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**Establishment of an artificial replication system for influenza  
virus and studies on the mechanism of the antiviral activities of  
the murine Mx1 protein on influenza virus**

**Huang, Taosheng, Ph.D.**

**City University of New York, 1992**

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**ESTABLISHMENT OF AN ARTIFICIAL REPLICATION SYSTEM FOR  
INFLUENZA VIRUS AND STUDIES ON THE MECHANISM OF THE  
ANTIVIRAL ACTIVITIES OF THE MURINE Mx1 PROTEIN ON  
INFLUENZA VIRUS**

by

**TAOSHENG HUANG**

**A dissertation submitted to the Graduate Faculty in  
Biomedical Sciences in partial fulfillment of the  
requirement for the degree of Doctor of Philosophy,**

**City University of New York**

**1992**

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**ABSTRACT****ESTABLISHMENT OF AN ARTIFICIAL REPLICATION SYSTEM FOR  
INFLUENZA VIRUS AND STUDIES ON THE MECHANISM OF THE  
ANTIVIRAL ACTIVITIES OF THE MURINE Mx1 PROTEIN TO  
INFLUENZA VIRUS**

by

Taosheng Huang

Advisor: Mark Krystal, Ph.D.

Until recently, influenza virus, as a negative sense RNA virus, had not been adaptable to many of the powerful molecular techniques available today. However, during the past few years, Dr. Palese and his colleagues in collaboration with Dr. Krystal have developed a system to study influenza virus replication and packaging. In their experiments, a DNA vector was used to produce an influenza virus-like RNA (*IVACAT-1*) *in vitro*. This RNA product has the 3' and 5' noncoding region of the NS gene flanking the antisense sequences of a coding region of the chloramphenicol acetyltransferase (CAT) gene. When mixed with purified polymerase prepared from influenza virus and transfected into cells, the RNA can be replicated and packaged by superinfecting influenza virus. This system now makes analysis of regulatory sequences in RNA feasible. However, the need for superinfecting influenza virus make it difficult to examine the functions of the individual viral proteins involved. To address this, a recombinant vaccinia virus-driven replication system for influenza

virus was developed, in which the synthetic influenza virus like RNA (*IVACAT-1*) was replicated and expressed by influenza virus helper proteins supplied through infection with recombinant vaccinia viruses. We found that the minimum subset of influenza virus proteins needed for specific replication and expression of the ribonucleoprotein was the three polymerase proteins (PB2, PB1, and PA) and the nucleoprotein (NP) and that addition of NS1 and NS2 did not have significant effects on replication and transcription in this system.

In the second part of this thesis, the antiviral mechanism of the murine Mx1 protein was studied. The murine Mx1 protein is an interferon-inducible protein which confers to cells the selective resistance to influenza virus infection. Using the recombinant vaccinia virus-driven replication system, we found that *IVACAT-1* RNP was expressed in Mx1 expressing cells infected with recombinant vaccinia viruses expressing influenza polymerase proteins (PB2, PB1, and PA) and NP.

In the third part of this thesis, we constructed a vector to express M1 protein of influenza virus. Expression of M1 protein was obtained at very high levels. Purification of M1 protein from bacteria was achieved through its solubility in acidic chloroform:methanol solution. Antibody raised against bacteria expressed M1 protein specifically recognized the matrix protein from influenza virus infected cellular extract. With this antibody, the intracellular localization was examined. We found that the M protein migrated to the nucleus during the course of virus infection. Bacterial-expressed M1 protein is able to inhibit viral polymerase activity in *in vitro* transcription reactions. Availability of antibody and a large amount of bacterial-

expressed M1 protein should allow further examination of the function of the M1 protein and the dissection of functional domains.

## **FORMAT OF THESIS**

This thesis was prepared according to new guidelines of the City University of New York which permit the direct incorporation of published research papers as chapters. Included in this thesis is a general introduction, published research articles as individual chapters as well as a general discussion. All references are pooled in order to avoid redundancy. The appendix contains studies carried out in Dr. Ming-Ta Hsu's laboratory. Copyright permission to use the published papers as a dissertation chapter is obtained from the relevant publishers.

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# TABLE OF CONTENTS

page

|                                                                                        |      |    |
|----------------------------------------------------------------------------------------|------|----|
| Approval page .....                                                                    | iii  |    |
| Abstract .....                                                                         | iv   |    |
| Note on Format of Thesis .....                                                         | vii  |    |
| Acknowledgements .....                                                                 | viii |    |
| Table of Contents .....                                                                | ix   |    |
| List of Figures.....                                                                   | xi   |    |
| I. Introduction                                                                        |      |    |
| Structure of Influenza Virus .....                                                     | 2    |    |
| Transcription of the Influenza Virus Genome .....                                      | 3    |    |
| Replication of the Influenza Virus Genome .....                                        | 6    |    |
| Functions of the Polymerase Proteins .....                                             | 8    |    |
| Functions of the nucleoprotein (NP) .....                                              | 10   |    |
| Nonstructural (NS1 and NS2) proteins .....                                             | 12   |    |
| Functions of the matrix protein (M1) .....                                             | 14   |    |
| Selective Resistance of Mx1 protein to Influenza virus.....                            | 17   |    |
| Development of an Expression/Rescue System for Influenza<br>Virus .....                | 24   |    |
| II. Determination of Influenza Virus Proteins Required for Genome<br>Replication ..... |      | 26 |

### **III. Expression of IVACAT-1 RNP in stable Mx1 cell line infected with recombinant vaccinia viruses expressing the influenza viral proteins**

|                             |    |
|-----------------------------|----|
| Abstract .....              | 45 |
| Introduction .....          | 46 |
| Materials and Methods ..... | 48 |
| Results .....               | 51 |
| Discussion .....            | 59 |

### **IV. Expression of the Influenza Virus Matrix Protein in Bacteria**

|                             |    |
|-----------------------------|----|
| Abstract .....              | 73 |
| Introduction .....          | 74 |
| Materials and Methods ..... | 76 |
| Results .....               | 80 |
| Discussion .....            | 84 |

### **IV. Discussion .....**

97

### **V. Significance .....**

105

### **VI. Appendix.....**

108

### **VII. List of Publications .....**

125

### **VIII. Bibliography .....**

126

## FIGURES

page

|                                                                                                                                                                         |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 1. Structure of influenza virus .....                                                                                                                                   | 2  |
| 2. Model of influenza viral mRNA synthesis .....                                                                                                                        | 4  |
| 3. Scheme of viral RNA synthesis of influenza virus .....                                                                                                               | 6  |
| 4. Schematic representation of the NS1 and the NS2 mRNA of<br>influenza A virus .....                                                                                   | 13 |
| 5. Minimum influenza virus proteins required for expression of the<br>synthetic ribonucleoprotein .....                                                                 | 38 |
| 6. Expression of <i>IVACAT-1 RNP</i> in 3PNP-4 cells and demonstration of<br>replication of synthetic RNP .....                                                         | 40 |
| 7. Effect of the NS1 and NS2 protein on expression of <i>IVACAT-1 RNP</i> .....                                                                                         | 42 |
| 8. Mx 1 protein inhibits expression of <i>IVACAT-1 RNP</i> when influenza<br>virus is used as helper .....                                                              | 61 |
| 9. Western blot analysis and inhibition of influenza virus multiplication<br>of Mx1- transfected Swiss 3T3 cells.....                                                   | 64 |
| 10. <i>IVACAT-1 RNP</i> was expressed in Mx1-expressing cells co-infected<br>with recombinant vaccinia viruses expressing influenza polymerase<br>proteins and NP.....  | 66 |
| 11. Expression of <i>IVACAT-1 RNP</i> in Mx1-expressing cells co-infected<br>with WSN and recombinant vaccinia viruses expressing the<br>influenza viral proteins ..... | 68 |

|                                                                                                                                                              |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 12. Effect of the polymerase proteins and NP on expression of<br>IVACAT-1 RNP.....                                                                           | 69  |
| 13. Construction of the expression vector for M1 protein of influenza<br>virus .....                                                                         | 87  |
| 14. Expression of M1 protein in bacteria.....                                                                                                                | 89  |
| 15. Purification of bacteria expressed M1 protein.....                                                                                                       | 91  |
| 16. Intracellular localization of M1 protein in influenza virus infected<br>cells.....                                                                       | 93  |
| 17. Inhibition of bacteria expressed M1 protein to the viral<br>polymerase.....                                                                              | 95  |
| 18. Effect of tunicamycin on the accumulation of adenovirus DNA and<br>SV40 DNA and on the incorporation of ( <sup>3</sup> H)-thymidine into Ad5<br>DNA..... | 117 |
| 19. Reversion of tunicamycin inhibition of adenovirus DNA replication<br>by N-acetylglucosamine .....                                                        | 119 |
| 20. Effect of tunicamycin on the accumulation of adenovirus DNA in<br>293 cells and on SV40 DNA accumulation in Cos-7 cells.....                             | 121 |
| 21. Effect of tunicamycin on the synthesis of adenovirus late RNA and<br>protein .....                                                                       | 123 |

**I.**

## **INTRODUCTION**

## INTRODUCTION

**Structure of Influenza Virus** Influenza viruses are members of orthomyxoviridae family. Based on the antigenic difference between their nucleoprotein (NP) and matrix (M) protein, influenza viruses are divided into three types (A, B, and C). Virion particles can be irregularly shaped or spherical, approximately 120 nm in diameter (Horns et al., 1960; Wrigley, 1979). The surface spikes are of three distinct types. As shown in Figure 1, the most prevalent surface

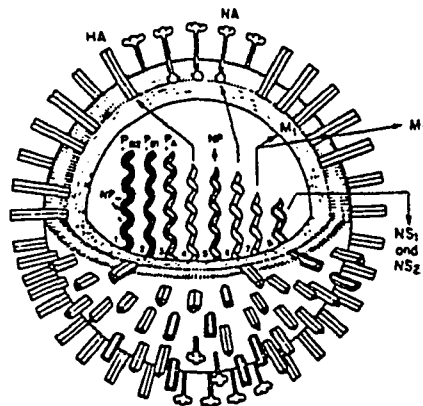


**Figure 1.** Structure of Influenza Virus (from Murphy and Webster, 1990)

proteins correspond to the hemagglutinin and neuraminidase components of the virus. The hemagglutinin spike appears rod shaped and the neuraminidase spike is mushroom shaped. The hemagglutinin and neuraminidase are attached by short

sequences of hydrophobic amino acids to a lipid envelope. There is no doubt that the M2 is also a component of virion membranes (Zebedee, S. L. and R. A. Lamb, 1988). However, compared with its relative abundance in virus-infected cells, this protein is underrepresented in the virion (Lamb et al., 1985). Under the lipid envelope lies the matrix protein. Within the matrix shell are single-stranded RNA molecules of negative polarity ( Inglis et al., 1976; Palese and Schuman, 1976; Ritchey et al., 1976, For review, see Palese, 1976) assembled with the nucleocapsid protein (NP) and three polymerase proteins (PB1, PB2, and PA). The influenza A, and B virus genome consists of eight segments, while influenza C virus has only seven. Two virus-coded nonstructural proteins (NS1 and NS2) are found in infected cells.

**Transcription of the Influenza Virus Genome** Transcription and replication of the influenza virus genome takes place solely in the nucleus (Herz et al., 1981). The mRNAs are transcribed from the virion RNAs (vRNA) by the virion-associated RNA-dependent RNA transcriptase. The basic mechanism has been elucidated in studies using an *in vitro* transcription system ( Braam et al., 1983). It has been postulated as follows: the first step involves the "snatching" of the cap structure off host-cell mRNAs (Bouloy et al., 1978; Plotch et al., 1979; Krug, 1981). For this reason, viral mRNA synthesis requires continuous RNA polymerase II function and is therefore inhibited by  $\alpha$ -amanitin (Mark et al., 1979). The viral cap-dependent endonuclease cleaves the capped cellular RNAs 10-13 nucleotides from their 5' ends

(Plotch et al., 1981), preferentially at a purine residue. These oligonucleotides then serve as primers for viral mRNA synthesis. Priming requires the presence of the 5'-methylated cap structure (Plotch et al., 1979; 1981; Bouloy et al., 1979). The methyl groups in the cap increase the priming activity (Bouloy et al., 1980). Of the primers, those containing a 3'-terminal A residue are preferred to those containing a 3'-

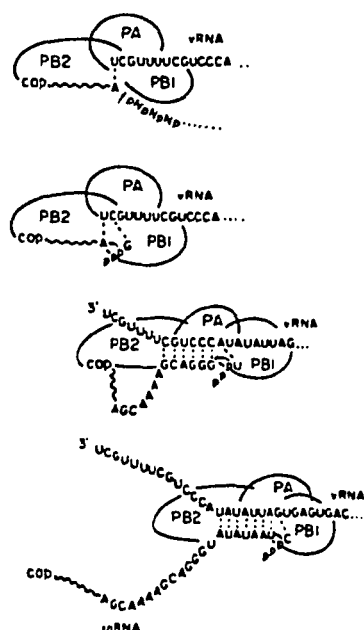


**Figure 2. Model of Transcription of Influenza Virus Genome (from Braam et al., 1983)**

terminal G residue (Plotch et al., 1981). Priming activity by capped RNAs does not require extensive base pairing between the primer and the 3' end of the template vRNA (Krug et al., 1980; Krug, 1983). For example, sequences lacking complementarity to the 3' end of vRNAs, such as capped 5'-terminal fragments of  $\beta$ -globin mRNA (14-23 nucleotides in length) and capped poly (A) or capped poly (AU) (1:1), are all effective as primers (Krug, 1981; Krug et al., 1980). Consequently, the stimulation of transcription is thought to result from a specific

interaction between the capped RNA and the viral protein(s) in the transcriptase complex. This interaction would require the recognition of the 5'-terminal methyl group of the primer RNA.

The second step of the transcription is the incorporation of a G residue onto the 3' end of the capped primer (Figure 2). This intermediate has been identified by incubating an unlabeled primer with the virion transcriptase complex in the presence of  $^{32}\text{P}$ -GTP as the only ribonucleoside triphosphate (Plotch et al., 1981). In this case, only the G residue was added and no incorporation of A, U, or C occurred in single triphosphate reactions (Plotch et al., 1981). Since the 3' terminal U residue is not used for transcription, it is thought that the 3'-terminal nucleotide of the primer interacts with this U residue. This may be why an A residue is the preferred base at the 3' end of the primer. After the addition of a G residue, transcription elongation follows.

The final steps of viral mRNA synthesis are termination and polyadenylation. The viral transcription terminates before the polymerase proteins reach the 5' end of vRNA (Figure 2). When hybrids between vRNA segments and viral mRNA were digested with single strand-specific nucleases, it was found that the viral mRNAs lack the sequence complementary to the 5' terminus of the vRNAs (Hay et al., 1977; Plotch and Krug, 1978). In this region, each of the eight vRNA segments contains a stretch of 5 to 7 uridine residues (Robertson, 1979; Skehel and Hay, 1978). This short U tract has been established as the site of poly (A) addition (Luo et al., 1991). Although it has been presumed that termination occurs at this site, it is not clear

whether poly(A) addition occurs by endonucleolytic cleavage of large transcripts, followed by polyadenylation or by stuttering of the polymerase complex and subsequent termination. However, recent data indicate that termination and poly A addition at least in part are the consequence of the secondary structure conformation of the viral RNP (Luo et al., 1991). Mutational analysis showed that both the uridine residue stretch and a double-stranded RNA panhandle structure formed by the 5'- and 3'- terminal nucleotide appeared to be required for polyadenylation. In terms of the panhandle structure requirement for polyadenylation, it is the secondary structure-dependent, but not sequence specific, since compensatory mutations at either end of the RNA which kept the panhandle structure allowed for mRNA synthesis, but mutations at only one end inhibited the mRNA synthesis. The results support the model that the RNA duplex structure serves as a barrier for the viral polymerase, causing it to stutter at the uridine residues, with the result of being the addition of a poly A tail.

**Replication of the Influenza Virus Genome.** Full-length complementary RNAs (cRNA) of the viral genome were first identified by hybridizing vRNA to labeled RNA from the infected cell. The resulting double-stranded RNA species were treated with S1 nuclease and analyzed by gel electrophoresis (Hay et al., 1977b). It was found that the hybrids of poly(A)<sup>-</sup> transcripts and vRNA migrated slower than those of the mRNA and vRNA and that the hybrids from poly A<sup>-</sup> transcripts were resistant to S1 nuclease treatment. These results suggested that

poly (A)<sup>-</sup> transcripts were complete copies of the vRNA segments (Figure 3). Several lines of evidence have supported this conclusion (Hay et al., 1977b). First, direct sequencing showed that the 3' end of cRNAs are complementary to the 13-nucleotide sequence found at the 5' termini of the vRNAs (Hay et al., 1982). Therefore, the

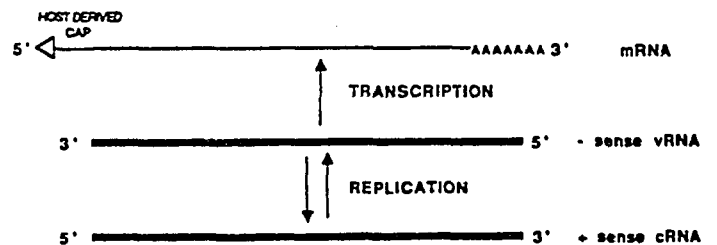


**Figure 3.** Scheme of Viral RNA Synthesis of Influenza Virus

termination and poly A addition signal which is used during viral mRNA synthesis must be suppressed during cRNA synthesis. In addition, cRNA synthesis occurs without the need for a primer (Hay et al., 1983). These transcripts have a non-methylated pppA 5'-terminus, and their 5' sequences are complementary to the 3' terminal sequences of the vRNA templates. Because they are complete copies of the vRNA segments, the cRNA functions as the template for vRNA synthesis. Consistent with this role, the full-length transcripts are not associated with polyribosomes but remain in the nucleus in the form of ribonucleoprotein (Hay et al., 1977a). Because

the complements of the 5' end of vRNA segments are present in the full-length transcripts, 5' and 3' ends can in theory form a panhandle structure similar to those formed by the vRNA (Hsu et al., 1987). It has been postulated that this panhandle structure serves as a recognition signal for the polymerase complex (Hay et al., 1977b; Luo et al., 1991).

**Function of the three Polymerase Proteins.** Influenza viral polymerase polypeptides, PB2, PB1 and PA are encoded by the three largest genome segments. These three polymerase proteins form a complex which has a number of enzymatic activities involved in transcription and replication of the influenza virus genome. Several groups have shown that the polymerases in the nucleus form a complex that is resistant to disruption by normal immunoprecipitation buffers (Detjen et al., 1987; Akkina et al., 1987). Almost all polymerase proteins exist as this complex, and there is little detectable free discrete polymerase protein.

To establish the role of each of the polymerase proteins in individual steps of viral RNA synthesis, cross-linking has been used to identify the polymerase protein which is associated with the cap structure and hence is the likely candidate for the viral cap-recognition protein. In one study, a capped RNA primer was labeled and was used as a radioactive probe to identify the cap recognition protein (Ulmanen et al., 1981). First, this radioactive capped RNA primer was incubated with viral core in the absence of ribonucleoside triphosphate. After irradiation with UV light and nuclease digestion, the labeled-cap was found to be crosslinked to the PB2 protein.

The specificity of crosslinking of PB2 to the labeled cap of the primer RNA was demonstrated since unlabeled RNAs that contained a cap structure were able to compete with the labeled cap. In another experiment, a photoreactive derivative of cap was used as an affinity label. Again, the PB2 protein became crosslinked to this probe and crosslinking was competitively and specifically inhibited by cap analogues (Blaas et al., 1982). Additional evidence for a function of the PB2 in cap recognition has been provided by a study of the *in vitro* transcription reaction catalyzed by temperature sensitive viral mutants mapped to the PB2 protein (Ulmanen et al, 1983). The two mutants, WSN ts1 and ts6, are defective in cap-binding at nonpermissive temperature, and consequently transcription is impaired.

To identify viral protein(s) catalyzing the chain elongation of transcription, viral cores were incubated with  $\alpha$ -<sup>32</sup>P GTP as the only ribonucleoside triphosphate. After UV irradiation and nuclease digestion, the radioactive G residue was found to be crosslinked to the PB1 protein in two dimensional gel electrophoresis (Ulmanen et al., 1981). This strongly suggests that a G residue is the first base added to the primer and that the PB1 protein catalyzes this reaction.

Although PA is in the polymerase complex, no specific role in replication and transcription has yet been found. In fact, experiments with temperature sensitive mutants mapped to PA show that the principle role of the PA protein in infected cells is not in viral mRNA synthesis, but rather in viral RNA replication. At nonpermissive temperature, cRNA and vRNA synthesis, but not viral mRNA synthesis, was inhibited (Krug et al., 1975; Mahy et al., 1981; Mowshowitz, 1981).

In contrast, for temperature sensitive mutants mapped to PB1 and PB2, both the viral mRNA and vRNA synthesis are inhibited immediately at the nonpermissive temperature.

