilized and the appropriate mixture was added to each tube as described below. Two μl of M13 sequencing primer (2.5 $\mu\text{g/ml}$ 17 mer d(GTAAAACGACGGCCAGT) New England Biolabs, Beverly, MA), 5 μl of template DNA (0.1 $\mu\text{g}/\mu\text{l}$), 1 μl 10X Hin buffer (66 mM Tris-HCl, pH 7.5, 66 mM MgCl_2 , 66 mM NaCl, 50 mM DTT) and 2 μl of sterile DDW were combined in an Eppendorf tube. The mixture was vortexed, centrifuged for 10 seconds and heated at 90°C for 3 minutes followed by incubation at room temperature for 30 minutes. Two μl of the above hybridization mix was added to each of the 4 reaction tubes. To the 4 reaction tubes containing the lyophilized radiolabeled material 1 μl of the appropriate dideoxytriphosphate (0.25 mM ddATP, 0.33 mM ddCTP, 0.33 mM ddGTP or 1.0 mM ddTTP) and 1 μl of the respective G, A, T and C mixes were added (see Table 3). Klenow fragment at 6 U/ μl (Bethesda Research Laboratories; Gaithersburg, MD) was diluted to 1 U/ μl in Klenow diluent (100 mM KPO_4 , pH 8.0 in 50% (v/v) glycerol) and 1 μl of the

diluted enzyme was added to each tube to start the reaction. The reactions were incubated at 37°C for 15 minutes and then 1 ul of 0.5 mM dATP was added to each tube. The incubation was continued for an additional 30 minutes. Seven ul of loading mix [80% (v/v) deionized formamide, 10 mM EDTA, pH 7.0, XC, 0.1% BPB] was added to each reaction. The samples were boiled for 3 minutes, chilled in an ice water bath for 1 minute and 2 ul per reaction was loaded onto the sequencing gel.

TABLE 3. DIDEOXY REACTION MIXTURES^{*}

	A	C	G	T
0.5 mM dCTP	7.5	1	10	10
0.5 mM dGTP	7.5	10	1	10
0.5 mM dTTP	7.5	10	10	1
10X Hin buffer	7.5	7.5	7.5	7.5

* Volumes in ul.

Sequencing gels

All sequencing gel solutions were made from a stock of 40% acrylamide (acrylamide to bisacrylamide ratio of 38:2). Dideoxy sequencing reactions were electrophoresed on 0.4 mm thick, 6% polyacrylamide gels cast between 40 x 20 x 0.5 cm siliconized glass plates. The gel solution consisted of 7.5 ml of 40% acrylamide, 23 g urea, 5 ml of 10X TBE, 21.5 ml of sterile DDW, 0.3 ml of 10% AP and 0.05 ml of TEMED. The 6% gel solution was poured between the glass plates and a "shark's" tooth comb was inserted. Following polymerization for 1 hour the comb was removed and the sample wells were washed out with buffer to remove any unpolymerized acrylamide. The gel was set up in an electrophoresis apparatus with 1X TBE buffer and 1.5 μ l of sample per well was applied. The gel was electrophoresed at 1200 V for the desired length of time. The plates were pried apart and the gel was fixed in 10% acetic acid, 10% methanol for 10 minutes and transferred to Whatman 3MM paper. The gel was dried and autoradiographed at -70°C for 12 hours and the sequence was read.

Computer analysis of nucleotide sequences

The complete nucleotide and predicted amino acid sequences of the influenza virus NS genes were stored and analyzed on an IBM370 computer at the University Computing Center of The City University of New York using published

programs (Staden, 1977, 1978).

Preparation of oligonucleotide primers for dideoxy sequencing

Influenza virus NS gene-specific deoxyribonucleotide primers, 14-20 bases in length, were synthesized using the Sam One automated DNA synthesizer (Biosearch, Inc.; San Rafael, CA). The phosphoramidite (Beacage and Caruthers, 1981) and the phosphotriester (Itakura et al., 1975; Khorana, 1979) procedures were used for solid phase oligonucleotide synthesis. A solid support (silica or controlled pore glass) with the 3' base of the desired sequence derivatized to it was used as the starting material. The nucleoside was attached to the support via its 3'-hydroxyl group and the 5' hydroxyl group of the nucleoside was derivatized to a dimethoxytrityl (DMTr) group. The oligonucleotide chain was elongated in the 3' to 5' direction.

a) Phosphoramidite method

Trichloroacetic acid diluted in methylene chloride was used to cleave the acid-labile DMTr group from the 5'-hydroxyl group of the nucleoside linked to the solid support. The DMTr cation is bright orange in an acidic solution and effluents containing this cation were collected after each detritylation step. The coupling.

efficiency was determined by spectrophotometric analysis at 498 nm of each detritylation step. Each nucleoside phosphoramidite to be added to the chain was activated and coupled with tetrazole. After coupling a reactive phosphate was produced by oxidation with a solution of water, pyridine and iodine in tetrahydrofuran. Any 5'-hydroxyl group that did not react with the activated phosphoramidite in the previous coupling was capped so it would not react in subsequent couplings. This capping procedure changed the unreacted 5' hydroxyls to acetate esters with acetic anhydride, dimethylaminopyridine in pyridine and tetrahydrofuran. Once cleavage of the second DMTr group occurred the coupling, oxidation, capping and cleavage cycle continued until the expected-length oligonucleotide was synthesized.

b) Phosphotriester method

In the phosphotriester method the condensation reaction was done in the presence of pyridine and N-methylimidazole. Triethylammonium salts of protected phosphodiester were the monomers used to extend the oligonucleotide. Mesitylene-2 sulfonyl chloride (MSCl) was the coupling reagent. No oxidation or capping steps were employed in the phosphotriester procedure.

Processing of oligonucleotide

After the oligonucleotide was synthesized the solid support was treated with 1 ml of concentrated NH_4OH at 55°C for 5 hours in order to cleave the oligonucleotide from the support and remove the protecting groups from the bases. The NH_4OH -treated supernatant fluid was transferred to another tube and lyophilized overnight. The pellet was resuspended in 300 μl of 0.3 M NaOAc. The sample was extracted twice with an equal volume of n-butanol and ethanol-precipitated. The oligonucleotide was lyophilized and resuspended in 200 μl of DDW. The concentration was determined spectrophotometrically at an absorbance of 260 nm. (One O.D. 260 nm unit of single-stranded oligonucleotide equals 35 $\mu\text{g}/\text{ml}$).

Purification of oligonucleotide

An equal volume of Maxam and Gilbert loading mix (80% (w/v) deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) XC and 0.1% (w/v) BPB) was added to a 150 μg aliquot of crude oligonucleotide. The sample was heated at 90°C for 2 minutes and loaded into a 3 cm long slot of a 1.5 mm thick, 12% polyacrylamide/TBE gel (26 x 16 cm) containing 7M urea. The oligonucleotide was electrophoresed at 500 V until the BPB tracking dye migrated close to the bottom. The gel was placed on a thin layer chromatography cellulose sheet containing a fluorescent indicator (Eastman Kodak; Rochester, NY) and the oligonucleotide band was

visualized by UV shadowing using a shortwave 254 nm UV lamp. The band was cut out of the gel and the oligonucleotide was eluted by incubating in 2 ml of 0.1 M NaCl at 37°C for 16 hours with agitation. The salt was removed from the oligonucleotide using a NACS ion exchange minicolumn (Bethesda Research Laboratories; Gaithersburg, MD). Four hundred ul of 1 M NaCl were added to elute the DNA from the column. The oligonucleotide was ethanol-precipitated, lyophilized and dissolved in 50 ul of DDW. The DNA concentration was determined spectrophotometrically before being used in dideoxy sequencing reactions.

Dideoxy sequencing of influenza virus NS RNAs

The NS gene sequences of B/Yamagata/1/73, clone 201 viruses and a partial sequence of the NS gene of A/turkey/Oregon/71 virus were obtained by the dideoxy chain termination method using purified viral RNA as template (Buonagurio et al., 1986a).

Sequencing primers were oligonucleotides, 14-16 bases in length, corresponding to positions 216-230 of the NS gene of A/turkey/Ore/71 virus and to positions 13-26, 86-101, 293-307, 645-660 and 965-980 of the B/Lee/40 virus NS gene (Briedes and Lamb, 1982). The template and primer were annealed and the mixture was divided into 4 dideoxy reactions. Each reaction consisted of all four deoxynucleoside triphosphates, one of which was alpha-³²P-

labeled and a base-specific dideoxynucleotide. In the presence of reverse transcriptase, the primer was extended to generate copies of the cDNA template specifically terminated at the 3' end by one of the 4 DNA bases. The polynucleotide chains were fractionated according to size by polyacrylamide gel electrophoresis under denaturing conditions and detected by autoradiography on Cronex X-ray film.

[Alpha-³²P]-dCTP (20 uCi; specific activity 3,000 Ci/mole; New England Nuclear; Boston, MA) was aliquoted to the A reaction tube and 20 uCi of [alpha-³²P]-dATP was aliquoted to the G, T, and C reaction tubes. The radioactive solution was lyophilized and 5 ul of the appropriate 2X cocktail mixture was added to each tube. The 2X mixes were prepared as shown in Table 4.

Virion RNA (5 ug from sucrose gradient-purified virus) and 200 ng of NACS-purified oligonucleotide primer were dissolved in 18 ul of DDW. The mixture was kept at 90°C for 3 minutes then cooled at room temperature for 5 minutes. Four ul of this mixture was added to each of the 4 reaction tubes. Reverse transcriptase at 20 U/ul (Molecular Genetic Resources; Tampa, FL) was diluted in RT diluent (10 mM KH₂PO₄, pH 7.2, 2 mM DTT, 0.2% Triton X-100, 10% (v/v) glycerol) to 4 U/ul. One ul of diluted RT was added to each tube and the reactions were incubated at 42°C for 30 minutes and then 1 ul of cold dNTP chase (1 mM each

of the 4 dNTPs in RT buffer) was added to each tube. The reactions were incubated an additional 30 minutes and 11 ul of Sanger loading mix was added per reaction. The samples were boiled for 3 minutes and loaded onto the sequencing gel. The gel was electrophoresed, fixed, and dried as described above. Then the gel was autoradiographed at -70°C for 2 hours and the sequence was read.

TABLE 4. 2X DIDEOXY REACTION MIXTURES

	A	G	C	T
RT Buffer	2X [*]	2X	2X	2X
dATP	200 uM	20 uM	20 uM	20 uM
dGTP	500 uM	200 uM	500 uM	500 uM
dCTP	20 uM	500 uM	200 uM	500 uM
dTTP	500 uM	500 uM	500 uM	200 uM
ddNTP	ddA, 25 uM	ddG, 25 uM	ddC, 25 uM	ddT, 25 uM
Label	20 uCi	20 uCi	20 uCi	20 uCi
alpha- ³² P	dCTP	dATP	dATP	dATP

^{*}2X RT Buffer: 100 mM Tris-HCl, pH 8.3; 120 mM NaCl; 40 mM DTT; 24 mM MgCl₂

The data presented in the following chapter were published previously in:

Laski, F.A., R. Belagaje, R.M. Hudziak, M.R. Capecchi, G.P. Norton, P. Palese, U.L. RajBhandary and P. Sharp. 1984. Synthesis of an ochre suppressor tRNA gene and expression in mammalian cells. EMBO J. 3:2445-2452.

It should be noted that this paper represents a collaborative effort and that only data measuring the suppressor tRNA activity using influenza virus were generated by myself.

Synthesis of an ochre suppressor tRNA gene and expression in mammalian cells.

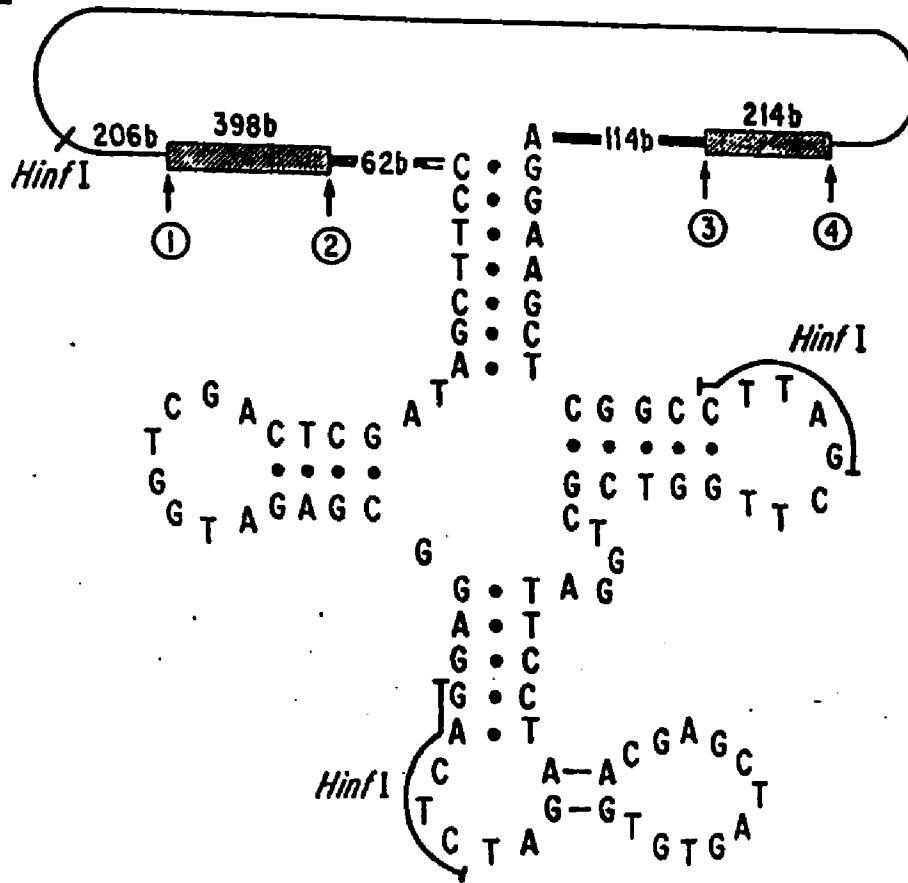
Results

Site-specific mutagenesis to generate a tRNA^{Tyr} (Su⁺) ochre gene

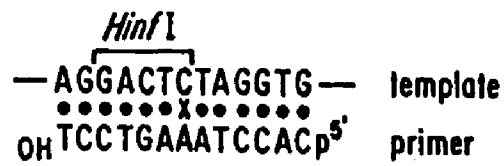
The sequence of the Xenopus laevis tRNA^{Tyr} (Su⁺) amber gene is shown in Figure 5A. This gene had previously been isolated by primer-directed mutagenesis (Laski, et al., 1982b) of the wild-type tRNA^{Tyr} gene. Using similar techniques an ochre suppressor tRNA gene was generated in an M13 vector by using a 13 base oligonucleotide in site-specific mutagenesis of the first position of the anticodon (Laski et al., 1982b). The single base mutation changes the anticodon to UUA which is complementary to the ochre termination codon, UAA (Figure 5B). The tRNA gene in M13-

