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NUCLEAR LOCALIZATION OF THE INFLUENZA A VIRUS NONSTRUCTURAL PROTEINS, NS1 AND NS2

by

Deborah Greenspan

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York

1988

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

NUCLEAR LOCALIZATION OF THE INFLUENZA A VIRUS NONSTRUCTURAL PROTEINS, NS1 AND NS2

by

Deborah Greenspan

Advisor: Peter Palese

The smallest RNA segment of influenza A viruses encodes the nonstructural proteins, NS1 and NS2. Although the genomic organization of these mRNAs is defined little is known about the functions of the proteins for which they encode. In this thesis, experiments were done to further characterize the NS1 and NS2 proteins of influenza virus. First, the nonstructural NS2 protein of influenza A/PR/8/34 virus was efficiently expressed in bacteria and monospecific antisera were prepared against the bacterially synthesized polypeptide. The antisera were used in immunofluorescence experiments to localize the NS2 protein to the nucleus during influenza virus infection. Before the availability of this reagent, the NS2 protein had erroneously been localized to the cytoplasm by cell fractionation studies.

Second, sequence analysis of the NS gene of A/turkey/Oregon/71 and an influenza B virus, clone 201, revealed nucleotide deletions resulting in truncated NS1 proteins approximately half the size of those of other influenza viruses. These shortened NS1 polypeptides were shown to retain their karyophilic pattern by immunofluorescence analysis thus indicating that large deletions in the carboxyl termini of these proteins can be tolerated without affecting the functional integrity of the polypeptides in tissue culture.

Finally, the NS1 protein has been shown to enter the nucleus of virus infected cells independent of any other influenza virus protein suggesting that this polypeptide contains within its sequence the information coding for its nuclear location. To define the nuclear signal of the NS1 protein, a series of recombinant SV40 vectors expressing deletion mutants or fusion proteins were constructed and these proteins were analyzed. Two nuclear localization signals were defined for the NS1 protein. One nuclear localization signal (NLS1) contains the stretch of basic amino acids Asp-Arg-Leu-Arg-Arg (codons 34-38). The second nuclear localization signal (NLS2) is defined within amino acid region 203-237. These signals are similar to previously defined nuclear signal sequences of other proteins. In addition, the presence of NLS 1 and NLS 2 in the NS1 protein may represent an example of signal redundancy within a protein.

FORMAT OF THESIS

This thesis was prepared according to the guidelines of the City University of New York. Included in this thesis is a general introduction, as well as published research articles as individual chapters. Chapter 2 contains a previously published article in its entirety whereas the article presented in chapter 3 has been edited to exclude work I was not involved in. Chapter 4 is an article that has been accepted to The Journal of Virology and chapter 5 contains previously unpublished data. Each chapter has a specific introduction, a materials and methods section, a results section and a discussion. In addition, a general discussion is included as the last chapter. The references for all the chapters are pooled.

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I. INTRODUCTION

A. Influenza A virus is a cause of respiratory disease in man and animals. The virus has been associated in the past with epidemic and pandemic outbreaks and remains a cause of significant morbidity and mortality today. The influenza A virus is well characterized although little is known about the proteins encoded by the RNA 8. The smallest RNA segment encodes two nonstructural proteins, the NS1 and the NS2 (Ritchey, et al., 1976b; Lamb and Choppin, 1979). This thesis presents experiments which investigate the nuclear localization of the influenza virus nonstructural proteins, NS1 and NS2.

Influenza viruses are members of the Orthomyxoviridae family. There are three types of influenza viruses: A,B and C. They can be distinguished by their antigenically distinct internal proteins: the matrix protein (M1), and the nucleoprotein (NP). Influenza A and B viruses have eight RNA segments whereas influenza C viruses contains only 7 RNA segments (Pons, et al., 1976; Ritchey, et al., 1976a; Palese, et al., 1980).

B. STRUCTURE OF THE INFLUENZA A VIRUS PARTICLE

The influenza A virus particle is pleiomorphic, with an average diameter of 80-120 nm by electron microscopy studies (Horne, et al., 1960; Wrigley, 1979). It contains lipids, eight structural proteins, and a segmented RNA genome of negative polarity. The 8 viral RNA segments encode at least 10 polypeptides as summarized in Table 1 (reviewed in Lamb, 1983). The virus particle has two types of surface glycoproteins, the hemagglutinin (HA) and

Table 1. Influenza A/PR/8/34 virus genome RNA segments and coding assignments*

RNA Segment	vRNA length	Protein encoded	Protein length	Protein function (references)
1	2341	PB2	759	component of polymerase complex;cap endonuclease (Plotch, et al., 1981; Ulmanen, et al., 1981)
2	2341	PB1	757	component of polymerase complex;initiation and elongation of transcription (Ulmanen, et al., 1981; Palese, et al., 1977)
3	2233	PA	716	component of polymerase complex (Braam, et al., 1983)
4	1778	HA	566	surface glycoprotein; receptor binding (Wilson, et al., 1981)
5	1565	NP	498	structural component of polymerase complex; part of RNP structure (Compans, et al., 1972)
6	1413	NA	454	surface glycoprotein; involved in virus release (Colman, et al., 1983)
7	1027	M1	252	virion structural protein (Compans, et al., 1970)
		M2	96	membrane associated glycoprotein; spliced mRNA (Lamb and Choppin, 1981; Lamb, et al.,1985)
8	890	NS1	230	nonstructural nuclear protein (Skehel, 1972; Young, et al.,1983)
		NS2	121	spliced mRNA (Lamb and Lai, 1980)

* Modified from Lamb (1983).

the neuraminidase (NA). The HA functions in the binding of the virus to host cell receptors containing sialic acid and thus mediates virus entry (Hirst, 1941; Klenk, et al., 1975). Penetration and uncoating of the virus involves the HA in a low pH-induced membrane fusion event (Huang, et al., 1980; White, et al., 1982). The NA is associated with the release and spread of the virus. It does this by hydrolyzing sialic acid from the cell surface, thus preventing the virus from becoming attached to the cell it is exiting (Schulman and Palese, 1977). In addition, particles with defective NA proteins aggregate due to the presence of sialic acid residues (Palese, et al., 1974). Finally, the NA may destroy sialic acid containing mucins which inactivate virus particles. These glycoprotein spikes are embedded in a lipid membrane, which is derived from the host cell surface, at the site of virus budding. Associated with the inner surface of the lipid membrane is the matrix protein, M1. The M1 protein is the most abundant protein in the virion and may provide structural integrity to the virus particle (Compans, et al., 1970). This protein may also function in the regulation of the viral polymerase complex (Zvonarjev and Ghendon, 1980; Mikheeva and Ghendon, 1983; Melnikov, et al., 1985; Ye, et al., 1987). Within the virus particle the RNA segments are associated with the nucleoprotein, NP, and the three polymerase proteins, PB2, PB1 and PA, which form the ribonucleoprotein (RNP) structure (Rochovansky, 1976; Kato, et al., 1985; Krystal, et al., 1986, Detjen, et al., 1987). This complex is associated with viral replication. The functions of the polymerase proteins have been elucidated by in vitro transcription studies, as well as by work on temperature sensitive mutants (McGeogh and Kitron, 1975; Krug, et al., 1975; Scholtissek and Bowles, 1975; Palese et al., 1977; Plotch, et al., 1981; Ulmanen, et al., 1981, Blass, et al.,

1982; Beaton and Krug, 1984). It is thought that the PB2 protein is involved in host cap binding, whereas the PB1 protein is associated with the initiation and elongation steps of transcription (Palese et al., 1977, Ulmanen et al., 1981; Ulmanen et al., 1983). The PA protein is known to be present in the polymerase complex, though its precise role is unclear. As indicated in Table 1, RNA segment 7 encodes two proteins, M1 and M2 (Lamb and Choppin, 1981; Lamb, et al., 1981). The mRNA for M1 is colinear with the virion RNA, whereas M2 mRNA is a spliced product (Lamb, et al.,1981). The M2 is a small glycoprotein found to be associated primarily with the surface membrane of virus infected cells and in small amounts with the virion (Lamb, et al., 1985; Zebedee, et al., 1985; Lamb, personal communication). Its function is not known though it has been associated with amantadine resistance. In addition to these structural proteins, two nonstructural proteins have been defined in influenza A virus infected cells, the NS1 and NS2.

C. NONSTRUCTURAL PROTEINS

The nonstructural proteins, NS1 and NS2, of influenza A virus infected cells are encoded by the smallest RNA segment (Lamb, 1983). Two mRNAs are transcribed from viral RNA 8; an unspliced mRNA, which codes for the NS1 protein, and a spliced mRNA which translates into the NS2 protein (Lamb and Lai, 1980). The genomic organization of these mRNAs is shown in Figure 1. These mRNAs, as with all influenza mRNAs, have host cell derived sequences at their 5' ends. In addition, the NS mRNAs share the first 56 nucleotides of the NS sequence. The NS2 mRNA is then spliced into a +1 reading frame at position 529 (Lamb and Lai, 1980). Therefore, the NS1 and

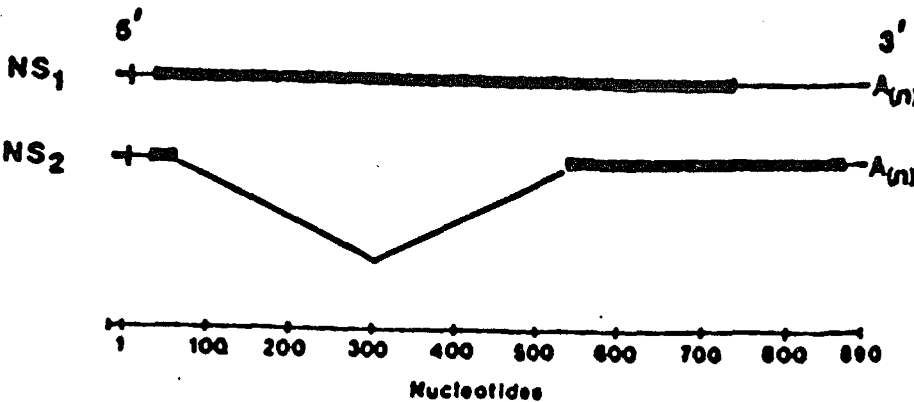

Figure 1: Genomic organization of the mRNAs derived from the NS gene (RNA 8) of influenza A virus.

At the 5' and 3' termini of the NS1 and NS2 mRNAs are non-coding regions, shown by thin lines. The coding regions of the two mRNAs are represented by thick lines. The NS2 mRNA is spliced. Starting at position 529, the NS2 mRNA is translated in a +1 reading frame relative to the NS1 mRNA. Upstream of the NS sequences are the heterogenous nucleotides derived from cellular mRNAs that are donated to the NS mRNAs, as well as to all other influenza mRNAs.

NS2 proteins encoded by these mRNAs have 10 residues in common at their amino termini, but differ over the rest of their amino acid sequences. The NS1 is an early protein found in the nucleus of virus infected cells (Briedis, et al., 1981; Young, et al., 1983). The NS2 protein is considered a late viral protein (Mahy, et al., 1980). Data are presented in this thesis which indicate that the NS2 protein localizes to the nucleus of virus infected cells.

The precise functions of the the NS1 and NS2 proteins are not yet known. Work with temperature sensitive mutants defective in RNA segment 8 of A/FPV/Rostock/34 indicates that lesions in this RNA lead to multiple effects including decreased HA, M and NS2 protein synthesis as well as a decrease in the accumulation of vRNA during infection (Wolstenholme, et al., 1980; Koennecke, et al., 1981; Robertson, et al., 1983).

D. VARIABILITY IN THE NS GENE OF INFLUENZA A VIRUS

Influenza A virus is unique in its ability to undergo rapid changes in its antigenic character. Widespread disease can occur due to the emergence of novel strains of virus. This may be the result of reassortment of the major surface antigen genes, the HA and the NA, with genes derived from influenza viruses present in animal reservoirs; a process known as antigenic shift. In addition to these large changes, point mutations may occur within the HA and NA genes. These mutations eventually accumulate, and the resulting variants are recognized less well by the host immune system. This process is referred to as antigenic drift (reviewed in Webster, et al., 1982). Variability is not restricted to the major surface antigens of the virus, but also occurs in the RNA encoding the nonstructural proteins, NS1 and NS2.

An evolutionary tree of the NS gene from influenza A virus strains isolated over 53 years was constructed in our laboratory based on sequence analysis. Mutations seemed to accumulate sequentially, and an evolutionary rate for the NS gene was determined to be approximately $2x10^{-3}$ substitutions/site/year (Buonagurio, et al., 1986). In addition to this evolutionary rate, which is 10^{6} times higher than that for germline genes in mammals, the NS1 protein encoded by these genes is pliant. Though the NS1 proteins of influenza A viruses are antigenically related, it is also known that the protein can tolerate nonsense mutations which lead to variability in the length of the protein's carboxyl terminus (Parvin, et al., 1983; Buonagurio, et al., 1986). The NS2 protein's coding region seems to be more highly conserved. In this thesis, work on the avian influenza virus A/Turkey/Oregon/71 is presented in chapter 3. This virus replicates well in tissue culture and eggs, yet codes for an NS1 protein one half the size of other influenza A NS1 proteins. The biological consequences of these deletions are unknown; it may be that the carboxyl terminal region of the NS1 is dispensable to its function. Some data that supports this theory is 1- the NS1 protein length varies with virus strain (Parvin, et al., 1983) and 2- sequence analysis of different virus strains indicates that the overlap region between the NS1 and NS2 allows for conservation of the NS2 amino acid sequence at the expense of the NS1 polypeptide (Lamb and Lai, 1980; Baez, et al., 1980).

E. SIGNAL SEQUENCES FOR COMPARTMENTALIZATION OF PROTEINS

The eukaryotic cell is surrounded by a plasma membrane and is

subdivided into compartments by intracellular membranes. The major components of this membranous system are the endoplasmic reticulum, the nuclear envelope, and the Golgi complex. Membrane bound organelles within the cell include chloroplasts, mitochodria, and lyzosomes. All cells contain carbohydrates, lipids, nucleic acids, and proteins. These proteins are compartmentalized within the organelles of a cell. In this thesis, studies were performed to investigate the information necessary for the nonstructural proteins, NS1 and NS2, to enter and accumulate in the nucleus of virus infected cells. NS1 proteins of different influenza A viruses have been localized to the nucleus of virus infected cells (Young, unpublished data). Extensive sequence data is available for the NS gene of various field strains (Baez, et al.,1980; Krystal, et al, 1983; Buonagurio, et al., 1980). The deduced amino acid sequences of the NS1 and NS2 proteins allow us to dissect the proteins' primary sequences and define the nuclear location signals. With this information, the nuclear localization signal for the NS1 protein was investigated and is described in chapter 4. Studies on the NS2 protein were also performed and are presented in chapter 5.

1. TRANSPORT TO THE CELL SURFACE

Over the years much work has been done on the characterization of information necessary for proteins to migrate to, and associate with, specific cellular components. A specific signal sequence is needed for membrane and secretory proteins to be transported across cellular membranes (Blobel and Dobberstein, 1975; Blobel, 1977). The signal can consist of up to 40 amino acids, usually at the amino terminus of the protein. It contains a polar, basic

amino-terminus followed by a hydrophobic core which is large enough to span the membrane (Watson, 1984). The signal recognition particle (SRP) (Walters and Blobel 1981) binds polysomes synthesizing pre-membrane or pre-secretory proteins and causes protein elongation to stop after approximately 80 residues. The SRP-polysome complex then binds the docking protein (DP) (Meyer, et al., 1982). The DP is an integral membrane protein of the rough endoplasmic reticulum. Once the complex binds the DP, the SRP is released and protein elongation is continued. The translocation of these proteins across the endoplasmic reticulum occurs cotranslationally, and usually the signal peptide is cleaved off. Although there are exceptions to this mechanism for protein insertion through the membrane, it is generally accepted as the pathway that most membrane and secretory proteins take to the cell surface. Studies are underway now in many laboratories to define the information necessary for the polarized transport that often occurs with membrane and secretory proteins.

2. TRANSPORT TO THE CHLOROPLASTS

Chloroplasts contain their own nucleic acid, but its genome is not large enough to encode all of its structural proteins. Therefore, approximately 80% of chloroplast proteins are encoded by nuclear DNA and translated from cytoplasmic mRNA (Chua and Gilham, 1977). These proteins are synthesized as precursor molecules on free ribosomes and are translocated into chloroplasts, post-translationally, in an energy-dependent matter (reviewed in Cashmore, et al., 1985). These precursors are cleaved to their mature size upon import (Smith and Ellis, 1979; Robinson and Ellis, 1984). Sequence

analysis of precursor and mature forms of chloroplast protein indicates the presence of amino-terminal extensions on the precursors which contain a preponderance of basic amino acids (Bedbrook, et al., 1980; Berry-Lowe, et al., 1982; Coruzzi, et al., 1983). This is thought to be important in the interaction of the transient amino-terminal peptide with the chloroplast envelope (Schmidt, et al., 1979; Pain, et al., 1988). These terminal extensions tend to lack hydrophobic residues that are characteristic of signal sequences for cotranslationally translocated secretory or membrane proteins. Chloroplasts are complex organelles that can be further divided into compartments. The amino terminal peptide extension of a protein destined for the chloroplast has also been shown to be involved in targeting the protein to the correct compartment within the chloroplast (Smeekens, et al., 1986).

3. TRANSPORT TO THE MITOCHONDRIA

Another organelle for which the protein transport has been well characterized is the mitochondria. In many respects, the import of proteins into the mitochondria is similar to that for chloroplasts. Most mitochondrial proteins are encoded by nuclear genes and synthesized in the cytoplasm. Proteins destined for internal mitochondrial compartments such as the matrix, the inner membrane or the intermembrane space, are generally synthesized as precursors with amino terminal pre-sequences, which are cleaved off upon entry into the mitochondria (reviewed in Schatz and Butow, 1983 and in Hay, et al., 1984). The pre-sequences, like those for chloroplast entry, are basic and hydrophilic in nature (Kaput, et al., 1982, Viebrook, et al., 1982, Horowich et al., 1984). Import of these precursors occurs posttranslationally

and is energy-dependent (Nelson and Schatz, 1979; Schleyer, et al., 1982; Kolansky, et al., 1982; Gasser et al., 1982). Not all mitochondrial proteins enter the organelle in this fashion, however. It has been shown that proteins of the outer membrane lack the transient pre-sequences and do not require an electrochemical potential across the inner membrane. Analysis of the 70kd protein of yeast mitochondrial outer membrane indicated that the first 41 amino acids of the protein were necessary for its correct targeting (Hurt, et al., 1985). This 41 amino acids can be divided into 2 regions. The first domain consisted of 11 residues which are predominately hydrophilic, and the second region is a stretch of 27 uncharged amino acids. It was determined that the first domain resembled a cleavable pre-sequence and functioned in targeting the protein to the mitochondria. The second domain acted as an anchoring sequence for the protein causing it to remain in the outer membrane. In the case of this 70kd outer mitochondrial membrane protein, cleavage of the signal was not necessary for transport.

4. TRANSPORT TO THE NUCLEUS

In eucaryotic cells, a membranous envelope surrounds the nucleus. This envelope is a double membrane that is contiguous with other cellular membranes, e.g., endoplasmic reticulum. At regular intervals the nuclear envelope's surfaces fuse to form nuclear pores. It is the nuclear pores which are the main routes through which nucleocytoplasmic exchanges occurs. These pores vary in size, depending on the cell type, but have been shown to have a radius of ~45 Å (Paine, et al., 1975). This pore size limits entry to the nucleus of proteins of a molecular weight larger than ~67,000 (Paine, et

al., 1975). Proteins below the size limit may enter the nucleus, but won't accumulate unless they are of nuclear origin (DeRobertis, 1983). Not all proteins below a molecular weight of 67,000 are found in the nucleus, and conversely, there are large proteins which are nuclear, e.g., SV40 large T antigen with a MW ~94,000 (Pope and Rowe, 1964). Factors other than the size of a protein seem to be involved in determining its nuclear localization.

Experiments on a mutant SV40 large T antigen were the first studies which allowed the definition of a signal sequence for a nuclear protein. SV40 large T antigen is mostly found in the nuclei of virus infected cells, although small amounts can be detected on the cell surface (Pope and Rowe, 1964; Soule, et al., 1980; Deppert and Walter, 1982: Ball, et al., 1984). A mutant SV40 large T antigen has been shown to localize in the cytoplasm of virus infected cells (Kalderon, et al., 1984a; Lanford and Butel, 1984). Sequence analysis of this mutant revealed a single point mutation. This mutation led to the replacement of the basic amino acid lysine with the neutral amino acid asparagine (Landford and Butel, 1984). This change occurs at amino acid 128 which is the second basic residue in a region where there are five consecutive basic amino acids. By cloning this region of the wild type large T antigen and then ligating it to the amino terminus of a protein not normally found in the nucleus, i.e., thymidine kinase, the authors were able to make a non-nuclear protein localize to the nucleus (Kalderon, et al., 1984a). These data suggest that this basic amino acid region is important for the large T antigen to enter the nucleus. Since this analysis, many nuclear signal sequences containing basic amino acid residues have been defined for other cellular and viral proteins (see Table 2). In addition to basic amino acid signal sequences, non-basic domains have also been shown to be involved in

the nuclear migration of certain proteins, such as the influenza A virus NP and the yeast matα2 protein.