**Function of the Nucleocapsid Protein (NP).** The first step in the replication of influenza genome is cRNA synthesis (see Figure 3). Because the cRNA synthesis requires protein synthesis *in vivo* (Banrett et al., 1979; Hay et al., 1982), it is likely that the newly synthesized virus-specific proteins are needed for either unprimed cRNA synthesis, antitermination, or both. To identify the protein(s), a modified *in vitro* system was established which catalyzed the synthesis of cRNA as well as viral mRNA (Beaton and Krug, 1984; 1986). Nuclear extracts collected from virus-infected cells at 6 hour post-infection synthesized cRNA in the absence of primer (Shapiro and Krug, 1988). Therefore, these nuclear extracts contain a factor(s) that is able to initiate cRNA synthesis without need of primer and antiterminate at the poly(A) site used for viral mRNA synthesis. Furthermore, the antitermination factor(s) could be separated from the viral nucleocapsid (RNP) by ultracentrifugation. Nucleocapsids were pelleted by ultracentrifugation and used in RNA synthesis reactions. These RNPs synthesized viral mRNA but little or no cRNA, indicating a virtual absence of the antitermination factor in the pellet. The supernatant fraction by itself had little activity in either viral mRNA or cRNA synthesis (Beaton and Krug, 1986). However, when the supernatant fraction was added to the pellet fraction, cRNA synthesis was restored, indicating that the supernatant fraction contained the factor(s) required for

antitermination at the poly (A) site.

Affinity chromatography was used to identify this antitermination factor (Beaton and Krug, 1986). The supernatant prepared as described above was depleted of individual viral proteins by incubation with protein A-Sepharose coupled to specific antibody. The protein A-Sepharose was then removed, and the resulting supernatant was added to the RNP pellet fraction and tested for antitermination synthesis. The supernatant deprived of the NP protein lost its ability to antiterminate, as no cRNA was synthesized. By contrast, antitermination activity was retained in supernatant deprived of the NS1 or NS2 protein (Beaton and Krug, 1986). These results suggest that the NP protein is required for antitermination during cRNA synthesis. However, the definitive experiment of adding NP back to the depleted supernatant fraction and rescuing activity has not been accomplished.

Synthesis of vRNA from cRNA also requires NP protein. Nuclear extracts from influenza virus-infected cells catalyzed vRNA synthesis *in vitro*. (Shapiro and Krug, 1988). Again, the nuclear extract was separated into a pellet fraction (containing RNPs) and supernatant fraction. The pellet fraction was able to synthesize a heterogeneous array of vRNAs of small size in the absence of supernatant fraction. The supernatant fraction by itself lacked any catalytic activity. However, when the two fractions were combined, discrete full-length vRNAs were synthesized again. Antibody-depletion experiments indicated that NP protein in the supernatant was also required for vRNA synthesis (Shapiro and Krug, 1988). In contrast, NS1 and NS2 are not needed for vRNA synthesis from cRNA.

The role of NP in cRNA synthesis was confirmed using WSN ts 56, a temperature sensitive mutant mapped to the NP protein (Shapiro and Krug, 1988). WSN ts 56 has been shown to be defective at nonpermissive temperature in the synthesis of cRNA (Shapiro and Krug, 1988) and vRNA (Scholtissek, 1978; Mahy et al., 1981; Shapiro and Krug, 1988), but not of mRNA (Krug et al., 1975; Scholtissek, 1978; Mahy et al., 1981). This indicates that the NP is required for both cRNA and vRNA synthesis *in vivo*. To verify that NP acted on cRNA synthesis, a nuclear extract was prepared from HeLa cells infected by WSN ts 56 at the permissive temperature 33°C (Shapiro and Krug, 1988). The activity of this extract in viral mRNA and cRNA synthesis was assayed at 33°C and 39.5°C (nonpermissive temperature). This extract synthesized cRNA at the permissive temperature. By contrast, at the nonpermissive temperature, this extract did not synthesize any detectable cRNA, though the synthesis of mRNA was not decreased at nonpermissive temperature (Shapiro and Krug, 1988). These results indicated that NP made by WSN ts56 mutant was inactive in antitermination at the nonpermissive temperature. However, this defect in NP did not affect mRNA synthesis at the nonpermissive temperature. Therefore, NP is required for the synthesis of cRNA and vRNA.

**Nonstructural Proteins (NS1 and NS2)** The nonstructural proteins, NS1 and NS2, are encoded by RNA 8 of influenza A and B virus (segment 7 for type C virus) (Lamb, 1983). Two mRNAs are transcribed from viral RNA 8 (Figure 4). The mRNA coding for the NS1 protein is an unspliced mRNA while the NS2 mRNA is

a spliced transcript. The rate of splicing is controlled solely by cis-acting sequences in NS1 mRNA itself (Alonso-Caplen and Krug, 1991). These two mRNAs share the first 56 nucleotides. After this sequence, the mRNA of NS2 has a deletion of 423 nucleotides, then rejoins the rest of the mRNA in a +1 reading frame (Lamb and Lai, 1983). Therefore, the NS1 and NS2 protein have 10 amino acids in common at their N-termini, but different over the rest of the amino acid sequences (Figure 4)

The precise functions of the NS1 and the NS2 protein have not been established yet. Work with temperature sensitive mutants defective in RNA 8 show that lesions in this RNA lead to multiple effects, including a decrease of HA, M, and NS2 protein synthesis as well as a decrease in the accumulation of vRNA during infection. However, conflicting results have been reported. In a different set of temperature-sensitive (*ts*) mutants of influenza virus with lesions in RNA segment 8, it was shown that the synthesis of the matrix protein (M1), hemagglutinin (HA),

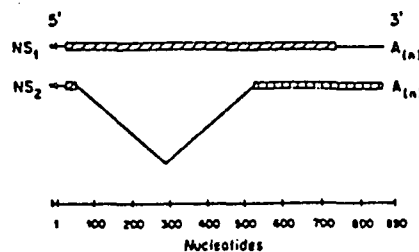


**Figure 4.** Schematic representation of the NS1  
and the NS2 mRNA of influenza A virus

and NS1 protein was greatly reduced at non-permissive temperature compared to that at permissive temperature. However, the levels of individual mRNAs including those of late proteins were almost the same as those at permissive temperature. The

results suggest that NS1 is involved in some post-transcriptional process relating to the synthesis of the late proteins and the NS1 protein (Hatada et al., 1990). In *in vitro* transcription reactions containing purified virion-RNP complexes, the addition of the NS1 protein did not effect RNA synthesis (Skorko et al., 1991). Studies on a mutant in NS2, *Wa-182*, showed that the mutation of NS2 protein affected the normal replication of the PA gene (Odagiri and Tobita, 1990). *Wa-182* is a reassortant of influenza virus A/WSN/33 whose NS gene was replaced with the NS gene from an influenza A/Aichi/68 mutant which contains two amino acid substitutions in the NS2 protein. When infecting cells at high-multiplicity, *Wa-182* was readily converted to defective interfering (DI) particles after only a single infection cycle. The defective interfering particles were devoid of the PA gene, but possessed several species of subgenomic RNA of the PA gene (Odagiri and Tobita, 1990). This results suggest that the NS2 protein may play a role in the PA gene synthesis.

**Function of the Matrix Protein (M1)** M1 protein is the most abundant protein in influenza virion, which is encoded by RNA segment 7. It is intimately associated with the lipid bilayer in such a manner that it is in close proximity to the hemagglutinin, neuraminidase and ribonucleoprotein. The N-terminus of the protein contains the lipophilic domain and C-terminus contains the domain that interacts with the transcriptase (Ye et al, 1989). Therefore, the M1 protein provides the structural integrity to the virion envelope.

M1 protein is localized in the nucleus where influenza virus replication and transcription occur. In *in vitro* transcription reaction, it was first shown by Zvonarjev and Ghendon that M1 protein downregulated the polymerase activity (Zvonarjev and Ghendon, 1980). In this particular experiment, purified virions (influenza A/PR/1/74) were treated with 1% NP 40 and 0.1% deoxycholate to disrupt the viral envelope and then viral RNPs were isolated by centrifugation in a linear 15-30 % glycerol gradient. Purified viral RNPs were used for *in vitro* transcription. The addition of M protein to the reaction mixture decreased the transcriptase activity by 40% (Zvonarjev and Ghendon, 1980). To locate the region of M1 protein responsible for down-regulating endogenous transcription, monoclonal antibodies to different epitopes of M1 protein were used. It was found that a monoclonal antibody specifically recognizing amino acid 129-164 could reverse transcription inhibition by M1 protein (Ye et al., 1989). Anti-idiotypic antibody to this region mimicked the action of M1 protein by inhibiting transcription *in vitro*. Transcription of influenza virus ribonucleoprotein was also inhibited by a nonodecapeptide with amino acid sequence corresponding to amino acid 90-108 of the M1 protein (Ye et al., 1989). This result suggests that amino acid sequences corresponding to 129-164 and 90-108 are responsible for inhibitory activity of M1 protein. Further investigation of the mechanism by which M1 protein inhibits the influenza viral polymerase activity has shown that M1 protein displayed a strong affinity for binding viral RNA and other extraneous RNA (Ye et al., 1989; Wakefield and Brownlee, 1989; Huang and Krystal, unpublished data). Single stranded RNA is preferred over double stranded RNA.

The complex of M1 protein and single stranded RNA has a pH optimum of 8.0, but is sensitive to disruption by salt at concentrations above 200mM. M1 protein binding saturates at a minimum of 1:200 nucleotides (Wakefield and Brownlee, 1989) and the binding activity was blocked by monoclonal antibody to amino acid sequences 90-108 of M1 protein. These results together suggest that M1 protein inhibits the transcription by binding to influenza viral RNA. To visualize more clearly the structural properties of this region of influenza virus M1 protein responsible for transcription inhibition and RNA binding activity, site specific mutagenesis analysis is needed.

Another function of M1 protein is its ability to modulate transport of influenza viral RNPs. Influenza virus replication and transcription occur solely in the nucleus. During the course of influenza virus infection, the virus first binds to cell surface sialic acid residues and is internalized by receptor-mediated endocytosis (Martin 1982, Marsh and Helenius, 1989). Penetration into the cytosol occurs by an acid-activated, HA-mediated fusion event between the virus membrane and the membrane of a late endosome. After release into the cytosol, the viral RNPs separate from M1 protein and are efficiently transported into the nucleus (Martin and Helenius, 1991). In the nucleus, the virus RNPs are transcribed and replicated. When newly synthesized NP and polymerase appear, they are efficiently target to the nucleus by their nuclear targeting signal. Virus RNPs are assembled in the nucleus and then transported into the cytosol. M protein, localizing in the nucleus of infected cells (Gregoriades, 1977; Maeno et al., 1977; Paterson et al., 1988; Huang and Krystal, unpublished data) was

speculated to have a role in RNP transport. However, the evidence has not been obtained until recently. Martin and Helenius found that export of virus RNPs from the nucleus to the cytoplasm is dependent of the viral M1 protein. M1 protein associated with newly assembled virus RNP in the nucleus and escorted them to the cytoplasm through the nuclear pores. In contrast, during the entry to host cytoplasm, M1 protein dissociated from the virus RNPs, thus allowing virus RNPs enter the nucleus. Amantadine was found to block the dissociation of M1 protein and thereby inhibit import of incoming virus RNP to the nucleus. These experiments provided the evidence that M1 protein modulates import and export of virus RNPs.

**Selective Resistance of Murine Mx1 Protein to Influenza Virus** The murine Mx1 protein is a 72 Kd nuclear protein (Horisberger et al., 1983; Dreiding et al., 1985). The Mx gene was originally found in the inbred mouse strain A2G, which has inborn resistant to influenza virus. Mice possessing this gene develop an efficient antiviral state only after exposure to  $\alpha/\beta$  interferon (Staeheli et al., 1984). *In vitro*, the antiviral state against influenza virus can be neutralized by antibodies specific to the murine Mx protein (Arnheiter and Haller, 1988). This indicates that the interferon (IFN)-induced murine Mx protein mediates resistance to influenza virus (Haller, 1981; Krug et al., 1985). For confirmatory experiments, transgenic mice carrying the murine Mx1 gene were found to possess resistance to influenza virus (Arnheiter et al., 1990).

The Mx specific antisera facilitated cloning of the cDNA encoding the Mx

protein. In a tour de force series of experiments, antisera against the murine Mx protein was first prepared by injecting Mx<sup>-</sup>BALB/c mice with extracts of interferon treated-cells from congenic Mx<sup>+</sup> mice (BALB A2G-Mx) (Staeheli et al., 1985). In order to clone cDNA coding for the murine Mx1 protein, poly A<sup>+</sup> RNA from Mx<sup>+</sup> embryo cells treated with IFN was size-fractionated by electrophoresis through a methylmercury agarose gel. The mRNA was extracted from the individual fractions and used for cell-free protein synthesis in a rabbit reticulocyte lysate. Mx protein was identified by immunoprecipitation with the antiserum. Fractions enriched with Mx mRNA was then used for cDNA synthesis. The cDNA library was screened by differential cloning hybridization using <sup>32</sup>P-labeled cDNA prepared from size fractionated mRNA of either IFN-treated BALB A2G-mx (Mx<sup>+</sup>) cell or IFN-treated BALB/c (Mx<sup>-</sup>) cells and a clone was isolated. Sequence analysis of this cDNA indicated that the Mx protein contains 631 amino acids and is extremely hydrophilic with a large number of charged amino acids. The Mx1 gene was mapped to mouse chromosome 16 and consists of 14 exons spread over more than 55 Kb of DNA (Hug et al., 1988). A number of inactive alleles were also identified from Mx<sup>-</sup> mice; one gene lacked three exons while another contained a nonsense mutation which halves the potential coding capacity of the Mx protein (Staeheli et al., 1988). A vector containing wild-type Mx1 cDNA was transfected into Mx<sup>-</sup> NIH 3T3 cells, and cells expressing the Mx protein were isolated. Cells containing large amounts of the Mx protein in the nucleus were protected against infection by influenza virus (Staeheli et al., 1986; Pavlovic et al., 1990). Cells containing either lower amounts of the Mx

protein or no Mx protein were not protected against influenza virus. This indicated that the Mx protein does not require other IFN-induced gene products in order to establish resistance to influenza virus. A second Mx gene was identified in mouse, and corresponding mRNAs were cloned (Staeheli et al., 1988; Staeheli and Sutcliffe, 1988). The putative Mx2 protein is 73% identical to Mx1. Mx2 cDNA transfected cells expressed the putative 76 KD mouse Mx2 protein. This protein accumulated in the cell cytoplasm (Zuercher et al., unpublished data). Transfected cells expressing Mx2 protein showed a high degree of resistance to VSV, but remained susceptible to influenza virus (Zuercher et al., unpublished data).

Recently, transgenic mice expressing the murine Mx1 protein under the control of an interferon-responsive element (Mx promoter itself) have been generated (Arnheiter et al., 1990). It is induced by interferon as well as by influenza virus. High responder lines produce the murine Mx1 protein locally at the site of initial viral replication and show little viral spread. Mice of low responder strains show more extensive viral spread. These results indicated that influenza virus infection induced interferon, which in turn induced the Mx protein thereby conferring to the mouse resistance to influenza virus.

The mechanism by which the Mx protein selectively inhibits the replication of influenza virus is unclear. Initially, it was reported that treatment of macrophage of Mx<sup>+</sup> mice with IFN  $\alpha/\beta$  prior to influenza virus infection led to the inhibition of the translation of apparently functional viral mRNAs in the cytoplasm, but did not affect the synthesis of viral mRNAs in the nucleus (Meyer and Horisberger, 1984).

However, this observation is controversial and probably incorrect. In other experiments, it was shown that viral mRNA synthesis was severely inhibited in Mx<sup>+</sup> mouse embryo fibroblasts treated with IFN (Krug et al., 1985). The levels of viral mRNA were analyzed by Northern blotting, both in the absence and in the presence of the protein synthesis inhibitor, anisomycin. In Mx<sup>+</sup> cells treated with IFN, the HA and polymerase protein mRNAs were undetectable and the level of the NS1 mRNA was extremely low. In the presence of anisomycin, viral mRNA synthesis is restricted to that which is catalyzed by the inoculum transcriptase. Additional experiments showed that the absence of most viral mRNAs and a very large reduction in the levels of the NS1 and M1 mRNA reflected an inhibition of viral mRNA synthesis (Krug et al., 1985). In contrast, IFN treatment of Mx<sup>-</sup> cells has little or no effect on either the level or the rate of synthesis of viral mRNA catalyzed by inoculum transcriptase. These results provide evidence that the Mx protein inhibits viral mRNA synthesis. Similar results have been obtained from studies on stable Mx protein-expressing cell lines (Zurcher et al., 1992).

In the nucleus, the Mx protein could act directly or indirectly to inhibit the viral RNA transcriptase. The Mx protein contains a putative guanine nucleotide-binding site (Horisberger et al, 1990). Recently, bacterial-expressed Mx1 has indeed been shown to have GTP binding activity. It was therefore suggested that the Mx protein might be a cap-binding protein or might activate a cellular cap-binding protein and this cap-binding activity might arrest the ability of virus to use cellular capped RNA as primers. To determine whether Mx<sup>+</sup> cells treated with IFN contain

newly induced cap-binding activities, UV cross-linking was used to identify proteins that specifically interact with the cap structure. A labelled cap structure was incubated with cellular extract from Mx<sup>+</sup> cells treated with IFN. After irradiation of UV light and digestion of pancreatic RNase, the samples were then analyzed on protein gels. The result showed that the arrays of nuclear cap-binding activities in Mx<sup>+</sup> and Mx<sup>-</sup> cells treated with IFN were not significantly different. In addition, no cap-binding activity associated with the Mx protein could be detected (Broni et al, 1990). These results suggest that the Mx protein might directly act to inhibit the viral transcriptase in the nucleus.

Use murine Mx1 cDNA as a probe, two human proteins with structural similarity to mouse Mx1 protein, human MxA and Human MxB, were identified (Aebi et al., 1989; Horisberger et al., 1990; Staeheli and Haller, 1985). In contrast to mouse Mx1, human Mx protein accumulated in cytoplasm of IFN-treated-cells (Staeheli and Haller, 1985; Pavlovic et al., 1990; Aebi et al., 1989). To test whether the human Mx protein possess antiviral potentials, the MxA cDNA were stably transfected into mouse 3T3 cells (Pavlovic et al., 1990). Stable human MxA-expressing cells conferred resistance to influenza virus as well VSV. Stable MxB cells remained susceptible to influenza virus and VSV. Thus, these results indicate that the naturally occurring human MxA protein might have antiviral potential, where MxB might lack antiviral activity.

Stable cells constitutively expressing MxA made it possible to determine the MxA-sensitive step of the influenza virus replication cycle. It was found that

influenza virus multiplication was blocked at a step following primary transcription. In contrast to the nuclear Mx1 protein, influenza virus-infected cells expressing the cytoplasmic human MxA protein accumulated all viral primary transcripts to normal levels (Pavlovic et al., 1992). These transcripts carried an apparently normal poly A tail and accumulated in the cytoplasm. Despite the synthesis of apparently primary transcripts in influenza virus infected cells expressing MxA, influenza viral genome amplification did not take place (Pavlovic et al., 1992), indicating that MxA inhibited either protein synthesis or a subsequent step of the viral multiplication and that the mechanism of inhibition for the human MxA protein differs from that the murine homolog. Stable cells constitutively expressing MxA were also used to determine the MxA-sensitive step of the VSV replication cycle. Primary transcription of the parental VSV genome was strongly inhibited in MxA-expressing cells (Staeheli and Pavlovic, 1991). Compared to control cells, infected cells expressing MxA contained more than a 50-fold lower pool of primary VSV L transcripts. MxA had a less pronounced effect on primary transcription of other VSV genes (Staeheli and Pavlovic, 1991). The principal site of MxA action against VSV therefore seems to be the level of viral mRNA synthesis. Since MxA inhibited the multiplication of VSV and influenza virus, it was interesting to learn whether this phenotype was due to a single or rather two distinct activities of MxA. The fact that MxA had no effect on influenza viral mRNA synthesis, but inhibited VSV mRNA synthesis already indicated two separate modes of action. This view was recently supported. A mutant form of MxA conferred resistance to influenza virus but not to VSV (Pavlovic and

Staeheli, personal communication). The mutant form of MxA differed from wild-type MxA by a single amino acid substitution near C-terminus. This observation suggests distinct mechanisms of MxA action against influenza virus and VSV.

Unlike the murine Mx1 protein which can function as an inhibitor of influenza virus only when present in the nucleus ( Noteborn et al., 1987; Zurcher et al., 1992), the human Mx A protein is cytoplasmic. In addition, the human MxA protein behaves like the nuclear mouse Mx1 protein after it is moved to the nucleus with the help of a foreign nuclear transport signal (Zurcher et al., 1992).

Comparative analysis may produce some clue as to the precise mechanism of inhibition by Mx1. There are two cellular proteins which have been identified to be the homologs of Mx1. One is the product of yeast vacuolar protein sorting gene, VPS1 (Rothman et al., 1990). Yeast cells contains a prominent vacuolar which is considered to be equivalent to the mammalian lysosome. The VPS1 gene encodes a protein required for sorting of newly synthesized vacuolar proteins from the secretory pathway. The other identified Mx homolog is rat dynamin (Obar et al., 1990). Dynamin forms periodic cross bridges between microtubules and converts them into highly organized bundles. The N-terminal region of about 300 amino acids is highly conserved among Mx1, VPS1 and dynamin. These suggest that the Mx1 protein might act during the transport of the parental virion nucleocapsids. To test this possibility, Mx<sup>+</sup> A2G embryo fibroblast cells were treated with IFN and infected with influenza virus in the presence of anisomycin. At two hours postinfection, the presence of both the Mx protein and of the parental nucleocapsids was determined

by double immunofluorescence. All cells expressing high levels of the Mx protein in the nucleus also contained parental virion nucleocapsids within the nucleus. The intensity of NP-specific rhodamine staining was indistinguishable from that observed in A2G cells not treated with IFN, indicating that the transport of parental virion nucleocapsids into the nucleus of A2G cells in the presence of the Mx protein was similar to the transport in its absence.