Figure 5. (A) Structure of M13-tT-(Su⁺) amber. M13-tT-(Su⁺) amber contains a 263 bp HaeII/HhaI fragment of X. laevis DNA flanked by SV40 sequences cloned into the HindIII site of M13mp5. The tRNA^{Tyr}(Su⁺) amber gene was constructed by the site-specific mutagenesis of the wild-type tRNA^{Tyr} gene (Laski et al., 1982b). The sequence corresponding to the mature tRNA^{Tyr} (Muller and Clarkson, 1980) and its intervening sequence as well as the HinfI sites that cover and immediately flank the anticodon region are shown. Descriptions of the recombination sites are as follows: 1, the HindIII site of SV40 (m. u. 0.65) with the HindIII site of the M13 mp5 polylinker; 2, the HhaI site of X. laevis DNA with the HhaI site of SV40 (m. u. 0.72); 3, the HaeII site of the X. laevis DNA was joined to the HaeII site of SV40 (m. u. 0.82); 4, the HindIII site of SV40 (m. u. 0.86) was joined to the HindIII site of the M13 mp5 polylinker. For description of recombination sites see Laski et al. (1982b). (B) Sequence of the oligonucleotide primer. The oligonucleotide used for site-specific mutagenesis is complementary to the tRNA^{Tyr}(Su⁺) amber gene except for one mismatch. Incorporation of the mutation results in an anticodon (UUA) complementary to an ochre codon and removes a HinfI site.

A



B



tT-(Su⁺) ochre was properly transcribed and spliced in a HeLa S100 extract as described (Laski et al., 1984).

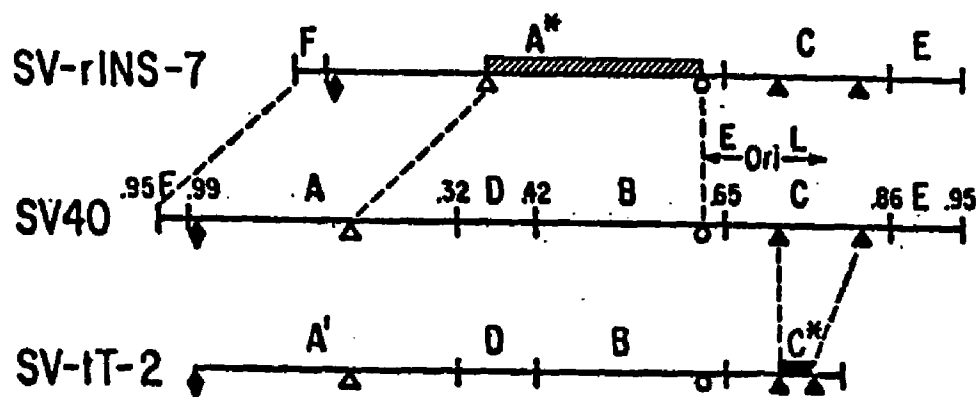
SV40-tRNA^{Tyr}(Su⁺) ochre recombinant

The DNA segment containing the tRNA gene was excised from the M13-tT-(Su⁺) ochre recombinant and cloned into the late region of SV40 (Figure 6). This construct [SV-tT-(Su⁺) ochre] is equivalent to that prepared previously with the SV-tT-(Su⁻) and SV-tT-(Su⁺) amber suppressor tRNA^{Tyr} genes (Laski et al., 1982a, 1982b). Virus stocks of all three SV40 recombinants were prepared by co-transfection of CV-1 cells with an SV40 rat preproinsulin recombinant, SV-rINS-7, which has a deletion/substitution in the early region of SV40 (Figure 6). About 1 week after co-transfection, the CV-1 cells underwent cytopathic degeneration and a virus stock was prepared (Laski et al., 1984).

Suppression of termination of influenza NS1 ochre codon.

While screening different field isolates of influenza viruses by migration in SDS polyacrylamide gels, Parvin et al. (1983) recognized that the NS1 polypeptides had a high level of heterogeneity. Subsequent sequencing of the NS gene segments from these viruses showed that the different migration patterns on polyacrylamide gels were the result

Figure 6. (A) HindIII restriction map of SV-rINS-7, SV40 and SV-tT-2. SV-tT-2 represents the structure of the tRNA^{Tyr}-SV40 recombinants that were used to make the SV-tT-(Su⁻), SV-tT-(Su⁺) amber and SV-tT-(Su⁺) ochre virus stocks. These tRNA^{Tyr}-SV40 recombinants are identical except for point mutations in the anticodon region of the tRNA^{Tyr} gene. Vertical lines represent positions of the HindIII sites in SV40 and map unit (m. u.) values of the sites are indicated. SV-rINS-7 (Horowitz et al., 1983) has an insertion of sequences from the rat preproinsulin gene and a deletion of sequences from the early region of SV40 creating the new restriction fragment A* (hatched box)(Laski et al., 1982a). SV-tT-2 has an insertion/deletion in the late region of SV40 creating the new restriction fragment C* and a deletion of late SV40 sequences (map unit 0.86-1.0) creating the new restriction fragment A'. The origin of replication (Ori) and the direction of early (E) and late (L) transcription are shown. Restriction sites used in construction of the recombinants: ▲, HhaI site; o, TaqI site; △, BclI site; EcoRI site. A, B, C, D, E and F indicate the different HindIII restriction segments of SV40. These recombinants have been previously described (Laski et al., 1982a, 1982b).



of size-differences in the carboxyl terminal region of the NS1 proteins (Parvin et al., 1983; Krystal et al., 1983; Buonagurio et al., 1986). Stop codons were found in six different positions in the NS genes, giving rise to NS1 polypeptides ranging in size from 202 to 237 amino acids. Examples for all three stop codons (amber-UAG, ochre-UAA and opal-UGA) were found. The polypeptide lengths predicted from these sequences were in close agreement with the observed mobilities of the viral NS1 polypeptides on SDS/6 M urea polyacrylamide gels. Furthermore, these observations suggested that large deletions in the carboxyl termini of the NS1 polypeptides of field virus isolates are tolerated without affecting their function during viral replication (Parvin et al., 1983).

A functional assay for the measurement of amber Su^+ (suppressor) tRNA activity was developed (Young et al., 1983b). The rationale for this assay is based on the ability to quantitate the readthrough product of the NS1 polypeptides of influenza viruses. If a suppressor tRNA is present than readthrough of the termination codon in the NS mRNA occurs, resulting in a longer NS1 polypeptide. In this way it was shown that cells engineered to express an amber Su^+ tRNA will suppress the amber stop codon of the influenza A/Cam/46 virus NS1 mRNA (Young et al., 1983b; Ho et al., 1986).

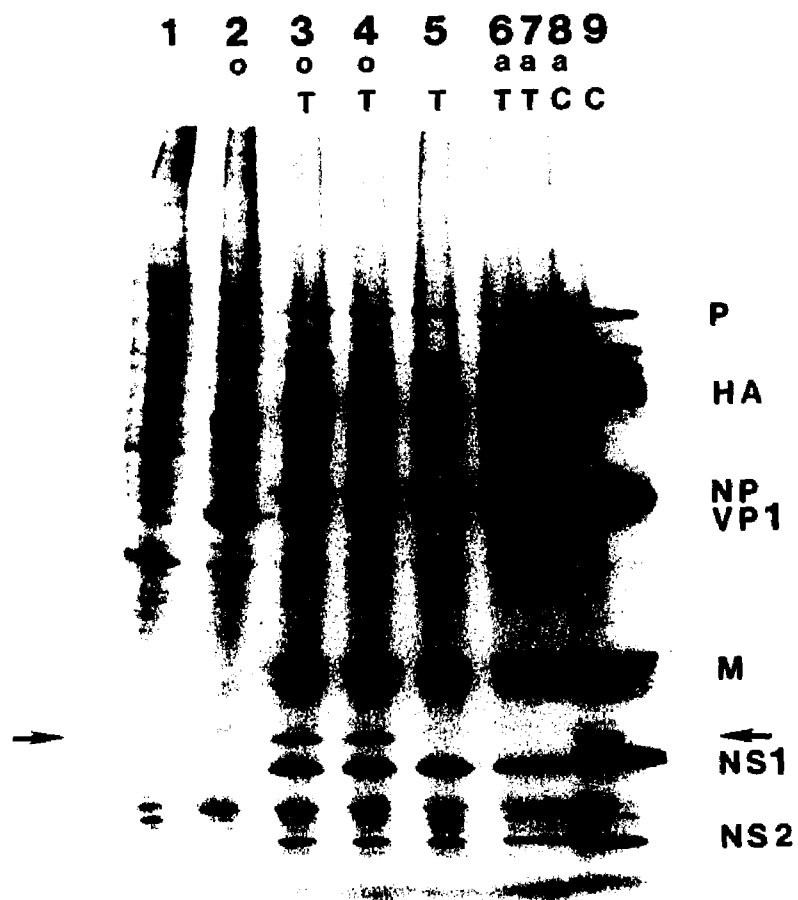
Previously an influenza (A/Cam/46) virus containing an

amber stop codon in the NS1 gene was used to quantitate the level of suppression with the SV-tT-(Su⁺) amber recombinant (Young et al., 1983a). The NS1 gene of influenza A/Tex/1/68 virus terminates in an ochre (UAA) codon and readthrough at this codon should result in a product which is 17 amino acids longer than the 220 amino acid normal protein (Parvin et al., 1983). To test the suppressor activity of the SV-tT-(Su⁺) ochre recombinant, CV-1 cells were infected with this virus stock and superinfected with influenza A/Tex/1/68 virus. The ³⁵S-methionine-labeled cell extract was separated by electrophoresis on polyacrylamide gels (Figure 7, lane 3) and its protein pattern was compared with that of cells infected only with A/Tex/1/68 virus (lane 5). A readthrough polypeptide of the anticipated molecular weight was observed only in extracts of cells co-infected with the (Su⁺) ochre virus (lane 3 indicated by arrow), but not in control CV-1 cells (lane 1) nor in cells that either had previously been infected with (Su⁻) SV40 recombinant, or the (Su⁺) amber virus (lanes 6 and 7, respectively; Table 5). The last result shows that, as expected, the amber suppressor activity did not suppress termination at ochre codons. Control experiments showed that SV-tT-(Su⁺) amber virus did suppress termination at the amber codon of the A/Cam/46 virus NS1 gene (lane 8).

The identity of the NS1 readthrough products following

Figure 7. Suppressor tRNA activity of SV40 (Su^+) ochre recombinant virus-infected cells as assayed by readthrough of influenza NS1 mRNA. CV-1 cells (1×10^6 cells) were infected with SV-tT-(Su^+) ochre virus or SV-tT-(Su^+) amber virus as indicated below. Thirty six hours later cells were superinfected with influenza virus A/Cam/46 or A/Tex/1/68 virus. After another six hours, cells were pulse-labeled for 1 hour using ^{35}S -methionine and then the cell extracts were subjected to electrophoresis on a gradient polyacrylamide gel as previously described (Young et al., 1983a). Lanes: 1, uninfected cells; 2, cells infected for 42 hours with SV-tT-(Su^+) ochre virus; 3, cells were first infected for 36 hours with (Su^+) ochre virus and then superinfected for 6 hours with influenza A/Tex/1/68 virus; 4, same as lane 3 except that concentration of SV-tT-(Su^+) ochre virus was two fold higher; 5, cells infected for 6 hours with A/Tex/1/68 virus; 6, cells were first infected for 36 hours with (Su^+) amber virus (Laski et al., 1982b) and then superinfected for 6 hours with influenza A/Tex/1/68 virus; 7, same as lane 6 except that the concentration of SV-tT-(Su^+) amber virus was two fold higher; 8, cells were first infected for 36 hours with (Su^+) amber virus and then superinfected for 6 hours with influenza A/Cam/46 virus; 9, cells infected for 6 hours with A/Cam/46 virus. 'o' and 'a' indicate the (Su^+) ochre and the (Su^+) amber SV40 recombinant

preparations, respectively. 'T' and 'C' identify influenza A/Tex/1/68 and A/Cam/46 virus infected cells, respectively. The molecular weights of the P, HA, NP, M1 NS1 and NS2 polypeptides are 82-86K, 77K, 56K, 28K, 23K and 14K, respectively. The left and right arrows indicate the positions of the NS1 readthrough products of A/Tex/1/68 and A/Cam/46 viruses, respectively.



amber or ochre suppression was confirmed by immunoprecipitation using a monospecific rabbit antiserum (Young et al., 1983b) made against NS1 protein synthesized in bacteria (data not shown). Optimal readthrough using the (Su⁺) ochre virus was found to occur if influenza virus infection followed a 36-48 h pre-infection with the SV40 recombinant (data not shown). Use of preparations of the (Su⁺) ochre virus and the (Su⁺) amber virus at a 2-fold higher concentration did not increase the level of readthrough (Figure 7, lanes 4 and 7, respectively).