These data on nuclear localization seem to indicate that this process differs from protein translocation to other cellular compartments. Nuclear proteins do not appear to have an amino terminal portion that is cleaved to produce mature proteins as is the case for most proteins translocated to the rough endoplasmic reticulum, for example. One may speculate that nuclear proteins need to have their "entry signal" present all of the time, even after nuclear entry. During mitosis the nuclear membrane structure breaks down and proteins can exit the nucleus. At this stage, nuclear proteins may be found in the cytoplasm. In order for these proteins to find their way back into the nucleus they need to retain their entry signal (DeRobertis, 1983).

Recently in vitro as well as in vivo assays have been used to elucidate the mechanism of a protein's specific entry and accumulation into the nucleus (Newmeyer and Forbes, 1988; Richardson, et al., 1988). The results of these studies indicate that transport of a protein into the nucleus can be divided into two steps: binding to the nuclear envelope and translocation through the nuclear pores. Binding of a nuclear protein seems to occur at the nuclear pores and is dependent on the presence of a nuclear signal sequence. ATP is required for the translocation of a protein into the nucleus. The first step is thought to occur quickly and is not inhibited by the addition of the lectin WGA. The translocation step is slower and can be inhibited by WGA. This suggests that the nuclear pore machinery involved in translocation contains N-acetylglucosamine residues and is physically separate from the signal sequence receptor since binding is unaffected by the WGA. These assay systems should allow for the further dissection of nuclear transport.

Table 2: Nuclear Signal Sequences

Protein	Signal	Reference
SV40 Large T antigen	PKKKRLV	Kalderon,et al., 1984b;Landford and Butel, 1984
Yeast MAT 2 protein	MNKIPIKDLLNPQ	Hall,et al.,1984
Influenza NP protein	QLVWMACNSAAFEDLRVLS	Davey,et al,1985
Yeast ribosomal protein	MSHRKYEAPRHGHLGFLPRLR	Moreland, et al. 1985
Polyoma Large T antigen	PKKAREDVSRKRPR	Richardson, et al., 1986
SV40 VP1 protein	APTKRKGS	Wychowski, et al., 1986
Xenopus Nucleoplasmin protein	RPAATKK* KPAAKK AKKKKLD PTKKGKG	Burglin and DeRobertis, 1987
Adenovirus E1A protein	KRPRP	Lyons, et al., 1987
Yeast Histone 2B	GKKRSKA	Moreland, et al. 1987
SV40 VP2 protein VP3 protein	PNKKKRK	Wychowski, et al.,1987, Gharakhanian et al., 1987

* Within a 50 amino acid region, 4 possible nuclear signal sequences were found.

II.

EXPRESSION OF INFLUENZA VIRUS NS2 NONSTRUCTURAL PROTEIN IN BACTERIA AND LOCALIZATION OF THE NS2 IN INFECTED EUCARYOTIC CELLS

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A. INTRODUCTION

The complete sequence of one influenza A virus (A/PR/8/34) is known, along with the nucleotide sequences of individual genes from numerous influenza A virus variants (reviewed in Lamb, 1983). Thus, much information is available on the structural organization of the genomes of influenza viruses, but less is known about the precise function(s) of the products encoded by these various genes. In particular, the function of the two nonstructural proteins NS1 and NS2, coded for by the shortest RNA segment (Ritchey, et al., 1976b; Inglis, et al., 1979; Lamb and Choppin, 1979), remains undefined. In this paper, we describe attempts to learn more about the smaller of these nonstructural proteins. The NS2 polypeptide was expressed in bacterial cells to generate large quantities suitable for producing antisera. The NS2 protein was partially purified, and monospecific antibodies against NS2 were prepared in rabbits. This reagent was then used to demonstrate that NS2 localizes in the nucleus of influenza virus-infected cells.

B. MATERIALS AND METHODS

1. PLASMIDS

Plasmid pAPR801, a pBR322 derivative containing a double-stranded cDNA of the A/PR/8/34 influenza virus NS gene, and plasmid pAS1/PR8, a pBR322 lambda phage chimeric also having the A/PR/8/34 NS gene, have been described previously (Baez, et al., 1980; Young, et al., 1983). Plasmid pA11-SVL2, a eucaryotic expression vector based on the simian virus 40 (SV40) late promoter, was obtained from George Khoury and has been previously described (Gruss, et al., 1982). pSVr-INS7, which contains an intact SV40 late region, was obtained from Philip Sharp (Laski, et al.,1982). Plasmids pA11-SVL2 and

its derivatives and pAPR801 were grown in Escherichia coli C600 cells. Plasmids pAS1/PR8 and pAS1/PR8-NS2 were replicated in E.coli N99 and N5151 cells. DNA manipulations, including restriction enzyme digestion, isolation and ligation of fragments, and transformation of E.coli with plasmid DNA, were done by standard procedures (Maniatis, et al., 1982).

Plasmid pAPR801 was digested with BstN1, and the resulting seven fragments were end-filled with the Klenow fragment of DNA polymerase I. HindIII linkers (GCAAGCTTGC) (P-L Biochemicals, Inc., Milwaukee, Wis.) were ligated to the fragments which were then digested with HindIII. The fragment coding for the portion of the NS2 gene, including nucleotides 527 to 890, was isolated from a 4% polyacrylamide gel. pAS1/PR8 was also digested with HindIII. This enzyme cuts the plasmid in two places. The fragments were then treated with bacterial alkaline phosphatase and used in a ligation reaction containing the isolated fragment of the NS2 gene. This preparation was used to transform E.coli (N99) cells, and ampicillin-resistant colonies were isolated. DNAs from several colonies were screened with various restriction enzymes, and several plasmids possessing the NS2-coding sequence in the correct orientation relative to the promoter were further screened for the ability to produce NS2 protein. Construction of the resulting plasmid pAS1/PR8-NS2 is diagrammed in Fig. 2.

2. NS2 PRODUCTION AND PARTIAL PURIFICATION

E.coli N5151 cells were transformed with pAS1/PR8-NS2 and grown to an optical density of 0.65 at 32°C. To induce the NS2 protein the temperature was quickly raised to 42°C (Rosenberg, et al., 1983). A 1-liter culture was then grown at 42°C for 2 hours. The bacteria were pelleted (20 min at 12,000 x g), suspended in phosphate-buffered saline(PBS), and centrifuged

again. The bacterial pellet was then stored at -70°C overnight. The NS2 protein was partially purified by a modification of the procedure used for NS1 protein (Young, et al., 1983). The bacterial pellet was thawed and resuspended in 40 ml of buffer A containing 50mM Tris-hydrochloride(pH 8), 2 mM EDTA, 0.1mM dithiothreitol, and 5%(wt/vol) glycerol, to which lysozyme was added at a final concentration of 0.2 mg/ml. The suspension was incubated on ice for 20 minutes and then centrifuged for 30 minutes at 12,000 x g. The pellet was resuspended in 10 ml of buffer A, and sodium deoxycholate was added to a final concentration of 0.05%. The mixture was then Dounce homogenized (20 strokes) and centrifuged for 45 min at 12,000 x g. The pellet was resuspended in 20 ml of buffer A and mixed with an equal volume of a buffer containing 10mM Tris(pH 7.2), 1% Nonidet P-40, 5% sodium deoxycholate, 0.1 M NaCl, and 1mM EDTA. This suspension was incubated on ice for 15 minutes, Dounce homogenized (20 strokes), and pelleted at 16,000 x g for 30 min. The extraction with sodium deoxycholate and Nonidet P-40 was repeated, and the pellet was resuspended in 6M guanidinium chloride- 1% 2-mercaptoethanol and centrifuged for 1 hour at 16,000 x g. The resulting supernatant contains partially purified NS2. This preparation was at least 50% pure NS2 as judged by gel electrophoresis, followed by Coomassie blue R-250 staining, and recovery of NS2 from the induced bacteria was at least 90%.

3.ANTISERUM PRODUCTION

Before immunization, the guanidinium chloride was removed from the partially purified NS2 preparation by dialysis against two changes of distilled water. This resulted in the formation of a precipitate which was pelleted at 8,000 x g for 15 minutes. Both the supernatant and pellet contained NS2 protein. The pellet was used for subcutaneous inoculation, and

the supernatant was used for intravenous inoculation. A sample of each fraction was run on a 7 to 14% polyacrylamide gradient gel. These gels were stained with Coomassie blue R-250, and the amount of NS2 present was quantitated by comparsion with known amounts of protein standards run on the same gel. Approximately 500 ug of NS2 protein in suspension was combined with an equal volume of Freund complete adjuvant. This was injected subcutaneously into a New Zealand White rabbit at eight sites along its back, and 4 weeks later this was repeated except that incomplete adjuvant was used. Finally, the animal was given an intravenous boost of ca. 15 ug of the NS2 protein (supernatant fraction; see above) 2 weeks after the last subcutaneous injection. The animal was bled 1 week after the final boost. In addition to this serum, an antiserum was raised against NS2 isolated by preparative gel electrophoresis of the pellet after Nonidet P-40 and sodium deoxycholate extraction. An immunization protocol similar to the one described was used to generate this antiserum.

4. CONSTRUCTION OF SV-NS VECTORS AND EXPRESSION OF NS1 AND NS2 PROTEINS IN CV-1 CELLS.

SV40 recombinants containing the influenza virus NS gene were obtained by insertion of a complete NS gene into the BamHI site of pA11-SVL2 (Inglis, et al.,1979). For this purpose, plasmid pAPR801 was digested with EcoRI; the fragments were end-filled, ligated with BglII linkers (CAGATCTG) (New England Biolabs, Beverly, Mass.), and digested with BglII. The cDNA containing the NS gene sequences was purified by polyacrylamide gel electrophoresis. pA11-SVL2 was digested with BamHI, which cleaves at a single site in the late region near the agnoprotein initiation codon of SV40 DNA (Gruss, et al., 1982). The digested pA11-SVL2 and the NS gene fragment

were ligated and used to transform competent E.coli 600 cells. Colonies containing the NS gene were identified by filter hybridization and screened with restriction enzymes to determine the orientation of the NS gene relative to the SV40 promoter.

The recombinant pA11-SVL2 vectors containing the NS gene (10 µg of DNA) were digested with XbaI to remove pBR322 sequences. After phenol extraction and ethanol precipitation, the mixture was religated without further purification. The ligated DNA was mixed with 10 µg of pSVr-INS7 (Laski, et al., 1982) which had been digested with EcoRI and religated to eliminate pBR322 sequences. pSVr-INS7 DNA is added to provide the late SV40 functions in cotransfected cells. The mixture was transfected into CV-1 cells with a modification (Parker and Stark, 1979) of the calcium phosphate precipitation procedure. Two weeks later, cells and medium were frozen and thawed three times and used as virus stocks.

5. IMMUNOPRECIPITATION

Confluent MDCK cell monolayers grown in 35-mm dishes were infected with 0.1 ml of egg-grown influenza viruses (A/PR/8/34, A/Japan/305/57, A/Udorn/72) for 1 hour at 37°C. At 6 hours postinfection, the cells were labeled with [^{35}S]methionine (200 uCi/ml) for 1 hour as reported previously (Parvin, et al., 1983). Confluent CV-1 cell monolayers in 35-mm dishes were infected with 0.1 ml of SV40 recombinant virus stocks. At 2 days postinfection, the cells were labeled with [^{35}S]methionine for 2.5 hours. After the labeling period, cells were lysed in 200ul of buffer containing 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride (pH 7.4), and 1mM phenylmethylsulfonyl fluoride. Samples (25ul) of labeled cell extracts were incubated overnight

with rabbit sera at 4^oC. Formalin-fixed Staphylococcus aureus cells (100 ul; Bethesda Research Laboratories, Rockville, Md.) were added, and the mixture was then incubated for 30 minutes at 4^oC. Samples were pelleted (2 minutes at 15,000 x g) at 4^oC and suspended in 250ul of 500mM LiCl-100mM tris-hydrochloride (pH8.5). They were then pelleted again and washed once with distilled water. Finally, samples were resuspended in loading buffer and electrophoresed on 7 to 14% polyacrylamide gradient gels containing 6M urea as described previously (Parvin, et al., 1983).

6.IMMUNOFLUORESCENCE

For the experiment shown in Figure 6, CV-1 cells were grown on eight-well multitest slides (Flow Laboratories, Inc., McLean, Va.) and were infected with influenza A/PR/8/34 and B/Lee/40 viruses at a multiplicity of infection of ca. 3. Cells were fixed with 3% formaldehyde in PBS (pH 7.4) and permeabilized with 0.05% Triton X-100. Slides were then incubated with rabbit anti-NS2 serum diluted 1:40 or 1:125 in PBS containing 5% goat serum, washed, and in the case of A/PR/8/34 virus infections, incubated with the mouse monoclonal anti-hemagglutinin antibody H28-E23 (Bachi, et al., 1984), likewise diluted into PBS with goat serum. Cells were then washed and exposed to a mixture of rhodamine-conjugated goat anti-rabbit immunoglobulin G (IgG) $F(ab)_2$ and fluorescein-conjugated goat anti-mouse IgG $F(ab)_2$ antibodies (Cappel Laboratories, Cochranville, Pa.) which were also diluted into PBS containing 5% normal goat serum. Cells were then washed, mounted, and observed and photographed in a Reichert-Jung Polyvar microscope equipped with epifluorescence and differential interference contrast optics.

For other experiments (see Figure 7 and 10; Table 3), cells were seeded

onto 13-mm cover slips at ca. 10^5 cells per cover slip. The next day these cells were infected and fixed with 1% formaldehyde for 1 hour at room temperature, followed by incubation with 0.1% Triton X-100 for 3 minutes. Cover slips were treated with 100ul of NS2 antiserum at 1:125 dilution or 1:50 dilution of NS1 antiserum (Young, et al., 1983) for 30 minutes at 37°C, washed, and stained with fluoresceinated goat anti-rabbit IgG (Cappell) at a 1:200 dilution for 30 minutes at 37°C. Cells were viewed on a Leitz fluorescence microscope.

7. ABSORPTION OF ANTISERA.

Antisera were absorbed by a solid-phase procedure. Purified NS1 or NS2 (50 ul at a concentration of 100 ug/ml in PBS) isolated from bacteria was adsorbed to wells of polyvinyl chloride microtiter plates for 1.5 hours at 37°C. Fifty microliters of borate buffer (0.02 M [pH8]; 0.9% NaCl, 0.05% NaN_3) with 1% bovine serum albumin was added and incubated an additional 1.5 hours at 37°C. Plates were washed three times with PBS. Antisera (50 ul of a 1:50 dilution in PBS plus 0.1% bovine serum albumin) were added to the wells and incubated 1.5 hours at 37°C. This step was repeated twice. The sera were diluted with 50 ul of 10% goat serum and used for immunofluorescence as described above.

C. RESULTS

1. CONSTRUCTION OF A PLASMID CONTAINING THE NS2 GENE.

The plasmid pAS1/PR8-NS2 was constructed to express the influenza A/PR/8/34 virus NS2 protein in bacteria. For this purpose, we modified plasmid pAS1/PR8 (Young, et al., 1983) which was previously used to express the gene for the NS1 protein: The NS2 mRNA in influenza virus-infected

cells differs from the NS1 mRNA by the absence of nucleotides 57 to 528, which are spliced out (Lamb and Choppin, 1979; Lamb, 1983). These sequences were removed from pAS1/PR8 by digestion of the plasmid with HindIII which retains nucleotides 5' to the splice site and religation with a DNA fragment containing the sequences of the NS2 mRNA 3' to the splice site (Figure 2). This construction resulted in an open reading frame colinear with the deduced coding sequence of NS2, with the exception of the ninth codon, which underwent a change from phenylalanine to leucine (see lower part of Figure 2).

2.EXPRESSION OF NS2 PROTEIN.

The pAS1/PR8-NS2 plasmid was used to transform N5151 E.coli cells. The cells were shifted to 42°C to induce the synthesis of NS2 protein. Cells were pulse-labeled for 5 minutes with[^{35}S]methionine and chased in unlabeled medium for varying amounts of time. Analysis of labeled cells before and after temperature shift by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography is shown in Figure 3. A new protein band appears in cells shifted to 42°C (lane 2) that is not present in cells grown at 32°C (lane 1). The new protein with a molecular weight of 12,000 is similar in size to the influenza virus NS2 protein. The amount of labeled NS2 protein after a 90 minute chase (lane 5) is similar to the amount of label after a 5 minute pulse (lane 2). Thus, the NS2 protein is synthesized at high levels in induced E.coli cells, and very little degradation of NS2 is observed over a 90 minute time course.

Figure 4 shows a Coomassie blue stained gel of transformed E.coli cells before a temperature shift (lane2) and after a 2 hour incubation at 42°C (lane 3). For comparsion, cells transformed with pAS1/PR8 and incubated at

Figure 2. The pAS1/PR8-NS2 plasmid was constructed to express only NS2-coding sequences in the absence of the complete NS1 sequence. The NS2 sequences 3' to the splicing junction (nucleotide 27 to 56) were derived from pAPR801 by BstN1 and HindIII digestion (Baez, et al., 1980). The sequences 5' to the splice junction (nucleotides 529 to 890) were derived from plasmid pAS1/PR8, which was used previously to express NS1 protein (Young, et al., 1983). The final construct, pAS1/PR8-NS2, contains a complete copy of the A/PR/8/34 NS2 gene except for a single nucleotide change that was introduced by the commercially prepared HindIII linkers. This resulted in an amino acid change in position 9 from phenylalanine to leucine, as shown in the lower portion of the figure. With this exception, the gene product expressed in bacteria has the same amino acid sequence as that predicted for NS2 in virus-infected cells. The numbering system of the nucleotides is based on the previously obtained sequence of the A/PR/8/34 NS gene (Baez, et al., 1980).

Figure 2:

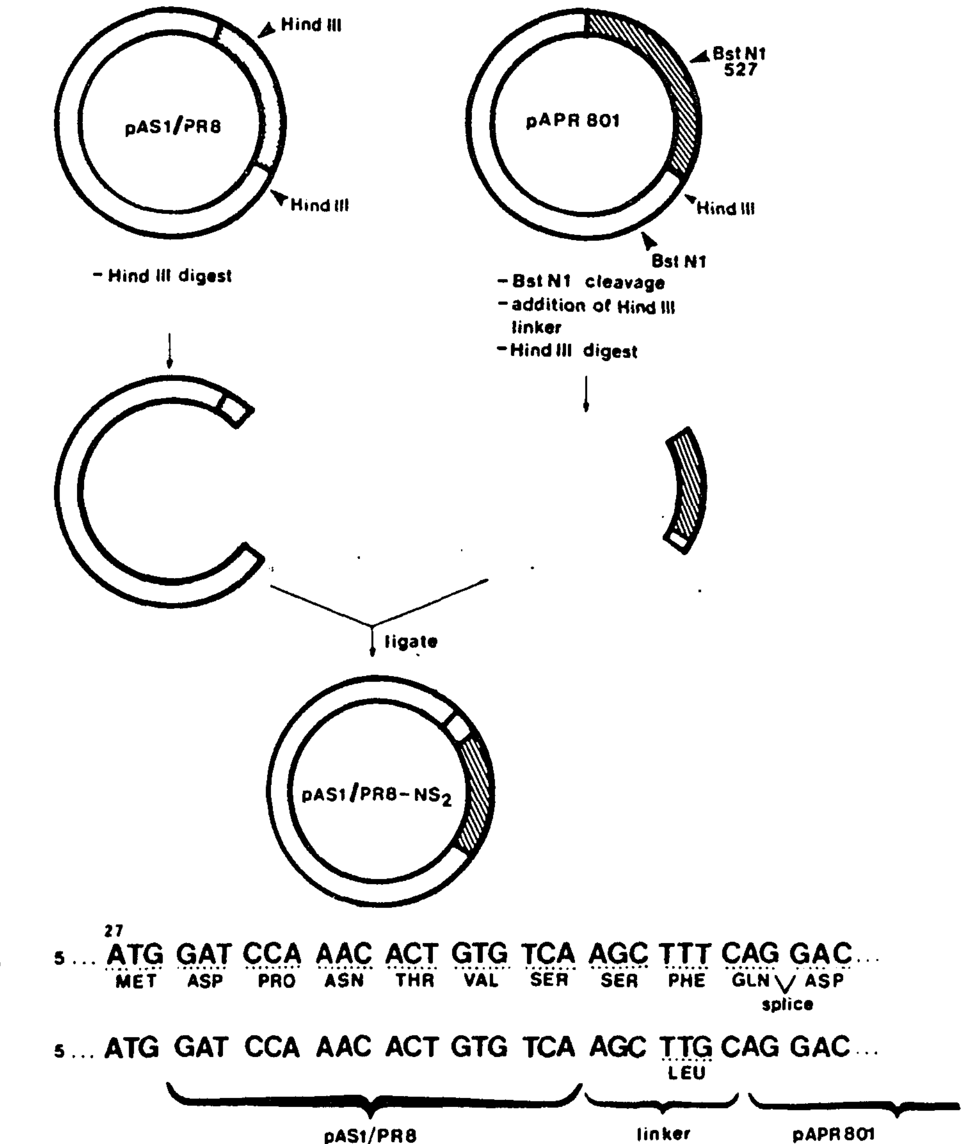


Figure 3. Biosynthesis and stability of NS2 protein in E.coli.