**Development of An Expression/Rescue System for Influenza Virus.** In recent years, great success in applying "reverse genetics" to the study of influenza virus has been achieved. Key to these experiments involves the ability to dissociate and purify the protein fraction of RNP and subsequently reassociate functional RNP using synthetic RNA. Briefly, the envelope of influenza virus is first disrupted with detergent and RNP is prepared by ultracentrifugation (Honda et al 1987; Parvin et al 1989). When this RNP complex is centrifuged through a CsCl-glycerol gradient, the viral RNA can be dissociated from the core proteins. RNA can then be added back to the core protein to reconstitute active RNP. The RNP proteins depleted of viral RNA are able to copy short cRNAs which are T7 transcript products of an appropriate plasmid DNA (Parvin et al., 1989). The termini of this RNA are identical to the 3' 15 nucleotides and the 5' 22 nucleotides conserved in segment 8 of influenza virus. These results indicate that the promoter lay solely within the 15 nucleotide of 3' terminus. The recognition and synthesis from this short cRNA were found to be specific for the promoter at the 3' terminal sequence and the panhandle

structure is not required.

Based on the promoter analysis, a system has been developed to study influenza virus replication and packaging (Luytjes et al., 1989). First, a DNA vector was used to produce an influenza virus-like RNA *in vitro*. The RNA product has the 3' and 5' noncoding region of the NS gene flanking the antisense sequences of a coding region of the chloramphenicol acetyl transferase (CAT) gene. When this synthetic RNA is mixed with purified polymerase to assemble ribonucleoprotein (RNP), complexes are formed which, after transfection, can be replicated and shown to express the CAT activity in influenza virus-superinfected cells. In addition, the influenza virus-like gene is packaged into the virus particle for several generations (Luytjes et al., 1989). These results indicated the 22 5' terminal and 26 3' terminal bases of influenza A virus are sufficient to provide the signals for RNA transcription, replication and packaging of RNA into influenza virus. Thus, this system allows analysis of the RNA signal such as transcription, replication, packaging (Luytjes et al., 1989), and polyadenylation (Luo et al., 1991). This approach is also open a new avenue to the construction of live attenuated viruses for use as vaccine (Enami et al., 1990; Muster et al., 1991). However, the need for superinfecting influenza virus makes it difficult to study the function of the viral proteins involved these processes. A major aim of this thesis is to refine this system so as to enable the study of the viral proteins.

**II**

**DETERMINATION OF INFLUENZA VIRUS PROTEINS  
REQUIRED FOR GENOME REPLICATION**

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### **Abstract**

**An artificial vaccinia vector driven replication system for influenza virus RNA has been developed. The minimum subset of influenza virus protein needed for specific replication and expression of the viral ribonucleoprotein (RNP) was found to be the three polymerase proteins (PB2, PB1 and PA) and the nucleoprotein (NP).**

The orthomyxoviruses are a group of enveloped viruses possessing a negative strand segmented RNA genome. The influenza A viruses contain eight RNA segments with more than half of the genome coding for proteins presumed to play active roles in the transcription and replication of the genomic RNA. The viral polymerase consists of a complex of three proteins (PB2, PB1 and PA) along with the nucleoprotein (NP), which is also involved in the assembly of the RNP molecule (Krug et al., 1989). It has been suggested that the two virus-encoded non-structural proteins, NS1 and NS2, play a role in replication due to their high level expression and their presence in the nucleus, which is the site of viral replication (Greespan et al., 1985, Krug et al., 1989, Lamb and Lai, 1984, Young et al., 1983). *in vitro* systems capable of transcribing mRNA from genomic RNP, in use for many years, have shown that the PB2 protein is responsible for binding to the cap structure of host mRNA and the PB1 protein is involved in elongation of mRNA molecules (Beaton and Krug, 1984, Blaas et al 1982, Blaas et al., 1982). However the slow development of *in vitro* replication systems and the limited availability of conditional lethal mutants has greatly hampered the ability to dissect the replication process. Limited knowledge stems from studies using virus-infected cell extracts (Beaton and Krug, 1984, Honda et al, 1988, Rio et al, 1985). From these studies, it has been shown that the presence of the nucleoprotein is required for full-length synthesis of the positive strand template and the subsequent copying of this template to create genomic vRNA (Beaton and Krug, 1986, Shipiro et al., 1987).

In recent years, vaccinia virus vectors have been developed to express foreign proteins for immunological studies and to explore structure-function relationships of specific proteins (Moss, 1988, Piccini and Paoletti, 1988, Piccini et al., 1987). Recombinant vaccinia vectors have also been used to express mixtures of viral proteins in an attempt to study virus replication, assembly and growth (Gotoh et al., 1989, Karacostas et al., 1989, Pattnaik and Wertz, 1990). In this report, mixtures of recombinant vaccinia viruses expressing individual influenza virus proteins have been used to artificially assemble a functional and specific replication system for influenza virus RNA. Previously, we have described an assay for the transcription, replication and rescue of a synthetic NS gene-like RNA or an intact viral gene in influenza virus-infected cells (Enami et al., 1990, Luytjes et al., 1989). In this system, synthetic RNA is added to purified viral polymerase to assemble viral-like RNP molecules (Parvin et al, 1989). These particles are then transfected into influenza virus infected cells and subsequently replicated by the proteins produced by the super-infecting influenza virus. Although this system is competent for the study of the various replication and packaging signals present in the viral RNA (through site-specific mutagenesis of the synthetic RNA) the need for super-infecting influenza virus makes it difficult to examine the functions of the individual proteins during replication. Here, we describe a completely artificial replication system where, rather than infecting cells with influenza virus, synthetic RNPs are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia vectors. In this way we show that the only influenza virus proteins essential for transcription and replication

of the template RNP are the three polymerase proteins and the nucleoprotein.

In order to facilitate analysis, a cDNA clone which can produce an influenza virus-like vRNA molecule coding for a reporter gene was used. This NS-like vRNA contains the antisense of the coding region of the chloramphenicol acetyltransferase gene (CAT) in place of the antisense coding regions for the nonstructural proteins, NS1 and NS2 (Luytjes et al., 1989). This recombinant RNA (*IVACAT-1*) was incubated with purified influenza virus RNP proteins and used in an attempt to develop a non-influenza virus dependent replication system. Mouse fibroblast C127 cells were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1987) and transfected one hour later with the *IVACAT-1* RNP. Mixtures of vectors expressing the three polymerases (PB2-VAC, PB1-VAC and PA-VAC) and the nucleoprotein (NP-VAC) were used. Replication and transcription of the synthetic RNP was assayed by analyzing cells for CAT activity after overnight incubation. Figure 5 examines the CAT activity present in cells initially infected with many of the possible mixtures of the 4 recombinant vaccinia viruses. Figure 5, lane 4 is a positive control in which the influenza A/WSN/33 virus was used in lieu of the recombinant vaccinia viruses. CAT activity is present in this sample as well as in cells infected with all four vaccinia vectors (Figure 5, lanes 8 and 10). Cells expressing any of the subsets of these four proteins did not produce detectable CAT protein (Figure 5, lanes 5-7,9,11). In addition, transfected RNA not incubated with the purified polymerase was also negative for CAT expression (Lane 2). Thus, the presence of the PB2, PB1, PA and NP proteins are all that is necessary and sufficient for RNP

expression and replication in this system.

A number of other cell lines were then tested as hosts for this vaccinia virus driven system. These include MDBK, Hela, 293 and L cells. In each case, CAT activity was observed only when cells were infected with vectors that express the 3 polymerase proteins and the NP. (data not shown) Previously, a cell line (designated 3PNP-4) was constructed which constitutively expresses low levels of the PB2, PB1 and PA proteins and high levels of the NP protein. These cells can complement the growth of ts mutants mapping either to the PB2 or NP gene segments (Krystal et al., 1986, Li et al., 1989). Since replication through recombinant vaccinia virus vectors is dependent only on these proteins, it was conceivable that this cell line may be able to amplify and express the synthetic CAT-RNP in the absence of any virus infection. However, when this experiment was attempted, no detectable CAT activity was obtained (data not shown). In order to investigate the reasons why this cell line did not support replication, mixtures of recombinant vaccinia viruses were used to infect 3PNP-4 cells. As expected, the addition of the four polymerase proteins supported the expression of CAT (Fig. 6A, lane 2). Figure 6A, lane 3 shows that the minimum mixture of vectors needed to obtain CAT activity in 3PNP-4 cells are those expressing only the PB1 and PA proteins. Therefore, the steady state levels of PB2 and NP proteins in 3PNP-4 cells are sufficient but the levels of PB1 and PA are below threshold for CAT expression in the absence of helper virus. This correlates with the complementation phenotype exhibited by these cells, since only the growth of PB2 and NP mutants and not PB1 and PA mutants is significantly complemented

at non-permissive temperature (Li et al., 1989).

Since the synthetic *IVACAT-1* RNA is of negative polarity, CAT can only be synthesized via transcription off the RNP molecule. Theoretically, detectable levels of CAT can be produced either through transcription off the transfected input RNP (equivalent to primary transcription) or through transcription off amplified RNA (necessitating RNP replication). Previous work using influenza virus infection to drive the expression of the CAT protein showed that detectable expression occurred only if the input CAT-RNP was replicated (Luytjes et al., 1989). This was shown by the use of a second CAT-RNA, *IVACAT-2*, which contains 3 mutations within the 12 bases at the 5' end of the viral RNA (Luytjes et al., 1989). This 12 base region is conserved among all eight gene segments in all influenza A viruses (Desselberger et al., 1980). This synthetic *IVACAT-2* RNP is transcribed *in vitro* by the influenza virus polymerase but it is not replicated and when transfected into influenza virus-infected cells CAT activity remains undetected (Luytjes et al., 1989). Therefore, primary transcription off the input RNA does not produce detectable levels of protein in influenza virus infected cells. Accordingly, we used this mutant RNA to examine whether the vaccinia vector-expressed influenza proteins induce CAT activity solely through primary transcription of the input RNP or allow for amplification through replication and subsequent transcription. C127 cells were infected with the recombinant vaccinia viruses and then transfected with either *IVACAT-1* or *IVACAT-2* generated RNPs. Figure 6B shows that low levels of CAT activity can be detected in cells transfected with *IVACAT-2* RNP (lane 2). When quantitated, 0.5-1% of the

chloramphenicol is converted to an acetylated form, compared to 0.2-0.4% in mock transfected lanes (not shown). However, much greater levels of activity are present in cells transfected with CAT-1 RNP (lane 1; routinely 15-50% conversion of chloramphenicol), indicating that amplification is occurring in these cells. Additional evidence for RNP replication comes from use of another synthetic CAT molecule, designated *IVACAT-9* (Luytjes, unpublished). This RNA contains, in 5' to 3' order, the entire 5' noncoding region of the NS gene, the CAT coding region in the plus sense, a short linker sequence and the entire 3' noncoding region of the NS gene. Although transcribed *in vitro* off a separate cDNA, this RNA represents the cRNA replicative intermediate of *IVACAT-1* RNA. Though this RNA can theoretically express CAT protein by direct ribosome translation, when packaged into RNP and transfected into C127 cells, no activity is detected (Fig. 6C, lane 1). However, when transfected into cells where viral proteins are supplied the mixture of recombinant vaccinia viruses, CAT activity is highly induced (Fig. 6C, lane 2). This implies that the influenza viral polymerase proteins can amplify the expression of CAT, presumably by the continued replication of this cRNA template and subsequent transcription of the vRNA. These data together provide strong evidence that the recombinant vaccinia virus-driven system is sequence-specific and that the RNP's are undergoing replication.

In the experiments described, neither the NS1 nor NS2 proteins were required for RNP replication. Although their function is not known it has been speculated that these proteins may play a major role in replication because both proteins are

synthesized in large amounts and are present in the nucleus (Greespan et al., 1985, Lamb and Lai, 1984, Young et al 1983). Therefore, it was tested whether addition of one or both of these proteins would have an effect on the levels of CAT expressed from the synthetic NS-like *IVACAT-1*. When vaccinia vectors expressing either the NS1 or NS2 proteins (or the two together) are added to the functional mixtures of four vaccinia vectors, the level of CAT gene expression is not significantly altered, indicating that in this system these proteins have little if any effect on *IVACAT-1* replication and expression (Fig. 7). Since the above experiment now requires the coinfection of up to six separate vaccinia recombinants in a single cell, protein expression in these cells was analyzed. Cells were infected with five or six vaccinia recombinants and labelled with  $S^{35}$  met for 3 hours at 3 hours post infection. Extracts were analyzed for NS1 and NS2 expression by immunoprecipitation with polyclonal rabbit antibodies and examined by PAGE (Gotoh et al., 1989, Shapiro et al., 1987). Figure 7B lanes 3 and 4 shows that NS1 and NS2 respectively are expressed when coinfecting with recombinants PB2-VAC, PB1-VAC, PA-VAC and NP-VAC and lane 5 shows that NS1 and NS2 are both expressed when all six vectors are used to infect a single cell. Additional experiments have also shown that the four influenza virus polymerase proteins are expressed (not shown). Therefore, it may be speculated that the NS1 and NS2 proteins are not directly required for the replication of this NS-like RNP. Previous work with mutants in the NS gene indicated that NS1 may function in late viral gene expression, vRNA synthesis and/or RNA splicing (Buonagurio et al., 1984, Shimizu et al., 1983, Smith and Inglis, 1985,

Wolstenholme et al., 1980). It should be noted that the genotype of *IVACAT-1* is NS-like, which is preferentially synthesized early in infection and may not come under the same controls as genes expressed later in infection (Shipiro et al., 1987). Another possibility which can not be ruled out is that a vaccinia virus protein(s) can replace a necessary function of these NS proteins, although upon inspection, no obvious similarities were found between either the NS1 or NS2 proteins and known vaccinia virus proteins. The contrasting properties of these two viruses also argues against a complementing vaccinia virus protein, as vaccinia is a large double-stranded DNA virus replicating exclusively in the cytoplasm while influenza virus is a negative sense RNA virus replicating exclusively in the nucleus. In addition, the replication of the synthetic RNPs occurs even in the presence of cytosine arabinoside (ara-C, data not shown), an inhibitor of replication and late gene expression in vaccinia virus (Cooper and Moss, 1978, Kaverin et al., 1975, Oda and Joklik, 1967).

This recombinant vaccinia vector dependent scheme possesses a number of advantages over the use of influenza virus infection to drive the replication of synthetic RNA. For one, since the expression of the viral proteins is completely artificial it will allow for a precise dissection of the processes involved in replication. Replication first involves the synthesis of positive sense template from the negative sense genomic RNA. This positive sense cRNA is then copied in order to amplify genomic sense RNP, which is then used for secondary transcription and/or packaging (Krug et al., 1989). The system described herein demonstrates that only the influenza viral PB2, PB1, PA and NP proteins are required for the expression and

replication of RNP. Another advantage of this vaccinia vector driven replication scheme is that since the influenza polymerase proteins are expressed from cDNA integrated into the vaccinia virus, the mutagenesis of the polymerase proteins becomes a feasible and powerful method to further analyze structure-function relationships of the viral polymerase proteins. Also, we are currently attempting to rescue infectious influenza virus through the transfection of mixtures of reconstituted viral RNPs and the use of vaccinia vectors as helper viruses.

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## FIGURE LEGENDS

**Fig. 5.** CAT expression in vaccinia virus-infected/*IVACAT-1* RNP transfected cells. Approximately  $10^6$  mouse C127 cells in 35mm dishes were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1987) at an M.O.I. of 5-10 for each vector. After 1.5 hours, synthetic *IVACAT-1* RNP was transfected into the virus-infected cells as described (Luytjes et al., 1989). Cells were incubated overnight, harvested and assayed for CAT activity according to standard procedures (Gorman et al., 1982). The assays contained .05 uCi [ $^{14}$ C] chloramphenicol, 20 ul of 40 mM acetyl-CoA (Boehringer) and 50 ul of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 hours. The labels under the lane numbers indicate the treatment of cells. Lanes 1-control; 2-naked RNA transfection (no polymerase added), no helper virus infection; 3-RNP transfection, no helper virus; 4-RNP transfection, influenza virus as helper; Lanes 5-11- RNP transfection, vaccinia virus vectors as helper viruses express the indicated influenza virus proteins.

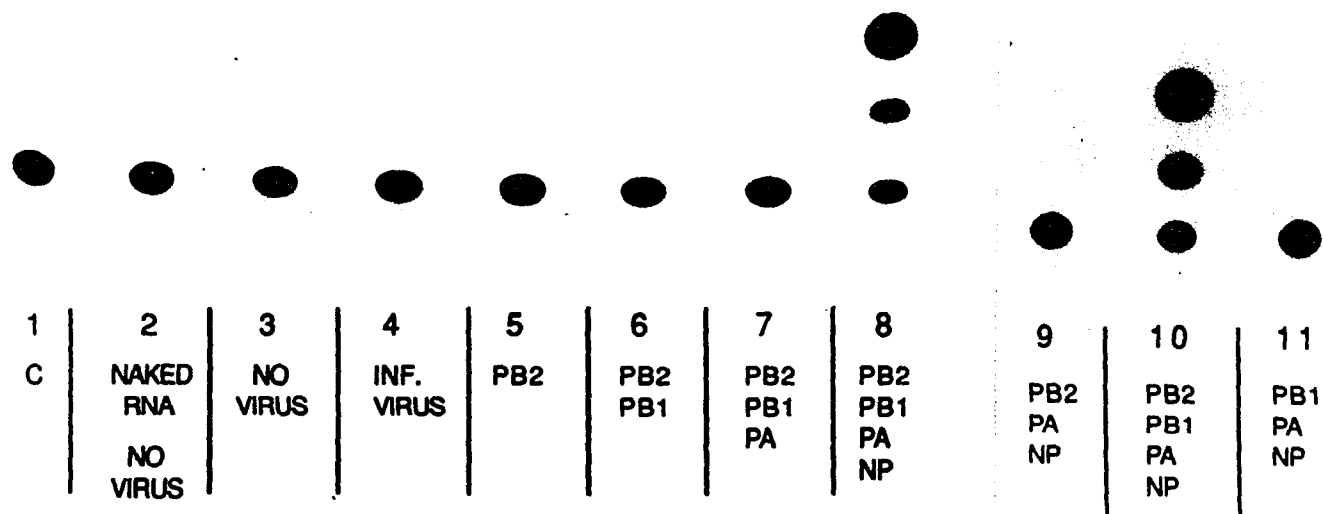


**Fig. 6.** A) 3PNP-4 cells were infected with vaccinia vectors expressing the PB2, PB1, PA and NP proteins (Lane 2) or various subsets of the four vectors, transfected with *IVACAT-1 RNP* and examined for CAT activity as described. Shown under each lane is the influenza viral proteins expressed in each sample. B) 293 cells were infected with the four required vaccinia vectors and transfected with synthetic RNP made using IVA-CAT-1 (lane 1) or IVA-CAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assays were performed. C) Recombinant vaccinia virus-infected C127 cells were transfected with synthetic RNP examined for CAT activity after overnight incubation. Lane 1: IVA-CAT-9 RNP transfected into uninfected cells. Lane 2: IVA-CAT-9 RNP transfected into cells infected with the four recombinant vaccinia vectors. Lane 3: IVA-CAT-1 RNP transfected into cells infected with the four recombinant vaccinia vectors.