In bacterial systems, ochre suppressors recognize both amber and ochre codons (Brenner and Beckwith, 1965). Therefore, it was of interest to test whether the SV-tT-(Su⁺) ochre virus would also suppress termination at amber codons in mammalian cells. Pre-infection of cells with the (Su⁺) ochre virus did not result in a readthrough of the amber codon of the NS1 gene of A/Cam/46 virus (Table 5). As mentioned above, under identical conditions, the SV-tT-(Su⁺) amber virus did suppress termination of the NS1 gene of A/Cam/46 virus and the (Su⁺) ochre virus was active in suppression of the NS1 gene of A/Tex/1/68 virus.

Discussion

Mammalian cells are not known to be permissive for translation of all three nonsense codons. Selection of

TABLE 5. Suppression of termination at NS1 ochre or amber codons. SV40 recombinants expressing suppressor tRNA genes were used to infect CV-1 cells and the amount of ochre and amber suppression was determined by quantitating the readthrough product of the NS1 polypeptide of influenza A/Tex/1/68 and A/Cam/46 virus, respectively (Fig. 7). (% suppression represents the amount of readthrough product of NS1 divided by the amount of NS1 and of the readthrough product of NS1). Conditions for infection of CV-1 cells were as described for Fig. 7. Suppression values represent the average of two independent experiments.

Table 5. Suppression of termination at NS1 ochre or amber codons

<u>Conditions</u>	Level of Suppression (%)	
	<u>A/Tex/1/68 NS1</u> (ochre)	<u>A/Cam/46 NS1</u> (amber)
CV-1	<1	<1
CV-1 infected with(Su^+) ochre SV40 recombinant	31.3	<1
CV-1 infected with (Su^+) amber SV40 recombinant	<1	32
CV-1 infected with (Su^-) SV40 recombinant	<1	<1

cell lines with suppressor activity by conventional methods of reversion of phenotype of nonsense mutants has so far not been successful. An alternative approach is the transformation of cells with tRNA genes with suppressor activity (Laski et al., 1984). Site-specific mutagenesis was used to alter the anticodon of a X. laevis tyrosine tRNA gene (Laski et al., 1982b).

Suppression activity at ochre codons was assayed using influenza A/Tex/1/68 virus. The (Su^+) ochre tRNA gene was introduced into cells as part of a replicating SV40 recombinant before infection by influenza virus. The level of suppression of termination at the ochre codon of the NS1 gene of influenza virus A/Tex/1/68 was examined. Cells pre-infected with the (Su^+) ochre SV40 recombinant synthesized a readthrough polypeptide at 30% efficiency. This results in the synthesis of high levels of (Su^+) tRNA and probably establishes near optimal conditions for suppression at nonsense codons. This level was comparable with that obtained under equivalent conditions with (Su^+) amber SV40 recombinant and influenza A/Cam/46 virus which terminates its NS1 gene with an amber codon (Young et al., 1983b). Thus amber and ochre codons are probably equally susceptible to suppression by tRNAs in mammalian cells. Detailed studies of suppression of nonsense codons in bacterial genes have revealed that the sequence context of the codon is important in specifying the efficiency of

readthrough (Bossi and Roth, 1980; Miller and Albertini, 1983; Bossi, 1983). It is difficult to judge the effect of context on the level of suppression of the influenza virus NS1 gene. However, it is possible that some ochre codons might be more efficiently suppressed under comparable conditions. In any case, a 30% level of readthrough is typical of strong yeast suppressors and adequate for most genetic studies (Piper, 1979).

In E. coli, ochre suppressors recognize both amber and ochre codons (Brenner and Beckwith, 1965) in accordance with the wobble hypothesis of Crick (1966). This is not the case in yeast where ochre tRNA suppressors are specific for ochre codons (Gilmore et al., 1971). This specificity is thought to be due to the modification of the U in the first position of the UUA anticodon to a base which cannot recognize amber codons by wobble pairing (Piper, 1979). The X. laevis tRNA^{Tyr} (Su⁺) ochre gene appears to be specific for ochre codons since pre-infection of CV-1 cells with an SV40 recombinant containing this gene did not result in suppression of termination at an amber codon of the NS1 gene.

The data presented in the following chapter are part of a manuscript:

Ho , Y.-S., G.P. Norton, P. Palese, A.M. Dozey and Y.W. Kan. 1986. Expression and function of suppressor tRNA genes in mammalian cells. Cold Spring Harbor Symp. for Quant. Biol. No. 51 (in press).

Expression and function of suppressor tRNA genes in mammalian cells.

Results

Construction and function of suppressor tRNA genes

Previously, Temple et al. (1982) site-specifically mutagenised the anticodon region of a normal tRNA gene in order to generate an amber suppressor tRNA gene. Briefly, they introduced an EcoRI fragment of DNA containing the human tRNA^{Lys} into M13mp7 phage and changed two nucleotides in the anticodon region by primer-directed mutagenesis. The tRNA^{Gln} gene was mutated in a similar manner in order to generate an amber suppressor tRNA gene (Ho et al., 1986).

Transient expression of suppressor tRNA genes in mammalian cells

The two suppressor tRNA genes and their normal counterparts were subcloned into the late region of SV40

between HhaI sites or HhaI and AccI sites, oriented in the same or opposite transcriptional direction as SV40 late transcription (Fig. 8). Virus stocks of each SV40 recombinant were grown up by cotransfecting CV-1 cells with SV40 DNA am404 (Rawlins and Muzyczka, 1980) which contains an amber mutation in the large T antigen (Ho et al., 1986).

In vivo suppression of the amber codon

A rapid quantitative assay has been developed to determine the level of suppressor tRNA activity in mammalian cells based on infecting cells with influenza A/Cam/46 virus (Young et al., 1983b). The amount of readthrough of an amber stop codon of the viral protein NS1. This assay was used to measure suppressor tRNA^{Lys} and tRNA^{Gln} activity in CV-1 cells. At 36 hours after infection of CV-1 cells with SV40 recombinant viruses carrying tRNA genes, the cultures were incubated with influenza virus A/Cam/46 for another 6 hours and pulse-labeled with ³⁵S-methionine. As a control, the tRNA^{Tyr(Su+)} gene [SV-tT-2(Su+)] previously constructed by Laski et al. (1982b) was included. An example of the results obtained is shown in Fig. 9. The readthrough of the amber codon corresponding to amino acid number 218 in the mRNA by suppressor tRNA^{Tyr}, tRNA^{Lys} or tRNA^{Gln} results in proteins that migrate to slightly different positions on SDS-polyacrylamide gels. Although the the readthrough

Figure 8. Map of the SV40 vector. The normal or suppressor tRNA genes were inserted into the late transcription region as indicated to produce the recombinants SV-Gln, SV-Gln(Su+), SV-Lys and SV-Lys(Su+). These genes were inserted either in the direction designated 1 or opposite to the direction designated 2 of late transcription.

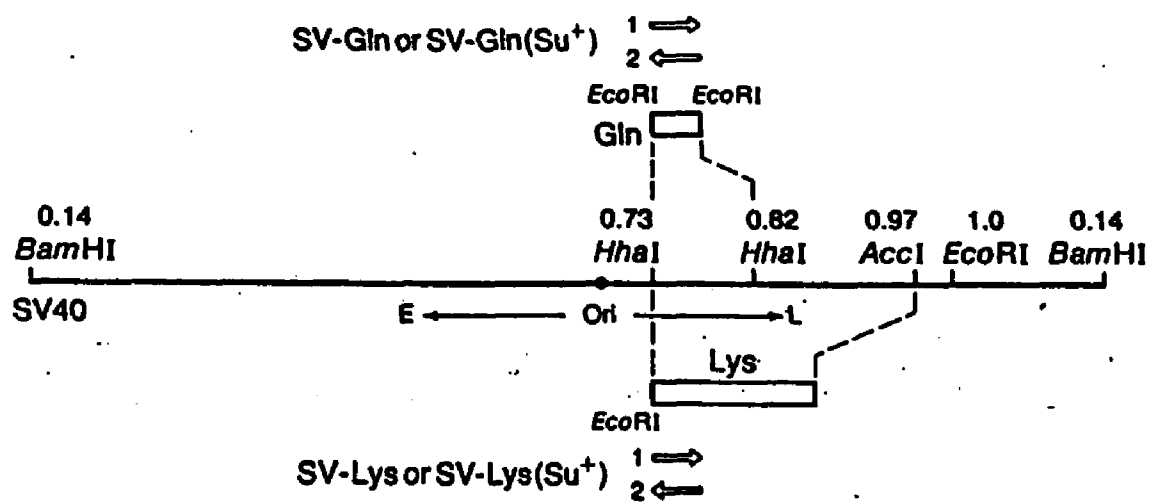
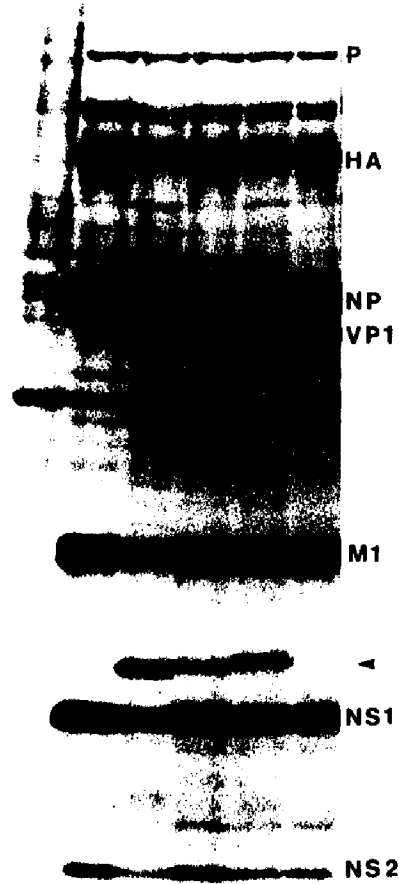


Figure 9. Suppressor tRNA activity of SV40 recombinant viruses in CV-1 cells. CV-1 cells (2×10^6) were infected with SV40 recombinants (10^8 PFU/ml) (0.1 ml); 36 hours later influenza A/Cam/46 virus was added (MOI=10). Cells were pulse-labeled for 1 hour with ^{35} S-methionine after an additional 6 hours. Total cell extracts were used for electrophoresis on a 7-14% gradient polyacrylamide gel (Young et al., 1983). Lane 1: mock-infected cells. Lane 2: Cells infected with influenza virus. Lane 3: Cells infected with SV40 recombinant SV-Gln(Su+)-1 and influenza virus. Lane 4: Cells infected with SV40 recombinant SV-Lys(Su+)-1 and influenza virus. Lane 5: Cells infected with SV40 recombinant SV-tT-2(Su+) and influenza virus. Lane 6: Cells infected with SV40 recombinant SV-Gln-1 and influenza virus as control. The arrow indicates the position of the NS1 readthrough product. The molecular weights of the P, HA, NP, M1, NS1 and NS2 polypeptides of the influenza virus are 82-86K, 77K, 56K, 28K, 23K and 14K, respectively. The band VP1 represents the major SV40 protein.

1 2 3 4 5 6



products were all 237 amino acids in length, differences in the amino acid sequences may result in subtle differences in protein-SDS interactions and then lead to migrational differences on gels.

The efficiency of suppression was measured by counting the radioactivity associated with the NS1 protein and its readthrough product. Suppressor tRNA activity was not detected in CV-1 cells infected with SV40 recombinant viruses carrying normal tRNA genes. The efficiency of suppression by the three suppressor tRNA genes in several experiments ranged from 30% to 50%. No suppression was detectable in cells infected with the SV40 recombinant virus carrying the tRNA^{Gln(Su+)} gene in an orientation opposite to the SV40 promoter.

Establishment of permanent cell lines containing different copies of functional suppressor tRNA^{Lys} genes

The dihydrofolate reductase (DHFR) gene was used as a selectable marker to introduce suppressor tRNA genes into Chinese hamster ovary (CHO) dhfr⁻ cells (Ho et al., 1986). Genes linked to a DHFR gene can be coamplified during the selection of methotrexate (MTX) resistance after transforming them into dhfr⁻ cells. MTX inhibits DHFR in a competitive manner and therefore indirectly inhibits the de novo synthesis of purines, thymidylate and glycine.

Exposure to high concentrations of MTX kills the majority of cells but $<10^{-6}$ cells have more than one copy of the DHFR gene and are able to proliferate in medium containing MTX.

CHO-K1 dhfr⁻ cells were transformed with the vector pMV104 that contained a cloned DHFR gene, (Subramani et al., 1981) or the vector into which was inserted a normal tRNA^{Lys} gene (pMV104-Lys) or a tRNA^{Lys(Su+)} gene [pMV104-Lys(Su+)] (Fig. 10, Ho et al., 1986). A transfection frequency of about 1 transformant per 10^4 cells was obtained on initial selection. The pools of transformants were then exposed to increasing concentrations of MTX (0.1, 1, 10 and 100 μ M) to select resistant colonies. Southern blot analysis of the genomic DNAs from CHO-K1 transformants was performed using a DNA fragment containing the human tRNA^{Lys} gene. It was found that as resistance to MTX was increased, the copy number of Su⁺tRNAs genes increased to 250-300 copies per cell (Table 6).