Bacterial cell cultures containing pAS1/PR8-Ns2 were induced for 30 minutes at 42°C and then pulse-labeled with [^{35}S]methionine was removed, and cells were maintained at 42°C in medium containing unlabeled methionine for the times indicated. At the end of the incubation, cell extracts were electrophoresed on 7 to 14% polyacrylamide gels containing 6M urea. Gels were dried and autoradiographed. Lane 1, Uninduced bacterial cell culture; lane 2, induced bacterial cell culture (chase time = 0 minutes); lane 3, induced bacterial cell culture (chase time = 30 minutes); lane 4, induced bacterial cell culture (chase time = 60 minutes); lane 5, induced bacterial cell culture (chase time = 90 minutes); and lane 6, influenza A/PR/8/34 virus-infected MDCK cells (pulse-labeled for 60 minutes with [^{35}S]methionine 7 hours postinfection[see the text]).

Figure 3:

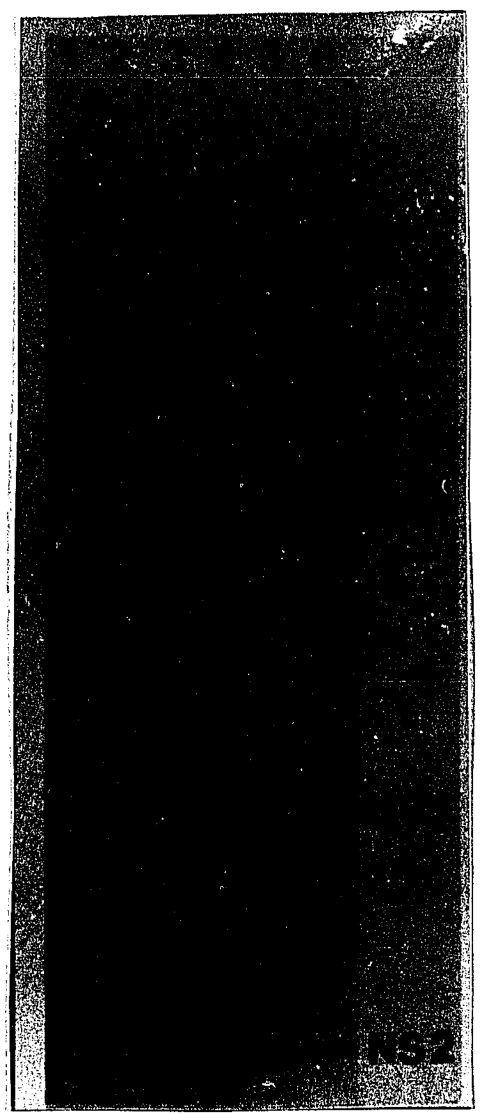
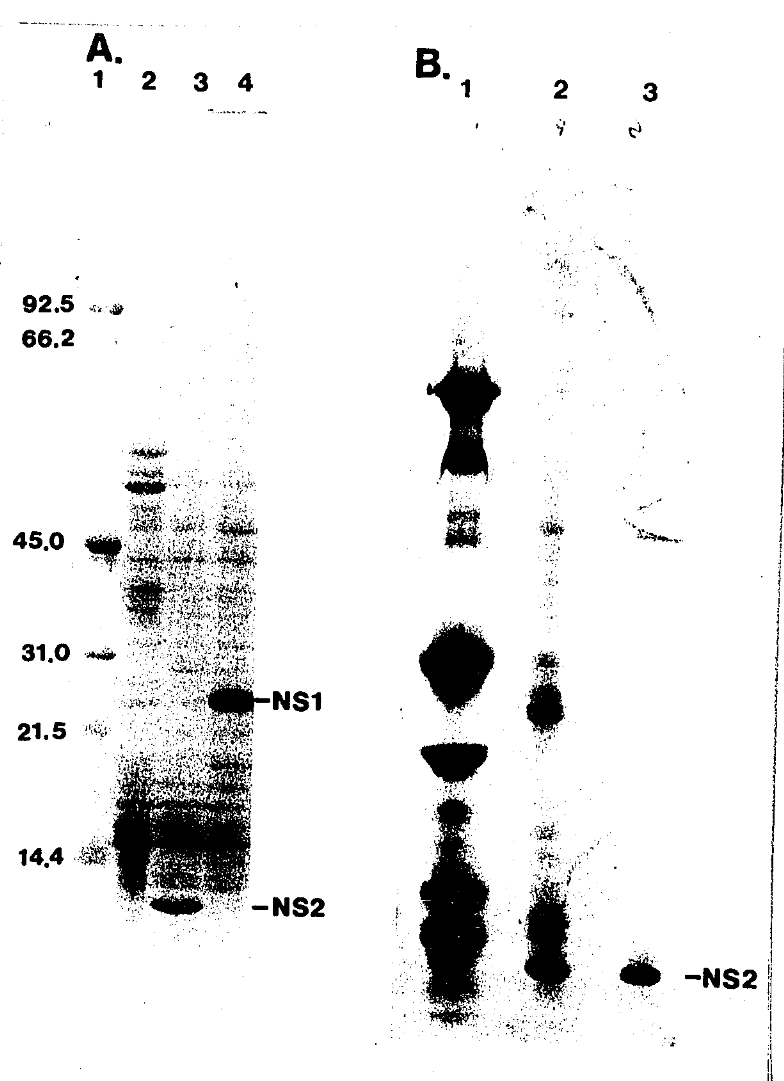


Figure 4.Expression of influenza virus NS2 protein in E.coli. (A) E.coli cells (N5151) transformed with either pAS1/PR8-NS2 or pAS1/PR8 (Young, et al., 1983) were grown at 32°C for 30 minutes; the temperature was then shifted to 42°C for induction. The equivalent of 1 ml of bacterial cell culture was separated on a 7 to 14% polyacrylamide gel containing 6M urea and stained with Coomassie blue R-250. Lane 1, Low-molecular-mass markers (14.4 to 92.5 kilodaltons); lane 2, uninduced bacterial cell culture; lane 3, bacterial cell culture containing pAS1/PR8-NS2 induced for 2 hours; and lane 4, bacterial cell culture containing pAS1/PR8 induced for 2 hours. (B) NS2 was purified from a bacterial cell culture containing pAS1/PR8-NS2 induced for 2 hours. The purified NS2 was dialyzed to remove guanidinium chloride. The resulting precipitate was pelleted and suspended in a volume of buffer equal to that of the supernatant. Equivalent amounts of supernatant and pellet were separated on a 7 to 14% polyacrylamide gel and stained with Coomassie blue R-250. Lane 1, Low-molecular-mass markers [same as in (A)]; lane 2, purified NS2 pellet fraction; and lane 3, purified NS2 supernatant fraction.

Figure 4:



42^oC to induce synthesis of NS1 are shown in lane 4. These results show that NS2 protein can be produced in large quantities in bacteria, similar to the production of NS1 (Young,et al., 1983). From data shown in Figure 4, we estimate the NS2 protein to be approximately 5 to 10% of total cellular protein.

3. PARTIAL PURIFICATION OF NS2.

The NS2 produced in E.coli during a 2 hour incubation at 42^oC was partially purified to raise antisera against NS2. Cells from a 1-liter culture were treated with lysozyme and then lysed with deoxycholate. The NS2 was not solubilized by the detergent and was pelleted at 12,000 x g. After further extraction of the pellet with deoxycholate and Nonidet P-40 to remove bacterial contaminants, the NS2 was then solubilized with guanidinium chloride. At this stage, the NS2 is ca. 50% of the total protein, and the yield (2 to 4 mg from a 1-liter culture) is nearly quantitative. When the guanidinium chloride was removed by dialysis, a precipitate formed. Both the precipitate and the resulting supernatant contained NS2 (Figure 4B, lanes 2 and 3). Both fractions were used for immunization of rabbits. Usually the NS2 remaining in the supernatant showed a further degree of purification after removal of the precipitate (lane 3).

4. REACTIVITY OF ANTISERA AGAINST NS2

The specificity of antisera raised against NS2 isolated from E.coli was tested in immunoprecipitation experiments. Influenza virus-infected cells were labeled with [^{35}S] methionine, solubilized with detergents, and reacted with antisera against either NS1 or NS2 purified from E.coli. The immune complexes were precipitated with fixed S.aureus cells and analyzed by sodium dodecyl sulfate gel electrophoresis and autoradiography (Figure 5). The anti-

NS2 serum specifically precipitated NS2 and not NS1 from cells infected with A/PR/8/34 virus (Figure 5A, lane 4), whereas the anti-NS1 serum specifically precipitated NS1 (lane 3) as shown previously (Young, et al., 1983). The cross-reactivity of antiserum against NS2 among influenza viruses of different subtypes is shown in Figure 5B. The antiserum raised against the NS2 of A/PR/8/34 virus expressed in E.coli precipitates the NS2 proteins of an H2 influenza virus (A/Japan/305/57; lane 3) and of an H3 virus (A/Udorn/72; lane 6). In further immunoprecipitation experiments, the antiserum against NS2 was also observed to react with the NS2 of A/Duck/Alberta/60/76 (H12) virus but not with that of an influenza B virus (B/Lee/40 virus; data not shown). Thus, the NS2 proteins of influenza A viruses belonging to different subtypes are antigenically related, whereas the NS2 protein of an influenza B virus is antigenically distinct.

5. IMMUNOFLUORESCENT LOCALIZATION OF THE NS2 PROTEIN.

The availability of specific antisera allowed us to investigate the intracellular localization of the NS2 protein during influenza virus infection. This was investigated with indirect immunofluorescence microscopy. CV-1 cells were infected with influenza A/PR/8/34 virus at a low enough multiplicity so that infected cells as well as uninfected cells serving as internal controls were present in the same microscopic field. The infected cells were identified by a monoclonal antibody against the hemagglutinin. Cells incubated for various periods of time after infection were labeled with rabbit anti-NS2 serum and rhodamine-labeled anti-rabbit IgG $F(ab)_2$ antibody. The viral hemagglutinin was labeled with a mouse monoclonal antibody and fluorescein-labeled anti-mouse IgG $F(ab)_2$ antibody. The results of these double-immunofluorescent labeling experiments are shown in Figure 6. The

Figure 5. Specificity of antiserum made to bacterially synthesized NS2. (A) Influenza A/PR/8/34 virus was used to infect MDCK cells. Six hours postinfection, the cells were labeled with [^{35}S]methionine for 1 hour, lysed, and used for immunoprecipitation. The experiment shown was performed with antiserum against NS2 purified by preparative gel electrophoresis. The same result was obtained with antiserum against NS2 purified by detergent extraction. Lane 1, Virus infected cell extract; lane 2, virus infected cell extract immunoprecipitated with normal rabbit serum; lanes 3 and 5, virus-infected cell extract immunoprecipitated with anti-NS1 antiserum; and lane 4, virus infected cell extract immunoprecipitated with anti-NS2 antiserum. (B) Influenza A/Japan/305/57 (H2N2) and A/Udorn/72 (H3N2) viruses were used to infect MDCK cells. Cells were labeled, extracted, and used for immunoprecipitation. Lane 1, Influenza A/Japan/305/57 virus infected cell extract; lane 2, influenza A/Japan/305/57 virus infected cell extract immunoprecipitated with normal rabbit serum; lane 3, influenza A/Japan/305/57 virus infected cell extract immunoprecipitated with anti-NS2 antiserum; lane 4, influenza A/Udorn/72 virus infected cell extract; lane 5, influenza A/Udorn/72 virus infected cell extract immunoprecipitated with normal rabbit serum; and lane 6, influenza A/Udorn/72 virus infected cell extract immunoprecipitated with anti-NS2 antiserum.

Figure 5:

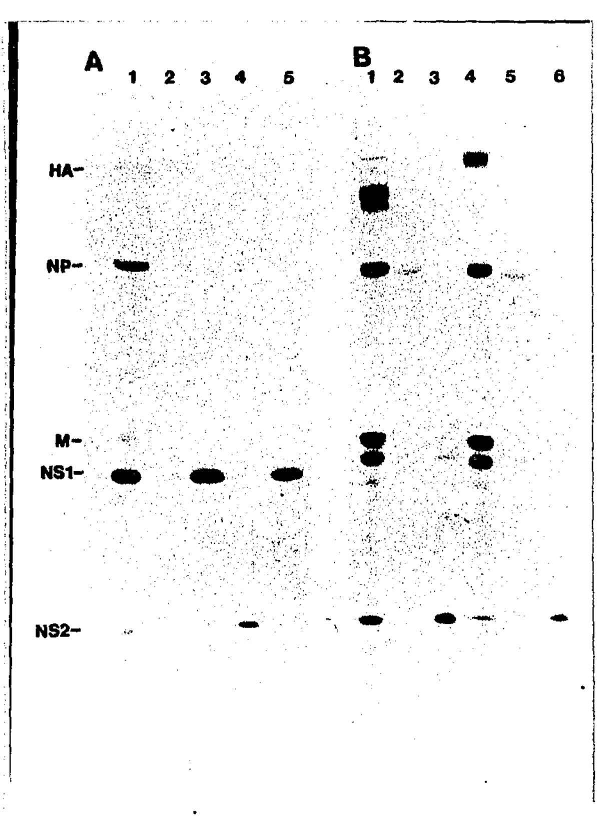
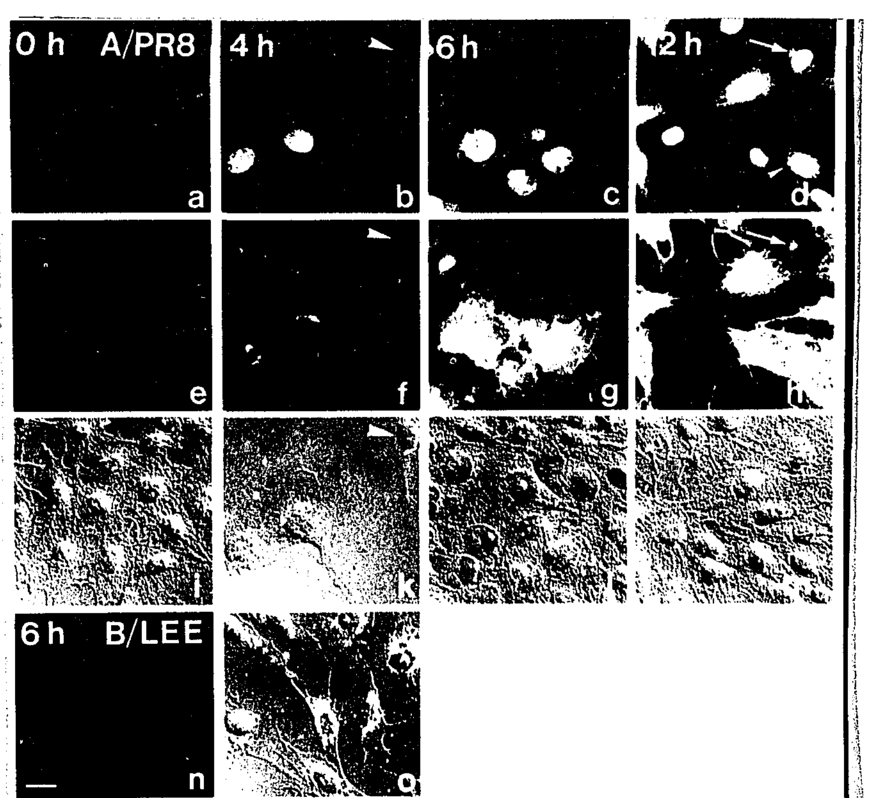


Figure 6. Intracellular localization of the NS2 protein. CV-1 cells were infected with influenza A/PR/8/34 or influenza B/Lee/40 virus, incubated at 37°C, and then fixed and processed for double immunofluorescence as indicated in the text. (a to d) Cells labeled wirh rabbit anti-NS2 antisera and rhodamine-conjugated goat anti-rabbit IgG antibodies. Cells were fixed after infection at the times indicated. (e to h) Same cells as in (a to d), labeled with mouse monoclonal antibody to hemagglutinin and fluorescein-conjugated goat anti-mouse IgG. In (i to m), the differential interference contrast images of the same fields are shown. (n and o) Cells infected with influenza B/Lee/40 virus, incubated for 6h, and labeled as in (a to d). Arrowheads in (b), (f), and (k) show that a cell not expressing hemagglutinin lacks NS2-specific immunofluorescence. Arrows in (d) and (h) show occasional perinuclear NS2-specific immunofluorescence which colocalized with that of hemagglutinin. Small arrowhead in (d) shows NS2-specific fluorescence along some sites of the plasma membrane.

Figure 6:



fluorescein and rhodamine fluorescence and differential interference contrast image of the same field are shown for each time point. Before infection, cells showed background immunofluorescence. With increasing time after infection, NS2-specific immunofluorescence (Figure 6a to d) became visible in those cells which also expressed the hemagglutinin (Figure 6e to h). Cells devoid of hemagglutinin staining showed no NS2 fluorescence (large arrowheads), and only occasionally a cell with little hemagglutinin immunofluorescence showed an appreciable intensity of NS2 immunofluorescence (Figure 6d and h). The predominant NS2-specific immunofluorescence was observed in the nucleus of infected cells but apparently not in the nucleoli. In some infected cells, particularly in later stages of infection, the NS2 immunofluorescence was also found in the perinuclear area of the cytoplasm (Figure 6d, small arrowhead) at similar sites in which the hemagglutinin was localized (Figure 6h, arrows). Cells infected with influenza B/Lee/40 virus show no NS2-specific immunofluorescence (Figure 6n and o).

6. IMMUNOABSORPTION OF ANTISERA.

To further characterize the specificity of the antisera in immunofluorescence experiments, the sera were subjected to immunoabsorption with purified NS1 and NS2 protein preparations. The results are shown in Table 3. The reactivity of anti-NS1 serum (Young, et al., 1983) with influenza A/PR/8/34 virus- infected CV-1 cells was removed by absorption with NS1 but not by absorption with NS2. Conversely, the reactivity of anti-NS2 was not altered by absoption with NS1 but could be absorbed with NS2. This indicates that the immunofluorescence observed with anti-NS2 serum is not due to cross-reactivity of the anti-NS2 serum with NS1.

TABLE 3 Immunofluorescence with antisera immunoabsorbed with purified NS1 or NS2 protein[a]

Treatment	Immunofluorescence reaction with:	
	Anti-NS1 serum	Anti-NS2 serum
None	+	+
NS1 absorption	−	+
NS2 absorption	+	−

[a] CV-1 cells infected with influenza A/PR/8/34 virus were fixed 6 h postinfection and used for immunofluorescence as described in the text. Fluorescence with treated sera was considered positive when the fluorescence intensity was not significantly different from that obtained with unabsorbed serum, as determined by the time required to expose ASA 200 film (automatically determined) with the light meter centered over the nucleus of an infected cell (45 ± 15 s). Fluorescence intensity was considered negative when it was similar to that obtained with mock-infected cells.

7. NS2-SPECIFIC IMMUNOFLUORESCENCE IN A/CAM/46 VIRUS-INFECTED CV-1 CELLS AND A/PR/8/34 VIRUS-INFECTED MDCK CELLS.

An intranuclear localization of NS2 was also observed in CV-1 cells infected with another virus, A/CAM/46 (Figure 7). Infected cells were incubated with antiserum against NS2 and stained with fluorescein-labeled anti-rabbit IgG antibody. The intense labeling of the nuclei of influenza A/CAM/46 virus-infected cells was comparable to that of A/PR/8/34 virus-infected cells. MDCK cells infected with A/PR/8/34 virus also showed NS2-specific immunofluorescence in their nuclei (Figure 7d). When infected MDCK cells were treated with Formalin and Triton X-100 alone (under conditions used for CV-1 cells), not all cells showed nuclear labeling (data not shown). However, when the treatment with Formalin and Triton X-100 was followed by incubation with cold acetone, nuclear fluorescence comparable to that of infected CV-1 cells was also seen in infected MDCK cells (Figure 7d).