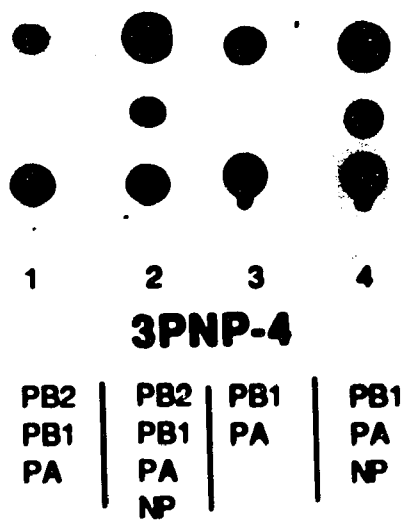
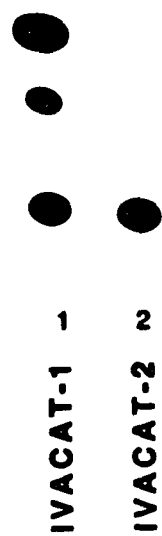
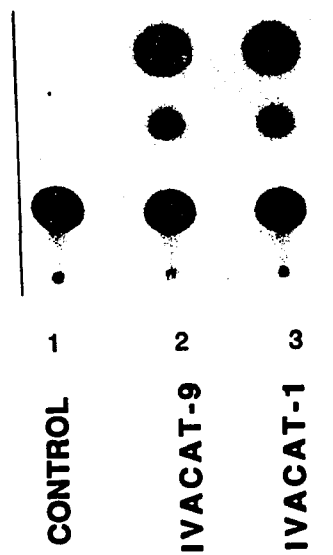
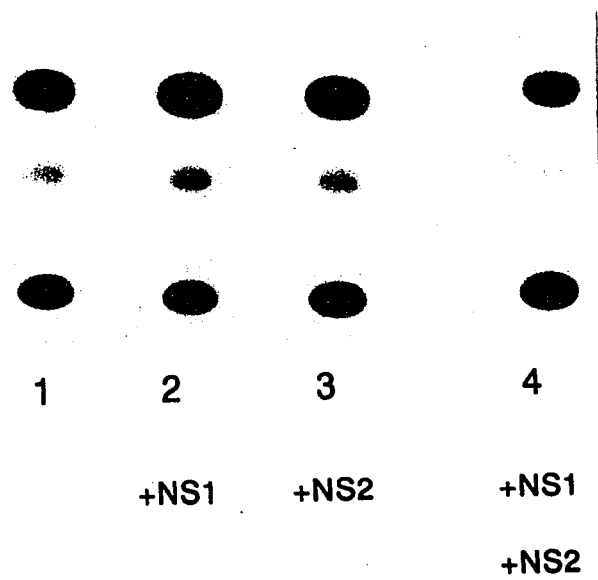
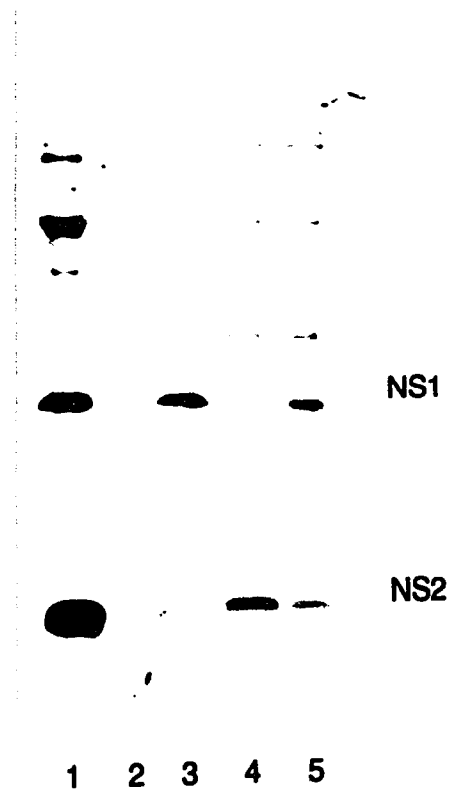
**A****B****C**

**Fig. 7.** NS1 or NS2 proteins do not alter expression levels of the NS-like CAT construct. **A)** C127 cells were infected with mixtures of vaccinia vectors, transfected with IVA-CAT-1 RNP and assayed for CAT activity after overnight incubation. Cells were infected with vectors expressing the following influenza virus proteins: Lane 1; PB2, PB1, PA and NP. Lane 2; PB2, PB1, PA, NP and NS1. Lane 3; PB2, PB1, PA, NP and NS2. Lane 4; PB2, PB1, PA, NP, NS1 and NS2. **B)** PAGE analysis of the expression of NS1 and NS2 in vector-infected cells. C127 cells in 35mm were infected Vac-PB2, Vac-PB1, Vac-PA, Vac-NP and either Vac-NS1, Vac-NS2 or the wild type WR vaccinia strain. Cells were analyzed for NS1 or NS2 expression by immunoprecipitation and electrophoresis on a 10% polyacrylamide gel. Lane 1; influenza virus infection control. Lane 2; the four vaccinia vectors plus WR strain. Lane 3; the four vectors plus Vac-NS1. Lane 4; the four vectors plus Vac-NS2. Lane 5; the four vectors plus Vac-NS1 and Vac-NS2.

**A****B**

### **III**

## **EXPRESSION OF IVACAT-1 RNP IN A STABLE Mx1 CELL LINE CO-INFECTED WITH RECOMBINANT VACCINIA VIRUSES EXPRESSING INFLUENZA VIRAL PROTEINS**

## ABSTRACT

The murine Mx1 protein is an interferon-inducible protein which confers selective resistance to influenza virus infection both *in vitro* and *in vivo*. The precise mechanism by which the murine Mx1 specifically inhibits replication of influenza virus is not known. Previously, sensitive replication systems for influenza virus ribonucleoprotein (RNP) have been developed, in which a synthetic influenza-like RNP is replicated and transcribed by influenza virus proteins provided *in trans*. Using these systems, the antiviral activity of the murine Mx1 protein was examined. IVACAT-1 RNP was expressed in Mx1-expressing cells co-infected with recombinant vaccinia viruses expressing influenza polymerase polypeptides (PB1, PB2, and PA) and NP.

## INTRODUCTION

Interferon treatment of cells will usually result in an antiviral state against a large number of viruses. Although not well characterized for most viruses, there are clearly multiple inhibitory pathways by which it could act on any given virus. For influenza virus, a proven pathway involves the interferon inducible murine Mx1 protein, which is a 72 Kd nuclear protein (Dreiding et al., 1985; Horisberger et al., 1983). This Mx1 protein has been shown to mediate specific resistance to influenza virus (independent of other interferon mediated effects) both *in vitro* and *in vivo* (Arnheiter et al., 1990; Krug et al., 1985; Pavlovic et al., 1990; Staeheli et al., 1984; Staeheli et al., 1986).

The mechanism by which the murine Mx1 protein selectively inhibits the replication of influenza virus is unclear. Initially, it was reported that treatment of macrophages obtained from Mx<sup>+</sup> mice with IFN-alpha/beta prior to influenza virus infection led to the inhibition of translation, but not the synthesis of viral mRNAs in the nucleus (Meyer and Horisberger, 1984). However, this observation has been controversial and conflicting data have also been reported. In another study, it was shown that when Mx<sup>+</sup> mouse embryo fibroblasts were treated with IFN-alpha/beta, viral mRNA synthesis in the nucleus was severely inhibited at the level of primary transcription (Krug et al., 1985). Furthermore, using transfected cell lines expressing

Mx1 protein in a constitutive manner, a strong inhibitory effect on influenza virus primary transcription was observed (Pavlovic et al., manuscript in preparation).

The ability to reconstitute active influenza virus RNP from purified proteins and synthetic RNA has contributed to new approaches in the study of negative strand RNA viruses (Parvin et al., 1989). The use of a synthetic viral-like RNA encoding the chloramphenicol acetyltransferase (CAT) gene allows for sensitive and quantitative estimates of replication and gene expression when introduced into virus-infected cells (Luo et al., 1991; Lutjes et al., 1989). Previously, we extended this system by establishing an artificial replication system, in which the synthetic model CAT gene is replicated and expressed by influenza virus proteins supplied *in trans* via infection with vaccinia virus recombinant vectors (Huang et al., 1990). In the present study, these systems have been used to examine the antiviral activity of the murine Mx1 in cells constitutively expressing different steady state levels of these proteins (Pavlovic et al., 1990). Use of recombinant vaccinia virus expressed proteins as helper permits a more detailed examination of the antiviral mechanism of the murine Mx1 protein. We found that IVACAT-1 RNA was expressed in Mx1 expressing cells co-infected with recombinant vaccinia viruses expressing the influenza virus polymerase proteins (PB2, PB1 and PA) and NP.

## **MATERIALS AND METHODS**

**Viruses and Cells.** Influenza A/PR/8/34 was grown in the allantoic cavity of 10-11 days old embryonated chicken eggs (Suguria and Ueda, 1980). Influenza A virus strain A/WSN/33 was grown and titrated in Maden-Darby bovine kidney (MDBK) cells. The stock of the FPV-B strain (Isal, 1978) of influenza A virus ( $3 \times 10^8$  PFU/ml) was prepared from supernatant of virus infected Swiss 3T3 cells (Pavlovic et al., 1990). Virus yield reduction assays were performed as previously described (Pavlovic et al., 1990). Recombinant vaccinia viruses Vac-PB2, Vac-PB1, Vac-PA, Vac-NP and VTF3 (Fuerst et al., 1980; Smith et al., 1986) were propagated in monolayer Hela cells and titrated in CV-1 cells. Transfected Swiss 3T3 cells constitutively expressing the murine Mx1 protein and control cells lacking Mx proteins were previously described (Horisberger et al., 1990; Pavlovic et al., 1990) or produced using the same technology. These cell cultures were passaged in 10% fetal serum-supplemented Dulbecco modified essential medium (DMEM) with 400 ug/ml of G418 as described (Pavlovic). Mouse embryo cells of Mx<sup>+</sup> phenotype were maintained as previously described (Staeheli et al., 1986).

**Interferon (IFN) and IFN treatment.** Mouse IFN- $\alpha/\beta$  (specific activity  $4.7 \times 10^5$  units per mg protein) was purchased from Lee Biomolecular (San Diego, California). Mx<sup>+</sup> mouse embryo fibroblasts were treated with IFN at a final concentration 1000 U per ml in DMEM containing 10 % fetal calf serum. The cells

were incubated for 15 hours before transfection of synthetic RNP and subsequent influenza virus infection.

**Preparation and transfection of Synthetic RNP.** Influenza virus core proteins were purified through a series of glycerol and CsCl/glycerol gradients (Parvin et al., 1989). This resulted in a pool of viral proteins consisting mainly of the NP protein along with minor amounts of active polymerase (PB2, PB1 and PA). Synthetic viral-like RNA encoding the CAT gene was generated through run-off transcription by bacteriophage T7 polymerase off HgaI digested pIVACAT-1 plasmid in standard reaction conditions (Huang et al., 1990; Luty et al., 1989).

The appropriate amounts of viral protein needed to assemble RNP was determined empirically. Synthetic RNP was assembled and transfected via calcium phosphate precipitation. In a typical experiment, 1  $\mu$ g RNA was suspended in 100  $\mu$ l 0.25 M  $\text{CaCl}_2$  along with 10  $\mu$ l NP/polymerase preparation and 2  $\mu$ l (60 units) RNasin (Promega). This solution was mixed with an equal volume 2x HeBS (350 mM NaCl, 100 mM Hepes, 1 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.05) and allowed to sit at room temperature for 20 minutes before addition to cells. Cells were left at room temperature for 15 minutes, and then 2 ml of appropriate growth medium was added. Following a 4 hour incubation at 37°C, cells were infected with either influenza virus or with recombinant vaccinia viruses (moi = 3 for each vector) and incubated at 37°C overnight. Cells were harvested and equivalent amounts of viral proteins (Lowry et al., 1951) were assayed for CAT activity via standard procedures (Gorman et al., 1982).

**Immunoprecipitation analysis.** Confluent monolayer cells were infected with recombinant vaccinia viruses at an m.o.i of approximately 3. At 3 hour post-infection, medium was removed, cells were washed twice with PBS and then labeled with 100 uCi of  $^{35}\text{S}$ -methionine in methionine-free medium. Following two hours of incubation at 37°C, cells were harvested and lysed in lysis buffer ( 0.5% NP-40, 25 mM DOC in PBS). Corresponding antibodies were incubated with the sample in Buffer A (190 mM NaCl, 50 mM Tris-Cl, (pH 7.4), 6 mM EDTA, 2.5% Triton X-100, 1-2 mM PMSF, 0.1% BSA and 1.5 mg/ml methionine) at 4°C overnight. Protein A sepharose beads (Sigma) were added to each sample and shaken for an additional 1.5-3 hours at 4°C. The protein-A sepharose beads were washed three times with buffer B ( 150 mM NaCl, 0.1% Triton X-100, 100 mM Tris-Cl (pH 8.3), 5 mM EDTA, 1-2 mM PMSF, 0.1% BSA, 1.5 mg/ml methionine) and once with PBS. After treatment with iodoacetamide at 37°C for 20 minutes, the samples were boiled in SDS loading buffer and resolved by 10% SDS-PAGE. The gel was fixed in glacial acetic acid, treated with 22% PPO in glacial acetic acid and dried before autoradiography was performed at -70°C.

**Western blot analysis.** Protein extracts were prepared by lysing cells in protein gel sample buffer (Laemm et al., 1970). Samples (200  $\mu\text{g}$  of protein per lane) were electrophoresed through 10% polyacrylamide gels, and the proteins transferred to nitrocellulose membranes. Immunostaining was carried out as previously described (Aebi et al., 1989) using AP5 antiserum (Meier et al., 1988). Bound antibodies were visualized with 0.1% of peroxidase-conjugated protein A.

## RESULTS

**Examination of the antiviral activity of the murine Mx1 protein .** In order to study the mechanism of inhibition by Mx1 at the molecular level, we have used a system in which a model RNA gene segment encoding the bacterial CAT gene (IVACAT-1) is assembled into RNP and transfected into influenza virus infected cells. The infecting influenza virus proteins provide help *in trans*, as the synthetic RNP is expressed, replicated and packaged into infectious virions (Luytjes et al., 1989). Monolayer Mx<sup>+</sup> fibroblasts originating from A2G-Mx mice (Staeheli et al., 1984) were left untreated or treated with 1000 U per ml of mouse alpha/beta interferon for 15 hours to induce the expression of the Mx1 protein. The cells were then transfected with reconstituted IVACAT-1 RNP through standard calcium phosphate transfection followed by influenza virus infection 2 hours later. Replication and expression of the transfected synthetic RNA was assayed by analyzing cells for CAT activity after overnight incubation. Figure 8A, (lane 1) shows that Mx<sup>+</sup> cells not treated with IFN were able to express significant levels of CAT activity, whereas the same cells pretreated with IFN do not support the expression of CAT (lane 2).

Recently, we have developed an artificial replication system in which recombinant vaccinia vectors expressing the influenza virus PB2, PB1, PA and NP

proteins can substitute for helper function previously supplied through influenza virus infection (Huang et al., 1990). Since the murine Mx1 protein is specific only for influenza virus (Pavlovic et al 1990; Staeheli et al., 1986), the influenza viral proteins expressed by these vaccinia recombinants should not be inhibited and would be expected to continually be transcribed and translated in the presence of the Mx1 protein. The possible effects of this continued expression on Mx1 activity can then be assayed by examining the expression of the synthetic CAT-RNP. In the absence of IFN treatment, the four vaccinia vectors supported CAT expression after transfection in Mx<sup>+</sup> and Mx<sup>-</sup> cells (data not shown). However, when the transfection/infection experiment was repeated in the presence of IFN treatment neither Mx<sup>+</sup> nor Mx<sup>-</sup> cells generated CAT activity (data not shown). This inhibition of RNP expression is presumably caused by IFN-induced inhibition of vaccinia virus.

In an effort to obviate the need for interferon induction, stable cell lines constitutively expressing the Mx1 proteins were used in subsequent experiments. We previously described the production of such cell lines (Pavlovic et al., 1990) which varied with respect to Mx1 expression (Fig. 9A) The degree with which each cell line inhibits influenza virus correlates with the level of Mx1 protein present (Fig. 9). When reconstituted IVACAT-1 RNP particles were transfected into these Mx1-expressing cell lines and subsequently infected with influenza virus, no detectable CAT activity was obtained (Figure 8B, lane 2,3). Thus, these cells behaved like cells cultured from Mx<sup>+</sup> mice treated with IFN (Figure 8A, lane 2). When the control cell

line (6.1), was used as host (Figure 8B, lane 1) high CAT activity was observed.

**IVACAT-1 RNP was expressed in Mx1-expressing cells infected with recombinant vaccinia viruses expressing influenza polymerase proteins and NP**

Since the transformed 3T3 cell lines express Mx1 protein in a constitutive fashion in the absence of interferon (Fig. 9A), these cells may be suitable hosts for the vaccinia driven influenza virus replication system. Murine Mx1-expressing and control cell lines were transfected with IVACAT-RNP and subsequently infected with recombinant vaccinia viruses expressing the PB2, PB1, PA and NP protein. As can be seen in Figure 10A lanes 2-4, all three Mx1 expressing cells can now support the replication and subsequent expression of RNP mediated CAT protein. However, the three cell lines still exhibit a certain degree of inhibition compared to control cells (lane 1). Also, the three cell lines consistently exhibit different levels of CAT activity compared to each other, with cell line 10.2 always displaying the lowest CAT activity, while cell line 23.3 displays slightly higher activity than cell line 27.2. Thus, this observation correlates well with the steady state levels of Mx1 and the reduction of inhibition of virus titer obtained after influenza virus infection. Cell line 10.2 exhibits the highest level of Mx1 expression and the most elevated degree of inhibition of virus growth titers while cell line 27.2 exhibits the least inhibition (Figure 9). To rule out the possibility that the variation in CAT activity between control and Mx1 expressing cell lines is due to differences in vaccinia virus-mediated expression of influenza virus polypeptides, these proteins were examined by

immunoprecipitation analysis. The murine Mx1 protein expressing cell line (23.3) and the control cell line (3T3 SV2neo 6.1) were infected with the mixture of 5 PFU each of the recombinant vaccinia vectors expressing the PB2, PB1 and PA proteins. Cell extract was incubated with antibody raised against the influenza virus PB1 protein and immunocomplexes were precipitated and analyzed in a 10 % PAGE-SDS gel. As shown in Figure 10B, these two cell lines produce similar amounts of the influenza virus polymerase proteins through infection by the recombinant vaccinia vectors. In addition, since the three polymerase proteins co-precipitate with antibody against PB1, the complex of the PB2, PB1 and PA proteins can be formed in these Mx1 expressing cells.

Although control cells (6.1) and the Mx1-expressing cells were all derived from Swiss 3T3 cells, it was formally possible that clonal variation may account for some of the results presented above. We had previously shown that different cell lines can vary in their ability to replicate and express the synthetic viral-like RNP and that this may correlate with transfection efficiency (Huang et al., 1990). To exclude this possibility, relative transfection efficiencies were measured using a plasmid containing the CAT gene downstream (in the sense orientation) of a T7 promoter. After transfection with this plasmid, these cells were then infected with 5 PFU/cell of a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (VTF-3)(Fuerst et al.,1986). The T7 polymerase expressed by the recombinant vaccinia virus should allow for the expression CAT protein off the transfected plasmid. Thus, relative transfection efficiencies can be measured via measurements

of CAT activity. As shown in Figure 10C, all cell lines behaved similarly. In dilution experiments, the variation seen between any two lines was less than 10 percent (data not shown).

**Does Mx1 act on the influenza viral proteins ?** As described above, the murine Mx1 protein normally does not allow for expression of the synthetic CAT-RNP. Even when the recombinant vaccinia virus system was used to express viral proteins, CAT activity was inhibited to a certain extent (Figure 10A). This suggested that Mx1 acts on the viral proteins required for IVACAT-1 expression. Therefore, the artificial replication system was next used to determine which of the viral protein(s) is possibly targeted by Mx1. Influenza virus (WSN strain) was used along with single recombinant vaccinia vectors to co-infect the Mx1-expressing cell line 23.3 which had been previously transfected with IVACAT-1 RNP. By itself, WSN virus would not provide enough helper proteins to enable detection of CAT activity in Mx1-expressing cells (see Figure 8B). Alternatively, infection of cells by less than the full complement of vaccinia recombinant viruses expressing the PB2, PB1, PA and NP proteins would not support replication and expression of RNP (Huang et al., 1990). However, CAT activity is present in 23.3 cells with simultaneous infection of the three recombinant vectors expressing PB2, PB1 and PA (Figure 11, Lane 6). In addition, a low CAT activity was also obtained when the recombinant vector expressing the PB2 protein was used in co-infection experiments with WSN virus (Lane 3).

Additional experiments were performed to determine if IVACAT-1 RNP was

expressed in 23.3 cells co-infected with WSN and recombinant vaccinia in other combinations. Confluent monolayers of the Mx1-expressing cell line 23.3 were transfected with the reconstituted IVACAT-1 RNP and co-infected 4 hour later with influenza A virus (WSN) and recombinant vaccinia viruses expressing the three polymerase (PB2, PB1, and PA) and NP in a variety of combinations. Expression of IVACAT-1 RNP was assayed by analyzing cells for CAT activity and results were quantitated through scintillation counting of reaction products. The results are shown in Figure 12A. A positive control (lane 17) in which cell line 6.1 was used in lieu of the Mx-expressing cells and a negative control, where 23.3 cells were infected with a recombinant vaccinia vector expressing the influenza hemagglutinin (lane 1) were also included. It seems that significant CAT activity (8-12 fold increase over lane 1) was only present in those cells co-infected with WSN and any combination including Vac-PB2 (Figure 12A Lanes 3, 6, 9, 10, 12, 13, 15, 16). Cells co-infected with WSN and other recombinant vaccinia vectors did not produce such a notable CAT activity (2-3 fold at best), irrespective of mixtures used. However, CAT activity was much higher in cells infected with vaccinia vectors expressing complete polymerase complexes (PB2, PB1 and PA; lanes 12, 16) than incomplete PB2-containing mixtures.

Since the PB2 protein might be a limiting factor in influenza virus infected cells, the general effect of overexpression of individual viral proteins during normal influenza virus infection was examined. The 6.1 cell line (Mx<sup>-</sup>) was transfected with reconstituted IVACAT-1 RNP and infected with WSN and a recombinant vector

expressing either of PB2, PB1, PA, NP or HA. The cells were then assayed for CAT expression after overnight incubation. Dilution cellular extract (1:50) were used so that CAT activity was measured in the linear range. As shown in Figure 12B, overexpression of PB2 resulted in a 2 - 3 fold increase in CAT activity over vac-HA infected cells while overexpression of PB1 and PA showed no effect.