Function of the suppressor tRNAs in CHO cells

The level of suppression in the CHO cell lines was determined by measuring the readthrough product of the NS1 protein after infection with influenza A/Cam/46 virus (Table 6). Suppression efficiency varied from nondetectable to 3.6% in the cell lines containing different copy numbers of the Su⁺tRNA genes.

Figure 10. Structure of the plasmid pMV104. The tRNA^{Lys} and tRNA^{Lys(Su+)} genes were inserted into this plasmid as indicated.

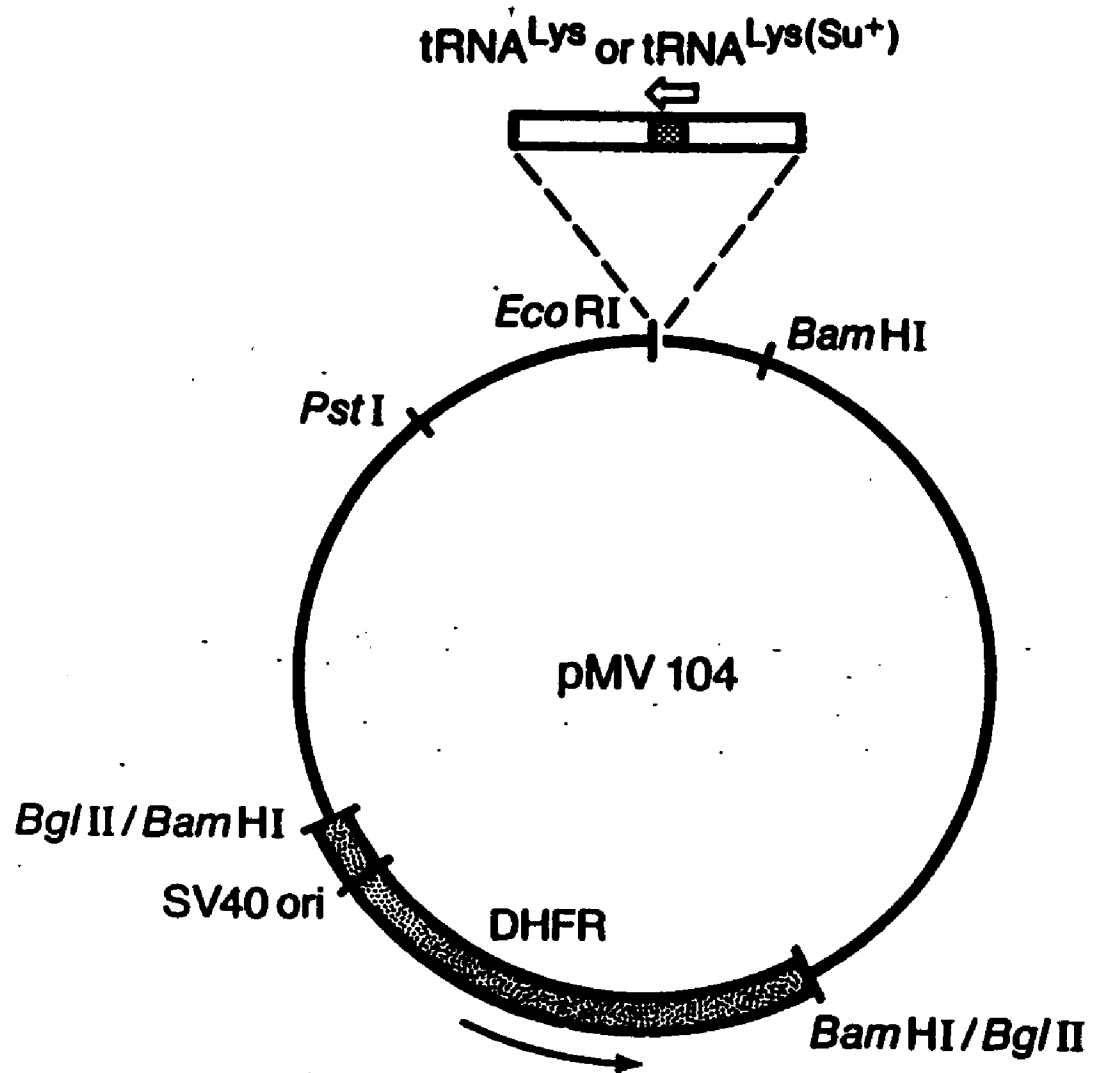


TABLE 6. Amber suppression in CHO cell lines expressing tRNA^{Lys (Su+)}

Methotrexate (μ M)	Lys (Su+) tRNA (copy number)	gene	Level of Suppression (%)
0	1		0.5
0.1	20		1.0
1.0	100		1.9
10.0	200		1.7
100.0	250-300		3.6

From: Ho et al., 1986.

Since the efficiency of suppression was much greater in SV40 Su⁺tRNA infected CV-1 cells than in stably transformed CHO-K1 cells, (approx. 40% vs. 3.6%) the steady state levels of suppressor tRNA^{Lys} in these cells was examined by Northern blot analysis using the ³⁵P-labeled tRNA gene and a ³²P-labeled oligonucleotide probe complementary to the anticodon region of the Su⁺tRNA^{Lys} (Ho et al., 1986). The result of that experiment indicated that the accumulation of suppressor tRNA^{Lys} in SV40 Su⁺tRNA^{Lys}-infected CV-1 cells was only slightly higher than that of the transformed CHO cell lines which contained approximately 300 copies of suppressor tRNA^{Lys} genes per cell. In addition, the endogenous tRNA^{Lys} levels in CHO-K1 and CV-1 cells were equal.

Caskey and Campbell (1979), reported that the efficiency of suppression may be a function of the extent of aminoacylated suppressor tRNA in the cells. In the present study the in vivo level of aminoacylated Su⁺tRNA^{Lys} was measured by quantitating the ratio of charged to uncharged tRNA. The result of this experiment revealed that 50% of the Su⁺tRNA^{Lys} is charged in the transiently infected CV-1 cells, but less than 10% of the Su⁺tRNA^{Lys} is charged in the CHO cell lines.

Discussion

Kan's group (Temple et al., 1982; Ho et al., 1986)

constructed functional human suppressor tRNA genes by site-specific mutagenesis of normal human tRNA genes. These Su^+ tRNA genes were efficiently expressed and suppressed $Beta^0$ thalassemia mRNA when injected into the nuclei of Xenopus oocytes. These studies showed that functional human amber suppressor genes can be constructed by site-specific mutagenesis of the anticodon region. Furthermore, these genes produced tRNAs capable of suppressing the amber codons in transient assays such as CV-1 cells. However, in high copy number in stably transformed CHO cells, these suppressor tRNAs are not charged efficiently and mediate only low levels of suppression. Transformation of other mammalian cell types with Su^+ tRNA genes may circumvent the problem of inefficient charging.

A possible application for the introduction of Su^+ tRNA genes lies in correcting genetic defects such as some types of Beta thalassemias that are due to nonsense mutations. Retrovirus vectors which contain suppressor tRNAs genes may be used to insert the Su^+ tRNA gene with high efficiency into bone marrow cells of Beta thalassemia patients. Expression of the Su^+ tRNA gene may lead to the expression of a full length globin product. It should be noted that retrovirus vectors have successfully been used in vitro to introduce appropriate genes into cells characterized by enzyme deficiency states such as adenosine deaminase-deficient severe combined immunodeficiency disease (Friedman

1985; Williams et al., 1986) Lesch Nyan syndrome (Miller et al., 1983, 1984) and Gaucher's disease (Choudary et al., 1986).

Natural suppressor tRNA activity in higher eukaryotic cells

Results

The suppressor tRNA assay was used to determine if naturally occurring suppressor tRNA activity exists in mammalian cells. Specifically, the assay for suppressor tRNA activity involves infection of cells with influenza viruses. Six hours later cells are labeled with ^{35}S -methionine for 1 hour and the cell extract is separated on a 7-14% polyacrylamide gel as described by (Young and Palese, 1979). The amount of NS1 and NS1 readthrough product is determined by scanning the exposed X-ray film or by directly determining the level of radioactive material in the excised gel bands in a scintillation counter.

Previously Hatfield et al. (1983) had isolated a gene coding for an opal suppressor tRNA from a chicken liver DNA library, but they did not determine whether this Su^+ tRNA is expressed and active during protein synthesis. Therefore, primary chicken kidney (PCK) cells were chosen in an attempt to detect suppressor tRNA activity in metabolically active cells. In addition, mammalian kidney (MDCK) cells were analyzed for the presence of naturally occurring suppressor tRNA activity. Different influenza viruses (A/USSR/77, A/FM/1/47, A/sw/Wisc/om/2, A/FW/1/50, A/PR/8/34, A/Cam/46, A/Berkeley/1/68 and A/Tex/1/68) were used to infect PCK or MDCK cells and infected cell

extracts were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 11). The influenza viruses were chosen to permit detection of amber, ochre as well as opal suppressor tRNA activity. The amino acid position of the 1st and 2nd nonsense codons in the NS1 reading frame of these viruses are indicated in table 7. Comparison of the uninfected PCK cell extract (lane 1) with virus-infected PCK cell extracts (lanes 2-9) (in the region of the NS1 polypeptide) did not reveal any NS1 readthrough products. Similarly NS1-readthrough products were not identified in any of the virus-infected MDCK cell extracts following protein gel electrophoresis (lanes 11-15). The level of suppression that can be reliably measured by densitometric tracings of films exposed to polyacrylamide gels is approximately 4%.

Therefore, in order to determine if natural suppressor tRNA activity exists at low levels in eukaryotic cells it was necessary to devise a more sensitive suppressor tRNA assay. A Western blot protocol described in materials and methods was utilized to search for naturally occurring suppressor tRNA activity. Following electrophoresis the proteins were transferred onto a DEAE-membrane and the membrane was incubated with anti-A/NS1 antibody (Young et al., 1983a) followed by incubation with ¹²⁵I-labeled goat anti-rabbit IgG.

Utilizing this Western blot assay, Influenza A/USSR/77,

Figure 11. Natural suppressor tRNA activity in PCK cells and MDCK cells. Six hours post infection with influenza virus cells were labeled with [³⁵S] methionine for 1 hour, lysed and cell extracts were analyzed on a 7-14% gradient polyacrylamide gel. Lane 1, uninfected PCK cell extract; lanes 2-9 influenza virus-infected PCK cell extracts; lane 10 uninfected MDCK cell extract; lanes 11-15 influenza virus-infected MDCK cell extracts; lanes 2 and 11, influenza A/USSR/77 virus-infected cell extracts; lanes 3 and 12, influenza A/FM/1/47 virus-infected cell extracts; lanes 4 and 13, influenza A/sw/Wisc/om/2 virus-infected cell extracts; lanes 5 and 14, influenza A/FW/1/50 virus-infected cell extracts; lanes 6 and 15, influenza A/PR/8/34 virus-infected cell extracts; lane 7, influenza A/Cam/46 virus-infected cell extract; lane 8, A/Berkeley/1/68 virus-infected cell extract; lane 9, A/Tex/1/68 virus-infected cell extract.

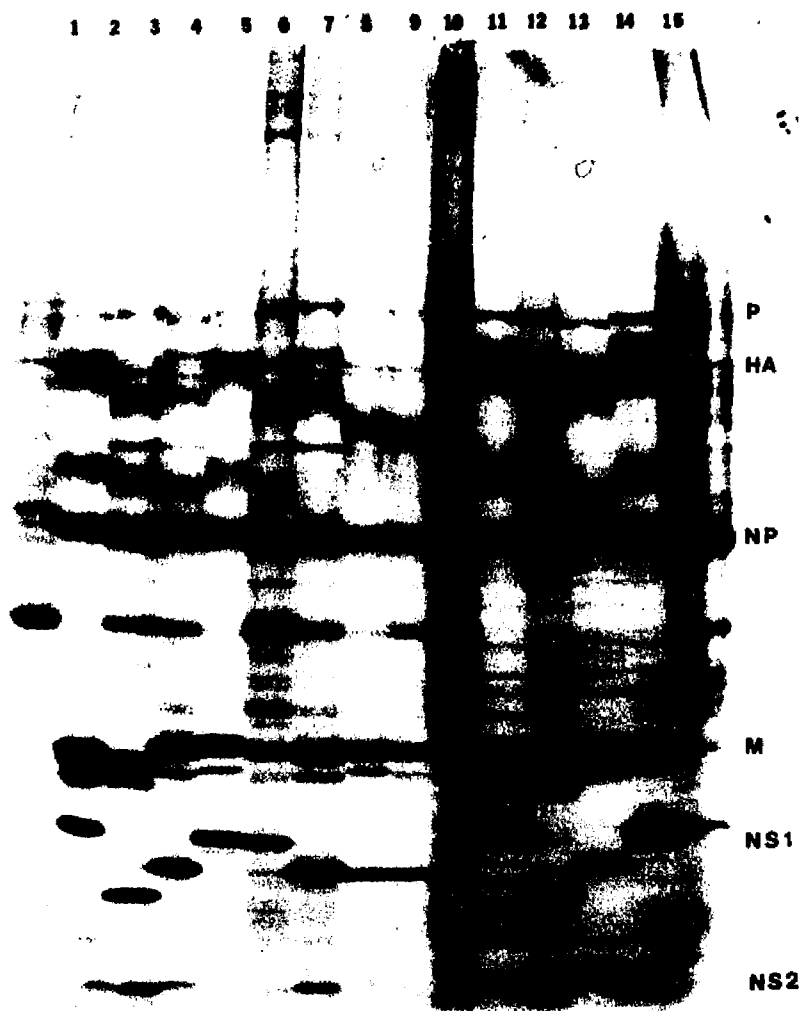


TABLE 7

Virus	NS1 Protein length ^a (amino acids)	1 st Stop codon ^b	2 nd Stop codon
A/USSR/77	237	TGA ₂₃₈	TAG ₂₅₀
A/FM/1/47	202	TGA ₂₀₃	TAG ₂₁₉
A/sw/Wisc/cm/2	218	TGA ₂₁₉	TGA ₂₃₁
A/FM/1/50	237	TGA ₂₃₈	TAG ₂₅₀
A/PR/8/34	230	TGA ₂₃₁	TGA ₂₃₈
A/Can/46	218	TAG ₂₁₉	TGA ₂₃₈
A/Berkeley/1/68	220	AAA ₂₂₁	TGA ₂₃₁
A/Tex/1/68	220	AAA ₂₂₁	TGA ₂₃₁

^aThe protein length was predicted from the nucleotide sequence (Parvin et al., 1983; Krystal et al., 1983).