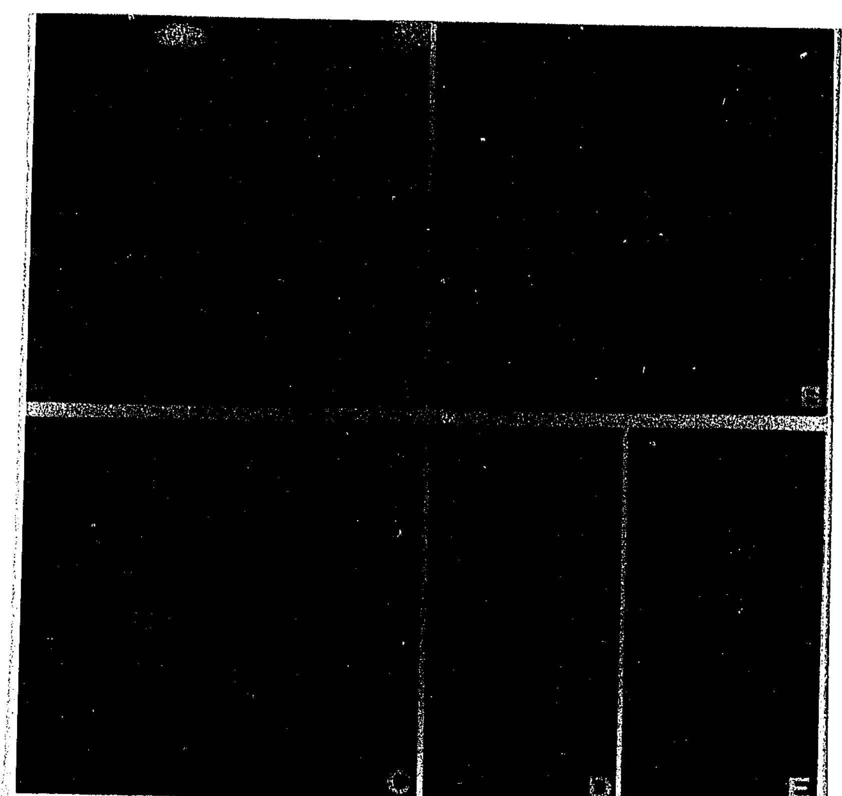
8. EXPRESSION AND LOCALIZATION OF NS1 AND NS2 PROTEINS IN CELLS INFECTED WITH RECOMBINANT SV40 VIRUSES CONTAINING THE NS GENE.

The SV40 vectors used to express the NS1 and NS2 proteins in CV-1 cells are shown in Figure 8. SVL2-801M and SVL2-801V contain a single copy of the NS gene, whereas SVL2-801MM contains two tandem copies of the gene in a head-to-tail orientation. SVL2-801M and SVL-801MM have the NS protein-coding region oriented in the mRNA sense with respect to the SV40 promoter; SVL2-801V contains the NS gene in the opposite orientation.

CV-1 cells were infected with the three SV40 virus stocks and were labeled with [^{35}S]mthionine 2 days postinfection, and the labeled proteins were analyzed by sodium dodecyl sulfate-gel electrophoresis and autoradiography (Figure 9A). The SVL2-801M (lane 3) and SVL2-801MM (lane

Figure 7: Localization of NS2 protein in influenza A/CAM/46 virus-infected CV-1 cells and in influenza A/PR/8/34 virus-infected MDCK cells. Virus-infected cells were fixed 8h postinfection and used for immunofluorescence as described in the text. (A) A/PR/8/34 virus-infected CV-1 cells. (B) A/CAM/46 virus-infected CV-1 cells. (C) Mock-infected CV-1 cells. (D) MDCK cells infected with A//8/34 virus. (E) Mock-infected MDCK cells. Fixation of MDCK cells with Formalin and Triton X-100 was followed by treatment with acetone at -20°C for 5 min.

Figure 7:



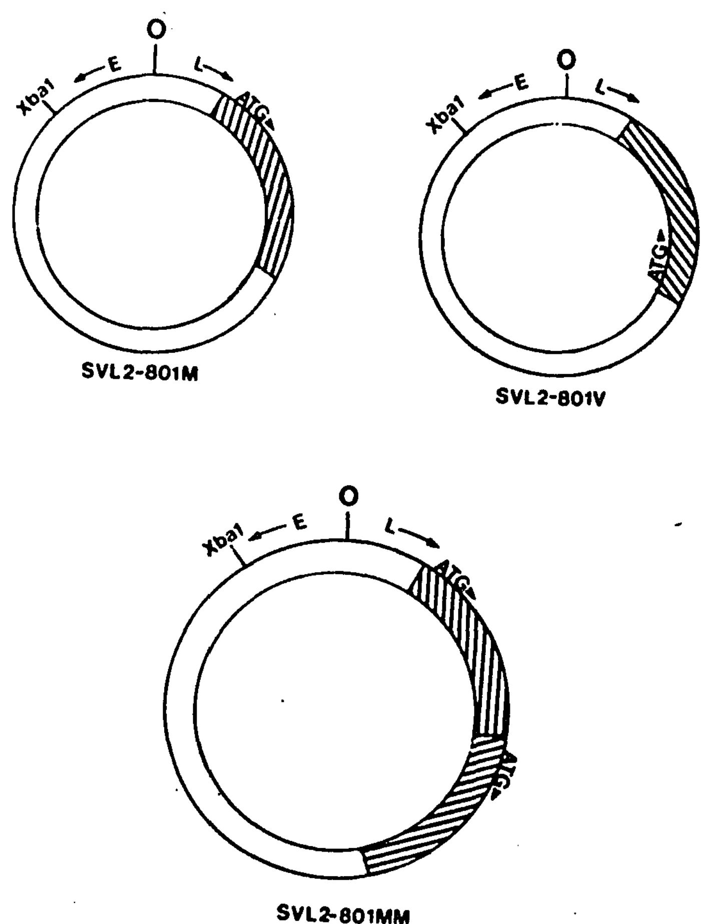
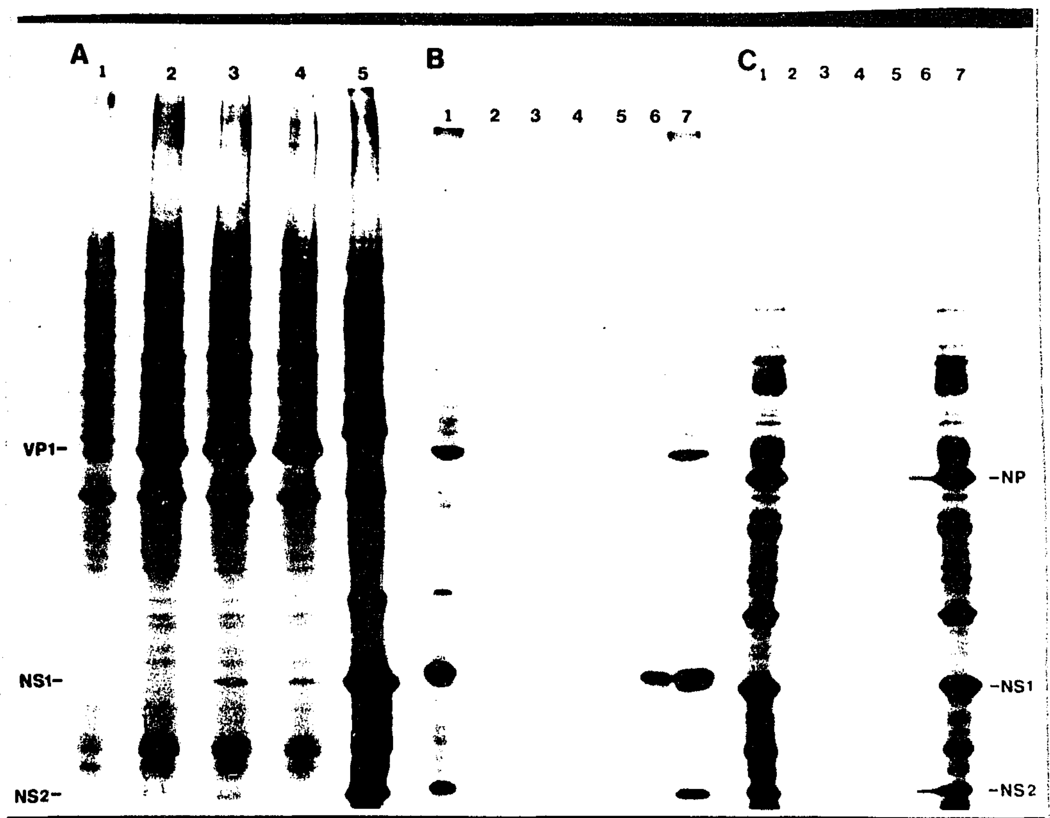


Figure 8: Structure of the SV40 recombinants. The NS gene was inserted into the late region of the SV40 vector pA11SVL2 (Gruss et al., 1982) as described in the text. The hatched regions represent the A/PR/8/34 NS gene. ATG indicates the position of the start codon for NS1 and NS2. The arrows indicate the direction of SV40-promoted transcription. It should be noted that these DNAs originally contained pBR322 sequences inserted at the indicated XbaI site. For transfection of CV-1 cells, these pBR322 sequences were removed by XbaI digestion followed by religation of the SV40 recombinant DNA.

Figure 9: Expression of NS1 and NS2 proteins in CV-1 cells with SV40 vectors. (A) Linear 7 to 14% polyacrylamide gel electrophoresis of infected CV-1 cells labeled for 3 h with [^{35}S]methionine at 2 days postinfection. Lane 1, Mock-infected CV-1 cells; lane 2, SVL2-801V-infected CV-1 cells; lane 3, SVL2-801M-infected CV-1 cells; lane 4, SVL2-801MM-infected CV-1 cells; and lane 5, influenza virus A/PR/8/34-infected CV-1 cells labeled for 1 with [^{35}S]methionine at 6h postinfection. The VP1 protein of SV40 virus and NS1 and NS2 protein are indicated. (B) Immunoprecipitation with anti-NS1 antiserum. Lanes 1 and 7, A/PR/8/34 virus-infected CV-1 cell extract; lane 2, immunoprecipitation of mock-infected CV-1 cells; lane 3, immunoprecipitation of SVL2-801V-infected CV-1 cells; lane 4, immunoprecipitation of SVL2-801M-infected CV-1 cells; lane 5, immunoprecipitation of SVL2-801MM-infected CV-1 cells; and lane 6, immunoprecipitation of influenza virus A/PR/8/34-infected CV-1 cells. (C) Immunoprecipitation with anti-NS2 serum. Lane 1 and 7, A/PR/8/34 virus-infected CV-1 cell extract; lane 2, immunoprecipitation of mock-infected CV-1 cells; lane 3, immunoprecipitation of SVL2-801V-infected CV-1 cells; lane 4, immunoprecipitaioin of SVL2-801M-infected CV-1 cells; lane 5, immunoprecipitation of SVL2-801MM-infected CV-1 cells; and lane 6, immunoprecipitation of influenza virus A/PR/8/34-infected CV-1 cells.

Figure 9:



4) viruses synthesized proteins with the same apparent molecular weights as NS1 and NS2 from influenza A/PR/8/34 virus-infected cells (lane 5). These protein bands were not observed in SVL2-801V (lane 2) virus-infected cells or in mock-infected cells (lane 1). These bands were identified as NS1 and NS2 by immunoprecipitation with specific antisera. Anti-NS1 serum (Figure 9B) precipitated NS1 from SVL2-801M and SVL2-801MM virus-infected cells (lanes 4 and 5) but not from cells infected with SVL2-801V (lane 3). Likewise, anti-NS2 serum precipitated NS2 from SVL2-801M and SVL2-801MM virus-infected cells (Figure 9C, lanes 4 and 5) but not from cells which were infected with SVL2-801V virus containing the NS gene in opposite direction to the SV-40 promoter (lane 3). Precipitation of labeled NP from extracts of A/PR/8/34 virus-infected cells (Figure 9C, lane 6) was not a consistent observation (cf. Figure 9C with Figure 4 and 9B).

The localization of NS1 and NS2 expressed by SV40 recombinant viruses was examined by immunofluorescence microscopy (Figure 10). The prominent nuclear fluorescence observed with influenza virus-infected cells labeled with anti-NS1 serum or anti-NS2 serum is also observed in cells infected with SVL2-801M virus (Figure 10A and B). Similar results were obtained with cells infected with SVL2-801MM virus (data not shown). In contrast, SV42-801V recombinant virus-infected cells showed background immunofluorescence with the anti-NS2 antiserum (Figure 10C).

D. DISCUSSION

Construction of pAS1/PR8-NS2 permitted efficient expression in bacteria of a polypeptide homologous to the NS2 of influenza virus-infected cells. This polypeptide remained insoluble after disruption in bacteria and was

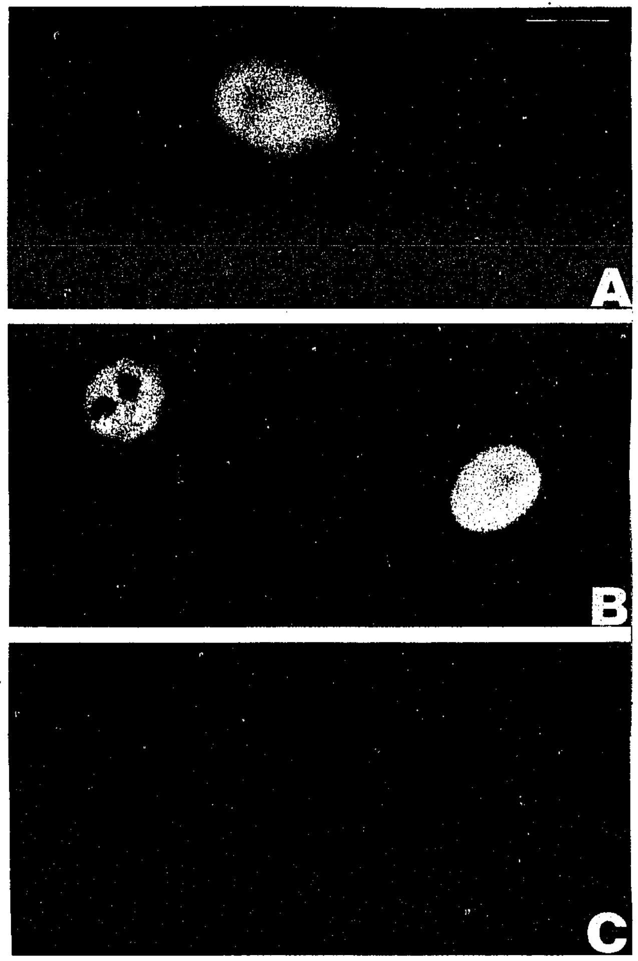


Figure 10: Immunofluorescence of SV-NS-infected CV-1 cells. Cells were fixed 2 days postinfection as described in the text. (A) SVL2-801M virus-infected cells incubated with anti-NS2 serum. (B) SVL2-801M virus-infected cells incubated with anti-NS1 serum. (C) SVL2-801V virus-infected cells incubated with anti-NS2 serum.

partially purified by subsequent detergent extractions. Rabbit antisera made against these NS2 preparations were shown by immunoprecipitation to be monospecific for NS2 proteins, but they broadly cross-reacted with NS2 polypeptides induced by a variety of influenza A viruses. NS2 polypeptides from H1, H2, H3, or H12 subtype virus-infected cells reacted with these antisera. This is not surprising since the most distantly related NS2 proteins (H1 and H 12) in this series have a greater than 80 % amino acid sequence homology (Baez, et al., 1981). On the other hand, the NS2 polypeptide of an influenza B virus, which lacks extensive sequence homologies with the corresponding A virus polypeptides (Briedis and Lamb, 1982), is not immunoprecipitated by these monospecific antisera. Although the bacterially expressed NS1 and NS2 polypeptides share eight amino acids at the amino terminal, the respective antisera made against these proteins do not cross-react in immunoprecipitation experiments with influenza virus-infected cell extracts and cell extracts of bacteria induced to synthesize NS1 (Young, et al., 1983) or NS2. Furthermore, immunoabsorption experiments revealed that the reactivity of anti-NS2 serum was not removed by absorption with a purified NS1 preparation.

The availability of an NS2-specific antiserum allowed us to reexamine the question of intracellular localization of the NS2 polypeptide in influenza virus-infected cells. Previous results were based on subcellular fractionation experiments which had produced conflicting data. Early work had suggested an intranuclear localization of a small nonstructural protein (Krug and Etkind, 1973). More recently, however, support was provided for the idea that the NS2 polypeptide of influenza virus-infected cells is associated with the cytoplasmic fraction throughout the replication cycle (Mahy, et al.,1980;

Bredis, et al., 1981). Our immunofluorescence data would seem to indicate a nuclear localization of the NS2. Differences in strains used in the studies may provide an explanation for the observed differences in NS2 localization. Alternatively, the cell fractionation techniques used may contribute to the complications observed in these experiments. Specifically, the NS2 polypeptide is a small molecule (ca. 120 amino acids) which could be removed from the nuclear fraction by some isolation procedures. Since in our immunofluorescence experiments the NS2 was predominantly localized in the nucleus regardless of cell type and influenza virus strain employed, it is likely that conflicting data in the literature are the result of differences in the isolation techniques used by various investigators.

Analysis of cells infected with SV40 recombinants carrying the NS gene of influenza A/PR/8/34 virus confirmed earlier results (Lamb and Lai, 1984) that splicing of NS-specific mRNAs also occurs with DNA-directed RNA transcription and that it is not dependent on the expression of the other influenza virus genes. The present study has not addressed the question of regulation of NS1-NS2 splicing, and no efforts were made to quantitate the expression of NS1-NS2 transcripts or their translation products. It should by noted, however, that comparable amounts of NS1 and NS2 were immunoprecipitated after pulse-labeling of SV40 recombinant virus-infected cells. No differences in the pattern of NS1-NS2 expression were observed whether the SV40 recombinants containing one or two full-length NS genes were used for infection. However, as expected, the SV40 recombinant
possessing an NS gene that was not under the SV40 promoter control did not direct the synthesis of either NS1 or NS2 proteins. Expression of the SV40 recombinants also allowed us to study the specific intranuclear localization of

NS1 and NS2 in the absence of influenza virus replication. Since these SV40 recombinant viruses express only one influenza virus gene, the possibility can be ruled out that nuclear localization of NS1 and NS2 is the result of a tight association of these proteins with the nuclear ribonucleoprotein complex of influenza viruses. (The ribonucleoprotein complex includes the nucleoprotein and some or all of the P proteins.) It would appear that the NS1 and the NS2 polypeptides, either alone or in conjuction with each other, carry the information for migration to the nucleus of infected cells. Recent studies have shown that the SV40 T antigen contains a sequence of five basic amino acids at positions 127 to 131 that can influence whether T antigen is localized in the nucleus or in the cytoplasm (Kalderon, et al., 1984; Lanford and Butel, 1984). Similar sequences are present in NS1 and NS2. For instance, four of five amino acids at position 84 to 88 in the NS2 sequence and positions 17 to 21 in the NS1 sequence of influenza A/PR/8/34 virus are basic amino acids (Krystal, et al., 1983). Another sequence that is rich in basic amino acids extends from positions 35 to 46 in the NS1 sequence. Thus it is possible that these sequences are involved in the nuclear localization of the NS proteins analogous to the SV40 T antigen. Efforts are currently underway to construct SV40 vectors which express NS1 or NS2 polypeptides separately. Such vectors may be helpful in exploring the question of whether NS1 and NS2 proteins of influenza viruses possess "signal" sequences specific for nuclear compartmentalization.

III.

INFECTIOUS INFLUENZA A AND B VIRUS VARIANTS WITH LONG CARBOXYL TERMINAL DELECTIONS IN THE NS1 POLYPEPTIDES

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A.INTRODUCTION

The influenza A virus genome contains eight single-stranded RNA segments of negative polarity. The smallest segment encodes two nonstructural proteins, NS1 and NS2. The NS1 mRNA is a colinear transcript of the virion RNA and the NS2 mRNA is derived from the NS1 mRNA through a splicing mechanism (for review, Lamb, 1983). The result of this processing event is an open reading frame for the NS2 protein that shares the N-terminal 10 amino acids with the NS1 protein. Beyond the splice site the remainder of the NS2 protein (11 amino acids) is translated in a +1 reading frame relative to that of the NS1 protein. The carboxyl termini of the NS1 proteins of the field isolates examined so far overlap the NS2 coding regions. SDS-polyacrylamide gel electrophoresis revealed migrational differences in the NS1 proteins of different strains and sequence analysis of the NS genes showed that point mutations in the RNAs resulted in nonsense codons in six different locations (Parvin et al., 1983; Krystal et al., 1983; Buonogurio et al., 1986b). The lengths of the NS1 polypeptides predicted from nucleotide sequences of these RNAs were in agreement with the migrational differences seen on SDS-polyacrylamide gel electrophoresis.