## DISCUSSION

The development of reverse genetics for the study of negative strand viruses has opened new avenues for the molecular analysis of these viruses (Luytjes et al., 1989; Park et al., 1991; Parvin et al., 1989). In terms of influenza virus this has allowed for the examination of cis-acting sequences within the genome (Luo et al., 1991; Parvin et al., 1989), the study of the viral proteins needed for replication (Huang et al., 1990) and the construction of new viruses (Enami et al., 1990). A feature of this influenza virus system is the use of a synthetic RNP molecule coding for the marker CAT gene. In this chapter, we use these systems to analyze the mechanism of inhibition of a specific inhibitor of influenza virus, the murine Mx1 protein. Although the antiviral activity of the interferon-inducible murine Mx1 protein to influenza virus has been documented for decades, the mechanism is still poorly understood. The murine Mx1 protein does not affect attachment or penetration of influenza virus as parental virion nucleocapsids are efficiently transported into the nucleus in the presence of the murine Mx1 protein (Broni et al., 1990). Previous work has suggested that the murine Mx1 protein inhibits the accumulation of primary transcripts off the parental nucleocapsid (Krug et al., 1985; Pavlovic et al, in preparation).

We first examined whether IVACAT-1 RNP could be expressed in Mx-expressing cells co-infected with influenza virus and recombinant vaccinia virus expressing the influenza viral proteins. Since the murine Mx1 protein is specific for influenza virus

and does not inhibit the expression of vaccinia virus proteins, recombinant vaccinia virus vectors expressing influenza virus proteins were employed (smith et al., 1987). Stable cell lines constitutively expressing the murine Mx1 were used to avoid the interferon stimulated but non-Mx related inhibition of vaccinia expression. IVACAT-1 RNP was indeed able to express in Mx1-expressing cells co-infected with recombinant vaccinia viruses expressing the three influenza virus polymerase and NP. However, the concentration-dependent decrease in CAT activity was observed in Mx1 expressing vs. control cell lines (Figure 10) as the levels of CAT activity observed in transfected/infected Mx1 cell lines were inversely proportional to the levels of Mx1 present in each cell line.

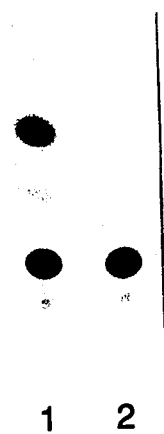
Further studies of cells co-infected with WSN and single vaccinia vectors showed that a low CAT activity was expressed in Mx-expressing cells co-infected with WSN and recombinant vaccinia virus expressing PB2. These results suggest that the PB2 protein is a possible target of Mx1. We can not, however, exclude the possibility that this effect is at least partially nonspecific since the PB2 protein by itself could boost expression of IVACAT-1 RNP in Mx- cells as shown in Figure 12B. In Mx cells, PB2 can stimulate CAT expression by 2-3 fold (Figure 12B) whereas in the 23.3 cell line, expression is stimulated 8-12 fold over other samples. However, we still can not rule out that the PB2 protein might just upregulate the system. In addition, cells expressing the three polymerases (Fig. 12A, lane 12) or the three polymerases and NP (Fig. 12A, lane 16) were able to produce CAT activity at much higher levels than those cells expressing subsets containing the PB2 protein (lanes 3,6,9,10,13,15). This

effect could be due to the fact that there are more active polymerase complexes present in cells infected with vectors expressing the three polymerase proteins than in Vac-PB2 expressed cells alone (Figure 12A, lanes 3,6,9,10,13,15). Additionally, experiments, such as a quantitative CAT assay, are needed to confirm the effect of PB2. In addition, the recombinant-vaccinia virus assay used in these studies can only examine questions of transcription or replication of viral RNP. It does not address other important aspects of viral infection, such as gene regulation, RNP transport or virus assembly. Therefore, it is possible that the Mx1 protein may act on later stages of infection as well. Finally, it should be kept in our mind that in Mx+ cells co-infected with influenza virus and recombinant vaccinia virus expressing the three influenza viral polymerase proteins, NP protein might be included by helper influenza virus or/and by purified polymerase used to constitute synthetic IVACAT-1 RNP. It is clear that further studies are needed to elucidate the molecular mechanism of antiviral activities by the Mx1 protein.

## FIGURE LEGENDS

**Figure 8A.** The murine Mx1 protein inhibits expression of IVACAT-1 RNP. 80% confluent monolayer Mx<sup>+</sup> cells (A2G mouse embryo fibroblast cells) were used for interferon induction and subsequent transfection/infection as described in the text. After overnight incubation, cells were harvested and assayed CAT activity by standard procedures (Gorman et al., 1982). The assays contained 0.05 uCi of <sup>14</sup>C chloramphenicol, 20 ul of 40 mM acetyl coenzyme A and cell extract in 0.25 M Tris-HCl buffer (pH 7.5). Incubation times were 2 hours. Lane 1; untreated cells: Lane 2; treated with IFN **B)** Control 6.1 cells (lane 1) and Mx1 expressing cell lines 23.3 and 27.2 (lanes 2 and 3 respectively) were transfected with IVACAT-RNP and infected with WSN at an m.o.i. of 3. CAT activity was examined as described in the text.

**A**



**B**

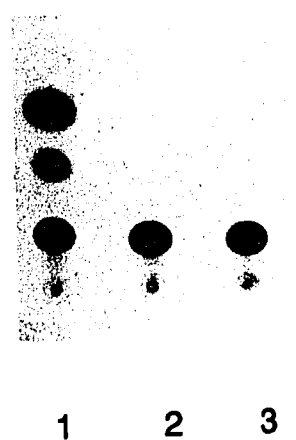


**Figure 9** **A)** Western blot analysis of Mx1 protein in transfected Swiss 3T3 cells. Protein extracts of the indicated Mx1 expressing cells lines were subjected to Western blot analysis using AP5 antiserum (Meier et al., 1988). Protein extracts of control (3T3-neo; the 6.1 cell line) and BALB.A2G-Mx mouse embryo fibroblasts (MEF) treated with IFN (+) or left untreated (-) were included as controls. **B)** Inhibition of influenza virus multiplication by Mx1 protein. The indicated cell lines were infected with 1 PFU of influenza virus (FPV-B) per cell. Samples of the culture supernatant were removed 24 hr postinfection and the viral titers were determined by the 50% tissue culture infective dose method.

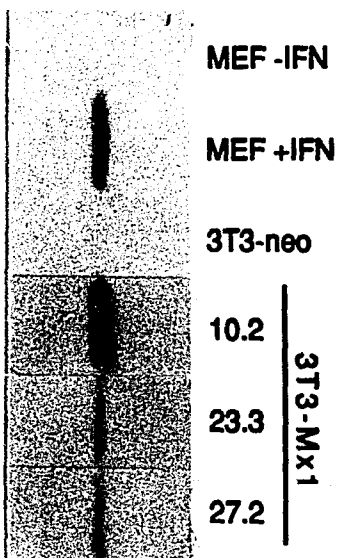
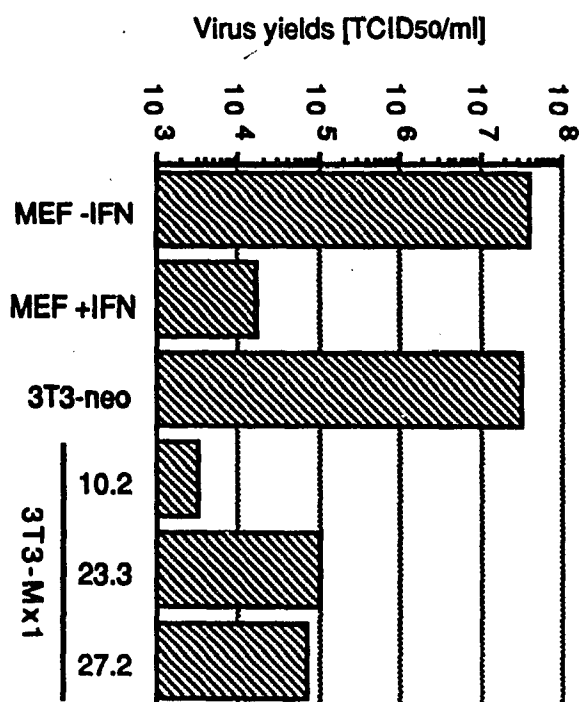
**A****B**

**Figure 10.** Expression of IVACAT-1 RNP in Mx1-expressing cells infected with recombinant vaccinia viruses expressing influenza viral polymerases and NP. A) Control 6.1 and Mx 1-expressing cell lines 3T3 23.3 (Lane 2), 27.2 (Lane 3) and 10.2 (Lane 4) were transfected with IVACAT-1 RNP and infected with the mixture of recombinant vaccinia viruses expressing the three polymerase and NP proteins. After overnight incubation, CAT activity was assayed after 2 hours incubation at 37°C. B) 6.1 and 23.3 cells were infected with the mixture of recombinant vaccinia vectors expressing the influenza viral proteins PB2, PB1, and PA. Cells were analyzed for expression of these proteins by immunoprecipitation with antibody to PB1 and electrophoresis on a 10% PAGE-SDS gel. C) Cells in 35 mm dishes were transfected with 2 ug of pIVACAT-9 DNA (10) by standard calcium phosphate transfection and infected with VTF-3 at an M.O.I. of 3 PFU per cell. The CAT activity was assayed after overnight incubation.

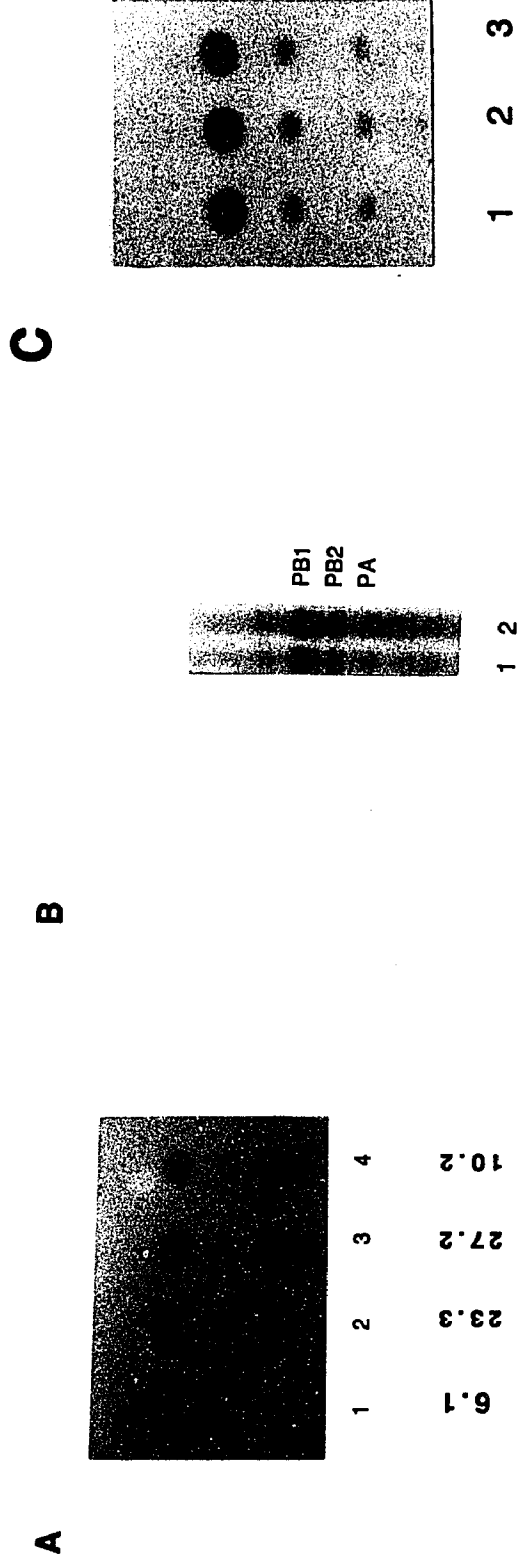
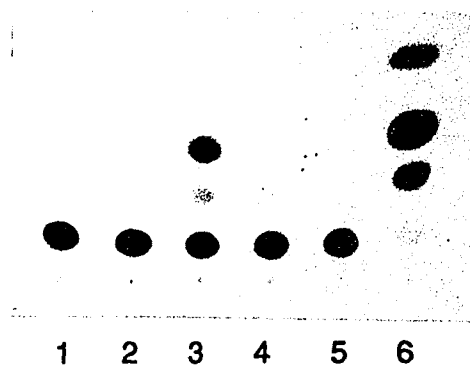


**Figure 11.** IVACAT-1 expression in the Mx1 protein expressing cells. The Mx1 protein expressing cells, 23.3, was transfected with IVACAT-1 RNP. 4 hours later, cells were co-infected with WSN and recombinant vaccinia virus expressing either HA (Lane 1), NP (Lane 2), PB2 (Lane 3), PB1 (Lane 4), PA (Lane 5) or the three polymerase proteins (Lane 6). Cells were incubated overnight and CAT activity examined assayed after a 2 hour incubation.



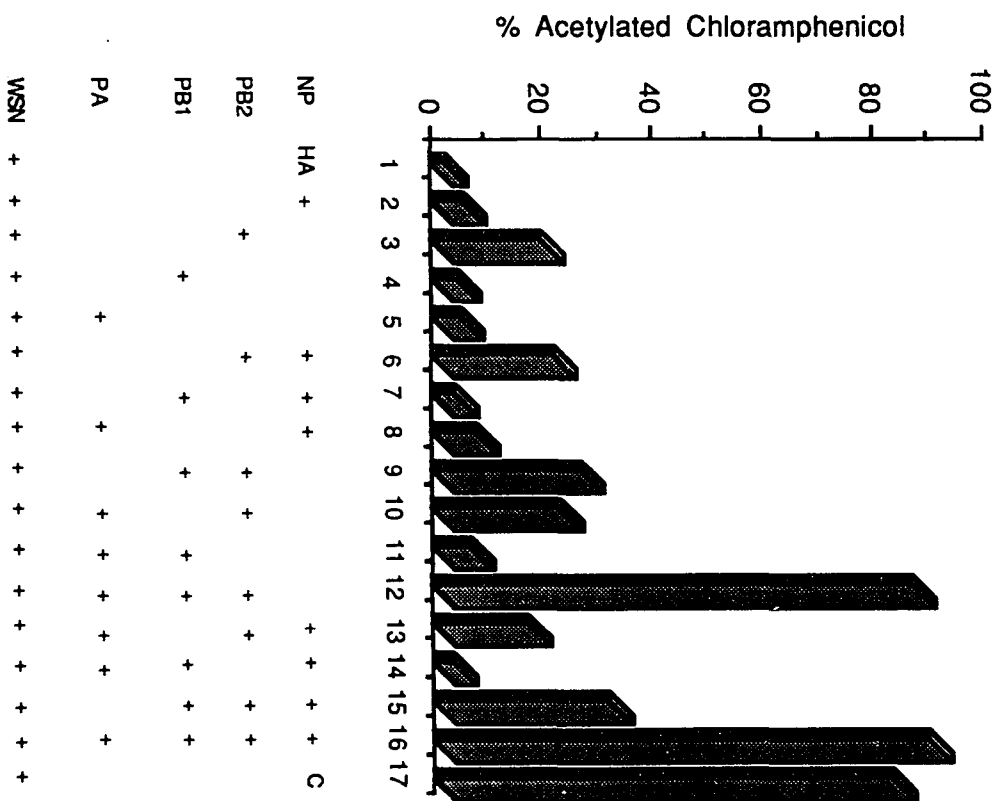
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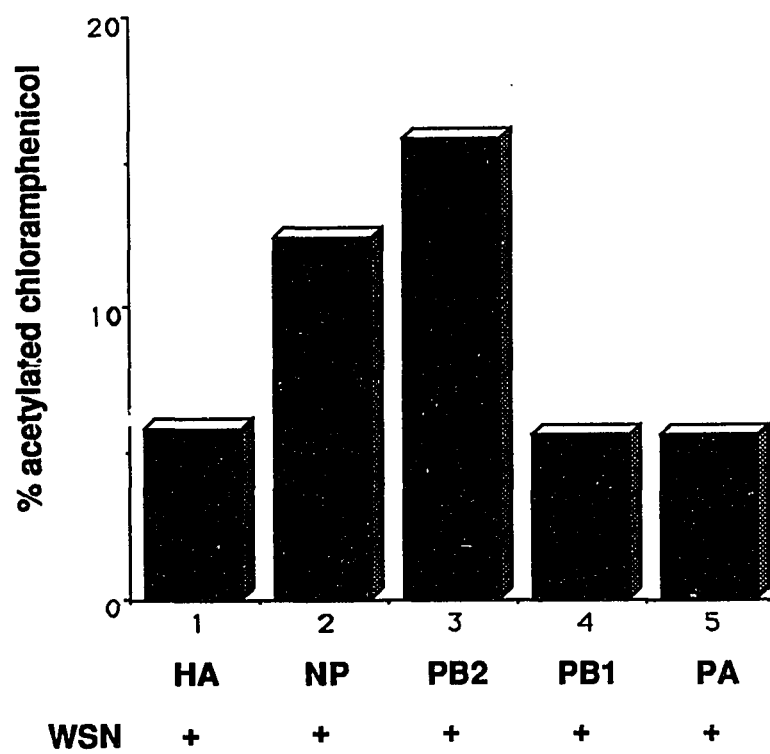
PB1

PA

WSN + + + + + +

**Figure 12A)** Effect of various mixtures on CAT expression. Cell line 23.2 was transfected with IVACAT-1 RNP for 4 hours, and then co-infected with WSN and recombinant vaccinia viruses expressing influenza viral proteins in a variety of combinations. Cells were assayed for CAT activity, products were excised from TLC plates and conversion rates calculated by scintillation counting. Labels under the lane numbers indicate the treatment of cells. Control cell line 6.1 was used in lane 17. **B)** Effect of vector expressed proteins during normal influenza virus infection. Confluent 6.1 cells were transfected with IVACAT-1 RNP as above. Cells were then infected with WSN virus and recombinant vaccinia vectors as indicated. Cells were harvested after overnight incubation and dilutions (1:50) of extracts were used for CAT assays. Results are presented in graphic form.





## **IV**

### **EXPRESSION OF THE INFLUENZA VIRUS MATRIX PROTEIN IN BACTERIA**

## **ABSTRACT**

A cDNA clone of the matrix gene from influenza A/PR/8/34 virus was used to construct a vector to express the matrix (M1) protein in bacteria. Expression of full length M1 protein was obtained at levels greater than 5 mg per liter. Purification of the bacterial protein was achieved through its solubility in an acidic chloroform:methanol solution, a unique property of the authentic viral protein. The purified matrix protein was used as antigen to produce the antibody in rabbits. The specificity of antibody was tested by immunoprecipitation with cellular extract of influenza-infected MDBK cells. Immunofluorescence analysis with this antibody showed that M protein migrates to nucleus during the replicative cycle and then export to cytoplasm. In *in vitro* transcriptase assays, bacteria expressed M1 protein inhibited viral RNA polymerase as well as the M protein purified from the virion did. Availability of these reagents should allow further study of the functions and the structure-function relationship of this protein.

## INTRODUCTION

The matrix protein (M1) of influenza virus is the most abundant protein in the virion, comprising about 40% of the total protein (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971). It is located at the inner surface of the lipid bilayer, and presumably acts as the "glue" between the envelope and the RNP core (Lamb 1983). It can be speculated that one domain of the matrix protein binds to the RNP core while another domain of the protein binds to the lipid bilayer or the surface glycoproteins, adding rigidity to the membrane and thus giving the virus its structural integrity. This latter property has been studied *in vitro*, as purified M1 can be reconstituted with lipid vesicles (Bucher et al., 1980; Gregoriades, 1980; Gregoriades & Frangione, 1981).

Another activity of the matrix protein *in vitro* is its ability to regulate transcription by inhibiting viral polymerase activity. Addition of purified matrix protein to isolated viral cores was shown to significantly inhibit the viral nucleoprotein (NP) activity *in vitro* (Zvonarjev & Ghendon, 1980; Mikheeva & Ghendon, 1983; Melnikov et al., 1985, Ye et al., 1989). Further studies with the use of protease digested fragments of M1 has localized this inhibitory region to a domain within a COOH-terminal 15-kd fragment (Ye et al., 1987,1989). Inhibitory effects of M1 protein is probably mediated through its RNA binding property (Ye et al., 1989; Wakefield and Brwnlee, 1989). Although the importance of this inhibitory

activity during influenza virus infection has not yet been demonstrated, it is significant that the matrix protein of vesicular stomatitis virus can also down regulate VSV transcription in vitro (Carroll & Wagner, 1979; Clinton et al., 1978; Wilson & Lenard, 1981). Recently, it was demonstrated that the matrix protein plays an important role in the transport of the virus ribonucleoprotein (RNP) particle (Martin and Helenius, 1991) in virus-infected cells. It was shown that export from the nucleus to the cytoplasm depended on the viral M1 protein. M1 associated with newly assembled viral RNPs in the nucleus and escorted them to the cytoplasm through the nuclear pores. In contrast, during the initial stages of virus infection, M1 protein dissociated from the RNPs, allowing them to enter the nucleus. The block of the dissociation event by amantadine prevents import of incoming RNPs into the nucleus (Martin and Helenius, 1991), thereby, inhibiting the virus infection.

As a step towards better understanding of the various functions of the matrix protein we have engineered a bacterial clone which expresses high levels of the matrix protein. This protein, like the authentic viral protein, retains the unique property of solubility in acidic chloroform:methanol, thus allowing for easy purification. Antibody generated with the purified bacteria expressed M1 protein recognized M1 protein of influenza virus infected cells in immunoprecipitation and immunofluorescence analysis. Availability of a large amount bacteria expressed M1 protein and its antibody should allow further analysis the functions of M1 protein.