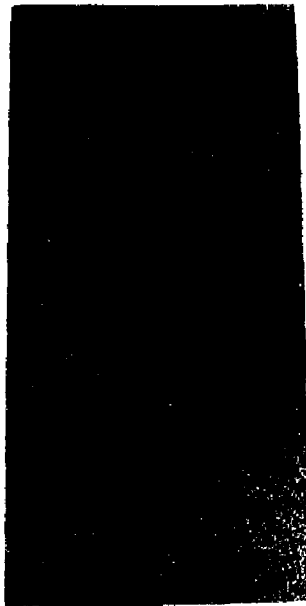
^bThe subscript denotes the codon in which the nonsense mutation occurs in comparison to the A/USSR/77 strain.

A/FM/1/47 and A/Cam/46 virus-infected chicken embryo fibroblast (CEF) cells were analyzed for opal and amber suppression of the NS1 polypeptide. The result of this experiment is shown in Fig. 12. The primary antibody also crossreacts with 2 higher molecular weight proteins of host cell origin (lane 1). This crossreactivity could be due to the polyclonal nature of the rabbit antiserum. No NS1 readthrough product was detected for any of these three influenza virus-infected cells (lanes 2-4). The sensitivity of this assay is such that a readthrough product at 0.1 to 0.5% of the level of the normal NS1 protein is reliably detected.

In addition, Chinese hamster ovary cells, CHO-K1 dhfr⁻ transformed with either the normal tRNA^{Lys} gene or the amber suppressor tRNA^{Lys} gene, were analysed by this assay. CHO-K1 cells transformed with a (Su⁺)tRNA^{Lys} gene had been cotransformed with a DHFR gene followed by selection with increasing concentrations of MTX. The Su⁺ tRNA activity of these different CHO-K1 cell lines was determined by infection with A/Cam/46 virus. The normal NS1 gene product expressed in A/Cam/46-infected cells is 218 amino acids while the readthrough of the amber stop codon results in a protein of 237 amino acids (Table 7). Infected cell extracts were electrophoresed on SDS gels and examined by Western blot analysis as described above (Fig. 13). Cells transformed with the normal tRNA^{Lys} genes did not reveal a

Figure 12. Natural suppressor tRNA activity in CEF cells. Twenty hours post infection, with influenza virus, CEF cells were lysed and cell extracts were electrophoresed onto a 7-14% gradient polyacrylamide gel and electrophoretically transferred onto a DEAE-membrane. The DEAE-membrane was incubated with anti-A/NS1 antibody (Young et al., 1983a) followed by incubation with ^{125}I -labeled goat anti-rabbit IgG. Lane 1, uninfected CEF cell extract; lane 2, influenza A/USSR/77 virus-infected cell extract; lane 3, influenza A/Cam/46 virus-infected cell extract; lane 4, influenza A/FM/1 virus-infected cell extract.

1 2 3 4



NS1

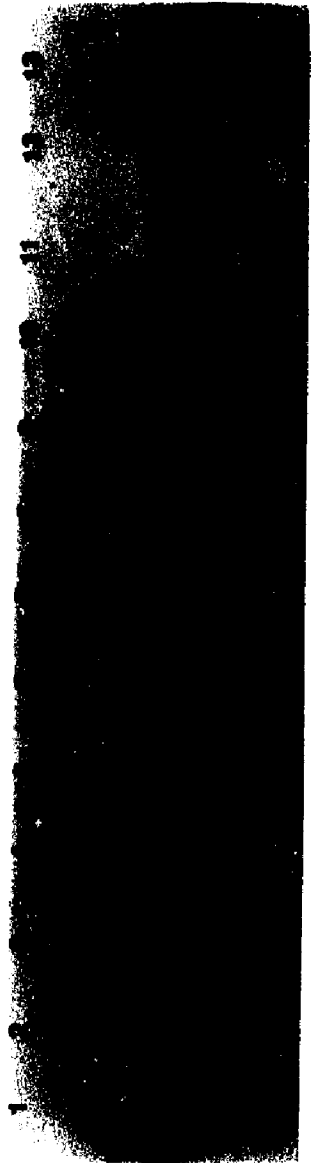
measurable level of suppression (sensitivity 0.1 to 0.5%). Also, cells which were transformed with tRNA^{Lys(Su+)} gene and not selected with MTX, did not show a detectable level of suppression. However, tRNA^{Lys(Su+)} gene transformed cells selected in the presence of 0.1 uM MTX consistently showed a level of suppression of approximately 1%. There were 20 copies of the tRNA^{Lys(Su+)} gene in CHO cells selected with 0.1 uM MTX versus only 1 copy of the tRNA^{Lys(Su+)} gene in CHO cells which were not selected with MTX (Ho et al., 1986). As tRNA^{Lys(Su+)} gene copy number increased from 1 to 20, the level of suppression increased from nondetectable to only 1%. Whereas, approximately a 20% level of suppression was detected in control CV-1 cells infected for 36 hours with SV-tT-2(Su+) recombinant virus and superinfected with influenza A/Cam/46 virus. In summary, if natural suppressor tRNA activity exists in CHO cells its activity is less than 0.1 to 0.5% which is the level of sensitivity in this system.

Discussion

Assays have recently been developed to detect suppressor tRNA activity in higher eukaryotic cells (Hudziak et al., 1982; Summers et al., 1983; Young et al., 1983; Capone et al., 1986). In this laboratory a suppression assay was established which is based on the tRNA-mediated readthrough of the NS1 polypeptide of influenza A/Cam/46 virus (Young et al., 1983). This

Figure 13. Natural suppressor tRNA activity in CHO-K1 cells transformed with a normal tRNA^{Lys} gene. Twenty hours post infection with influenza A/Cam/46 virus, CHO cells were lysed and cell extracts were electrophoresed on a 7-14% gradient polyacrylamide gel. The NS1 and NS1 readthrough proteins were analyzed according to the Western blot technique described in the legend to Fig. 12. Lanes 1-4, virus-infected CHO tRNA^{Lys} cell extract; lanes 5-8, virus-infected CHO tRNA^{Lys(Su+)} cell extract; lanes 9-12, CHO tRNA^{Lys(Su+ 0.1 uM MTX)}; Lane 13, CV-1 cells infected for 36 hours with SV-tT-2(Su+) recombinant virus and superinfected with influenza virus, (1/5 dilution of the virus-infected cell extracts); lanes 1, 5 and 9 undiluted virus-infected cell extracts; lanes 2, 6 and 10, 1/5 diluted virus-infected cell extracts; lanes 3, 7 and 11, 1/25 diluted virus-infected cell extract; lanes 4, 8 and 12, 1/125 diluted virus-infected cell extract.

NSN



quantitative assay to measure suppressor tRNA activity in a variety of systems is made possible by a combination of factors. i) Many eukaryotic cells appear to synthesize influenza virus-specific proteins following infection with egg-grown virus, concomitant with the shut-off of host-cell protein synthesis. ii) The NS1-readthrough protein is likely to be stable since influenza virus field isolates have been found to contain NS1 polypeptides of different lengths. iii) The level of the suppressed as well as the nonsuppressed NS1 gene products can be measured in the same sample and therefore this ratio should accurately reflect the suppressor tRNA activity in a particular cell (assuming every cell in the population exhibits comparable suppressor activity). iv) Preinfection with SV40 recombinants or expression of the Su^+ tRNA genes in transformed cells does not appear to interfere with influenza virus gene expression.

Within the last few years natural suppressor tRNAs have been identified in rabbit reticulocytes (Geller and Rich, 1980) and bovine liver (Diamond and Dudock, 1981). Also, genes coding for Su^+ tRNAs have been isolated from chicken liver and human DNA libraries (Hatfield et al., 1985; O'Neill et al., 1985). However, it is not known whether these Su^+ tRNA genes are expressed and possess activity in metabolically active cells. I utilized the suppressor tRNA assay of Young et al. (1983) in order to address this

question, but could not detect any activity in PCK or MDCK cells which was sensitive to a level of approximately 4%. I increased the sensitivity of the assay by the use of a Western blot procedure. Although the sensitivity of the assay was improved by a factor of 10, no evidence for natural suppressor tRNA activity was found using either CEF or CHO cells.

Recently, another approach to detect amber, opal and ochre suppressor tRNA activity was reported (Capone et al., 1986). It involves the use of DNA-mediated transfection of a gene coding for chloramphenicol acetyltransferase (CAT) Gorman et al., (1982). Capone et al. (1986) used site-specific mutagenesis to introduce nonsense codons into the E. coli CAT gene. These mutant CAT genes are then employed to transfect cells and the expression of the enzymatic CAT activity permits quantitation of the amount of suppression in different mammalian cells. It is hoped that this assay in conjunction with the protocols described here may be used in the future to answer the question whether natural suppressor tRNA activity plays a role in the expression of genes in higher eukaryotic cells.

The following paragraphs are taken from a manuscript which has been accepted for publication.

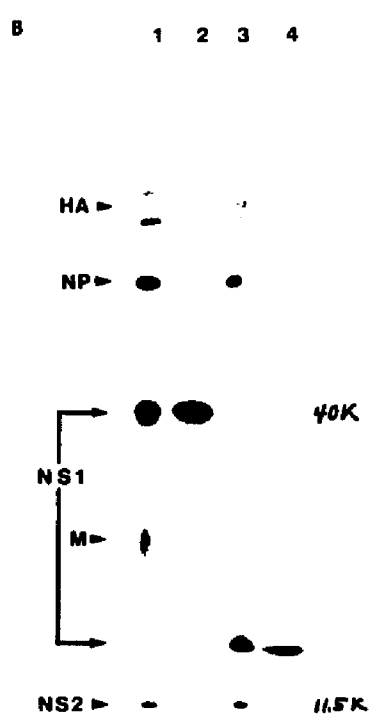
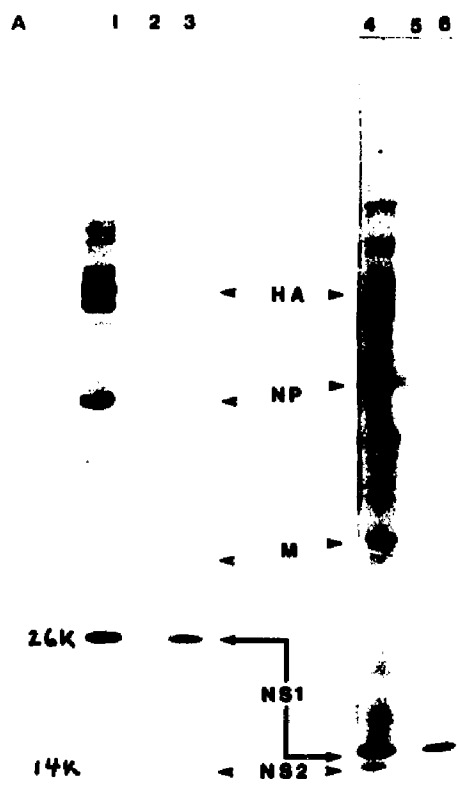
Norton, G.P., T. Tanaka, K. Tobita, S. Nakada, D.A. Buonogurio, D. Greenspan, M. Krystal and P. Palese. 1986. Infectious influenza A and B virus variants with long carboxyl terminal deletions in the NS1 polypeptides. Virology (in press).

Results

Identification of NS1 protein in influenza A/turkey/Oregon/71 virus infected cells

Protein gel analysis of influenza A/turkey/Ore/71 virus-infected MDCK cells did not reveal a protein band in the position expected for the NS1 protein of influenza A virus-infected cells (Fig. 14A). However, a labeled protein band of apparent 14.8 K MW migrating near the NS2 protein was detected in the A/turkey/Ore/71 virus-infected cells and not in A/PR/8/34 virus cell extracts (Fig. 14A). In order to verify that this faster moving polypeptide is the NS1 protein, monospecific anti-A/NS1 antibody was used to immunoprecipitate the NS1 proteins of A/turkey/Ore/71 and A/PR/8/34 virus-infected cells. As shown in Fig. 14A, the 14.8 K polypeptide of A/turkey/Ore/71 virus-infected cells was specifically precipitated by anti-A/NS1 antibody. This result suggests that this fast moving protein band in

Figure 14. Identification of NS1 bands in influenza A and B virus-infected cells. Six hours post infection, MDCK cells were labeled with ^{35}S -methionine for 1 hour, lysed and cell extracts were analysed on 7-14 % gradient polyacrylamide gels either directly or following immunoprecipitation. A) Immunoprecipitation of NS1 proteins of influenza A/PR/8/34 and A/turkey/Oregon/71 virus-infected cells. Apparent molecular weights for the NS1 and NS2 proteins of A/PR/8/34 virus-infected cell extracts are indicated on the left-hand side of Fig. 14A. Lanes 1, 2, and 3: influenza A/PR/8/34 virus-infected cell extracts; lanes 4, 5, and 6: influenza A/turkey/Oregon/71 virus-infected cell extracts; lanes 1 and 4, whole virus-infected cell extracts; lanes 2 and 5, virus-infected cell extracts immunoprecipitated with normal rabbit serum; lanes 3 and 6, virus-infected cell extracts immunoprecipitated with anti-A/NS1 antiserum; B) Immunoprecipitation of NS1 proteins of influenza B/Lee/40 and clone 201 virus-infected cells. Lanes 1 and 2, B/Lee/40 virus-infected cell extracts; lanes 3 and 4, clone 201-infected cell extracts; lanes 1 and 3 contain whole virus-infected cell extracts; lanes 2 and 4 contain virus-infected cell extracts following immunoprecipitation with anti-B/NS1 antiserum. Apparent molecular weights for the NS1 and NS2 proteins of B/Lee/40 virus-infected cell extracts are shown to the right of Fig. 14B.



influenza A/turkey/Ore/71 virus-infected cells corresponds to the NS1 protein of other influenza A viruses. Immunoprecipitation experiments using anti-NS2 antiserum revealed that the fastest moving band is indeed the NS2 protein in the A/turkey/Ore/71 virus-infected cell extract (data not shown).