The influenza B virus genome also comprises eight segments and it was shown that the structural features of the NS gene of influenza B virus are similar to those of the A viruses (for review, Lamb, 1983; Air and Compans, 1983). The NS1 and NS2 proteins contain 281 and 122 amino acids, respectively, and 11 amino acids at the n-terminus of NS1 and NS2 are shared. As found for influenza A virus NS1 proteins the NS1 proteins of different influenza B viruses have also shown migrational differences on polyacrylamide gels following electrophoresis (Oxford et al., 1983). In

addition, Tanaka et al. (1984) reported that a laboratory-derived influenza B virus, clone 201, induced an aberrant NS protein.

In the present study we demonstrate that influenza virus A/turkey/Oregon/71 codes for an NS1 protein half the size of that of other influenza A virus NS1 proteins. A similar gene structure was found for the NS gene of an influenza B virus resulting in a influenza B virus NS1 protein of less than half the size of that of other B virus NS1 proteins. In both instances the C-terminus of the NS1 proteins does not overlap with the unique region of the NS2 polypeptide. This leads to a nontranslated region between the NS1 and the main body of the NS2 coding regions. The lengths of these regions are 120 nucleotides in the A/turkey/Oregon/71 virus NS gene and 294 nucleotides in the B virus clone 201 NS gene.

B. MATERIALS AND METHODS

1. VIRUSES AND CELLS

Influenza viruses A/turkey/Oregon/71 (H7N5), A/PR/8/34 (H1N1), B/Lee/40, and B/Yamagata/1/73 were grown in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C (A viruses) or 35°C (B viruses). The mutant influenza virus B/610B5B/201 (clone 201) was accidentally derived from a coinfection experiment in tissue culture involving B/Yamagata/1/73 and A/Aichi/2/68 viruses in the presence of anti-A (H3N2) virus antibody (Tanaka et al., 1984). Clone 201 was propagated in Madin Darby canine kidney (MDCK) cells. Viruses were purified using sucrose density gradient centrifugation and the RNA was extracted as reported previously (Ritchey et al., 1976). MDCK cell cultures were used for viral protein analysis of virus infected cells and for isolation of influenza B virus NS1 protein. African green monkey (CV-1) cells were used for immunofluorescence experiments of

B viruses, in order to reduce the possible cross-reactivity of host cell proteins with the anti-B/NS1 antiserum made from the NS1 protein synthesized in B/Lee/40 virus infected MDCK cells.

2. ANALYSIS OF VIRAL POLYPEPTIDES

Confluent monolayers of MDCK cells in 35-mm dishes were infected with approximately 10 PFU/cell of influenza virus (A/turkey/Ore/71, A/PR/8/34, B/Lee/40, clone 201) for 1 hr at 37°C (A viruses) or 33.5°C (B viruses). At 6 hr postinfection cells were pulse-labeled with [^{35}S]methionine (200 uCi/ml) for 1 hr as reported previously (Ritchey et al., 1976). Cell lysates were either used directly or immunoprecipitated employing monospecific rabbit antisera made against bacterially expressed A virus NS1 proteins (Young et al., 1983). Monospecific rabbit antiserum against B virus NS1 protein was prepared using NS1 protein isolated from protein gels as described below. Treated and untreated cell extracts were separated by electrophoresis on a 7-14% linear gradient sodium dodecyl sulfate-polyacrylamide gel (Young and Palese, 1979).

3. PRODUCTION OF INFLUENZA B VIRUS ANTI-NS1 ANTISERUM

Confluent monolayers of MDCK cells in 35-mm dishes were infected for 16 hr with B/Lee/40 virus at a multiplicity of infection (m.o.i.) of 10 at 33.5°C. Whole cell lysates were prepared and electrophoresed on SDS-polyacrylamide gels (Young and Palese, 1979.) Gel slices containing the NS1 protein band were excised from the gel and placed in dialysis tubing and electroeluted overnight (100V, 8mA) at 4°C. The amount of NS1 protein was quantitated by comparison with known amounts of protein standards run on the same protein gel. Approximately 10 ug of NS1 protein in suspension was combined with an equal volume of Freund's complete adjuvant and was used for subcutaneous injection into New Zealand White rabbits at four sites along

the back. Three weeks later this procedure was repeated except that incomplete adjuvant was used. The animals were boosted at 2-week intervals for an additional month and bled 1 week after the final boost.

4. IMMUNOFLUORESCENCE

For the experiments shown in Figure 12, MDCK of CV-1 cells were grown on 13-mm coverslips at ca. 10^5 cells per coverslip. The next day these cells were infected for 8 and 20, hr with influenza A and B viruses, respectively, at an m.o.i. of 10. Influenza A virus-infected cells were fixed with 1% formaldehyde for 1 hr at room temperature, followed by incubation with 0.1% Triton X-100 for 5 min, and treated with acetone at -20 C for 5 minutes (Greenspan et al., 1985). Influenza B virus-infected cells were fixed with acetone for 10 min at -20°C. The cells were treated with 35ul of A virus anti-A/NS1 antiserum at 1:5 dilution or with a 1:40 dilution of B virus anti-B/NS1 antiserum for 30 min at 37°C. Cells were washed and then stained with fluoresceinated affinity purified goat anti-rabbit IgG (Cappel) at a 1:100 dilution for 30 min at 37°C. Cells were viewed on a Leitz fluorescence microscope.

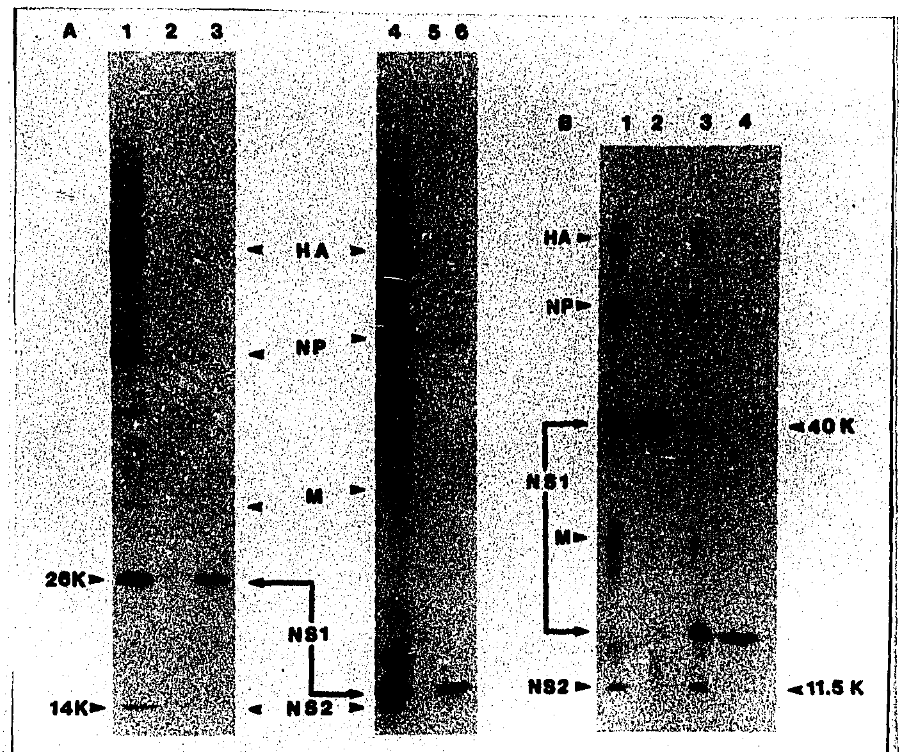
C. RESULTS

1. 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 (Figure 11A). However, a labeled protein band of apparent 14.8K MW migrating near the NS2 protein was detected in the A/turkey/Ore/71 virus-infected cells and not in the A/PR/8/34 virus cell extracts (Figure 11A). In order to verify that this faster moving

Figure 11: Identification of NS1 bands in influenza A and B virus-infected cells. Six hours postinfection, MDCK cells were labeled with [^{35}S]methionine for 1 hr and lysed and cell extracts were analyzed 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. 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 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 an anti-B/NS1 antiserum.

Figure 11:



polypeptide is the NS1 protein, monospecific anti-A/NS1 antibody was used to immunoprecipitate the NS1 protein of A/turkey/Ore/71 and A/PR/8/34 virus-infected cells. As shown in Figure 11A, the 14.8K polypeptide of A/turkey/Ore/71 virus-infected cells was specifically precipitated by anti-A/NS1 antibody. The result suggest that this fast moving protein band in influenza A/turkey/Ore/71 virus-infected cells corresponds to the NS1 protein of other influenza A viruses. Immunoprecipitation experiments using anti-NS2 antiserum (Greenspan et al., 1986) revealed that the fastest moving band is indeed the NS2 protein in the A/turkey/Ore/71 virus-infected cell extract (data not shown).

2. NUCLEAR LOCALIZATION OF THE TRUNCATED NS1 PROTEIN OF A/TURKEY/ORE/71 VIRUS

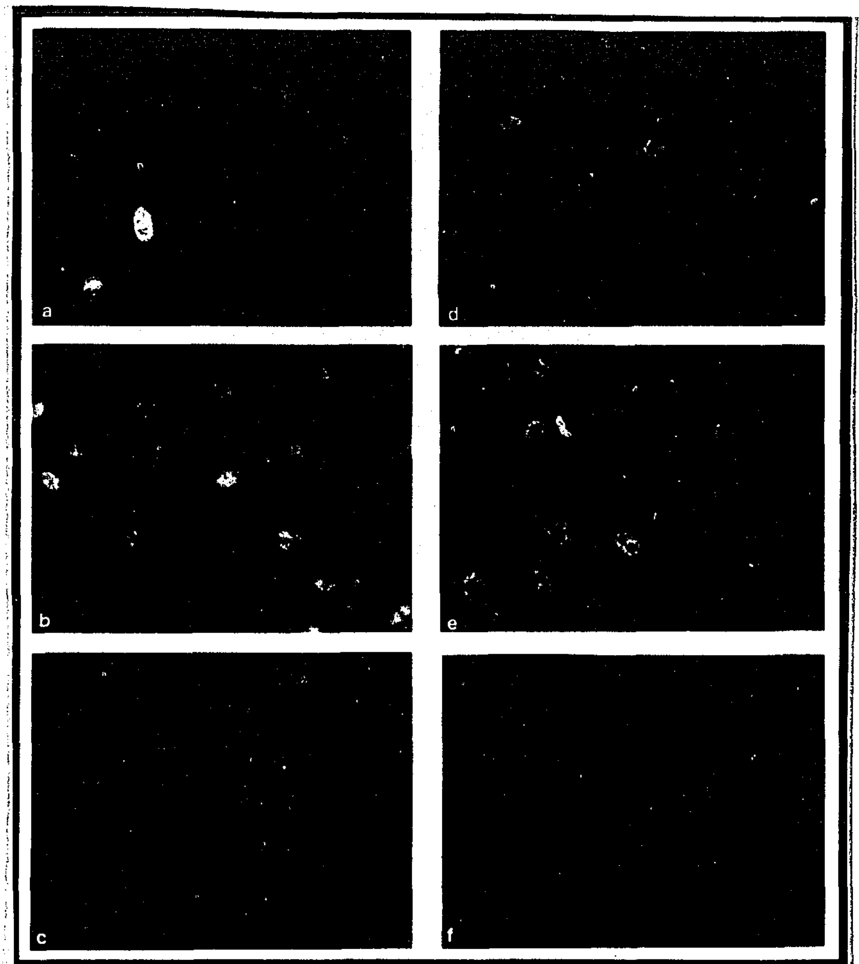
Since the NS1 protein in influenza A virus-infected cells is localized in the nucleus (for review Lamb, 1983; Young et al.,1983), we examined A/turkey/Oregon/71 virus-infected cells for the presence of the NS1 protein using indirect immunofluorescence analysis. Figure 12 shows that the 124 amino acid long NS1 protein of A/turkey/Ore/71 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 hr 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).

3. 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-

Figure 12: Localization of NS1 protein in influenza A/turkey/Oregon/72, A/PR/8/34, B/Lee/40, and clone 201 virus-infected cells. Virus-infected cells were fixed at 8 and 20 hr 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.

Figure 12:



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 15.6K from clone 201-infected cell extract, as shown in Figure 11B.

4. 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 (Figure 12). 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 Figure 12. This pattern was confirmed in a time course experiment (6, 8, 10, 12, 16, and 20 hr postinfection) (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.

D. DISCUSSION

Extensive heterogeneity in the length of the NS1 polypeptides of different influenza A viruses had been demonstrated previously (Krystal et al., 1983; Parvin et al., 1983; Buonagurio et al., 1986b). The A/FM/1/47 virus was shown to code for and 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 viruses

revealed a 10 base deletion at nucleotide positions 377-386 . The result of this deletion was a frame shift 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. In 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 (Figure 13A). 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 has 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 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 Helfer (1972) reported that A/turkey/Ore/71 virus was responsible for up to 1% of the 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 of 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 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. 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 (Figure 13B) is similar to that of A/turkey/Ore/71 virus NS gene (Figure 13A) 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 the 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,

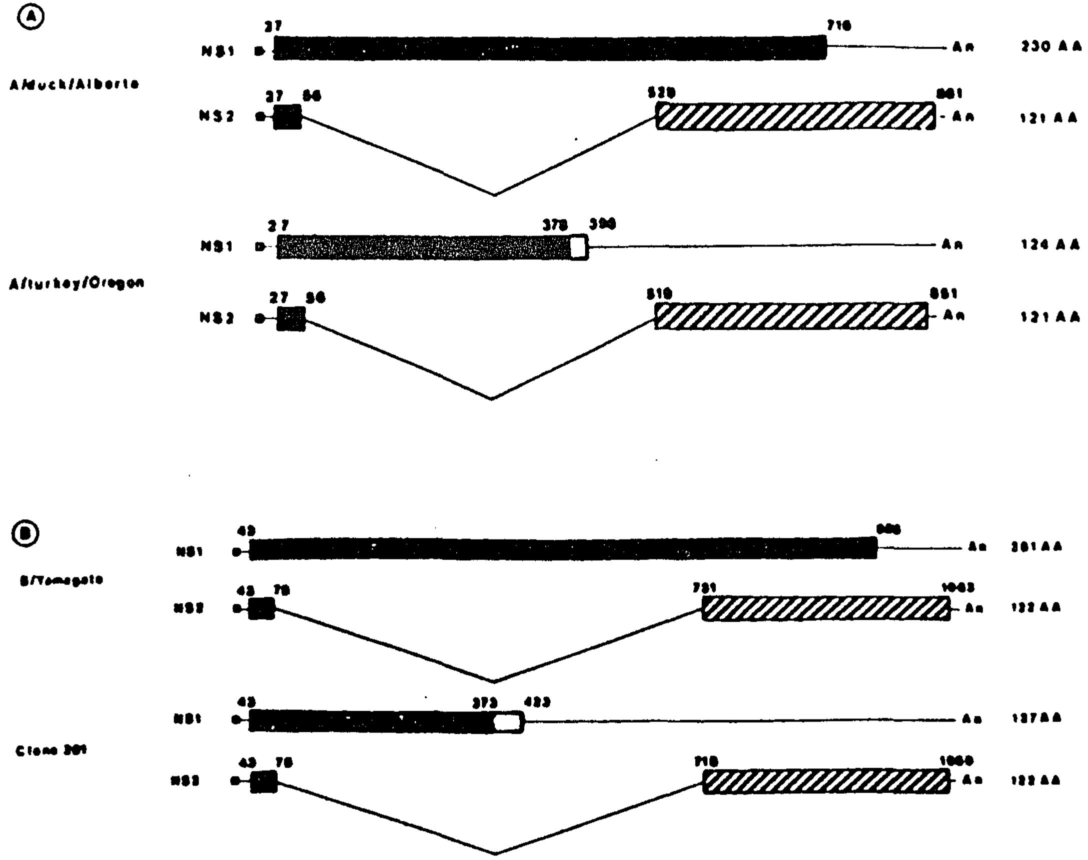


Figure 13: Comparison of NS mRNA structures. (A) A/turkey/Oregon/71 virus and A/duck/Alberta/60/76 virus NS genes. The NS1 specific mRNA of

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 11 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 acids 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 apppears 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 indicate intervening (spliced out) sequences. Numbers identify 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.

we find that the truncated NS1 polypeptide of clone 201 has the same karyophilic pattern as that of B/Lee/40 virus (Figure 12). 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. 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%.

IV.

TWO NUCLEAR LOCATION SIGNALS IN THE INFLUENZA VIRUS NS1 NONSTRUCTURAL PROTEIN

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A.INTRODUCTION

Sequences have been characterized which can direct proteins to discrete cellular compartments, e.g. endoplasmic reticula, mitochondria, chloroplasts, and most recently the nucleus (Schatz and Butow, 1983; Hay, et al., 1984; Watson, 1984; Cashmore, et al., 1985; Smith, et al.,1985). Nuclear location signals consisting of short stretches of basic amino acids have been defined for many proteins, including the SV40 T antigen (Kalderon, et al., 1984a; Kalderon, et al., 1984b; Landford and Butel, 1984) and VP1 protein (Wychowski, et al., 1986) rat glucocortocoid receptor (Picard and Yamamoto, 1987), nucleoplasmin (Dingwall, et al., 1987) and adenovirus E1A protein (Lyons, et al., 1987). Conversely, non-basic amino acid domains have also been implicated in the nuclear migration of certain proteins, such as the influenza NP protein (Davey, et al., 1985) and the yeast mat 2 protein (Hall, et al., 1984). In addition, it has been reported that some proteins, such as polyoma large T antigen, contain two regions within their sequences which may act as nuclear localization signals (Richardson, et al., 1986). The NS1 and NS2 proteins of influenza virus infected cells are nuclear proteins (Lazarowitz, et al., 1971; Krug and Etkind, 1973; Mahy, et al., 1980; Briedis, et al., 1981; Young, et al., 1983; Greenspan, et al., 1985). When CV-1 cells are infected with a recombinant SV40 virus containing the NS gene, both the NS1 and NS2 proteins are expressed and migrate into the nucleus (Greenspan, et al., 1985). Additionally, an NS1 protein which is expressed from a recombinant vaccinia virus enters and accumulates in the nucleus of infected cells independent of other influenza virus proteins (Smith, et al., 1987). In this system, splicing does not occur and the NS2 protein is not detectable. It therefore seems likely that the information necessary for the nuclear location

of NS1 is encoded within its sequences.

In a continuing effort to examine the functional domains of the NS1 polypeptide, we have expressed altered NS1 proteins in an attempt to define its nuclear signal. In this study, we have identified two different domains containing nuclear localization signals. Either region alone is sufficient to direct the NS1 protein to the nucleus, but in the absence of both of these regions the protein is localized in the cytoplasm.

B.MATERIALS AND METHODS

1. PLASMIDS AND CELLS

The cDNAs of the NS genes of A/PR/8/34 and A/Alaska/6/77 were cloned into the Eco R1 site of pBR322 and are designated pAPR801 and PQ7, respectively (Baez, et al., 1980; Buonagurio, et al., 1986); plasmid pSPR1 is derived from pSP64 and pSP65 (Krystal, et al., 1986); pAll-SVL2 is an SV40 late region expression vector (Gruss, et al., 1982; Greenspan, et al., 1985). Plasmid pMCα19, a pBR322 derivative which contains the cDNA of the chimpanzee α - globin gene, was obtained from Stephen Liebhaber (Liebhaber and Begley, 1983). All plasmids were grown in Escherichia coli HB101 cells. DNA manipulations including restriction enzyme digestion, ligation of DNA fragments and transformation of E.coli with plasmid DNA, were done by standard procedures (Maniatis, et al., 1982).