## MATERIALS AND METHODS

**Construction of the Expression Vector.** The cDNA coding for the M gene segment from A/PR/8/34 virus inserted into the EcoRI site of plasmid pSP65 was used as the parent clone (Young et al, 1982). Briefly, the plasmid was digested with RsaI, which cuts at nucleotides 25 and 29 of the M gene as well as in plasmid sequences. The large M gene fragment was isolated and ligated with the duplex forming synthetic oligonucleotides 5'GATCTGTAAACCGAGGTCGAAACGTACGT 3' and 5' ACGTACGTTTCGACCTCGGTAAACA 3'. This synthetic fragment restores lost coding sequence and creates a sticky end for insertion into the BamHI site of pAS1 (Maniatis et al., 1982). The reconstructed M gene was inserted the BamHI/SalI window of pAS1/PR8-NS<sub>2</sub> (Young et al. 1983), using a SalI site present in the pSP65 linker sequence. The resultant clones were first checked by sequence analysis (Chen & Seeburg, 1986), and then used for expression studies.

**Synthesis and Purification of Matrix Protein.** Plasmid containing *E. coli* N5151 cells (which constitutively expresses a temperature sensitive cI repressor) were induced to express protein as previously described (Greenspan et al., 1985). Briefly, samples were grown at 32°C and induced to make protein at 42°C for times ranging from 5 minutes to 2 hours. For S<sup>35</sup> methionine labelling experiments M9 media was used and for large scale culture cells were grown in L broth. For purification of bacterially expressed matrix protein, the method of Gregoriades (1973) was used.

Cells were induced for two hours at 42°C, harvested, washed and incubated in 20 ml of a 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8.0) solution with lysozyme for 30 min. Matrix protein was then extracted by the method of Gregoriades (1973). Two volumes of chloroform:methanol (2:1) were added and the proteins were extracted into the interphase. The interphase was harvested and twenty milliliters of an acidic chloroform:methanol solution (1.8:1 with 0.04N HCl) was added and the solution shaken for 10 minutes. The mixture was pelleted at 11,000 g for 10 minutes and the supernatant was removed and ether precipitated (3 volumes). The precipitate was pelleted, dried and resuspended in 10% NP40 at 55°C for 15 min.

**Preparation of Antibody and Immunofluorescence Analysis of Infected Cells.** The bacteria expressed M1 protein was used as antigen in order to produce high-tittered specific antisera in rabbits. The immunization protocol was as follows: rabbits were inoculated with 0.5 mg protein in Freund's Complete Adjuvant (FCA) subcutaneously at day 0, 0.5 mg subcutaneously in incomplete FCA at day 14 and I.V. boosted with 0.1 mg protein at day 28. Rabbits were bled on day 39. Antisera was further purified by passage over an affinity column made with A/PR/8/34 disrupted virions (gift of Dr. J. Schulman) and used for immunofluorescence analysis of virus infected cells. A/WSN/33 virus was used to infect MDCK cells on coverslips at a multiplicity of approximately 1. At 2 hour intervals, cells were removed and fixed (Greenspan et al., 1988). Briefly, fixation was accomplished by: (1) washing cells in PBS, (2) incubation in 1% formaldehyde/PBS for 1 hour; (3) PBS wash; (4) incubation with 0.1% Triton/PBS for 5 minutes; (5) PBS wash; (6) treatment with

acetone:methanol (1:1) at  $-20^{\circ}\text{C}$  for 5 min; (7) PBS wash. Cover slips were stained by incubation at  $37^{\circ}\text{C}$  for 30 min with a 1:10 dilution of affinity pure serum in PBS. Cells were then washed with PBS and treated with fluorescein or rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Biomedical, Inc.) for 30 min at  $37^{\circ}\text{C}$ . Samples were washed, mounted and viewed on a Zeiss fluorescent microscope, model D-7082.

**Preparation of RNP** Influenza A/PR/8/34 was grown in the allantoic cavity of 10-11 days old embryonated chicken eggs (Sugiria and Ueda, 1980). Influenza virus RNPs were prepared as described previously (Plotch et al., 1981). Briefly, RNP cores were prepared by disrupting virions (1 mg/ml) in 1% NP40, 10 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 1.25 mM DTT. After incubation at  $30^{\circ}\text{C}$  for 30 min, Mixture were subjected to centrifuge in an SW 50.1 rotor at 45,000 rpm for 4 hour through a 70%-30% discontinuous glycerol gradient. The samples were fractionated and analyzed in 10% SDS-PAGE. Fraction enriched in RNPs were used for *in vitro* transcription reaction.

**Transcription Reactions.** Viral cores were purified through detergent treatment and glycerol gradient centrifugation as described (Plotch et al., 1981). Unless indicated, transcription reactions were performed as follows: 10 ug of viral cores and various amounts of either viral M1 or bacteria expressed M1 protein in 0.25% NP-40 were added to a buffer containing 10mM Tris-HCl (pH 8.0), 1mM DTT, 80mM NaCl, 7.5mM  $\text{MgCl}_2$ , 0.4mM ApG, 0.5mM ATP, GTP and UTP, 0.1mM CTP and 10 uCi gamma- $^{32}\text{P}$ -CTP. Reactions were incubated at  $31^{\circ}\text{C}$  for 1 hour,

terminated by addition 10% TCA and 10 ug of tRNA. Samples were incubated on ice for 30 min and TCA-insoluble RNA precipitated. Radioactivity was measured in scintillation counter.

## RESULTS

**Expression of the Matrix Protein in Bacteria.** The bacterial vector system used was the pAS1 expression system (Maniatis et al., 1982), where expression is driven by the strong but repressible  $P_1$  promoter of Lambda. As the gene of choice (minus the methionine codon) is inserted in phase into a unique BamHI site immediately downstream of an ATG codon, the expressed protein does not contain additional vector coded amino acids. This system has been used successfully for the expression of many diverse proteins, including the NS1 and NS2 proteins of influenza virus (Young et al., 1983, Greenspan et al., 1985). As described earlier, the expression clone was constructed by replacing a short restriction fragment with a synthetic oligonucleotide (Figure 13) containing a BglII sticky end for insertion into the BamHI site of pAS1. The resultant clone should express an intact M protein, except for a single amino acid change of Ser to Asp at position 2. The resultant clones were first checked by sequence analysis (Chen & Seeburg, 1986), and then used for expression studies. Plasmid containing *E.coli* N5151 cells (which constitutively expresses a temperature sensitive cI repressor) were induced to express protein as previously described (Greenspan et al., 1985). Briefly, samples were grown overnight at 32°C in M9 media supplemented with amino acids. The next day, samples were diluted and grown for an additional four hours, transferred to M9 media with amino acids (minus methionine) and induced at 42°C for 30 minutes. Ten microcuries of

S<sup>35</sup> methionine was then added for 5 minutes and samples were TCA precipitated and analyzed by SDS-PAGE. The results are shown in Figure 14. As can be seen in Lanes 5, 7 and 9, plasmid containing cells express after induction a protein which migrates identically to virus-infected cell matrix protein. This is also seen in coomassie brilliant-blue stained gels when bacterial cells grown in rich media are induced and compared on SDS-PAGE gels to purified virion protein (not shown). The clone shown in Lanes 8 and 9, designated pAS1/M9, was used for further study.

**Purification of the Bacterial Expressed Matrix Protein.** Proteins expressed in bacteria should display properties analogous to the authentic protein in order to substitute for the authentic protein in biological assays. The matrix protein of influenza A virus has the unique property of being soluble in an acidic chloroform:methanol solution. This property is presumably due to long stretches of neutral and hydrophobic amino acids present in the polypeptide (Gregoriades, 1973). This provides a simple method for purifying the matrix protein from virus or virus-infected cells (Gregoriades, 1973). Therefore, we tested whether the bacterial expressed matrix protein was soluble in acidic chloroform:methanol and could be purified in this manner. One liter of bacteria was induced and extracted with solvent. The final precipitate was pelleted, resuspended in 10% NP40 and analyzed by SDS-PAGE. Figure 15 shows that the bacterial expressed matrix protein is indeed purified to near homogeneity by acidic chloroform:methanol extraction. Quantitation experiments indicate that this protein is expressed at levels greater than 5 mg per

liter of induced bacteria. It should be noted that in certain preparations, silver-stained gels revealed the presence of additional less intense bands migrating faster than the bacterial matrix protein. As their presence is variable between preparations, we presume that these are at least in part the result of degradation products of the matrix protein.

**Immunofluorescence Analysis** The bacterial matrix protein was then used as antigen in order to produce high-tittered specific antisera in rabbits. Antisera was then tested in immunoprecipitation and immunofluorescence analyses. As expected, antisera was specific for viral induced matrix protein in immunoprecipitation assays (not shown). Rabbit antisera against the bacterial matrix protein was further purified by passage over an affinity column made with A/PR/8/34 disrupted virions (gift of Dr. J. Schulman) for use in immunofluorescence studies. A/WSN/33 virus was used to infect MDCK cells on coverslips at a multiplicity of infection of approximately 5-10. At 2 hour intervals, cells were removed, fixed and stained. At 2 hours post-infection (p.i.), little fluorescent staining is evident (not shown). Positive fluorescent staining of the virus encoded matrix protein is apparent at 4 hours p.i. (Figure 16A). At this time point fluorescence is evident mainly in the nucleus of infected cells, although some light diffuse cytoplasmic staining is also apparent. At 6 hours p.i. (Figure 16B), matrix protein is still predominantly in the nucleus, although diffuse cytoplasmic staining is clearly visible. Evidence that the matrix protein is actually in the nucleus rather than perinuclear comes from the fact that at 4 and 6 hour p.i. dark

patches (possibly nucleoli) can be viewed in the nucleus of many infected cells, reminiscent of NS1, NS2 and NP immunofluorescence (Young et al., 1983, Greenspan et al., 1985, Li et al., 1986). Also, by 6 hours p.i., a number of cells exhibit an intense punctate pattern of fluorescence in the cytoplasm. By 8 hours p.i., the entire cell is brightly fluorescent and the punctate cytoplasmic staining is more pronounced and evident in all cells (Figure 16C). Double immunofluorescent staining of cells with the matrix protein antisera and monoclonal antibodies against viral NP protein indicates that the NP and M1 proteins are co-localized within this punctate pattern (not shown). Thus, this may be indicative of viral cores migrating toward the plasma membrane for release.

**Studies on transcriptase inhibition.** A well studied property of the viral matrix protein is its ability to inhibit the viral polymerase *in vitro*. In order to use bacterial expressed proteins to study this property we tested whether bacterial M1 protein is as efficient as viral M1 in down regulating transcription of viral cores. Purified viral cores were incubated with increasing amounts of viral or bacterial matrix protein in the presence of primer and ribonucleotides. Reactions were incubated for 1 hour, terminated by addition of transfer RNA and immediately TCA precipitated. Figure 17 shows that the bacterial expressed protein inhibits core transcription at concentrations equivalent to the viral protein. As has been reported in earlier studies (Zvonarjev and Ghendon, 1980), inhibition is not complete, with levels ranging from 50%- 75% inhibition between experiments.

## DISCUSSION

M1 protein of influenza virus has an important structure role in the virion. It is located on the interior side of the lipid bilayer, forming a shell which surrounds the nucleocapsid (Schulze, 1970). In addition, M1 protein has ability of inhibiting the polymerase activity of influenza virus (Ye et al., 1987; Zvonarjev and Ghendon, 1980). Complete removal of M protein from the polymerase complex results in greatly enhanced polymerase activity (Kato et al., 1985). Recently, Martin and Helenius demonstrated that M1 protein modulated the bidirection transport of virus RNP.

In this report, a bacterial expression vector was constructed using the pAS1 system. In this system, expression of interesting gene is under the control by a strong and repressible  $P_L$  promoter of Lambda phage. cDNA of M1 protein (without the initial codon) was inserted into a unique BamH1 site immediately downstream of an ATG codon. Vector construction was achieved by replacing a short fragment of cDNA of M1 protein with synthetic oligonucleotide which contained a Bgl II sticky end for insertion into the BamH I site of vector. The resultant clone would express a full-length M1 protein with a single amino acid change of Ser to Asp at the second amino acid of N-terminus. Constructed plasmid was transformed into *E. coli* N5151 cells, which expresses a temperature-sensitive cI protein. Plasmid containing cells were first grown up at 32°C. At this temperature, cI protein expressed by host cells

(*E. coli* 5151) is functional and thus represses promoter activity of expression vector. After the samples were shifted to nonpermissive temperature, the cI protein is inactive, allowing a large quantity of M1 protein was produced.

The bacterial-expressed M1 protein displays the unique property of the viral analogous, that being soluble in an acidic chloroform:methanol solution. This facilitated the purification of M1 protein from bacteria. Using this method, we purified M1 protein from bacteria to near homogeneity (Figure 15). In order to increase the solubility of the bacteria expressed M1 protein, it is important to completely remove ether from the protein pellet following ether precipitation by the speed-vacuum and to resuspend the protein pellet in NP-40 containing solution. To make high-tittered specific antibody, purified bacteria expressed M1 protein was used to immunized rabbits. Antisera was tested for its specificity by performing the immunoprecipitation analysis. As expected, antisera could specifically recognize the influenza viral M1 protein from influenza virus-infected MDBK cells.

With affinity purified antibody, we examined the intracellular localization of the M protein. Clearly, M1 protein localizes to the nucleus during influenza virus infection. Initially, in influenza A/WSN/33 infected cells, little fluorescent staining was seen. Positive fluorescent staining of the viral M1 protein is apparent at 4 hour p.i. (Figure 16). At this time point, fluorescence was very intense in the nucleus of infected cells. As shown in Figure 16, the M1 protein is nuclear rather than perinuclear. In later stages of infection, cell staining exhibit an intense punctate pattern of fluorescence in the cytoplasm. Double staining of infected cells at 8 hour

p.i. with antibody to NP and bacterial-expressed M1 protein showed that the NP and M1 protein are co-localized within this punctate pattern. This result suggests that viral cores migrate toward the cytoplasm membrane. Our data also showed that the addition of bacterial-expressed M1 protein to the *in vitro* transcription reaction decreased viral transcriptase activity in a similar fashion to authentic viral M1 protein. Availability of a large amount of M1 protein expressed in bacteria should allow further study of the various functions of M1 protein. Site-specific mutagenesis should allowed the definition of the functional domains of this protein.

## FIGURE LEGENDS

Figure 13. Construction of the expression vector for M1 protein The cDNA coding for the M gene segment from A/PR/8/34 virus inserted into the EcoRI site of plasmid pSP65 was used as the parent clone. The plasmid was digested with RsaI, which cuts at nucleotides 25 and 29 of the M gene as well as in plasmid sequences. The larger matrix gene fragment was isolated and ligated with the duplex forming synthetic oligonucleotides 5'GATCTGTTAACCGAGGTCGAAACGTACGT 3' and 5' ACGTACGTTTCGACCTCGGTAAACA 3'. This synthetic fragment restores lost coding sequence and creates a sticky end for insertion into the BamHI site of pAS1. The reconstructed M gene was inserted the BamHI/SalI window of pAS1/PR8-NS<sub>2</sub> using a SalI site present in the pSP65 linker sequence. The resultant clones were first checked by sequence analysis and then used for expression studies.

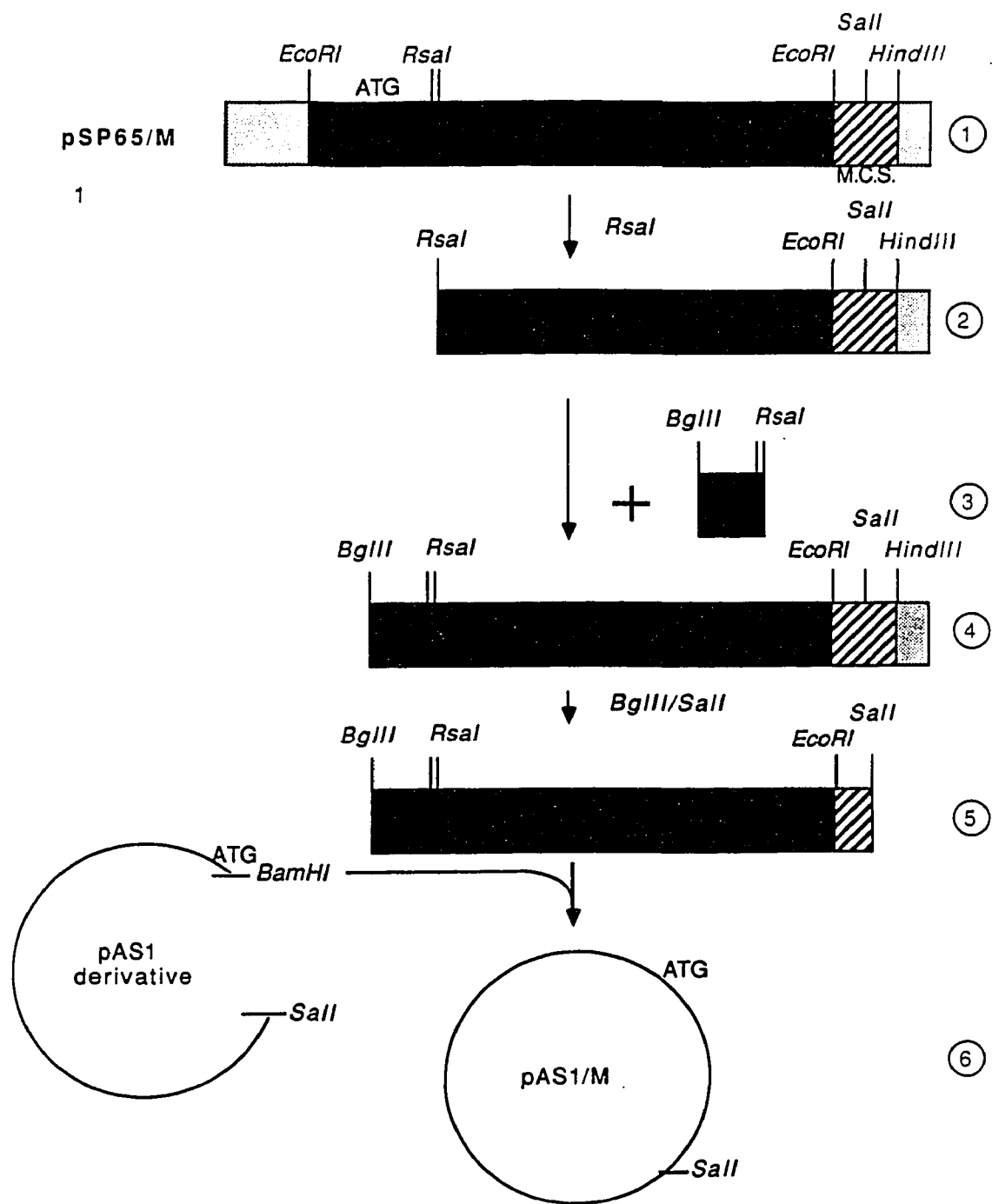


Figure 14. A) Expression of M1 in bacteria. Plasmid transformed E.coli N5151 cells were grown at 32°C and induced at 42°C for times ranging from 5 minutes to 2 hours. Bacterial proteins were labelled with S<sup>35</sup> methionine and analyzed by 7-14% SDS PAGE. Lane 1; A/PR/8/34 infected MDCK cell lysate labelled with S<sup>35</sup> methionine (Viral proteins are indicated). Lane 2; uninduced bacteria containing a pAS1 vector designed to express the influenza virus NS1 protein. Lane 3; induced bacteria expressing the NS1 protein. Lanes 4-9; three independent clones containing a pAS1/M vector labelled either before induction (lanes 4,6,8) or after induction (lanes 5,7,9). For S<sup>35</sup> methionine labelling experiments M9 media was used and for large scale culture cells were grown in L broth.

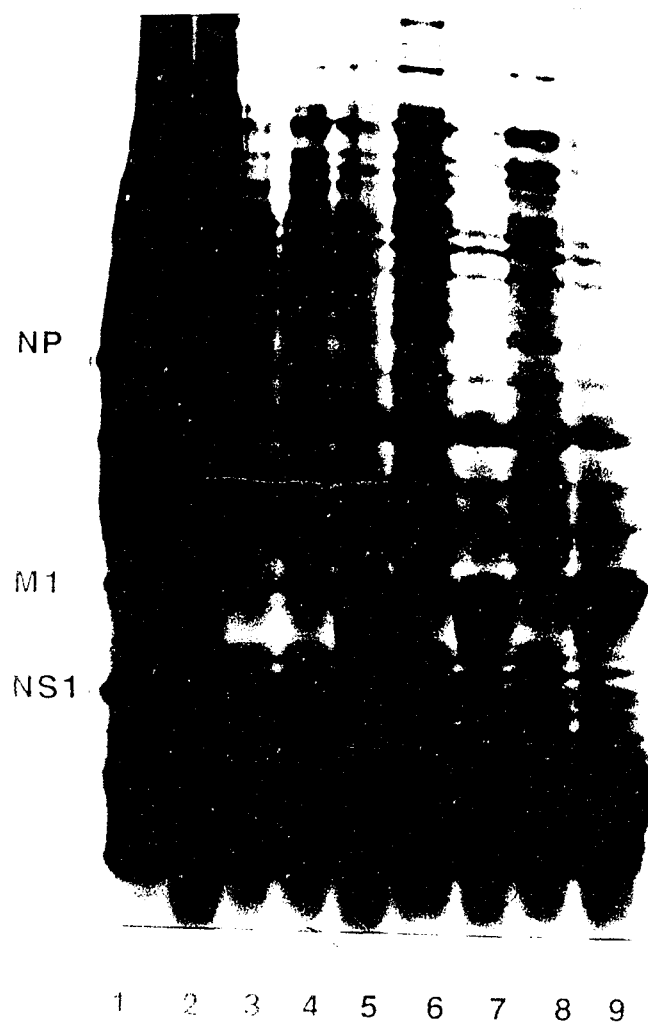


Figure 15. Purification of bacteria expressed matrix protein. Plasmid-containing bacteria were grown up at 32°C and induced for two hours at 42°C. Cells were then harvested and treated with lysozyme in 20 ml of a 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8.0) solution for 30 min. Matrix protein was then extracted with two volumes of chloroform:methanol (2:1) and the proteins were extracted into the interphase. The interphase was harvested and 20 ml of an acidic chloroform:methanol solution (1.8:1 with 0.04N HCl) was added and the solution shaken for 10 minutes. The mixture was pelleted at 11,000 g for 10 minutes and the supernatant was removed and ether precipitated (3 volumes). The precipitate was pelleted, dried and resuspended in 10% NP40. Purified protein was analyzed in 7-14% SDS-PAGE and stained with coomassie brilliant blue. Lanes 1-3; molecular weight marker proteins containing 2, 4 and 8 micrograms of each protein, respectively. Lanes 4,5; 0.67 and 2 micrograms of purified A/PR/8/34 virus. Lanes 6-9; twofold increases of acidic chloroform:methanol isolated protein. Molecular weights of standard proteins are indicated.