NS gene sequence of influenza A/turkey/Oregon/71 virus

Earlier reports revealed that the NS genes of influenza A viruses direct the synthesis of NS1 proteins of 202 to 237 amino acids in length (Parvin et al., 1983; Buonagurio et al., 1986b). In order to examine the possibility that a point mutation resulted in a stop codon leading to an A/turkey/Ore/71 virus NS1 protein approximately half the size of those of other influenza A viruses, we cloned and sequenced this gene. Comparison of the nucleotide sequence of the NS gene of A/turkey/Ore/71 virus with those of other influenza A viruses showed that this NS gene is most closely related to the NS gene of A/duck/Alberta/60/76 virus (Baez et al., 1981; Porter et al., 1980; Krystal et al., 1983; Nakajima et al., 1984; Lamb and Lai, 1980; Buonagurio et al., 1986b). The alignment of these two sequences revealed that a 10-nucleotide deletion had occurred in the turkey virus NS gene resulting in a frameshift of the NS1 reading frame (Fig. 15A). Since positions 376 and 386 are both thymidines, the deletion

Figure 15. Comparison of nucleotide and deduced NS1 and NS2 amino acid sequences of NS genes. (A) NS genes of influenza A/turkey/Oregon/71 and A/duck/Alberta/60/76 viruses. The complete nucleotide sequence of A/turkey/Oregon/71 virus NS gene has been determined by the M13 cloning system. In order to confirm the deletion site we also sequenced this region by the dideoxy chain termination method using virion RNA as a template. Influenza A/duck/Alberta/60/76 virus NS gene (Baez et al., 1981) is shown as the reference sequence and all nucleotide sequences are presented in the plus sense strand. Arrows indicate the putative splice sites generating the NS2 mRNA. The dashed lines indicate the 10-nucleotide deletion in the A/turkey/Oregon/71 virus NS gene. Deduced amino acid sequences of the NS1 and NS2 proteins of A/duck/Alberta/60/76 are also shown in the single letter code and amino acid changes in the A/turkey/Oregon/71 virus NS1 protein are shown below the nucleotide sequence. It should be noted that the first 10 amino acids are shared between the NS1 and NS2 proteins. Asterisks indicate stop codons in the reading frames of NS1 and NS2 proteins. (B) NS genes of influenza B/Yamagata/1/73 and clone 201 viruses. Nucleotide sequences were obtained by the dideoxy chain termination method using viral RNA as template, synthetic oligonucleotide primers, and reverse transcriptase. Under the assumption that there are few

changes among the B virus NS genes, we used a synthetic nucleotide primer corresponding to nucleotide positions 13 to 26 of the B/Lee/40 virus NS sequence (Briedis and Lamb, 1982) for priming at the 3' end of the virion RNA. Successful priming on both B/Yamagata/1/73-and clone 201-specific viral RNA suggested a high degree of homology among B virus NS genes in this region. In analogy to B/Lee/40 virus NS sequence, the initiation codon for the NS1 polypeptide is placed at position 43-45. All nucleotide sequences are presented in the plus sense strand. The dashed line indicates a 13-nucleotide deletion of clone 201 resulting in a frame shift in the NS1 coding region as indicated by the amino acid sequence below the nucleotide sequence. Arrows indicate the putative splice sites generating the NS2 mRNA. The first 11 amino acids are shared between the NS1 and NS2 polypeptides. Asterisks indicate stop codons in the reading frames of the NS1 and NS2 proteins.

A/duck/Albarta/60/76 M D S N T I T S F Q V D C Y L
 A/turkey/Oregon/71 AGCAAGGAGGCTGCAAAATGATTA ATG GAC TCC AAC ACC TCG TTT CAG GTA GAT TCC TAT CTA
 15
 71
 W H I R K L L S M R D M C D A P F D D R L R R D Q K A I
 TGG CAC ATA AGA AAG CTG CTC AGC ATG AGA GAC ATG TGT GAT GCT CCC TTT GAT GAT AGA CTC AGG AGA GAT CAA AAA GCA TTA
 43
 155
 K G R G S T L G L D L R V A T M E G K K I V E D I L K S
 AAG GGC AGA GGC AGC ACA CTT GGG CTC GAC CTA CGA GTG GCT AGC ATG GAA GGC AAA AAG ATT GTT CAG GAC ATC CTA AAG AGT
 71
 239
 E T D E N L K I A I A S S P A P R Y I T D M S I E I S
 CAG AGC GAT GAA AAT CTC AAG ATT GCA ATT GCA TCC AGC CCT GCT CCT CGG TAC ATT ACT GAT ATG AGC ATA GAG GAA ATA AGC
 99
 323
 R E W Y M L M P R Q K I T G G L M V K M D Q A I M D K R
 AAG GAA TGG TAC ATG CTC ATG CCC ACA CAG AAG ATA ACA GGG GGC CTG ATG ATG AAA ATG GAT CAG GGC ATT ATG CAC AAG AGG
 127
 407
 I T L K A N F S V L F D Q L E T L V S L R A F T D D G A
 ATA ACA CTC AAA GCA AAT TTC TCT GTC CTA TTC GAT CAA CTG CAG ACA TTA GTC TCA CTG AGG GCT TTC ACA GAT GAT GGC GGC
 155
 491
 I V A E I S P I P S M P G N S T E D V K N A I G I L I G
 ATT GGA GCT GAA ATA TCT CCC ATT CCC TCT ATG CCA GAT TCT ACA GAG GAT CTC AAA AAT GCA ATT GCA ATC CTC ATC GCT
 26
 575
 D L N G M Y T Q P E R L K Y R D S L G E S V M R M G D
 C L E W M D N S I R A S E N I O P F A M G C I R D E N G C
 GGA CTT GAA TGG AAT GAT AAC TCA ATT CGA GCG TCT GAA AAT ATA CAG AGA TTC GCT TGG GGA ATC CGT GAT CAG AAT GGC GGA
 54
 211
 639
 L H S L Q S R N A T W R E E L S Q K F E E I R W L I A
 P L P P E Q K R Y M A R R V F S E V
 CCT CCA CTC CCT CCA AAG CAG AAA CCG TAC ATG GCG AGA ACA GTT GAG TCA GAA GTT TCA A GAA ATC AGA TGG TTA ATT GCA
 81
 210
 741
 E C R N I L T K T E M S P E Q I T F L Q A L Q L L L E V
 GAA TGC AGA AAC ATA CTA ACC AAA ACT GAG AAC AGT TTC GAG CAG ATA ACA TTC TTG CAA GCA TTG CAA CTC TTA CTT GAA CTT
 109
 825
 E S E I R T F S F Q L I
 GAG AGT CAG ATA AGG ACA TTT TCT TTT CAG CTT ATT TAG TACTAAACACCCCTTTCTACT
 121
 890

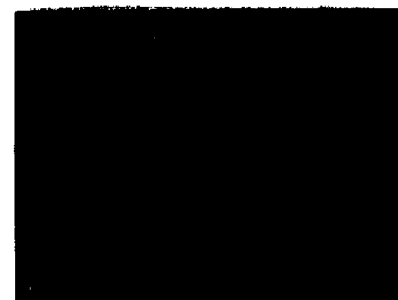
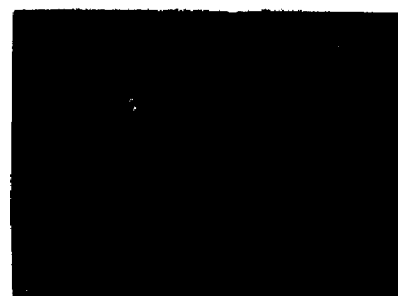
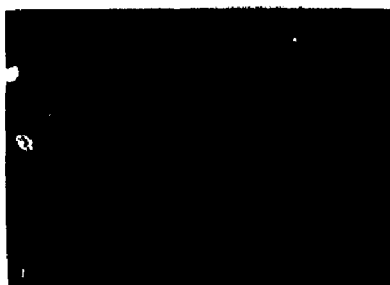
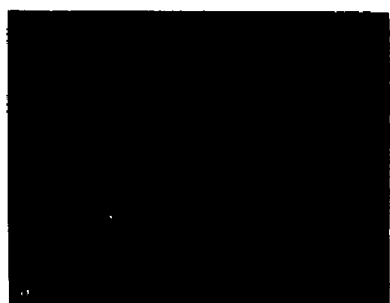
B/Yasagata/1/73
 Clone 201
 M A D N M T T T O I E V G P G A T N A T I M F 23
 ATG GCG GAC AAC ATG ACC ACA ACA CAA ATT GAG GTG GGT CCG GGA GCA ACC AAT GCG ACT ATA AAC TTT 111
 E A G I L E C Y E R L S N Q R A L D Y P G O D R L M F L 51
 GAA GCA GGA ATT TTG GAG TGC TAT GAA AGG CTT TCA TGC CAA AGA GCC CTT GAC TAC CCG CTA GAC CCG CTA AAC AGA CTA 195
 K R K L E S R I K T H N K S E P E S K P M S L F E R E A 79
 AAG ACA AAA TTA TCA ACA ATA AAG ACT CAC AAC AAA ACT GAG CCG GAA AGT AAA AGG ATG TCT CTT GAA GAG AGA AAA GCA 279
 I G V X M M K V L L F M N P S A G I E G P E P Y C N E M 107
 ATT GCG GTA AAA ATG ATG ATG AAA GTG CTC CTA TTT ATG AAC CCA TCT GCT GGA ATT GAA GCG TTT GAG CCA TAC TGT ATG AAA AAT 363
 S S N S N C P N C M W T D Y P P T S G F C L D D I E E E 135
 TGC TGC AAT AGC AAC TGT CCA AAG TGC AAT TGC ACC GAT TAC CTT CCA ACA TCA GCA AAG TGC CTT GAT GAC ATA GAA GAA GAA 447
 T A I G P I T L Q H Q E S A L M T 127
 P E N V D D P T E I V L M D M N N K D A R Q K I E E E V 163
 CCG GAG AAT GTT GAT GAC CCA ACT GAA ATA GTA TTA AGG GAC ATC AAC AAC AAA GAT CCA AGG CAA AAG ATA AAA GAG GAA GTA 531
 N T Q K E C K F N L T I R P D I K M V L S L R V L V M C 191
 AAC ACT GAG AAA GAA GGG AAG TTC CCG TTG ACA ATA AAA AGG GAT ATA CCG AAT GTG TTG TCC TTG ACA GTG TTG GTA AAC GGA 615
 T F L E H P N G Y K S L S T L H R L N A Y D O E G B L V 219
 ACA TTC CTC AAG CAC CCG AAT GGA TAC TCC TTA TCA ACT CTG CAT AGA TTG AAT GCA TAT GAC CAG ACT GGA AGG CTT GTT 699
 A K L V A T D D L T V E D E E D G H R I L N S L P E R F 29
 CCG AAA CTT GGT GCT ACT GAT GAT CTT ACA GTG GAG GAT GAA GAA CAT GGC CAT CCG ATC CTC AAC TCA CTC TTC GAG CCG TTT 783
 M K D I Q S O P E Q L E L R N E S Y P M L V K S T D Y H 57
 N E G A N S K P I R A A E T A V C V L S Q F G Q E N R L S 275
 AAT GAA GGA CAT TCA AAG CCA ATT CCA AGC CCA GCT GAA ACT GCG GTG GGA GTG TTA TCC CAA TTT GGT CAA GAG CAC CCA TTA TCA 867
 O R R E T I R L V T E E L Y L L S K R I D D N I L F H 84
 P E E G D N 281
 CCA GAG GAG GCA GAC AAT TAG A CTG GTT ACG GAA GAA CTT TAT CTT TTA ACT AAA AGA ATT GAT CAT AAC ATA TTG TTC CAC 949
 E T V I A N S S I I A D H I V S L S L L E T L Y E M E D 112
 AAA ACA GTA ATA GCT AAC AGC TCC ATA ATA GCT GAC ATG ATT GTA TCA TTA TTA TTG GAA ACA TTG TAT GAG ATG AAG GAT 1033
 V V E V Y S R Q C L 122
 GTG GTT GAA GTG TAC AGC AGG CAG TGC TCA ATTAAATATATATCTCTGTACTACT 1096

may be placed either between positions 376 to 385 or between positions 377 to 386. A stop codon at position 409 to 411 (according to the numbering system of A/duck/Alberta/60/76 virus NS gene) results in an NS1 protein of 124 amino acids, which is in agreement with the migration of the protein band identified on polyacrylamide gels (Fig.14A). It should be noted that the deletion in the NS1 coding region should not influence the structure of the spliced NS2 mRNA. This is also suggested by the appearance of an NS2 specific protein band in A/turkey/Oregon/71 virus infected cells which migrates on polyacrylamide gels in the position of that of other NS2 proteins (Fig. 14A). 12 nucleotide differences (8 in the NS1 coding region and 4 in the NS2 coding area) are found between the A/turkey/Ore/71 and A/duck/Alberta/60/76 virus NS genes (Fig.15A). All 12 substitutions are silent and do not alter the amino acid sequences of either the NS1 or the NS2 proteins in the two viruses.