2.CONSTRUCTION OF NS1 DELETION MUTANTS

Synthetic oligonucleotides were prepared using either a Biosearch SAM 1 oligosynthesizer or an Applied Biosystems model 380B synthesizer. For the

construction of pNS- Δ 2-7, which lacks codons 2 to 7 of the NS gene, plasmid pAPR801 was used. Plasmid pAPR801 was digested with HindIII (Figure 14), the fragments were end-filled using DNA polymerase I large fragment, and EcoRI linkers containing an initiation codon (CATTACGAATTCGTAATG) were added. The EcoRI fragment containing the desired NS sequences was isolated on agarose gels using DEAE paper (Winberg and Hammarskjold, 1980) and cloned into the EcoRI site of plasmid pSPR1. Constructions pNS- Δ 2-51 and pNS- Δ 2-81 were generated by digestion of plasmid pQ7 with either XbaI or NcoI, respectively. XbaI and NcoI cut within codon 51 and 81, respectively, in the NS gene (Figure 14). The fragments created by Xba I digestion were end-filled whereas the Nco I digested DNA was treated with S1 nuclease. These DNAs were then ligated to the EcoRI linker described above. Following EcoRI digestion of the DNA the fragments containing the 3'portion of the NS sequences were isolated and independently cloned into the EcoRI site of pSPR1. The fourth construct, pNS- Δ34-38, was created by digestion of pQ7 with EcoRI to release the NS insert. The isolated insert was digested with Sau3AI (Figure 14) and fragments were recloned into EcoRI digested pSPR1. This allows removal of an internal 15 basepair Sau 3AI fragment. The NS insert isolated from EcoRI digested pQ7 was also ligated directly into pSPR1 to create pNS-Ala. pNS-Ala was then used to generate the fifth construct, pNS-Δ 82-237. For this purpose plasmid pNS-Ala was NcoI digested, end- filled and religated. This causes a frameshift mutation in the NS1 coding sequence after codon 81. All plasmid DNAs were checked for correct construction by restriction enzyme analysis and partial RNA sequencing using SP6 polymerase (Parvin, et al., 1986). All deleted genes were first engineered into the EcoRI site of pSPRI so that the NS

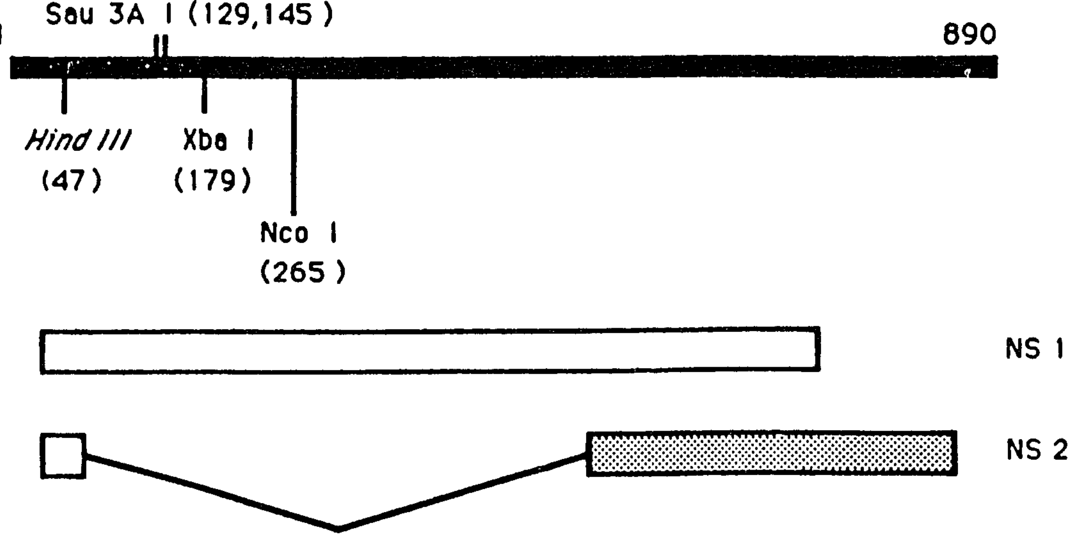


Figure 14: Restriction enzyme sites used in construction of NS gene mutants.

The restriction enzyme sites that were used for the construction of plasmids expressing NS1 deletion mutants and NS1-α-globin fusion proteins are shown. The solid line indicates the cDNA of the 890 nucleotide NS gene of influenza virus. The nucleotide position of the restriction enzyme sites is given within the parenthesis. The Hind III site is present only in pAPR801, which contains a cDNA copy of the NS gene of influenza A/PR/8/34; the XbaI site is found only in pQ7, a recombinant pBR322 containing the cDNA of the A/Alaska/6/77 NS gene. The organization of the NS1 and the NS2 proteins are also shown. The NS2 protein shares the first 10 amino acids with the NS1 protein (open boxes) and the carboxyl terminal sequences are derived from a plus one open reading frame (stippled box).

inserts could then be released by BamHI digestion (Krystal, et al., 1986). Deletion mutants pNS-Δ 2-7, pNS-Δ 2-51, pNS-Δ2-81, pNS-Δ34-38 and pNS-Δ 82-237 were BamHI digested to liberate the cDNA inserts and the isolated inserts were ligated into BamHI digested pA11-SVL2 to generate the recombinant SV40 vectors pSV-NS-Δ2-7, pSV-NS-Δ2-51, pSV-NS-Δ2-81, pSV-NS-Δ34-38 and pSV-NS-Δ82-237, respectively.

3.CONSTRUCTION OF NS1-α-GLOBIN CHIMERAE

pNS-Ala and pNS-Δ 34-38 were digested with EcoRI to release the cDNA inserts. The inserts were end-filled and BglII linkers were added (CAGATCTG)(New England Biolabs, Beverly, Mass.). After digestion with BglII to remove excess linkers, the DNAs were then digested with NcoI, which cuts the NS sequence after nucleotide 265 (codon 81). The fragments encoding the 5' end of the NS gene were isolated. Plasmid pMCα19, containing the entire α-globin cDNA sequence, was NcoI and MboI digested. NcoI cuts within the α-globin initiation codon while MboI cuts downstream of the stop codon within vector sequences. The isolated NS and α-globin containing fragments then were ligated with BamHI digested pA11-SVL2 to generate the recombinant SV40 vectors pSV-NSglobin-fpI(1-81) and pSV-NSglobin-fpII(1-33/39-81).

4.CONSTRUCTS ENCODING NS1 PROTEINS WITH CARBOXYL-TERMINAL DELETIONS

In order to obtain vectors that express NS proteins with COOH terminal deletions pSV-NS-Δ34-38 was digested with HpaI. This enyzme cuts in SV40 sequences downstream of the NS sequences. The DNA was then treated for various times with Bal 31 exonuclease and synthetic BamHI linkers (TAGCTAACTAGGATCCTAGTTAGCTA) containing stop codons in all 3 reading

frames were ligated to the DNA. The DNA was then KpnI (a single KpnI site is located upstream of the NS sequences) and BamHI digested and fragments from 600 to 900 bp in length were isolated. This mixture of DNA fragments was cloned into KpnI/BamHI digested pA11-SVL2. The exact end of the coding region in each deletion mutant was determined by double-stranded DNA sequencing (Chen and Seeburg, et al., 1985).

5.EXPRESSION OF RECOMBINANT NS1 PROTEINS

For transient expression, approximately 4 x 10^5 COS-1 cells were seeded on coverslips which were placed in a 60mm tissue culture dish. The next day 5 to 10 μg of one of the recombinant SV40 derived expression vectors was transfected into cells using either the calcium phosphate precipitation procedure described by Parker and Stark (Parker and Stark, 1979) or that modified by Chen and Okayama (Chen and Okayama, 1987). Recombinant SV40 virus stocks were also made and used in this study. These stocks were made by cotransfection of CV-1 cells with the recombinant SV40 NS DNAs and an early region deletion mutant, pSVr-Ins7, as described previously (Greenspan, et al., 1985). The protocol requires removal of pBR322 plasmid sequences from pSV-NS constructions by digestion with XbaI. However, since the A/Alaska/6/77 NS gene contains an internal XbaI site, recombinant pA11-SVL2 vectors containing this gene were digested with AhaII instead of Xba I to eliminate the pBR322 sequences.

6.IMMUNOFLUORESCENCE

Either subconfluent CV-1 monolayers infected with recombinant SV40 virus or transfected COS-1 cells were used for immunofluorescence. Cells were fixed 48 or 72 hrs. post-infection/transfection as described below (Greenspan, et al., 1985). Briefly, cells were treated with 1% formaldehyde in

PBS(pH 7.5) for 1 hour, washed with PBS, and permeabilized with 0.1% Triton X100 in PBS for 5 minutes. This step was followed by methanol/acetone (1:1) treatment at -20°C for 5 minutes. Samples were stored at 4 °C in PBS until stained. Coverslips were stained by first incubating at 37°C for 30 minutes with a 1:100 dilution of rabbit anti-NS1 serum in PBS containing 5% normal goat serum. Cells were then washed with PBS and treated with fluorescein conjugated goat anti-rabbit IgG (Cappel Biomedical Inc.) for 30 minutes at 37 °C. Samples were washed,mounted and viewed on a Zeiss fluorescent microscope model D-7082.

C.RESULTS

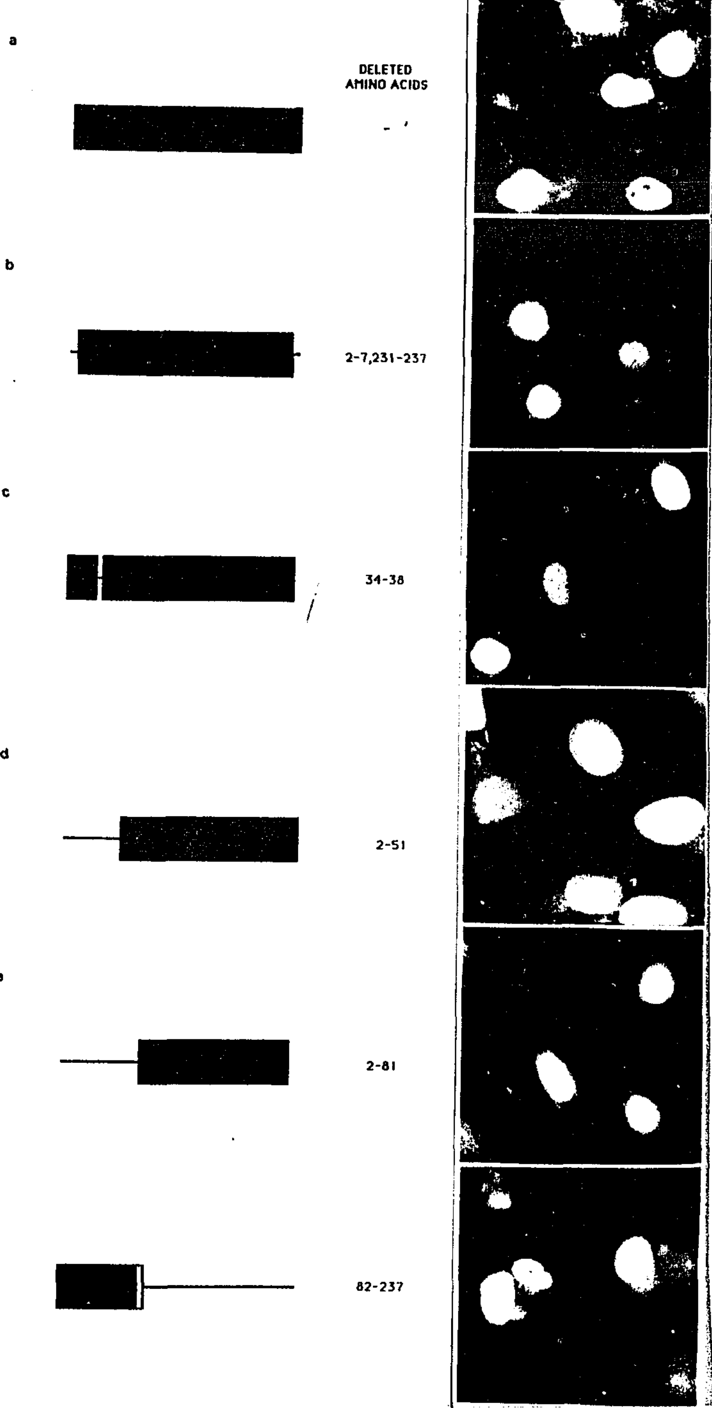
1.EXPRESSION OF DELETION MUTANTS OF THE NS1 GENE

The NS1 protein has been expressed in recombinant SV40 virus infected CV-1 cells and was shown to localize in the nucleus (Greenspan, et al.,1985). In this follow up study, mutants with deletions in the NS1 coding region were constructed, and the effects of these deletions on the protein's migration were investigated (Figure 15). First, it was thought that since both the NS1 and NS2 proteins localize to the nucleus in virus infected cells, a nuclear localization signal may be present in the region common to both proteins. The NS1 and NS2 polypeptides are encoded by overlapping reading frames which share 10 amino acids at their amino termini but differ over the rest of their sequences (Figure 14) (Lamb, 1983). In order to obtain a deletion mutant of NS1 lacking the amino terminal amino acids, plasmid pSV-NS-Δ2-7 was constructed as described in Materials and Methods. Plasmid pSV-NS-Δ2-7 then was used to obtain recombinant SV40 virus stocks. CV-1 cells were infected with these recombinant SV40 virus stocks and labeled with 35 S-methionine three days post infection. Cell lysates were analzyed by sodium

Figure 15: Expression of deletion mutants of the NS1 gene.

A schematic diagram illustrating the expressed sequence from each construct is shown. Solid boxes represent expressed proteins. The thin lines show the deleted portion of the protein and the numbers on the side of each protein indicate deleted amino acids. Immunofluorescence data of recombinant SV40 virus infected CV-1 cells are shown beside each construction. Cells were fixed at 72 hrs. post infection and stained with polyclonal monospecific rabbit anti-NS1 serum (Young, et al., 1983). Viruses used were a) SV-NS b) SV-NS-Δ 2-7 c) SV-NS-Δ34-38 d) SV-NS-Δ2-51 e) SV-NS-Δ2-81 f) SV-NS-Δ82-237. In panel f, the NS1 of SV-NS-Δ 82-237 infected cells contains an additional 11 nonviral amino acids, as shown by the open box.

Figure 15:



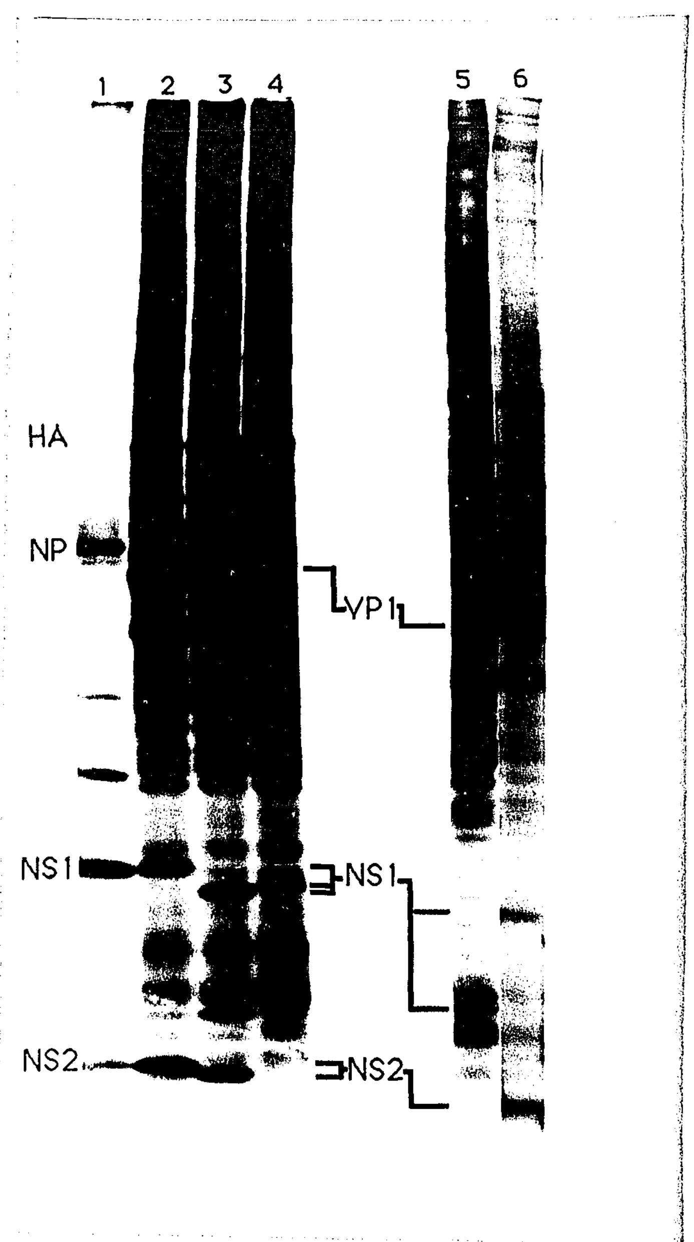
dodecyl sulfate gel electrophoresis and autoradiography (Figure 16). Cells infected with SV-NS-Δ 2-7 virus (Figure 16,lane 3) express NS1 and NS2 proteins which migrate slightly faster than wild type NS1 and NS2 proteins (Figure 16, lanes 1 and 2). The faster migration of these proteins is explained by the deletion of amino acids 2-7 in the mutant proteins. The truncated NS1 and NS2 proteins can be immunoprecipated with specific polyclonal anti-serum raised to bacterially synthesized NS1 and NS2 proteins (Young, et al., 1983; Greenspan, et al., 1985), respectively (data not shown). An additional band which migrates slightly slower than the NS2 protein is seen in lane 3 (indicated by arrow). This protein band is immunoprecipitated with antiserum to the NS1 and presumably represents a breakdown product of the mutant NS1 polypeptide. CV-1 cells infected with SV-NS-Δ2-7 virus were also analyzed by indirect immunofluorescences experiments. The truncated NS1 protein, like full length NS1 protein, was localized to the nucleus (Figure 15,a and b). Therefore removing amino acids 2-7 of the NS1 protein does not alter the protein's cellular location. The truncated NS2 protein was also found in the nucleus of SV-NS-Δ2-7 infected cells (data not shown).

Plasmid pSV-NS-Δ 34-38 has an internal deletion of 15 nucleotides in the NS1 coding sequence (Figure 15c). This region, amino acids 34-38, was chosen because it is similar to the basic amino acid signal sequence of SV40 T antigen (Kalderon, et al., 1984a; Kalderon, et al., 1984b). In addition, these amino acids are conserved among all influenza A virus NS1 proteins examined. The NS1 protein expressed in SV-Δ 34-38 virus infected cells migrates faster than wild type NS1 and slower than the NS1 in SV-NS-Δ 2-7 infected cells (Figure 16, lanes 4,1 and 3). The NS2 protein was not detected in several experiments although the coding sequence of the protein should not be

Figure 16: SDS - polyacrylamide gel electrophoresis of viral proteins.

CV-1 cells were infected with virus and pulse labeled for 1 hour with 35 S-methionine at 5 hrs. p.i. for influenza virus or 72 hrs. p.i. for SV40 recombinant viruses. Samples were electrophoresed on 7 to 14% polyacrylamide gels (Parvin, et al., 1983). Gels were dried and autoradiographed. Influenza and recombinant SV40 viruses used for infection were: lanes 1) A/PR/8/34; 2) SV-NS; 3) SV-NS- Δ2-7; 4) SV-NS-Δ34-38; 5) SV-NS-Δ2-51; 6) SV-NS. Arrow indicates a presumed breakdown product of the NS1 protein present in SV-NS-Δ2-7 infected cells.

Figure 16:



affected in the SV-NS-Δ34-38 construct. This observation was not examined further. When virus infected cells were analyzed by immunofluoresecence, the NS- Δ 34-38 protein was found to localize in the nucleus (Figure 15c), presenting the same staining pattern as wild type NS1 (Figure 15a).

Since the amino acids deleted in NS-Δ 34-38 are located in a very polar area of the protein, it was thought that a larger deletion in this domain may be necessary to see an effect on the nuclear location of NS1. Construction of pSV-NS-Δ 2-51 resulted in a protein missing amino acids 2-51 (Figure 15d). Lysates of CV-1 cells infected with SV-NS-Δ 2-51 virus have a band that corresponds to the expected molecular weight of the truncated NS1 protein (Figure 16, lane 5). NS2 protein is not detected in these virus infected cells (lane 5). This is expected since pSV-NS- Δ2-51 is missing the NS2 donor splice site (at nucleotide 57 of the wild type NS gene). When infected cells were analyzed by immunofluorescence the mutant NS1 protein was again found to localize in the nucleus despite the deletion of amino acids 2 to 51 (Figure 15d).

The next experiment was designed to examine the nuclear localization of NS1 mutant proteins with even larger deletions. In fact, constructs pSV-NS-Δ 2-82 and pSV-NS- Δ 82-237 allowed for separate expression of the COOH-terminal and the NH2-terminal domains of the protein (Figure 15, e and f). Virus SV-NS- Δ 2-81 expresses an NS1 protein which is missing the first 81 amino acids whereas SV-NS- Δ82-237 expresses only amino acids 1-81 plus an additional 11 amino acids (HGLHTCFAIHN), which are derived as a consequence of a frameshift caused by endfilling of the Nco I site. Cells infected with these deletion mutants were analyzed by immunofluorescence (Figure 15, e and f). In both instances the protein was found to be localized

to the nucleus. Since these recombinant viruses independently express the NS1 amino-terminal sequences (1-81) and the NS1 carboxyl-terminal sequences (82-237), it seems likely there is at least one signal present in each mutant protein. Therefore, more than one karyophilic signal should exist in the full length A/Alaska/6/77 NS1 protein.