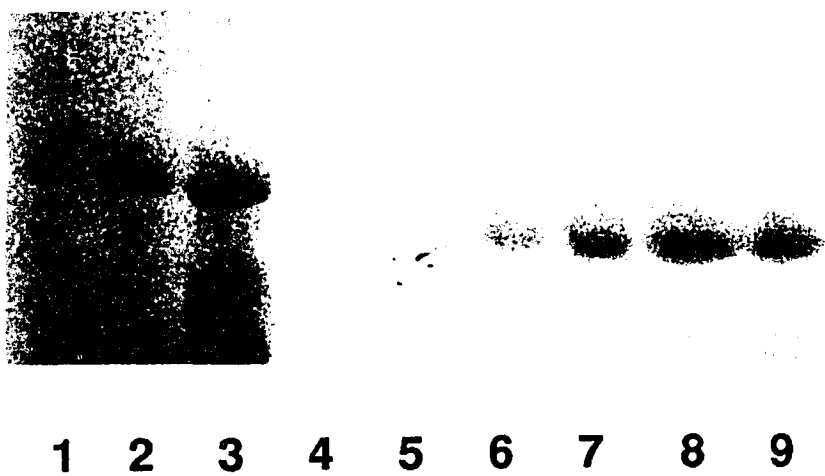
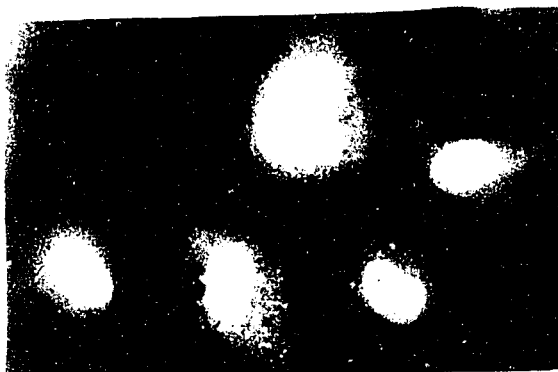


**Figure 16. Immunofluorescence analysis of A/WSN/33-infected MDCK cells.**

**Affinity purified rabbit anti-bacteria expressed M1 protein was used to stain cells at 4 (A), 6 (B) and 8 (C) hours post infection. The secondary antibody used was rhodamine labelled goat-anti-rabbit IgG. A/WSN/33 virus was used to infect MDCK cells on coverslips at a multiplicity of approximately 1. At 2 hour intervals, cells were removed and washed with PBS and fixed with 1% formaldehyde/PBS for 1 hour. Then, cells were incubated with 0.1% Triton/PBS for 5 minutes and treated with acetone:methanol (1:1) at  $-20^{\circ}\text{C}$  for 5 min. Cover slips were stained by incubation at  $37^{\circ}\text{C}$  for 30 min with a 1:10 dilution of affinity pure serum in PBS. Cells were then washed with PBS and treated with fluorescein or rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Biomedical, Inc.) for 30 min at  $37^{\circ}\text{C}$ . Samples were washed, mounted and viewed on a Zeiss fluorescent microscope, model D-7082.**



5



6

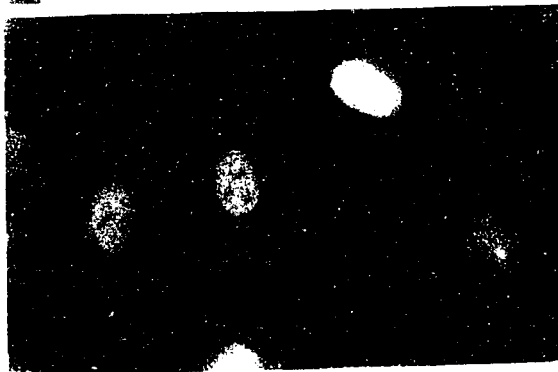
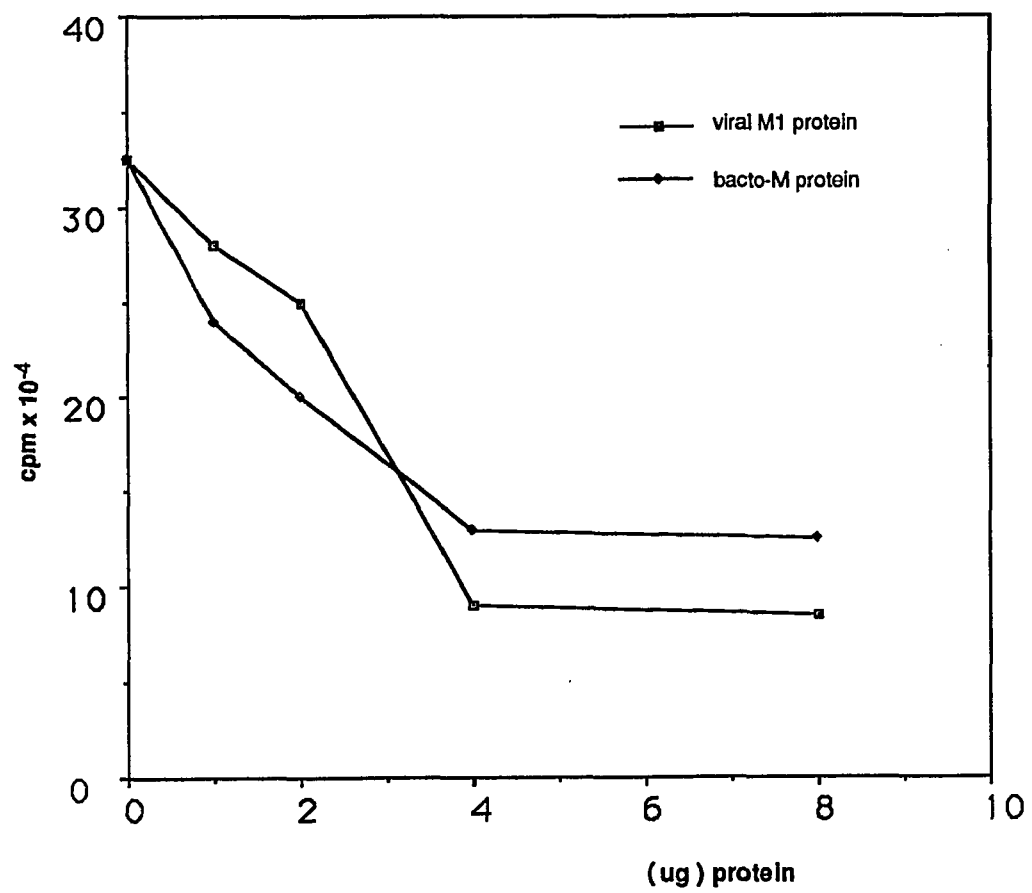


Figure 17. Transcriptase inhibition assay. Reactions were carried out as described in text. 10 ug of viral cores and various amounts of either viral M1 or bacteria expressed M1 protein in 0.25% NP-40 were added to a buffer containing 10mM Tris-HCl (pH 8.0), 1mM DTT, 80mM NaCl, 7.5mM MgCl<sub>2</sub>, 0.4mM ApG, 0.5mM ATP, GTP and UTP, 0.1mM CTP and 10 uCi gamma-<sup>32</sup>P-CTP. Reactions were incubated at 31°C for 1 hour, terminated by addition of 0.6 ml of sodium pyrophosphate (100 uM) and 10 % Trichloroacetic acid (TCA). Carrier tRNA was then added to the reaction mixture, and the samples were kept on ice for 20 min, and the acid-insoluble RNA was collected by filtration through glass filters (GF/A; Whatman, Inc., Clifton, N.J.). The radioactivity in the filter paper was counted by scintillation counter.



## V. DISCUSSION

Although influenza virus is a very important pathogenic virus, the slow development of *in vitro* replication systems and the limited availability of conditional lethal mutants have hampered the ability to understand the replication process. Recently, a system has been established in which an influenza NS-like RNA was synthesized from DNA vector (Luytjes et al., 1989). This RNA contains the antisense of the coding region of chloramphenicol acetyltransferase (CAT) in place of the antisense coding sequences for the nonstructural proteins (Luytjes et al., 1989). When mixed with purified influenza RNA polymerase (mainly NP protein and small amount of polymerase), RNA is assembled into functional RNP molecules (*IVACAT-1 RNP*). After transfection into cells superinfected with influenza virus, this recombinant RNP (*IVACAT-1*) was replicated and packaged by the superinfecting influenza virus. This is a very powerful system to study the cis-acting regulatory sequences of transcription, replication, packaging (Luytjes et al., 1989), and polyadenylation (Luo et al., 1991). This approach also opens a new avenue for the construction of recombinant viruses (Enami et al., 1990; Li and Palese, personal communication). However, as intact influenza virus is needed, it is difficult to examine the functions of individual proteins. Chapter 2 of this thesis described a completely artificial replication system. In this system, an influenza NS-like RNA (*IVACAT-1*) was also mixed with purified influenza polymerase for assembly into RNP and then transfected into cells which were subsequently infected with the

mixture of recombinant vaccinia viruses expressing the PB2, PB1, PA, and NP protein (Smith et al., 1987). Expression of the synthetic RNA was monitored by analyzing the cells for CAT activity. Therefore, the PB2, PB1, PA, and NP proteins are all that is necessary and sufficient for RNP expression (Figure 5).

In influenza virus infected cells, viral proteins are initially produced via primary transcription. Subsequently, RNA replication ensues and the bulk of the viral proteins are produced from amplified template. This is known as secondary transcription. Theoretically, the CAT protein we detected could have been obtained from synthetic RNP either via primary transcription off the transfected input synthetic RNP or via transcription off replicated RNA. To test whether our synthetic RNA is replicated, further experiments were performed. First, a mutated RNA (*IVACAT-2*) was used to make synthetic RNP, in which there were three mutations at the 5'-end. This RNA template is active for transcription *in vitro* but does not replicate (Luytjes et al., 1989). When this mutant RNP was transfected into recombinant vaccinia virus infected-cells, CAT activity was undetectable. The results indicate that primary transcription off the input RNA does not produce detectable CAT activity. Because the mutation at the 5'-end destroys the panhandle structure formed by the complementary sequences of at the 3'- and 5'- end which appear to be required for the mRNA synthesis (Luo et al., 1991), additional evidence for *IVACAT-1* RNP replication was obtained from the use of another synthetic CAT RNA (*IVACAT-9*). *IVACAT-9* RNA contained the entire the 5' noncoding region of the NS gene, the coding region of the CAT gene and the 3' noncoding region of

the NS gene, all in the plus sense. This RNA represents the cRNA intermediate of *IVACAT-1* RNA. When this RNP was transfected into cells in which influenza viral proteins PB2, PB1, PA, and NP had been supplied by infection with recombinant vaccinia viruses, CAT activity was detectable (Figure 6C, lane 2). To exclude the possibility that CAT activity was obtained by direct ribosome translation of *IVACAT-9*, a negative control was done in the absence of infection of recombinant vaccinia virus infection. These data together provided strong evidence that the synthetic RNP was undergoing replication and that the minimum subset influenza viral proteins needed for replication and transcription was the three polymerase proteins (PB2, PB1, and PA) and NP.

As influenza viral proteins are produced by individual recombinant vaccinia vectors, it now becomes possible to examine the function of the viral proteins. Two nonstructural proteins, NS1 and NS2, are present at high levels in the nucleus of influenza virus infected cells. Since the nucleus is where influenza virus genome replication and transcription occur, it was speculated that these proteins play a role in replication (Greenspan et al., 1985; Lamb and Lai, 1984, Young et al, 1983; Odagiri and Tobita, 1990). However, as shown in chapter 2, neither the NS1 nor NS2 protein was necessary for synthetic RNP replication. To test whether these two proteins had positive or negative regulatory activities, vaccinia viruses expressing the NS1 and/or NS2 proteins (Smith et al., 1987) were added to the mixture of the vaccinia viruses expressing the PB2, PB1, PA, and NP. As shown in Figure 7, addition of these two proteins did not alter the level of CAT activity significantly

(Figure 7A), though the proteins were expressed at high levels as examined by immunoprecipitation analysis (Figure 7B). Therefore, in this system the NS1 and NS2 proteins are not directly involved in the replication of the synthetic RNP. Although a vaccinia virus protein(s) could supply an analogous function of the NS1 and NS2 proteins, There are no similarities between the nonstructural proteins and known vaccinia proteins. In addition, vaccinia virus is a cytoplasmic double-strand DNA virus and the replication of synthetic RNP occurs even in presence of cytosine arabinoside which inhibits replication and therefore synthesis of late proteins of vaccinia virus (data not shown).

In the recombinant vaccinia-driven replication system described in chapter 2, all influenza viral proteins required for synthetic RNP replication are produced by DNA vectors. Therefore, mutagenic analysis would be now feasible and should allow for examination of the structure-function relationship of these proteins. To facilitate this analysis, a modified vaccinia-driven replication system is currently under study. We substituted VTF3 and plasmid pT7PB2 for the vaccinia virus expressing the PB2 protein. VTF-3 is a recombinant vaccinia virus expressing the T7 polymerase while pT7PB2 is a plasmid vector containing a T7 promoter upstream from a copy of the PB2 gene of influenza A/PR/8/34. When cells were transfected with *IVACAT-1 RNP* and plasmid DNA of pT7PB2 and then infected with the mixture of recombinant vaccinia viruses expressing PB1, PA, NP, and the VTF3 vector, CAT protein was indeed expressed in these cells (Huang and Krystal, unpublished data). Hopefully, this modified vaccinia-driven replication system would allow us to define

the functional domains of PB2, PB1, PA and NP. In addition, this system may allow for a precise dissection of the processes involved in replication and transcription and determine whether synthesis of the different types of viral RNA (vRNA, cRNA, and mRNA) are catalyzed by distinct polymerase complexes (Krug et al., 1989)

Another advantage of the vaccinia-driven replication system is for the study of antivirals. We have used it to study the mechanism of inhibition by the murine Mx1 protein. Although antiviral activity of the interferon-inducible murine Mx1 protein to influenza virus has been documented for a considerable length of time, the mechanism is still poorly understood. The antiviral activity of the Mx1 protein to influenza virus infection is independent of other interferon-inducible cellular proteins (Staeheli et al., 1984; Staeheli et al., 1986; Pavlovic et al., 1990). The murine Mx protein does not affect attachment or penetration of influenza virus as parental virion nucleocapsids are efficiently transported into the nucleus in the presence of the murine Mx protein (Broni et al., 1990). However, the murine Mx protein in mouse embryo fibroblasts led to the inhibition of viral mRNA synthesis (Krug et al., 1985). Since the Mx protein acts on such an early stage of replication, it is very difficult to study its mechanism further because almost undetectable levels of viral proteins are produced. Since the Mx protein does not inhibit expression of vaccinia virus proteins, our artificial replication system could presumably produce sufficient quantity of influenza viral proteins even in the presence of the Mx protein. Using the vaccinia-driven replication system, we examined the possibility that the Mx1 directly inhibited the viral transcriptase in stable Mx1-expressing cells (Pavlovic et al., 1990).

As shown in Figure 10, when murine Mx1-expressing cells were transfected with *IVACAT-1 RNP* and subsequently infected with recombinant vaccinia viruses expressing the PB2, PB1, PA, and NP, synthetic RNP could be replicated and transcribed as analyzed by the production of CAT protein in these cells. However, a certain degree of inhibition was also observed as compared to control cells. This inhibition correlated well with the reduction of influenza virus titers obtained after influenza virus infection. The higher the level of Mx1 expressed, the lower CAT activity was obtained (Figure 9 and Figure 10). The difference of CAT activity was neither due to the variation of the transfection efficiency of synthetic RNP nor due to the difference of the level of influenza virus proteins expressed by recombinant vaccinia viruses. As shown in external control and immunoprecipitation analysis, all these cell lines possessed similar transfection efficiency and expressed equivalent amount of influenza viral proteins. These data together indicate that overexpression of the influenza viral polymerase protein and NP by recombinant vaccinia viruses can support expression of *IVACAT-1 RNP* in Mx1 cells.

To identify the possible viral protein target of the murine Mx1 protein, influenza virus was used along with single recombinant vaccinia vectors to co-infect the stable Mx1-expressing cells. By itself, influenza virus would not provide enough helper protein to enable detection of CAT activity in *IVACAT-1 RNP* transfected Mx1-expressing cells (Figure 8). Alternatively, infection of recombinant vaccinia virus expressing less than the full complement of influenza viral proteins PB2, PB1, PA, and NP would not support replication and expression of synthetic *IVACAT-1 RNP*.

(Figure 5). However, Co-infection of Mx1 expressing cells with influenza virus and recombinant vaccinia vectors expressing the three polymerase proteins led to the detection of CAT activity. In addition, a low CAT activity was also expressed in Mx1 cells co-infected with WSN and recombinant vaccinia virus expressing PB2 alone. This result suggests that the PB2 protein is possibly a target of Mx1. However, PB2 also boost the expression of IVACAT-1 RNP in Mx- cells, therefore, further studies are needed to examine the specificity of PB2 effect.

M1 protein of influenza virus is the most abundant protein in influenza virion and has an important structure role. M1 protein as a nuclear protein downregulates the influenza virus polymerase activity *in vitro*. Recently, it was demonstrated that M1 protein involved in the import and export of virus RNP. To define the structure-function relationship, we constructed an expression vector for M1 protein. M1 protein was obtained at high levels. Purification of the bacterial-expressed M1 protein was achieved through its solubility in an acidic chloroform:methanol solution. Antibody against bacterial-expressed M1 protein specifically recognized the viral analogue. using this antibody, we examined the intracellular localization of M1 protein in influenza virus-infected cells. We found that M1 protein appeared in the nucleus at 4 hours p.i.. In later stages of infection, cells displayed an intense punctate pattern. Double staining of infected cells with antibody to bacteria expressed M1 and the viral NP protein showed that the NP and M1 protein were co-localized within this punctate. These data indicate that bacterial-expressed M1 protein possesses the properties of the viral M1 protein. Availability of M1 protein

and its antibody should allow further study the function of M1 protein and to define the structure-function relationship.

## VI. SIGNIFICANCE

Influenza virus is an important human pathogen, frequently causing widespread disease and a significant loss of life. Elucidation of the mechanism of replication and transcription has the potential of leading to approaches for the control of influenza virus. Although much has been learned about replication and transcription of influenza virus, there is still much which is unknown. A major obstacle was that influenza virus, as a negative sense RNA virus, had not been adaptable to many of the powerful molecular techniques in use for DNA viruses and positive sense RNA viruses. These include construction of viral mutants through mutagenesis of infectious clones and the development of *in vitro* replication systems based on recombinant DNA methodology. Therefore, the lack of viable systems and the limited availability of conditional lethal mutants have greatly hampered our ability to understand the replication process. The initial aim of this thesis was an attempt to develop an influenza virus-specific replication system whereby viral proteins were produced through a DNA source. This system possesses a number of advantages. First, since the viral proteins come from specific recombinant cDNA molecules, it allows expression of influenza viral proteins in various combinations. Hence we can study the role of individual influenza viral proteins, particularly those involved in the replication and transcription. Second, mutagenesis of the influenza viral cDNA would now be feasible which could allow for the analysis of structure-

function relationship of the viral proteins. Third, a non-influenza virus dependent replication system may produce a means for studying the mechanism of action of known and newly discovered antiviral agents (for example the murine Mx1 protein). As with all viruses, the search for specific inhibitors of viral replication has been seriously hampered by the fact that virus and infected cells share much of the biochemical apparatuses needed to synthesize protein and nucleic acid. However, in the case of influenza virus, replication and transcription of virus genes are catalyzed by viral RNA-dependent RNA polymerases. The murine Mx protein found in some mouse strains is interferon-induced and confers resistance to influenza virus infection presumably through inhibition at an initial stage of transcription. Elucidation of the mechanism by which the Mx protein confers this resistance to influenza virus might be an approach to finding more efficient and specific antivirals. M1 protein of influenza virus is a structural protein. In *in vitro* transcription reaction, M1 protein downregulates the polymerase activity. Recently, it was demonstrate that M1 protein modulate the transport of influenza viral RNP. Defining of the structure-function relationship would provide valuable information of designing antivirals and elucidating the mechanism of the nuclear transport.