Nuclear localization of the truncated NS1 protein of A/turkey/Ore/71 virus.

Since the NS1 protein in influenza A virus-infected cells is localized in the nucleus (for review Lamb 1983; Young et.al., 1983), we examined A/turkey/Oregon/71 virus-infected cells for the presence of the NS1 protein using indirect immunofluorescence analysis. Fig. 16 shows that the 124 amino acid long NS1 protein of A/turkey/Ore/71

Figure 16. Localization of NS1 protein in influenza A/turkey/Oregon/71, A/PR/8/34, B/Lee/40 and clone 201 virus-infected cells. Virus-infected cells were fixed at 8 and 20 hours post infection for A and B viruses, respectively and used for immunofluorescence as described in the text. a) A/turkey/Oregon/71 virus-infected MDCK cells. b) A/PR/8/34 virus-infected MDCK cells. c) uninfected MDCK cells. d) B/Lee/40 virus-infected CV-1 cells. e) clone 201 virus-infected CV-1 cells. f) uninfected CV-1 cells. The magnification of the cells is 25X.



virus also accumulates in the nucleus as is the case for the A/PR/8/34 virus NS1 protein. A time-course experiment (6,8,10 and 12 hours postinfection) confirmed the nuclear accumulation of the A/turkey/Ore/71 virus NS1 protein during the entire replication cycle of the virus (data not shown).

NS1 protein of influenza B virus

Tanaka et al.,(1984) reported that cells infected with a laboratory-isolated influenza B virus mutant, clone 201, lacked a protein band which migrated on SDS-protein gels at the position of the NS1 protein of the parental B/Yamagata/1/73 virus. In order to examine whether clone-201 - infected cells directed the synthesis of a protein related to that of the B/Lee/40 virus NS1 polypeptide, a B virus NS1-specific antiserum was prepared and used for immunoprecipitation analysis. Anti-B/NS1 antibody precipitated a polypeptide of apparent MW of 15.6 K from clone 201-infected cell extract, as shown in Fig. 14B.

NS gene sequences of influenza B/Yamagata/1/73 and clone 201 viruses

Comparison of the NS gene sequences of clone 201 and the parental B/Yamagata/1/73 viruses revealed no nucleotide changes except for a 13-base deletion between positions 374 and 386 (Figs. 15B). This deletion resulted in a frame shift of the NS1 coding region of clone 201 virus,

directing the synthesis of an NS1 protein of only 127 amino acids in length (Fig. 15B). It should be noted that only the first 110 amino acids of the NS1 proteins are shared by B/Yamagata/1/73 and clone 201 viruses and that the C-terminal 17 amino acids of clone 201 are read from the +1 reading frame.

Although the 3'-terminal sequences of the vRNA were not positively identified in the NS genes of B/Yamagata/1/73 and clone 201 viruses, we assume that based on our priming experiments these genes have the same 3' terminal gene structure as the B/Lee/40 virus NS gene. If this is correct the NS gene of B/Yamagata/1/73 virus is 1096 nucleotides and that of clone 201 only 1083. It appears that the splice sites of both NS genes are the same as those identified for B/Lee/40 virus (Briedis and Lamb, 1982). Comparison of the B/Yamagata/1/73 and B/Lee/40 virus NS genes reveals 60 nucleotide changes, resulting in 22 NS1 amino acid differences and 3 NS2 amino acid changes (data not shown).

Nuclear localization of truncated NS1 protein of clone 201

As was demonstrated for the NS1 proteins of influenza A virus infected cells, the NS1 protein of influenza B viruses appeared to accumulate in the nucleus of virus-infected cells. This was shown by indirect immunofluorescent experiments of B/Lee/40 virus-infected

cells using an anti-B/NS1 antiserum (Fig. 16). In addition, we showed that the NS1 protein in clone 201 virus-infected CV-1 cells also was localized in the nucleus as shown in Fig. 16. This pattern was confirmed in a time course experiment (6,8,10,12 16, and 20 hours post infection) (data not shown). In order to reduce possible cross-reactivity of host cell proteins with the anti-B/NS1 antiserum made from NS1 protein expressed in MDCK cells, we performed all immunofluorescence experiments in monkey (CV-1) cells.

Discussion

Extensive heterogeneity in the length of the NS1 polypeptides of different influenza A viruses had previously been demonstrated (Krystal et al., 1983; Parvin et al., 1983; Buonagurio et al., 1984; Buonagurio et al., 1986b). The A/FM/1/47 virus was shown to code for an NS1 protein of only 202 amino acids while that of the A/Alaska/6/77 virus was found to code for an NS1 protein of 237 amino acids in length. In this paper we report that a field isolate of influenza A virus, A/turkey/Ore/71, encodes a truncated NS1 polypeptide of only 124 amino acids. Comparison of this NS gene with that of other influenza A virus NS genes revealed a 10-base deletion at nucleotide positions 377-386 (Fig. 15A). The result of this deletion was a frameshift in the NS1 coding region of

segment 8 leading into an ochre stop codon 8 triplets downstream of the deletion. Since the last 7 amino acids are not usually found in this region of NS1 proteins, it is possible that only the N - terminal 117 amino acids are sufficient to provide the virus with a functional NS1 polypeptide.

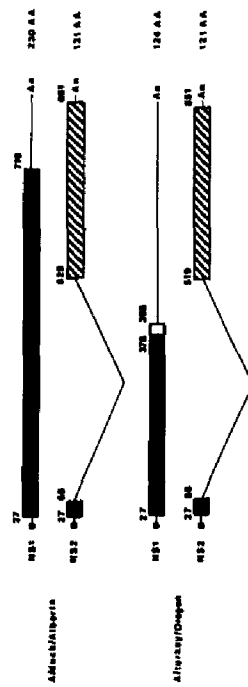
Unlike other influenza A virus strains, the A/turkey/Ore/71 virus possesses an NS gene in which the sequences coding for the carboxyl terminus of the NS1 polypeptide and the main body of the NS2 polypeptide do not overlap. As a matter of fact, there is a nontranslated region of 120 bases between the end of the NS1 coding region and the 3'-splice site of the NS2 transcript (Fig. 17A). Winter et al.(1981) have forwarded the hypothesis that the NS1 and NS2 cistrons may not have overlapped in the influenza virus progenitor strain, but were contiguous on the virion RNA. If this hypothesis is correct, then overlapping of the NS1 and NS2 proteins may have resulted from mutations in the stop codon of the NS1 polypeptide resulting in a readthrough into the NS2 reading frame.

Earlier work by several investigators had shown that the NS1 polypeptides of different influenza A viruses have a nuclear location in virus-infected cells (for review Lamb, 1983; Young et al., 1983). The fact that the short NS1 polypeptide of A/turkey/Ore/71 virus also migrates to the nucleus of infected-cells indicates that an NS1

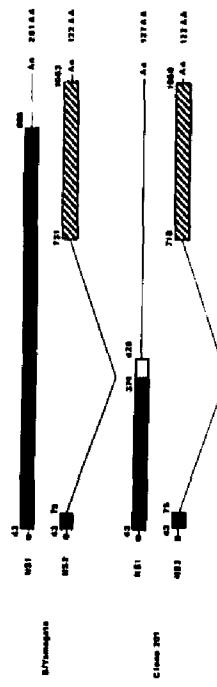
Figure 17. Comparison of NS mRNA structures. (A) A/turkey/Oregon/71 virus and A/duck/Alberta/60/76 virus NS genes. The NS1 specific mRNA of A/duck/Alberta/60/76 virus can code for a 230 amino acid long polypeptide (solid bar). The spliced NS2 specific mRNA encodes a polypeptide which shares the first 10 amino acids with that of NS1 (solid bar). The remaining 111 amino acids are translated in a +1 reading frame (hatched bar). In contrast, the NS1 specific mRNA of A/turkey/Oregon/71 virus codes for a 124 amino acid long polypeptide of which 117 amino acids are colinear with that of the A/duck/Alberta/60/76 virus NS1 mRNA (solid bar). The remaining 7 amino acids are derived from a +1 reading frame (stippled bar). The spliced NS2 specific mRNA of A/turkey/Oregon/71 virus appears to retain the structure of that of other influenza A viruses. (B) clone 201 and B/Yamagata/1/73 virus NS genes. The NS1 specific mRNA of B/Yamagata/1/73 virus codes for a 281 amino acid long protein (solid bar). The spliced NS2 specific mRNA codes for a protein which shares the first 11 amino acids with that of NS1 (solid bar) and the remaining 111 amino acids are translated in a +1 reading frame (hatched bar). The NS1 specific mRNA of clone 201 codes for a 127 amino acid long protein, 110 amino acid are colinear with B/Yamagata/1/73 (solid bar) and 17 amino acids are derived from a +1 reading frame (stippled bar) as a result of a 13 nucleotide deletion. The spliced NS2 mRNA

specific structure of clone 201 appears to have a structure similar to that of other influenza B viruses. The small squares at the 5' end and the An at the 3' end of mRNAs represent caps and poly A tails, respectively. The thin lines at the 5'-and 3'-termini of the NS1 and NS2 mRNAs represent noncoding regions. The v-shaped thin lines in the NS2 mRNAs represent intervening (spliced out) sequences. Numbers indicate positions along the NS gene (+ sense). The amino acid lengths of the deduced NS1 and NS2 polypeptides are indicated at the right of the diagram.

A



B



polypeptide of approximately half the size (as compared to that of other NS1 proteins) may provide the information required for nuclear localization of this protein.

A/PR/8/34 and A/turkey/Ore/71 viruses were grown to comparable titers in embryonated chicken eggs and both viruses readily formed plaques in MDCK cells with EID_{50} /PFU ratios of approximately 10 (data not shown). Furthermore, Beard and Halfer (1972) reported that A/turkey/Ore/71 virus was responsible for up to 1% deaths in infected turkeys, and that egg production dropped to 30% of its original level in infected-flocks. Therefore deletion of approximately half of the carboxyl terminus in the NS1 protein of influenza A viruses may not prevent efficient replication of virus in nature, embryonated eggs, or tissue culture.

Tanaka et al., (1984) reported on an aberrant NS protein encoded by influenza B virus clone 201 which was derived in the laboratory from B/Yamagata/1/73 virus. In order to elucidate the molecular defect in this mutant, we sequenced the NS genes of clone 201 and its parental B/Yamagata/1/73 virus. In comparing the NS gene nucleotide sequence of these two viruses we noted a 13-base deletion between nucleotide positions 374 and 386 in the clone 201 NS gene, which resulted in a frame shift in the NS1 coding region and a subsequent amber stop codon 18 codons downstream of the deletion (Fig. 15B). Our data concerning

clone 201 suggests an NS1 protein of only 127 amino acid-length, which is less than half of that found for the NS1 proteins of influenza B/Lee/40 (Briedis and Lamb, 1982) and B/Yamagata/1/73 viruses (281 amino acids). The structural organization of influenza B virus clone 201 NS gene (Fig.17B) is similar to that of A/turkey/Ore/71 virus NS gene (Fig. 17A) leaving a nontranslated region of 294 nucleotides between the NS1 and NS2 coding regions. In tissue culture, clone 201 reveals a growth rate which is similar to that of wild type influenza B/Yamagata/1/73 virus (Tanaka et al., 1984). Thus the infectivity of this virus is not compromised by a large deletion in the carboxyl terminal region of the NS1 protein.

As reported for the A virus NS1 proteins, we now find that influenza B virus NS1 proteins also localize in the nucleus of infected cells. In addition, we find that the truncated NS1 polypeptide of clone 201 has the same karyophilic pattern as that of B/Lee/40 virus (Fig. 16). Clone 201's carboxyl terminal 17 amino acids (derived from the +1 reading frame) are not found in the corresponding region of the NS1 polypeptide in B/Yamagata/1/73 or B/Lee/40 viruses (Fig. 15B). It is thus possible that only 110 out of 281 amino acids in the NS1 polypeptide of influenza B viruses are necessary for the functional capability of this protein.

In conclusion, the NS1 protein of influenza A and of

influenza B viruses tolerates large carboxyl terminal deletions without affecting its nuclear localization and also without compromising the infectivity of these viruses. We find it interesting that infectious influenza A and B viruses code for NS1 proteins which may vary in length by as much as 100 %.

Examination of influenza virus gene products expressed by vaccinia virus recombinants.