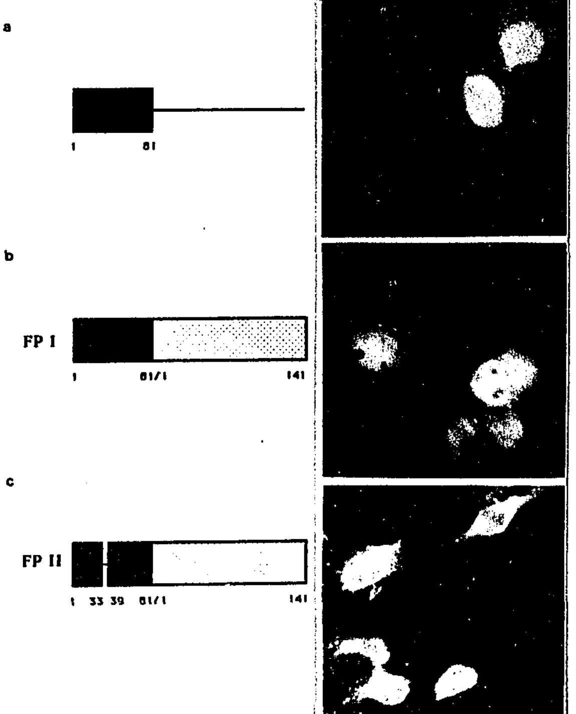
2.EXPRESSION OF NS1-α-GLOBIN CHIMERIC PROTEINS

The next step was to define the nuclear signal sequence present in the polypeptide region containing amino acids 1-81. Since the introduction of deletions in the 81 amino acid fragment could adversely affect the protein's stability, fusion proteins were expressed using the chimpanzee α-globin. The latter protein is normally cytoplasmic but is small enough to allow for diffusion into the nucleus (Richardson, et al., 1986). Two constructs were generated. One, (pSV-NSglobin-fpI), contained the first 81 codons of the NS1 gene fused to the entire α-globin coding region. The second construct (pSV-NSglobin-fpII) codes for the same chimeric protein except it lacks codons 34-38 in the NS1 region. Sequences are joined at NcoI sites (CCATGG) present at codon 81 (met) of the NS1 gene and at the initiation codon of α-globin gene. The fusion proteins were transiently expressed in COS-1 cells and localized by immunofluorescence microscopy (Figure 17). The fusion protein expressed from pSV-NSglobin-fpI is found to localize in the nucleus (Figure 17b), as does the NS1 protein expressed from SV-NS-Δ 82-237 (Figure 17a). In contrast, when the fusion protein missing NS1 amino acids 34- 38 is expressed in COS-1 cells, NS1 specific immunofluorescences is observed mainly in the cytoplasm (Figure 17c). We thus conclude that removal of the amino acids Asp-Arg-Leu-Arg-Arg in the amino terminal NS1 fragment alters the location of the fusion protein. This domain will be referred to as nuclear

Figure 17: Expression of NS1-α-globin chimeric proteins.

Fusion proteins containing NS1 sequences upstream of α-globin sequences were constructed. a) The truncated NS1 protein of pSV-NS-Δ82-237 contains amino acids 1-81 as well as an addtitional 11 amino acids, as described in the text. b) FPI is expressed from pSV-NSglobin-fpI and codes for NS1 amino acids 1-81 (solid box) fused with α-globin sequences (stippled box). c) FPII is expressed from pSV-NSglobin-fpII. This protein is the same as FPI except it is missing the NS1 sequences encoding amino acids 34-38. The full length α-globin sequences are present in FPI and FPII. Cos-1 cells were transfected with each construct and used for indirect immunofluorescence.

Figure 17:



localization signal 1, NLS 1.

3.DETERMINATION OF NUCLEAR LOCALIZATION SIGNAL 2, NLS2

Following the identification of NLS 1, attempts were made to identify a second nuclear localization signal in the carboxyl-terminal region. Bal-31 nuclease digestion was used to remove carboxyl-terminal coding sequences of the NS1 gene from plasmid pSV-NS-Δ 34-38. NS1 proteins expressed from these deletion mutants are missing NLS 1 as well as a defined number of amino acids from the carboxyl terminus. pSV-NS-Δ 34-38/175-237, pSV-NS-Δ 34-38/203-237 and pSV-NS-Δ34-38 encode proteins which terminate after NS1 amino acids 174, 202, and 237, respectively. The NS1 protein encoded by pSV-NS-Δ 34-38/203-237 has an extra serine residue at the carboxyl-terminal as a result of the addition of the synthetic stop codon linker. The location of the truncated NS1 proteins expressed by these constructs was determined by immunofluorescence of transfected COS-1 cells (Figure 18). The NS1 protein expressed from pSV-NS-Δ 34-38 localizes to the nucleus (Figure 18c) as shown before (Figure 15f). However, the NS1 protein expressed from pSV-NS-Δ 34-38/175-237 was found to be cytoplasmic (Figure 18a) as was the NS1 protein expressed from pSV-NS- Δ 34-38/203-237 (Figure 18b). Therefore nuclear localization signal 2, NLS 2, is defined between amino acids 203 and 237.

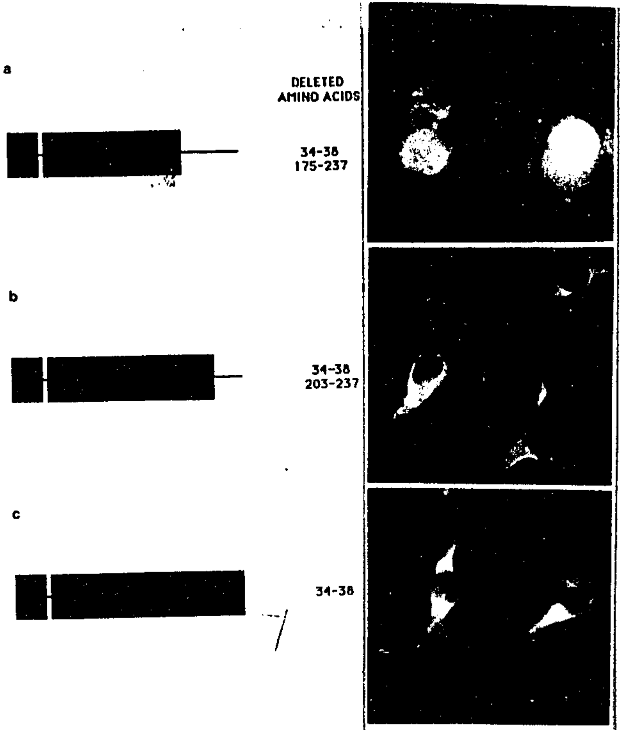
D. DISCUSSION

Since the pores in the nuclear envelope have a functional radius of 45 Å (Paine, et al., 1975), it is thought that proteins with a molecular weight less than 67,000 daltons can passively diffuse through these nuclear pores (Paine, et al., 1975). The NS1 protein is 26,000 daltons and theoretically could

Figure 18: Expression of NS1 proteins with carboxyl-terminal deletions.

Plasmid pSV-NS-Δ34-38, containing a cDNA of an NS1 protein missing amino acids 34-38, was used to generate carboxyl-terminal deletions. The NS1 proteins were expressed in a transient system in COS-1 cells. Cells were fixed 72 hrs. post-transfection and stained with anti-NS1 serum to localize the truncated proteins. The mutant NS1 protein encoded by each construction is diagrammed and indirect immunofluorescence is shown for cells transfected with a) pSV-NS-Δ34-38/175-237 b) pSV-NS-Δ34-38/203-237 c) pSV-NS-Δ34-38.

Figure 18:



passively diffuse into the nucleus. However, if diffusion alone accounted for the NS1 protein's cellular location one would expect it to be equally distributed throughout the cell rather than accumulate in any one compartment. As this is not the case with the NS1 protein, specific nuclear signal sequences must be present within the polypeptide (DeRobertis, 1983). Expression of influenza virus NS1 deletion mutants and NS1-α-globin fusion proteins have been used to identify two amino acid regions (NLS 1 and NLS 2) that affect the protein's cellular location. Truncated NS1 proteins with small deletions (NS-Δ 2-7 and NS-Δ 34-38) or large deletions (NS-Δ2-51, NS-Δ 2-81 and NS-Δ 82-237) were expressed and found to localize to the nucleus. Since mutant NS proteins NS-Δ82-237 and NS-Δ1-81 contain no overlapping sequence, at least two distinct nuclear signal sequences are present within the NS1 protein. Two nuclear signal sequences have been reported for a number of viral and cellular proteins including the polyoma T antigen (Richardson, et al., 1986), rat glucocorticoid receptor (Picard and Yamamoto, 1987) and the yeast matα2 protein (Hall, et al., 1984; Hall and Johnson, 1987).

The expression and localization of fusion proteins enabled us to define NLS 1. This signal contains amino acids Asp-Arg-Leu-Arg-Arg. At the present time we cannot eliminate the possibility that this short amino acid region comprises a portion of a larger signal disrupted by the removal of these amino acids. This domain of the NS1 protein is highly charged and conserved in all influenza A virus isolates studied, including avian and swine species (Figure 19)(Baez, et al., 1980; Baez, et al., 1981; Krystal, et al., 1983; Nakajima, et al., 1984; Buonagurio, et al., 1986). It is also highly conserved within the influenza B virus NS1 protein (Figure 19)(Briedis and Lamb, 1982; Yamashita, et al., 1988). The homology of the NLS 1 among the NS1 proteins

A/HT/24269/85	(H3N2)													R				
A/Alaska/6/77	(H3N2)													R				
A/Udorn/72	(H3N2)																	
A/Berkeley/1/68	(H2N2)																	
A/AA/6/60	(H2N2)																	
A/Denver/1/57	(H1N1)																K	
A/HT/23284/85	(H1N1)																K	
A/HT/18515/84	(H1N1)																K	
A/Maryland/2/80	(H1N1)																	
A/USSR/90/77	(H1N1)																	
A/FW/1/50	(H1N1)																	
A/FM/1/47	(H1N1)																K	
A/Bellamy/42	(H1N1)																	
A/PR/8/34	(H1N1)																	
A/WSN/33	(H1N1)	D	A	P	F	L	**D**	**R**	**L**	**R**	**R**	D	Q	K	S	L	R	G
A/Swine/Iowa/15/30	(H1N1)																	
A/FPV/Rostock/34	(H7N1)																	
A/Turkey/Oregon/71	(H7N3)					D									A		K	
A/Duck/Alberta/60/76	(H12N5)					D									A		K	
B/Lee/40		D	Y	P	G	Q	D	R	L	H	R	L	K	R	K	L	E	S

Figure 19: Comparison of NLS 1 sequences of influenza virus NS1 protein.

Amino acids 28 -44 of the NS1 protein of A/WSN/33 is used for comparison with those of other influenza A virus isolates (Baez, et al., 1980; Baez, et al., 1981; Krystal, et al., 1983, Nakajima, et al., 1984; Buonagurio, et al., 1986). The region defined as NLS 1 is highlighted. The homologous region in B/Lee/40 NS1 (Briedis and Lamb, 1982), amino acids 46-50, is underlined.

of influenza A and B virus is noteworthy since these proteins differ by over 80% of their amino acid sequence and are antigenically distinct (Young, et al., 1983). It is tempting to hypothesize that nuclear localization of the protein during the virus life cycle is critical and that there are tight constraints upon the amino acid sequence of this region.

The expression of NS1 proteins with carboxyl-terminal deletions has allowed for the definition of NLS 2 to within the region encompassing amino acids 203 - 237 (figure 20). This domain contains a short stretch of basic amino acids at residues 216-221. This stretch of amino acids, Pro-Lys-Gln-Lys-Arg-Lys is similar to other known nuclear signal sequences, as shown in Figure 20. NLS 2 is present in most influenza A virus isolates. Of 25 human, avian and swine isolates examined, 18 were found to contain the basic amino acid stretch (216-221) of NLS 2. Variability in the length of the NS1 proteins (Parvin et al., 1983; Buonagurio, et al., 1986) results in the absence of NLS 2 in certain naturally occurring isolates. For example, the NS1 of the avian virus A/Turkey/Oregon/71 has only 124 amino acids (Norton et al., 1987) and the human isolate A/FM/1/47 has only 202 amino acids (Krystal, et al., 1983). Although the NLS 2 is not an absolute requirement for protein function of the NS1 protein, the existence of multiple nuclear signal sequences in one protein may effect the kinetics of entry into the nucleus (Richardson, et al., 1986). At the present time a comparative study of the kinetics of migration of the NS1 protein from different viruses has not been done. In addition it has been postulated that a protein may enter the nucleus by either specific and selective entry or by diffusion followed by specific binding to nondiffusible nuclear components (DeRobertis, 1983). It has not been determined which is the functional mechanism for the NLS1 and NLS2

domains of the NS1 protein.

A common theme of known nuclear signal sequences is the presence of a proline residue before or after a stretch of basic amino acids. It is believed that this proline residue is important in the three-dimensional structure of the signal (Smith, et al., 1985). In the case of the NS1 protein a conserved proline is found three amino acids upstream from the basic NLS 1 sequence. Also, within the NLS 2 there is a region of basic amino acids preceded by a proline. Thus the NLS 1 and NLS 2 are very similar to the known nuclear localization signals of SV40 T antigen (Kalderon, et al., 1984a; Kalderon, et al., 1984b; Landford and Butel, 1984), polyoma T antigen (Richardson, et al., 1986), SV40 capsid polypeptides VP1, VP2, and VP3 (Wychowski, et al., 1986; Gharakhanian, et al., 1987; Wychowski, et al., 1987), and yeast ribosomal protein L3 (Moreland, et al., 1985) (figure 20). Conversely, in a few proteins, nuclear signal sequences have been defined which are not comprised of basic amino acids (Hall, et al., 1984; Davey, et al., 1985). For example, an accumulation signal has been defined for the influenza virus nucleoprotein, NP, which contains only one basic amino acid and no prolines (Davey, et al., 1985). Therefore, the NP signal is very different from NLS 1 and NLS 2 of the NS1 protein. It is interesting that such different signals could exist in nuclear proteins of the same virus.

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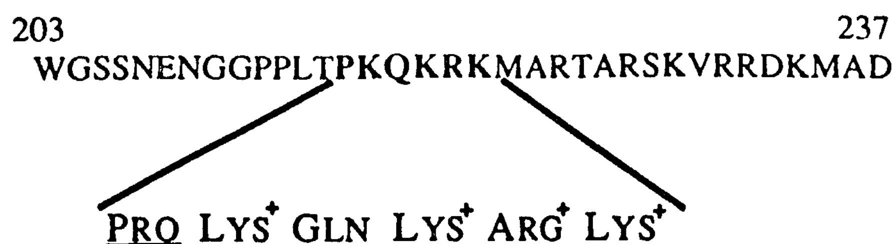


SV40 T antigen	PRO LYS$^+$ LYS$^+$ LYS$^+$ ARG$^+$ LYS$^+$ MET
SV40 VP1	PRO THR LYS$^+$ ARG$^+$ LYS$^+$ GLY SER
SV40 VP2/3	PRO ASN LYS$^+$ LYS$^+$ LYS$^+$ ARG$^+$ LYS$^+$
Polyoma T antigen	PRO LYS$^+$ LYS$^+$ ALA ARG$^+$ GLU ASP VAL SER ARG$^+$ LYS$^+$ ARG$^+$ PRO ARG$^+$
Yeast L3 protein *	PRO ARG$^+$ LYS$^+$ ARG$^+$

Figure 20: Amino Acid Sequence of Nuclear Localization Signal 2, NLS 2.
The sequence of the NS1 protein of influenza virus A/Alaska/6/77 is shown. Amino acids 203 to 237 are indicated in the single letter code. The sequence 216-221 is enlarged and compared with previously published signal sequences of other nuclear proteins. Basic amino acids are marked with plus signs and proline residues are underlined (Kalderon, et al., 1984a; Kalderon, et al., 1984b; Landford and Butel, 1984; Moreland, et al., 1985; Richardson, et al., 1986; Wychowski, et al., 1986; Wychowski, et al., 1987). * - only a portion of the reported yeast L3 protein signal is shown (Moreland, et al., 1985).

V.

STUDY OF THE NUCLEAR LOCALIZATION SIGNAL OF NONSTRUCTURAL NS2 PROTEIN OF INFLUENZA A VIRUS

A. INTRODUCTION

The NS2 protein of influenza A virus infected cells is found in the nucleus by cell fractionation studies (Krug and Etkind, 1973) as well as by indirect immunofluorescence as shown in chapter 2. The cDNA of the NS2 has been engineered into a recombinant vaccinia virus and the protein has been expressed (Smith, et al., 1987). In this system, the NS2 is found in the nucleus of recombinant vaccinia virus infected cells, in the absence of any other influenza virus protein. The protein's nuclear location is therefore not dependent on other influenza virus proteins. The NS1 and NS2 proteins are encoded by the same genomic RNA segment. These NS proteins have 10 amino acids in common at their amino terminus, but differ over the rest of their sequences. The NS2 protein is encoded by a spliced mRNA where its carboxyl terminal sequences are derived from a +1 reading frame (Figure 1) (Lamb and Lai, 1980). We have shown in chapter 4 that removing codons 2-7 of the NS gene did not affect the nuclear location of the NS2 protein. Therefore, the nuclear signal sequence of the NS2 protein most likely is within its unique sequences.

This chapter outlines the experiments performed in an attempt to define the information, within the NS2 polypeptide, necessary for its cellular location.

B. MATERIALS AND METHODS

1. PLASMIDS:

Plasmid pAS1/PR8-NS2 is a pBR322 derivative which contains λ phage control elements as well as the NS2 cDNA. Its construction is described in chapter 2. DNAs pA11-SVL2 and pA11-SVL2-801m are SV40 late region expression vectors; the latter containing a cDNA of the NS gene of

A/PR/8/34 influenza virus.

2. MUTAGENESIS:

A partial cDNA of the NS2 (missing codons 1-7) was subcloned into M13 mp18. This construction was then used for mutagenesis of the NS2 coding region. The Amersham oligonucleotide directed in vitro mutagenesis system was used. An oligonucleotide mixture, 5'GTCTTCAAACTTC(T/C)TTATT(C/G)TA 3', was synthesized on an Applied Biosystems (380 B) synthesizer. This mixture contained both the wild type and an altered nucleotide for the second base of codons 75 and 77. Therefore 4 possible oligonucleotides were present in the mix. Briefly, the M13 recombinant phage containing the partial NS2 clone was used to infect E.coli TG1 cells and the single stranded DNA was isolated. The phosphorylated oligonucleotide mix was annealed to the single stranded DNA template. E.coli polymerase Klenow fragment and T4 DNA ligase were added in the presence of dATP, dTTP, dGTP and dCTP-S to allow synthesis of a second strand and then ligation. The double stranded DNA was then separated from any single stranded DNA by filtration. The non-mutant strand of the ds DNA was then nicked with Nci I. This enzyme will nick the non-mutant DNA at its recognition sites but will not cut the mutant strand due to the presence of the thionucleotide dCTP-S. The DNA is then treated with exonuclease III which will partially digest the non-mutant strand that has been nicked. Finally, DNA polymerase I is added to make a complete second strand. This double stranded DNA is then used to transform competent TG 1 cells. Individual plaques were picked, grown, the single stranded DNA was isolated and then sequenced.

3. CONSTRUCTION OF EXPRESSION VECTORS:

The double stranded DNA from the recombinant M13 infected cells was

isolated. The construction of the expression vector is outlined in Figure 21. The Hind III - Bam HI fragment was isolated from recombinant M13 containing the cDNA for wild-type NS2, or mutant NS2. This fragment contains the NS2 cDNA except for codons 1-7. DNA pA11-SVL2-801m was digested with Kpn I, which cuts once in the SV40 sequence upstream of the NS cDNA, and with Hind III, which cuts the NS gene within codon 8. This Kpn I/ Hind III fragment was gel purified and will provide the NS2 initiation codon and amino acids 2-7. Vector pA11-SVL2 was Kpn I and Bam HI digested. These three fragments were then ligated together and used to transform competent HB101 cells. Colonies were screened by Grunstein and Hogness hybridization and the DNA of those colonies that were positive were checked by restriction enzyme analysis.

4. EXPRESSION OF NS2 PROTEIN IN RECOMBINANT SV40 VIRUS INFECTED CELLS:

Recombinant SV40 virus stocks were prepared as described in chapter 4. These virus stocks were then used to infect CV-1 cells. Cells were fixed and stained for indirect immunofluorescence 72 hrs post-infection as described in chapters 2 and 4.