## **VIII. APPENDIX**

**Inhibition of DNA Replication of Adenovirus  
type 5 and Simian Virus 40 by Tunicamycin**

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**Summary** Addition of tunicamycin, a glycosylation inhibitor, to SV40 infected CV-1 cells or Ad5 infected HeLa cells at the beginning of infection was found to inhibit the accumulation of viral DNA at late times after infection. However, tunicamycin did not block viral DNA replication when added to the infected cells at late times after infection. The inhibitory effect of tunicamycin was partially reversible in the presence of acetylglucosamine, suggesting that the effect was due to glycosylation. In spite of diminished amounts of viral DNA accumulated at the late phase of infection in the presence of tunicamycin, the transcription rate of Ad5 late RNA and the amount of adenovirus late proteins and mRNA were not significantly affected by tunicamycin treatment. The inhibitory effect of tunicamycin on Ad5 DNA replication was much reduced in 293 cells which provide E1a gene products in trans. Similar observation was obtained for the replication of SV40 DNA in Cos-7 cells which provide SV40 early gene products in trans. These results suggest that tunicamycin inhibits a glycosylation event induced by the early gene products of Ad5 and SV40 viruses during the early phase of infection.

Limited coding capacity necessitates small DNA viruses, such as simian virus 40 (SV40) and human adenoviruses, to require some host replication factors for the replication of their DNA. Accordingly, these viruses acquire the ability to activate host DNA replication machinery and induce transient cell DNA replication during the early phase of infection (Acheson, 1980; Flint, 1980; Flint, 1986). The viral gene products of SV40 and human adenoviruses responsible for the induction of host replication machinery have been identified. In SV40 it is the sequence located in the aminoterminal one third domain of the large T antigen that is found to induce host DNA synthesis (Chow and Martin, 1975; Hiscott and Defendi, 1979; Pipas and Nathans, 1983; Soprano et al., 1983). Transfection experiments using a plasmid containing either the Ad5 E1a 12S or 13S mRNA have implicated the 12S gene products in the stimulation of cell DNA synthesis (Moran and Zerler, 1988; Nerkajima et al., 1987; Stabel et al., 1985). The 12S mRNA, however, is required for the replication of the cell and viral DNA in growth-arrested cells (Montell et al., 1984; Spindler et al., 1985). Although these studies have identified the viral proteins involved in the induction of host DNA synthesis, little is known about how these early viral gene products modulate cell replication machinery.

Studies using inhibitors of glycosylation or temperature-sensitive mutants defective in N-glycosylation have indicated that a glycosylation event is crucial for the progression from G1 to S in mammalian and yeast cells (Arnold and Tanner, 1982; Nishikawa et al., 1980; Popolo et al., 1986). In the present report we investigated

whether glycosylation is also required for the replication of viral DNA replication in SV40- and Ad5-infected cells. Tunicamycin at a concentration of 5 ug/ml was found to inhibit the accumulation of Ad5 or SV40 DNA at 24 hours post-infection when the drug was added to HeLa or CV-1 cells at the time of infection or at one hour before infection with Ad5 and SV40, respectively (Fig. 17, Panel A, Lane c and Lane a; Panel B, Lane d and Lane b, respectively). The amount of viral DNA harvested at 24 hours post-infection in the cultures and treated with tunicamycin at the time of infection was reduced to approximately 20% and 5% respectively of the untreated Ad5 and SV40 infected cells. The effect of the drug decreased when the drug was added after infection. There was no effect when the drug was added after adenovirus DNA replication had begun at 6 hours post-infection (lane f). Treating infected cells for an equivalent amount of time, from 24 to 48 hours post-infection with tunicamycin, did not affect the accumulation of viral DNA (data not shown). The inhibition of adenovirus DNA replication was also shown by the reduced incorporation of  $^3\text{H}$ -thymidine into Ad5 DNA during a two hour pulse labeling at 24 hours post-infection (Fig. 18, panel C). However, the reduction of  $^3\text{H}$ -thymidine incorporation was the result of reduced amount of viral DNA in the drug-treated cells. The amount of  $^3\text{H}$ -thymidine incorporated per Ad5 DNA was essentially the same in the drug-treated and the control samples as determined by comparing the densitometric intensities of Ad5 DNA bands in fluorography and in the ethidium bromide stained gel. These results indicated that tunicamycin did not significantly affect the rate of residual Ad5 DNA replication in the drug-treated HeLa cells.

Whether the residual viral DNA replication represents subpopulation of infected cells resistant to tunicamycin can not be determined by the present data.

The inhibition of Ad5 and SV40 DNA accumulation by tunicamycin could be partially reversed by pretreating cells with 2 ug/ml of acetylglucosamine for 1 hour before the addition of tunicamycin (Fig.19, Lane d). Acetylglucosamine has been shown to partially reverse the inhibition of protein glycosylation by tunicamycin (Cates et al., 1984). This result therefore suggests that the inhibition of viral DNA replication is due to the interference of a glycosylation step during viral infection.

Since the inhibitory effect of tunicamycin was not observed when the drug was added after viral DNA replication has started, we suspected that the biochemical step inhibited by tunicamycin was mediated by the early functions of Ad5 or SV40. To test this possibility, we studied the effect of tunicamycin on Ad5 and SV40 DNA accumulation in 293 and Cos-7 cells, respectively. These two cell lines express Ad5 pre-early E1a genes and SV40 T antigen respectively (Gluzman, 1981; Graham et al., 1977; Jones and Shenk, 1979). If a glycosylation reaction is mediated by the viral early gene products, then tunicamycin should have no effect on viral replication because this reaction presumably has already taken place in 293 and Cos cells before viral infection. This was indeed observed: addition of tunicamycin at the time of infection or one hour before infection did not affect significantly Ad5 or SV40 DNA accumulation at 24 hours post-infection (Fig.20). The possibility that these cells for some reason were resistant to tunicamycin was eliminated by the observation that replication of influenza virus in 293 cells was inhibited by tunicamycin (data not

shown). These results suggest that early viral gene products could reverse the effect of tunicamycin on viral DNA replication perhaps because the glycosylation reactions mediated by these early gene products have already taken place before the addition of the drug. However, it can not be excluded at the present time that the lack of inhibitory effect observed in these two virus transformed cell lines is not related to the viral early gene products but is an intrinsic property of these transformed cells.

Ad5 and SV40 late gene expression is tightly coupled to the onset of viral DNA replication (Acheson, 1980; Flint, 1980; Flint 1986). Since tunicamycin inhibited the onset of viral DNA replication, we sought to determine whether the expression of viral late genes was also affected. Northern blot analysis indicated that the amount of late mRNA in the tunicamycin treated cells was indistinguishable from the untreated control culture (Fig. 21, panel A). Consistent with the normal amount of late mRNA in the Northern blot, both the quantity and the quality of <sup>35</sup>S-methionine pulse-labeled Ad5 late proteins in the drug-treated and the control cultures were essentially indistinguishable. (Fig. 21, panel B)

To determine whether the transcription rate of Ad5 late RNA was affected by tunicamycin treatment, we analyzed the synthesis of Ad5 late RNA using the *in vitro* nuclear run-on assay. Ad5 nascent RNA was labeled by <sup>32</sup>P-UTP in the Ad5-infected HeLa cell nuclei at 24 hours post-infection. The labeled nascent RNA from the control and tunicamycin treated cultures was hybridized to Southern-blotted Ad5 HindIII or KpnI restriction fragments. The hybridization efficiency of the two RNA preparations with DNA in excess was essentially the same (Fig. 21, panel C),

suggesting that the transcription rate of adenovirus late RNA also was not affected by tunicamycin treatment. Thus, despite a decrease of 80 % of Ad5 DNA at 24 hours post-infection, the transcription and the accumulation of adenovirus late mRNA appeared normal in the tunicamycin treated cells.

In the present report we showed that tunicamycin inhibited the onset of SV40 and Ad5 DNA replication when the drug was added at the time of infection. No inhibition was observed if the drug was added to the cells after the onset of viral DNA replication or in cells constitutively expressing viral early genes. These results suggest that a glycoprotein induced by the early genes of Ad5 or SV40 is important for the onset of viral DNA replication. This observation is interesting because a glycoprotein has been found to be required in the transition from G1 to S phase and the onset of DNA replication in yeast (Arnold and Tanner, 1982; Popolo et al., 1986; Vai et al., 1987) and in mammalian cells (Engstrom and Larsson, 1988; Ishii and Volpe, 1987; Larsson, 1985; Nishikawa et al., 1980). Since SV40 and adenovirus require at least some host replication factors to replicate their DNA, the activation of the glycoprotein crucial for the onset of host DNA replication by the viral early gene products may be a strategy adapted by these viruses. However, we do not know whether the glycoprotein important for the onset of viral DNA replication is the same glycoprotein needed for cell DNA replication. In *S. cerevisiae*, a membrane-associated glycoprotein of molecular weight of 100 kd has been implicated in the regulation of the onset of cell DNA replication (Klebl et al., 1984). We are currently investigating if a similar glycoprotein is induced by adenovirus and SV40 early gene

products. Because of possible side effects, interpretation of drug induced effects always has to be viewed with some reservation. In the case of involvement of glycoprotein in G1 to S progression, most of the studies have been carried out using tunicamycin which is a quite specific inhibitor of the formation of dolichyl-P-P-N-acetylglucosamine (Schwarz and Datema 1980; Savage and Baur, 1983), an intermediate for N-glycosylation. One study, however, uses temperature sensitive mutant in N-glycosylation to study the role of glycosylation in cell cycle progression (Klebl et al., 1984). This study essentially confirms the analyses using metabolic inhibitors. Thus although we could not completely rule out the possibility that the observed inhibition of viral DNA replication is due to unknown side-effect of the drug, the parallel between viral and cell DNA replication suggests that glycosylation is indeed involved in the onset of SV40 and Ad5 DNA replication. Furthermore, the lack of effect of tunicamycin in 293 and Cos cells tends to rule out non-specific cytotoxic effect of the drug. However, other experimental approaches are needed to confirm the present observations.

An interesting phenomenon of the effect of tunicamycin on Ad5 replication is the observation that the transcription rate of Ad5 late genes and the accumulation of late mRNA and proteins were not affected by tunicamycin despite a significant decrease in the amount of the accumulated Ad5 DNA. This result is somewhat surprising since adenovirus late gene expression has been found to be coupled with the onset of viral DNA replication (Shaw and Ziff, 1982). Thomas and Mathews also showed that the synthesis of late RNA required the new viral DNA made by

DNA replication (Thomas and Mathews, 1980). The observation presented here suggests either that the accumulation of viral templates for late gene transcription was not affected by tunicamycin or that there was a compensatory increase of transcription rate per template in tunicamycin treated cells. Since Ad5 DNA replication in the presence of tunicamycin was suppressed to 20 % of the control, it is also possible that the residual viral DNA synthesis is sufficient for supplying the templates for late RNA synthesis.

If the templates for late gene transcription is not decreased by tunicamycin then the percentage of Ad5 transcription complexes in the intracellular viral nucleoprotein complexes should be greatly increased in the tunicamycin treated cells. On the other hand, compensatory transcription would result in the increased in the number of nascent RNA chains in Ad5 transcription complexes. These two possibilities can be distinguished by the analysis of adenovirus transcription complexes from Ad5-infected cells in the electron microscope (Wolgemuth and Hsu, 1981).

**Figure 18.** Effect of Tunicamycin on the accumulation of Ad5 DNA (panel A) and SV40 DNA (panel B), and on the incorporation of  $^3\text{H}$ -thymidine into Ad5 DNA (panel C). Panel A: agarose gel electrophoresis of adenovirus DNA extracted from infected HeLa cells at 24 hour postinfection and digested with Hind III. Lane a, 5 ug tunicamycin/ml added 1 hour before infection. Lane b, control without tunicamycin. Lane c-f, tunicamycin added at 0, 1, 3 and 6 hour after infection, respectively. Lane g, molecular weight marker. Panel B: agarose gel electrophoregram of SV40 DNA purified from SV40 infected-CV-1 cells. Lane a, DNA molecular weight marker. Lane c, control without drug added, Lane b and lane d-f, virus infected cells were treated with 5 ug/ml of tunicamycin at 1 hour before (lane b) and 0, 3 and 6 hour postinfection respectively (c-f). I, supercoiled DNA, II nicked-circular DNA. Panel C: Fluorograph of  $^3\text{H}$ -thymidine pulse-labeled Ad5 DNA synthesized in the presence or absence of tunicamycin treatment. The viral DNA was digested with HindIII restriction endonuclease and separated in 1% agarose gel is shown. Lane C, control without drug. Lane T, treated with 5 ug of tunicamycin at the time of infection.

**A**

a b c d e f g

**B**

a b c d e f

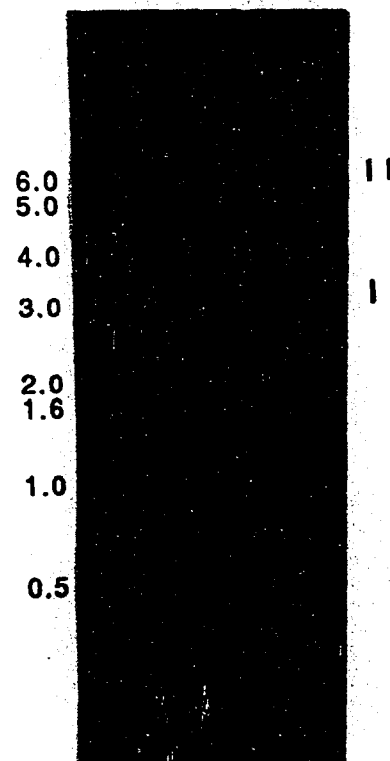
**C**

**Figure 19.** Reversion of tunicamycin inhibition of adenovirus DNA replication by N-acetylglucosamine. Viral DNA was purified and digested with Hind III. Lane a, control. Lane b-d, 5 ug/ml of tunicamycin was added at the infection time. Lane c and Lane d, 2 ug/ml of N-acetylglucosamine was added at the time of infection and 1 hour before the infection.

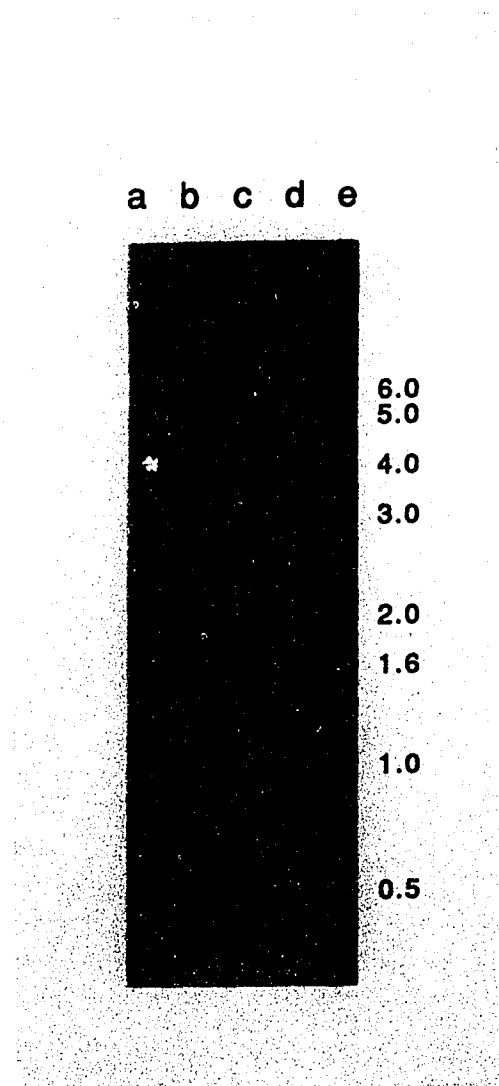
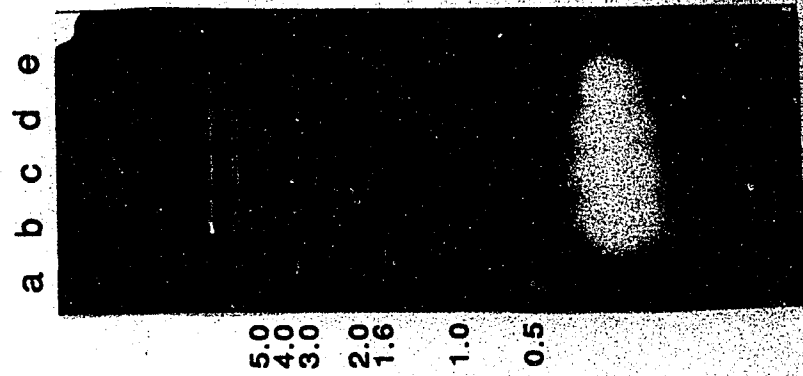


**Figure 20.** Effect of tunicamycin on the accumulation of adenovirus DNA in 293 cells and on SV40 DNA accumulation in COS-7 cells. Monolayer 293 and COS-7 cells were infected with Ad5 and SV40 respectively. Panel A, Adenovirus DNA purified from virus infected 293 cells and digested with Hind III. Lane a, DNA molecular weight marker, Lane b, control without tunicamycin, Lane c-e, tunicamycin added at 0, 3 and 6 hour postinfection, respectively. Panel B. SV40 DNA purified from SV40 infected COS-7 cells. Lane a, tunicamycin added one hour before infection; lane b, control without tunicamycin, Lane c-e, 5 ug/ ml tunicamycin added at 0, 3 and 6 hour postinfection, respectively.

**A**



**B**

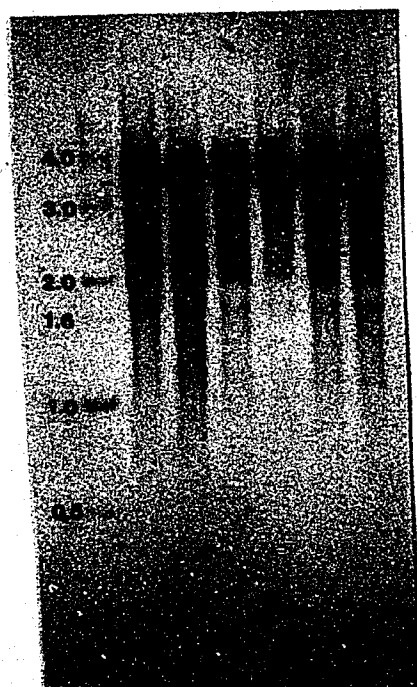


**Figure 21.** Effect of Tunicamycin on the synthesis of adenovirus late RNA and proteins. Panel A: Northern blot analysis of hexon mRNA extracted at 24 hours post infection using Ad5 HindIII-A fragment (50.1-73.6) as a probe. Lane a, 1 kb DNA marker; lane b, 5 ug/ml tunicamycin added 1 hour before infection. Lane c, control, no tunicamycin, Lane d-g, 5 ug/ ml of tunicamycin added at 0, 1, 3 and 6 hour after infection, respectively. Panel B: SDS polyacrylamide gel electrophoretic analysis of <sup>35</sup>S-methionine pulse-labeled adenovirus late proteins synthesized in the absence (Lane c) or the presence of 5 ug/ml tunicamycin added at the time of infection (lane t). At 24 hours post infection, cells were pulse labeled with 50 microcurie of <sup>35</sup>S-methionine in methionine-free medium for two hours. At the end of labeling period, cells were collected by centrifuge and lysed with protein electrophoresis sample buffer. After boiling for 5 minutes the samples were electrophoresed in a 10% polyacrylamide protein gel. The gel was fluorographed using 0.7M sodium salicylate.

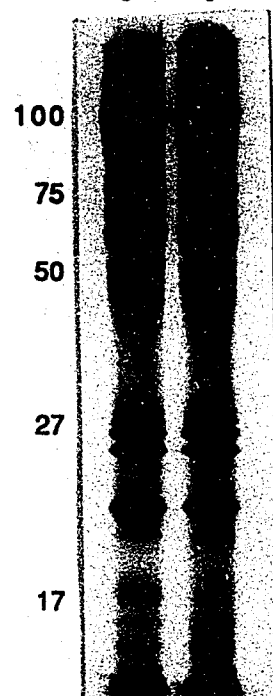
Panel C: Southern blotting analysis of nascent Ad5 RNA synthesized in the nuclear run-on reaction. Hela cells infected with Ad5 were treated with (Lane a and Lane b) and without (Lane c and Lane d) tunicamycin. At 24 hours post infection, the cytoplasmic membrane was lysed. Transcription reaction was proceeded as described under Materials and Methods. <sup>32</sup>P-UTP labeled nascent RNA was used to probe the Ad5 DNA which had been digested with Hind III (Lane a and Lane d) or Kpn I (Lane b and Lane c) and transferred to Nytran papers.

**A**

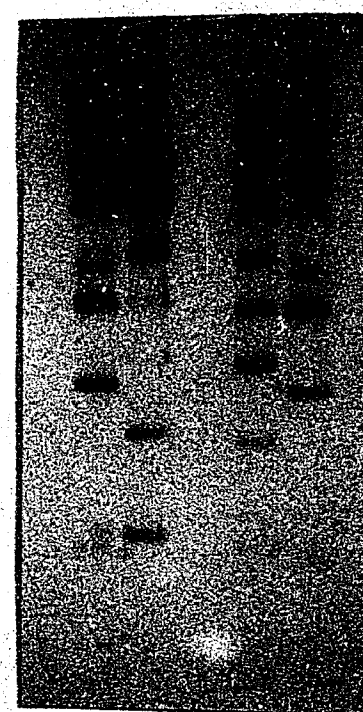
a b c d e f g

**B**

c t

**C**

a b c d



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