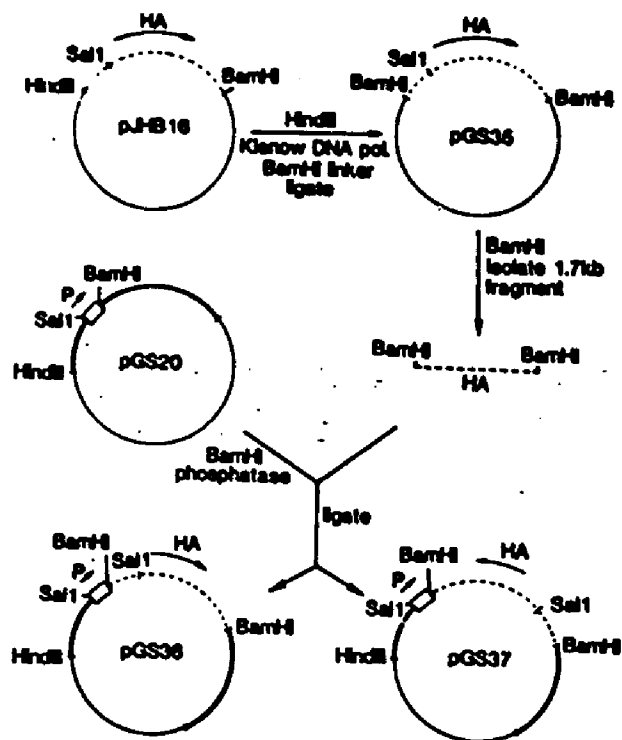
Results

All vaccinia virus recombinants containing cloned cDNA copies of influenza virus genes were constructed by Drs. G. L. Smith and B. Moss at the National Institutes of Health. Three vaccinia virus recombinants (V36, V63, and V64) (Fig.18), contain cloned HA genes derived from influenza viruses. These vaccinia recombinant viruses were obtained by constructing plasmids which contained the influenza virus HA genes and the vaccinia virus TK promoter inserted into the TK gene of vaccinia virus. The plasmids then were used to transfect cells already infected with vaccinia virus to allow homologous recombination between the TK sequences of the plasmids and of vaccinia virus (Smith et al., 1983). V36 (vInf1) possesses a cDNA copy of the influenza A/Japan/305/57 virus HA gene (Smith et al., 1983) and both the V63 and the V64 recombinant contain the cloned HA gene (Young et al., 1983c) of influenza A/PR/8/34 virus. The HA genes of V36 and V64 are in the orientation for mRNA transcription, but in the V63 recombinant the HA gene is in the opposite orientation with respect to the promoter.

The vaccinia virus vectors were first tested for their ability to express the cloned HA gene. Western blot

Figure 18. Construction of recombinant plasmids^a.
Influenza HA DNA; , vaccinia transcriptional regulatory
sequence (P) including RNA start site; , flanking
vaccinia virus DNA including interrupted TK gene;
, plasmid DNA.

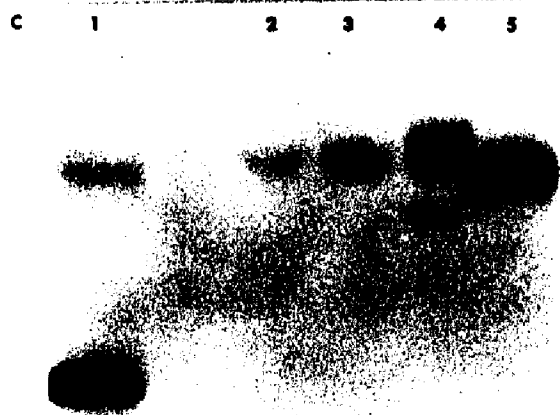
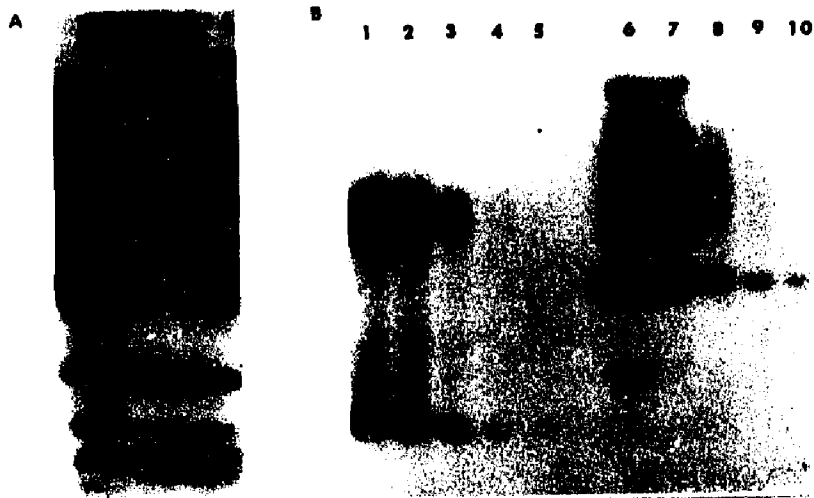
(^aSmith et al., 1983).



analysis was used to detect the PR8 HA in V64-infected cells (Fig. 19A). In order to estimate the amount of HA protein produced by the vaccinia-HA vector, V64 or A/PR/8/34 virus-infected cell lysates were diluted and analyzed by Western blotting following polyacrylamide gel electrophoresis. The V64 vaccinia recombinant virus produced levels of HA protein approximately 1/5 to 1/2 of that seen in cells infected with influenza A/PR/8/34 virus (Fig. 19B) and it migrated noticeably slower on gels than the HA protein synthesized by influenza virus (Fig. 19B). A possible explanation for this is the absence of neuraminidase activity in the vaccinia virus recombinant. This could result in an HA protein which contains neuraminic acid residues. In order to determine if neuraminic acid was present, the infected cell lysate was analyzed before and after treatment with neuraminidase of C. perfringens prior to Western blot analysis. Figure 19C shows that vaccinia virus-recombinant HA protein contains neuraminic acid because treatment with neuraminidase reduced the molecular weight of the protein. As a control, addition of exogenous neuraminidase to cells infected with influenza virus A/PR/8/34 did not affect the migration of the HA protein (Fig. 19C). Furthermore, V64-produced HA protein was located on the cell surface as detected by immunofluorescence analysis and hemadsorption (Fig. not shown). Recently, Mackett et al. (1985) also reported that

Figure 19. Characterization of the HA protein produced by vaccinia virus recombinant V64 which contains the cloned HA gene of influenza A/PR/8/34 virus. (A) Lane 1, CV-1 cells were infected with influenza virus A/PR/8/34 and six hours post infection cells were labeled with ^{35}S -methionine for 1 hour; lane 2 and 3, CV-1 cells were infected for 24 hours with either vaccinia recombinants V63 (lane 2) or V64 (lane 3). Cells were lysed and the amount of HA protein produced by A/PR/8/34, V63 and V64 viruses was examined by a Western blot assay using polyclonal rabbit anti-A/PR/8/34 virus antiserum as described in the Materials and Methods section. The presence of two bands that flank the HA protein band in lane 3 are most likely vaccinia virus protein bands since they also appear in lane 2. (B) CV-1 cells were infected with A/PR/8/34 virus (lanes 1-5) and vaccinia recombinant virus V64 (lanes 6-10). At 24 hours post infection, cells were lysed and the infected-cell extract was analyzed by Western blotting. Lanes 1 and 7, 1:5 dilution; lanes 2 and 8, 1:10 dilution; lanes 3 and 9, 1:20 dilution; lanes 4 and 10, 1:40 dilution; lanes 5, 1:80 dilution; lane 6, undiluted. (C). Mouse C127I cell monolayers were infected with influenza A/PR/8/34 virus or V64 virus and then incubated for 24 hours. The virus-infected cell extracts were treated with (lanes 3 and 5) or without (lanes 2 and 4) neuraminidase of C. perfringens and examined by Western blot analysis using monoclonal

anti-A/PR/8/34 virus HA antiserum. Lane 1, ^{35}S -methionine 6-7 hour-labeled A/PR/8/34 virus-infected cells; lanes 2 and 3, A/PR/8/34 virus-infected cells; lanes 4 and 5, V64 virus-infected cells.



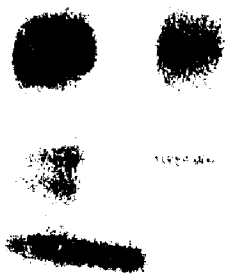
the vaccinia HA-recombinant virus HA gene product is processed and inserted into the plasma membrane.

In the case of V36, Smith et al. (1983) had previously reported that the HA protein was expressed in infected cells, glycosylated and transported to the cell surface. Western blot analysis and neuraminidase treatment of the HA protein produced by V36 revealed results similar to that presented above for V64 (data not shown).

A number of other vaccinia virus recombinants containing influenza virus genes were also tested for their ability to express cloned influenza virus genes. Five other vaccinia virus recombinants (V69, V76, V77, V78, and V81) contained the cloned NP, PB2, PB1, PA, and NS genes, respectively, derived from influenza A/PR/8/34 virus (Smith and Moss, unpublished data). All five influenza genes were correctly oriented with respect to the translocated vaccinia virus promoter. The NP and NS1 proteins were detected in infected cells by Western blot analysis following gel electrophoresis. Antisera used for detection contained rabbit antibodies made against whole virus and against bacterially expressed A virus NS1 and NS2 proteins (Young et al., 1983a; Greenspan et al., 1985) (Fig. 20 lanes 2 and 7). The V69 and V81 produced levels of nucleoprotein and NS1, respectively, which were equal to those seen in influenza A/PR/8/34 virus-infected cells (Fig. 20 lane 1). However, no P proteins were detected

Figure 20. Characterization of influenza virus HA, NP, PB2, PB1, PA and NS1 proteins produced by vaccinia virus recombinants V64, V69, V76, V77, V78 and V81, respectively, that contain the cloned genes from influenza A/PR/8/34 virus. 24 hours post infection with influenza A/PR/8/34 or vaccinia recombinant viruses CV-1 cells were lysed and cell extracts were electrophoresed in a 7-14% linear gradient polyacrylamide gel and electrophoretically transferred onto a DEAE-membrane. The membrane was then developed with a polyclonal antisera mixture specific to the viral proteins of influenza A/PR/8/34 virus. Lane 1, A/PR/8/34 virus-infected cell extract; lane 2, V64 virus-infected cell extract; lane 3, V69 virus-infected cell extract; lane 4, V76 virus-infected cell extract; lane 5, V77 virus-infected cell extract; lane 6, V78 virus-infected cell extract; lane 7, V81 virus-infected cell extract; lane 8, 5 ug purified A/PR/8/34 virus.

1 2 3 4 5 6 7 8



HA

NP

M1

NS1

by this immunoblot assay (Fig. 20 lanes 4, 5, and 6), most likely because the antiserum contained a paucity of P protein-specific antibodies. Also, the NS2 protein was not detected in V81 infected-cell lysates (Fig.20 lane 7). This could be explained by the fact that there is no evidence for splicing in vaccinia virus (Moss, 1985).

Discussion

Recently, recombinant vaccinia virus has been used successfully to express foreign genes such as herpes simplex virus thymidine kinase, glycoprotein D (Panicali et al., 1982; Paoletti et al., 1984), influenza virus hemagglutinin, influenza virus nucleoprotein (Panicali et al., 1983; Smith et al., 1983a; Yewdell et al., 1985), hepatitis B virus surface antigen (Smith et al., 1983b; Paoletti et al., 1984), Plasmodium knowlesi sporozoite antigen (Smith et al., 1984), rabies virus glycoprotein (Kieny et al., 1984, Wiktor et al., 1984) and vesicular stomatitis virus G and N proteins (Mackett et al., 1985). These vaccinia recombinant viruses were able to induce both humoral and cell-mediated immune responses as well as to protect the infected host against disease when challenged with live virus. Thus, there is potential for the application of vaccinia virus recombinants as live virus vaccines against both human and animal infectious agents.

In addition, Smith and Moss (1983) reported that up to 25 Kb of foreign DNA can be stably integrated into vaccinia virus vectors. Therefore vaccinia virus might be useful as a vector to deliver multiple vaccine antigens. Perkus et al. (1985) tested this hypothesis by inserting the genes coding for the hepatitis B surface antigen, herpes simplex virus glycoprotein D and influenza virus hemagglutinin into a single vaccinia virus genome. Using this vaccinia recombinant the authors were able to produce antibodies reactive to all three foreign antigens in inoculated rabbits.

A portion of my thesis deals with the analysis of influenza virus proteins expressed from vaccinia recombinant viruses that contain influenza virus-specific cDNA copies. Since vaccinia virus induces over 100 different proteins (Belle Isle et al., 1981), it would be difficult to detect influenza virus gene products in vaccinia recombinant virus-infected cells by SDS polyacrylamide gel analysis of [³⁵S]-methionine-labeled cell extracts. Therefore, Western blot analysis of vaccinia recombinant virus-infected cell extracts was used to examine expression of the influenza virus gene products. Comparison of the HA proteins made by the vaccinia HA-recombinant virus and influenza virus, using polyacrylamide gel electrophoresis and Western blotting revealed a difference in apparent molecular weights. The absence of

neuraminidase activity in the vaccinia HA recombinant virus suggested that the vector-produced HA protein contained neuraminic acid residues and that addition of exogenous neuraminidase would influence the migration of the slower migrating protein on SDS polyacrylamide gel electrophoresis. This proved correct since the molecular weight of the HA protein expressed with V64 was reduced by treatment with neuraminidase from C. perfringens (Fig. 19C).

The NP and NS1 proteins were identified in vaccinia recombinant viruses containing cloned DNA copies of the corresponding NP and NS gene segments (figure 20). In the vaccinia NS-recombinant-infected cell extract the synthesis of the NS2 protein was not detected. Since the NS2 protein is translated from a spliced mRNA species and there are no known examples of vaccinia virus mRNAs that are spliced (Moss, 1985), this finding is not unexpected. In addition, vaccinia virus does not have a nuclear stage during viral replication. In order to direct the splicing of NS2-specific message from the NS1 messenger RNA, it seems likely that nuclear factor(s) are needed in infected cells.

I was not able to detect expression of the P proteins in vaccinia virus recombinants containing the polymerase genes of influenza A/PR/8/34 virus (V76, V77 and V78). Since specific antisera for each polymerase component has recently been used for the demonstration of vaccinia virus-

mediated expression of the three P genes (Jones et al., 1986) it is assumed that the antisera preparation employed here did not show high enough sensitivity for detection in our system.

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