C. RESULTS

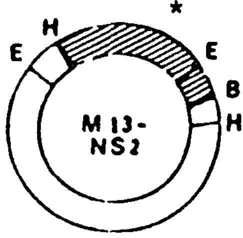
1. MUTAGENESIS OF THE NS2 cDNA IN M13:

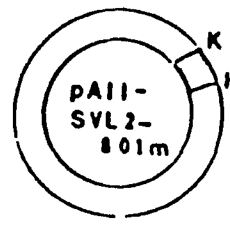
To define a nuclear signal sequence for the NS2 protein, site specific mutagenesis was employed. An incomplete NS2 cDNA in M13 was altered at codons 75 and 77 in two separate clones. This region was chosen for study because of its homology to the nuclear accumulation signal of the influenza virus NP protein (Davey, et al., 1985). The oligonucleotide used for mutagenesis introduced nucleotide changes which lead to codon 75 encoding a

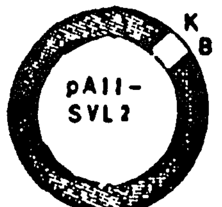
Figure 21: Construction of NS2 expression vectors.

M13-NS2 contains an incomplete cDNA of the NS2 gene. The NS2 gene in this plasmid is missing codons 1-7. The plasmid was digested with Hind III and Bam HI. This cuts the plasmid into three fragments: a large Hind III/ Hind III fragment of M13 sequences, a small Bam HI/Hind III fragment containing M13 polylinker sequence and a Hind III/BamHI fragment containing the cDNA of NS2. The Hind III/Bam HI fragment ( ) was gel purified. The asterisks (*) indicates the position of the point mutations for the mutant NS2 constructions (see Figure 22). pAII-SVL2-801 contains a complete cDNA of the A/PR/8/34 NS gene. This DNA was digested with KpnI and Hind III. KpnI digests the plasmid in the SV40 sequences upstream to the NS cDNA. The Hind III cuts the NS cDNA within codon 8. The small KpnI/ Hind III fragment ( ) was gel purified and will provide codons 1-7 for the NS2 gene. Vector DNA pAII-SVl2 was digested with KpnI and Bam HI. The two gel purified fragments and the digested vector were ligated together overnight and then the DNA mixture was used to to transform E.coli HB101 cells. The resulting plasmid, pA11-SVL2-NS2 is shown on the bottom of the figure. This plasmid contains a complete cDNA copy of the NS2 gene. B = Bam HI site, E= Eco RI site, H = Hind III site, K = Kpn I site.

Figure 21:







- digest plasmid with Hind III and Bam HI
- gel purify Hind III/ Bam HI fragment

- digest plasmid with Kpn I and Hind III
- gel purify KpnI/Hind III fragment

-digest plasmid with Kpn I and Bam HI

Gly instead of a Glu and codon 77 specifying a Thr in place of the Arg found in wild type NS2 (Figure 22). M13 phage plaques were amplified and the single stranded DNA was sequenced using specific primers. Clones containing the mutations were grown in large quantities and the double stranded form of the M13 was isolated. The mutated portion of the NS2 cDNA was cloned into SV40 late region expression vector, pA11-SVL2. The construction of recombinant SV40 vectors containing the wild type NS2 cDNA or the NS2 mutant cDNAs is described in materials and methods and Figure 21. These DNAs were then used to transfect CV-1 cells to make recombinant SV40 virus stocks.

2. EXPRESSION OF THE NS2 PROTEIN IN RECOMBINANT SV40 VIRUS INFECTED CELLS:

The NS2 proteins were expressed in CV-1 cells infected with recombinant SV40 virus stocks. Cells were fixed 72 hours post-infection and stained for indirect immunofluorescence experiments using a rabbit polyclonal anti-NS2 serum. In Figure 23 panel a, the wild-type NS2 protein is localized to the nucleus. In addition, the mutant NS2 proteins, NS2/x75 Glu→Gly and NS2/x77 Arg→Thr, are also localized to the nucleus, Figure 23 panels b and c respectively.

D. DISCUSSION

The NS2 protein of influenza A virus has been shown to enter and accumulate in the nucleus of virus infected cells (chapter 2) as well as in the absence of other influenza virus proteins (Smith, et al., 1987). Therefore it seems likely that the protein encodes a signal for it's nuclear location. The NS2 protein has a molecular weight of 14 Kd and could theoretically diffuse into the nucleus. If, as in the case for the NS1 protein, diffusion accounted

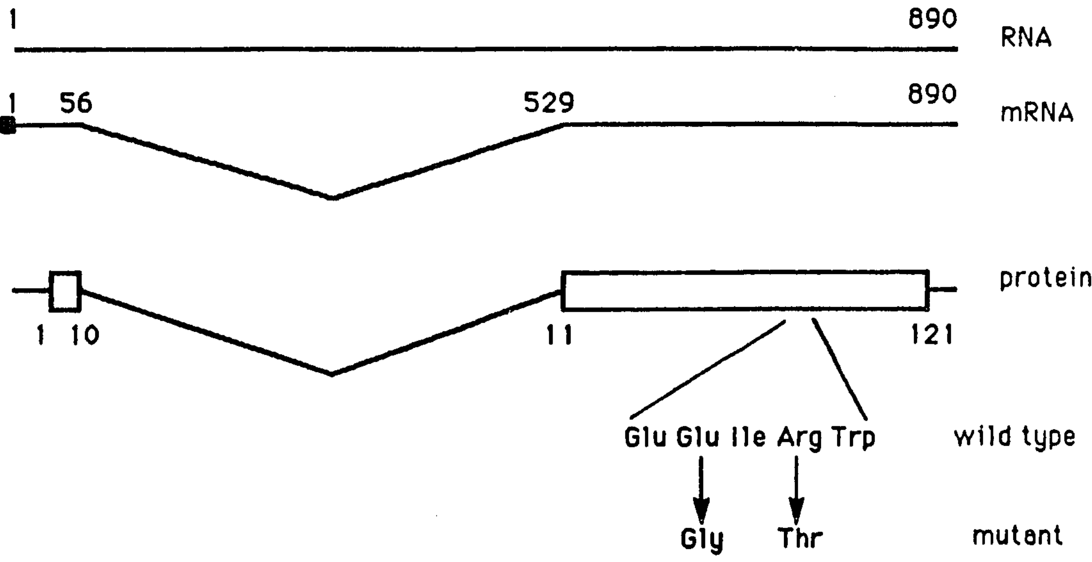


Figure 22: Genomic organization of the NS2 coding sequence.

The NS gene contains 890 bases. The NS2 mRNA is spliced and contains NS sequences 1-56 and 529-890. The first ten amino acids of the NS2 are encoded by the nucleotides 27-56. The remaining 111 amino acids are translated from a +1 reading frame starting at nucleotide 529. In the mutagenesis experiments amino acid 75 was changed from a Glu to a Gly and amino acid 77 was mutated from an Arg to a Thr.

for the NS2 protein's cellular location one would expect the protein to be present throughout the cell and not concentrated in one compartment (DeRobertis, 1983).

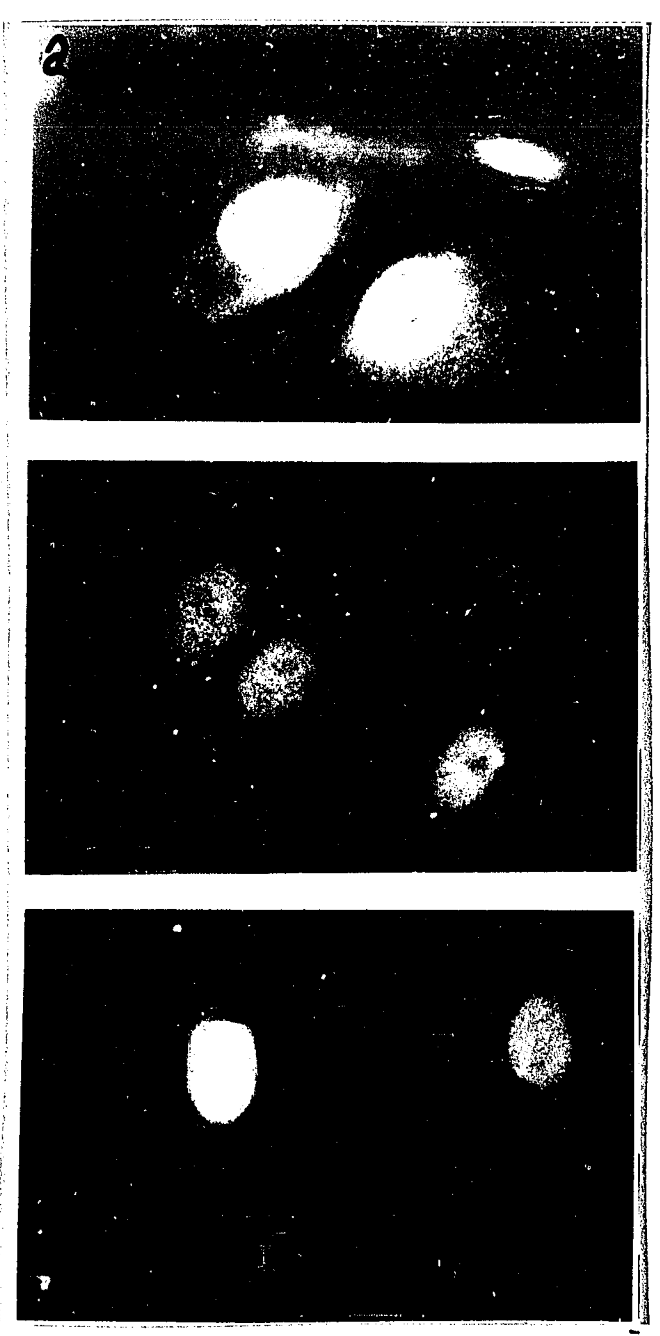
Previously, nuclear signal sequences have been defined for many proteins. A characteristic of these signals is the presence of basic amino acids (for a list of published nuclear signal sequences see Table 2). A notable exception to this is the accumulation signal defined within the amino acid region 327-345 of the influenza A virus NP protein (Davey, et al., 1985). This region includes the polypeptide sequence Phe-Glu-Asp-Leu-Arg-Val-Leu. This sequence is interesting because it is conserved in the NP proteins of influenza A, B, and C viruses (Nakada, et al., 1984). The NP proteins are antigenically distinct and along with the matrix protein, M1, is used to subtype viruses. A region of the NS2 protein, codons 73-79, Phe-Glu-Glu-Ile-Arg-Trp-Leu was found to have a striking homology to the accumulation signal of the NP protein. In addition, amino acids 73-79 were well conserved among NS2 proteins of different virus isolates. Therefore, mutagenesis of the cDNA encoding this region of the NS2 protein might alter the protein's nuclear location. We chose to change two of the charged residues within the region of interest and isolated two separate clones, each with one mutation. Both mutant NS2 proteins localized to the nucleus as did the wild type A/PR/8/34 NS2 protein (Figure 23). These experiemnts do not allow a positive identification of a nuclear signal sequence for the NS2 protein. It may be that one amino acid change in the NS2 protein in this domain can be tolerated.

Figure 23: Expression of NS2 protein in recombinant SV40 virus infected cells.

Cells were infected with virus and 72 hrs. post infection they were fixed and stained for indirect immunofluorescence using anti-NS2 serum.

(a) Virus infected cells expressing wild type NS2 protein. (b) Virus infected cells expressing NS2/x75 Glu→Gly. (c) Virus infected cells expressing NS2/x77 Arg→Thr.

Figure 23:



VI. DISCUSSION

RNA segment 8 of the influenza A virus encodes two nonstructural proteins, NS1 and NS2 (Ritchey, et al., 1976b; Inglis, et al., 1979; Lamb and Choppin, 1979). The NS1 is characterized as an early protein found in the nucleus of virus infected cells (Mahy, et al., 1980; Young, et al., 1983). The NS2 protein is a late viral protein (Mahy, et al., 1980) shown to localize to the nucleus of virus infected cells (chapter 2). Chapter 2 outlines experiments in which both the NS1 and NS2 genes were expressed in recombinant SV40 virus infected cells. This system allowed us to study the expression of the NS1 and NS2 proteins independent of all other influenza viral proteins. Both nonstructural proteins were found to be nuclear, as determined by indirect immunofluorescence. These data established that in order to localize to the nucleus the NS1 and NS2 proteins did not need to associate with the following influenza viral proteins: M1, NP, PB1, PB2, or PA. Since the NS1 and NS2 proteins have been separately expressed and found to be nuclear, it is likely that the nonstructural proteins each encode, within their polypeptide backbone, the information necessary for their nuclear localization. The individual expression of the NS1 and NS2 proteins, in a recombinant vaccinia virus system, confirms our data that these proteins enter the nucleus independent of one another (Smith, et al.,1987).

Further studies defined the information present, within the NS1 protein, which determines its nuclear location. Signal sequences have been characterized for other nuclear proteins (see Table 2). A feature common to many of these signals is a stretch of basic amino acids and a proline residue. In Chapter 4, two nuclear signal sequences for the NS1 protein are defined by the expression of NS1 deletion mutants and NS1-α- globin fusion proteins.

One nuclear location signal, NLS1, contains the amino acid sequence Asp-Arg-Leu-Arg-Arg (codons 34-38). A proline residue is found three amino acids upstream from this region. A second nuclear localization signal, NLS2, was defined within the amino acid region 203-237. Within this domain, there is the amino acid sequence Pro-Lys-Gln-Lys-Arg-Lys (codon 216-221). This sequence is very homologous to previously defined signal sequences for other nuclear proteins.

One advantage of studying the influenza virus NS1 protein is the great amount of sequence data available for the NS1 gene of different virus strains (Baez, et al., 1980; Krystal et al., 1983; Buonagurio et al., 1986). Of nineteen human, avian, and swine isolates analyzed, all contain the NLS1. NLS1 is even found within the NS1 of the avian virus A/turkey/Oregon/71 despite an internal deletion of ten nucleotides which results in a frame shift mutation and a truncated NS1 protein of 124 amino acids. This protein is approximately one half the size of other known NS1 proteins, yet the virus grows to high titers in embryonated chicken eggs. The truncated NS1 protein can be localized to the nucleus in virus infected cells, and thus, contains a nuclear signal sequence, i.e., NLS 1.

Even more interesting is that NLS1 seems to be conserved in the NS1 protein of influenza B virus (Briedis and Lamb, 1982; Yamashita, et al., 1988). This is striking since these proteins differ by greater than 80% of their sequences and are antigenically distinct (Young, et al., 1983). NLS 1 is also present in the mutant influenza B virus clone 201. The NS gene of this virus also contains an internal deletion which causes a frameshift, in the mRNA, downstream of the region which encodes the conserved NLS1 (Chapter 3). The NLS1 is present in the truncated NS1 protein which has been localized to

the nucleus of virus infected cells. Another virus with an internal deletion in the NS gene is the laboratory derived variant CR43-3 (Buonogurio et al., 1984). This virus encodes an NS1 protein missing amino acids 66-77, yet it still migrates to, and accumulates within, the nucleus. These data establish that there exists tight constraints on the sequence of the NLS1 and suggest that the NLS1 is critical for the function of the NS1 protein during the virus life cycle.

In contrast to NLS1, NLS2 is not present in all strains examined. Sequence data for the NLS2 region is available for 25 isolates of which eighteen contain the basic amino acid stretch of NLS2 (codon 216-221). For example, the NS1 protein of A/turkey/Oregon/71 is truncated and contains only 124 amino acids, and thus, does not contain NLS2. The exact role of NLS2 is unclear, although it has been postulated that the presence of more than one nuclear signal sequence may have an effect on the kinetics of nuclear entry (Richardson, et al., 1986). At this time, no data on the comparative kinetics of the NS1 protein's nuclear entry are available. The presence of two signals in the NS1 protein may be a example of signal redundancy.

Synthesis and purification of large amounts of NS2 protein allowed us to prepare polyclonal monospecific anti-NS2 sera. Antisera specific for the NS2 protein was previously unavailable. With this reagent we were able to localize the NS2 protein to the nucleus of the cell during an influenza virus infection. Prior to this work, the location of the NS2 protein in virus infected cells was controversial (Krug and Etkind, 1973; Mahy et al., 1980; Briedis, et al., 1981). Using indirect immunoflourescence to localize the NS2 protein we have eliminated the possibility of nuclear proteins leaking into the

cytoplasm, a problem which may have confounded cell fractionation studies (Mahy, et al., 1980; Briedis, et al., 1981).

Experiments outlined in Chapter 5 attempt to define a nuclear signal sequence for the NS2 protein of influenza A virus. In contrast to the NS1 protein, analysis of the amino acid sequence of the NS2 protein revealed no conserved basic amino acid region. Instead, amino acids 73-79, Phe-Glu-Glu-Ile-Arg-Trp-Leu were found to have striking homology with the nuclear accumulation signal previously defined for the influenza virus NP protein which includes the sequence Phe-Glu-Asp-Leu-Arg-Val-Leu (Davey, et al., 1985). Site specific mutagenesis of this region at codons 75 and 77 in the NS2 was performed. Two separate NS2 constructions, each with one amino acid change, was expressed in recombinant SV40 virus infected cells. The mutated NS2 proteins were found to localize to the nucleus as did the wild type NS2 protein. From these data we have not positively identified a nuclear signal sequence for the NS2 protein. If this amino acid region is responsible for the NS2 protein's cellular location then the nonstructural proteins would possess very different nuclear signal sequences. This might mean that these proteins enter the nucleus by different mechanisms.

Possible mechanisms for nuclear entry and accumulation have been postulated which include 1 - selective entry through the nuclear envelope and 2 - nonselective entry followed by binding to nondiffusible components of the nucleoplasm (DeRobertis, 1983). Although no attempt was made to determine the mechanism of nuclear entry for the NS1 protein we can derive some information on a possible mechanism from our data. If the cellular location of the NS1 protein were due to nonselective entry into the nucleus succeeded by binding to nuclear components then removal of NLS1 and NLS2 would lead

to the NS1 protein localizing in both the nucleus and the cytoplasm. Our immunofluorescence data indicate that the NS1 protein is found predominately in the cytoplasm when NLS1 and NLS2 are removed (Figure 18). Although this data may indicate that the second postulated mechanism is unlikely we have no positive data in support of hypothesized mechanism #1. In order to prove that selective entry of the NS1 protein through the nuclear envelope occurs, additional experiments would be necessary. The α- globin protein was specifically chosen for construction of fusion proteins because we did not know whether the NS1 protein's nuclear location was due to an active entry process or diffusion. The fusion proteins expressed containing α - globin sequences were small enough to diffuse into the nucleus and hence, these data can not differentiate between the two proposed mechanisms for nuclear location. Proteins that are larger than 70 kd cannot diffuse into the nucleus; therefore, a fusion protein with a molecular weight $\geq$ 70,000 found to be nuclear would indicate an active transport process. Besides additional fusion protein experiments, a more quantitative assay might be able to tell us the relative distribution of the NS1 protein within the cellular compartments and help in defining the mechanism for nuclear transport. From our data it seems as though the NS1 protein may enter the nucleus by an active transport mechanism. Since a region of the NS2 protein has homology to the accumulation signal of the NP protein the NS2 protein's nuclear location may be due to diffusion into the nucleus followed by specific binding. This scenario would mean that the NS1 and NS2 proteins enter thenucleus of virus infected cells by different mechanisms.

VII. SIGNIFICANCE

The nuclear localization of the influenza A virus nonstructural proteins, NS1 and NS2, was studied. We expressed the nonstructural NS2 protein of A/PR/8/34 virus in bacteria. This protein was synthesized in large amounts, purified, and then used to produce polyclonal monospecific anti-NS2 serum. Antisera against the NS2 protein was previously unavailable as the protein is present in very small quantities in virus infected cells. This reagent allowed for the localization of the NS2 protein to the nucleus in virus infected cells, by indirect immunofluorescence. Prior to these experiments, there had been controversy over the cellular location of the NS2 protein.

The NS1 and NS2 proteins were expressed separately in recombinant SV40 viruses and found to localize to the nucleus. These recombinant SV40 viruses are important tools since they enabled us to determine that the NS1 and NS2 proteins localize to the nucleus independent of all other influenza viral proteins. Therefore, nuclear location signals exist within the amino acid sequence of the nonstructural proteins.

Nuclear localization signals were defined for the NS1 protein. These signals are similar in sequence to previously defined signals of other nuclear proteins. We found two nuclear signal sequences which function in the NS1 protein although both are not always present within the protein. The presence of two signals in the NS1 protein may be an example of signal redundancy within a protein.

Since the nonstructural proteins are found in the nucleus during the influenza virus life cycle it would be interesting to determine whether they are involved in viral transcription or replication, which are both nuclear

events. When a biological assay is developed for the NS1 and NS2 proteins, the recombinant SV40 viruses expressing mutant NS proteins, described in this thesis, will be valuable for defining functional domains.

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