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Characterization of cis-acting elements of influenza A virus RNA

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CHARACTERIZATION OF cis-ACTING ELEMENTS OF INFLUENZA A VIRUS RNA

by

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ABSTRACT

CHARACTERIZATION OF cis-ACTING ELEMENTS OF INFLUENZA A VIRUS RNA

by

Xingqiang Li

Advisor: Professor Peter Palese, Ph.D.

Influenza A virus is an enveloped virus that contains a segmented RNA genome of negative polarity. My work has centered on studying cis-acting elements of influenza virus RNAs. First, an *in vitro* RNA synthesis system was established to analyze the promoter required for virion RNA (vRNA) synthesis. Substitution mutations were introduced into each of the first 13 positions of the 3' noncoding sequence of a complementary RNA (cRNA) template, and the effects of these mutations on vRNA synthesis were determined. The first 11 nucleotides of the 3' noncoding sequence were found to contain the minimum promoter required for vRNA synthesis. It was also found that addition of extra nucleotides at the 3' end decreased the promoter activity of the templates, indicating that the influenza virus polymerase does not recognize an internal promoter efficiently.

The mutations in the model RNAs were also examined *in vivo* using a ribonucleoprotein (RNP) transfection system. In contrast to the *in vitro* system, it was found that the majority of mutations at the 3' terminal sequence significantly decreased or abolished expression of the model

RNAs containing the chloramphenicol acetyltransferase (CAT) reporter gene. These results suggest that the cRNA promoter overlaps other essential cis-acting elements required for CAT expression *in vivo*.

The second part of my studies on cis-acting elements concerned a further analysis of the polyadenylation signal of influenza A virus RNAs. Earlier work had shown that a stretch of uridines near the 5' end of the virion RNAs and the RNA duplex made up of the 3' and 5' terminal sequences adjacent to the U stretch are involved in polyadenylation. I have further characterized the polyadenylation signal of influenza virus RNAs using the RNP transfection system. I found that (1) the optimal length of the U stretch is 5 to 7 uridine residues; (2) the sequence upstream of the U stretch at the 5' end is not involved in polyadenylation; and (3) the optimal distance between the 5' end and the U stretch is 16 nucleotides. The combination of these specific features defines the polyadenylation site of influenza virus RNAs.

DEDICATION

This dissertation is dedicated to my wife, Jianghao Chen,
and my father and mother.

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FORMAT OF THESIS

This thesis is prepared according to the guidelines of Graduate School of Biological Sciences, Mount Sinai School of Medicine, City University of New York. The thesis has a general introduction with literature review, two papers as chapters and a short discussion/outlook section. Copyright permission for the published paper has been obtained from the publisher.

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INTRODUCTION

Influenza virus, an enveloped animal virus, causes an acute infectious respiratory disease and remains a constant threat to human health. Studies on the understanding of the replication of influenza viruses have been hampered because influenza viruses contain a negative strand RNA genome which has been refractory to genetic manipulation approaches. However, the recent establishment of reverse genetics techniques for influenza virus has opened new avenues for studying influenza viruses. One of the experimental advances concerns the analysis of cis-acting elements which are essential in regulation of viral gene expression. Another aspect is rescue of transfectant viruses which have novel properties and may find use either as new influenza virus vaccines, or as vectors to express foreign epitopes and proteins.

This thesis describes studies aimed at defining the cis-acting elements which regulate viral gene expression. Chapter 1 reviews the current knowledge of the proteins required for influenza A virus replication and describes recent advances made in studying the essential cis-acting elements of influenza virus RNAs. Chapter 2 describes in detail my experiments on the mutational analysis of the promoter required for virion RNA synthesis. Chapter 3 summarizes my results on the characterization of the polyadenylation signal of influenza virus RNAs.

CHAPTER 1

Overview

The genome of influenza A virus contains eight single-stranded RNA segments of negative polarity (Palese, 1977). The negative sense virion RNAs (vRNA) are the templates for viral messenger RNA (mRNA) synthesis which is primed by 5' capped fragments derived from host-cell mRNAs. The viral mRNAs terminate at a stretch of U residues 17-22 nucleotides away from the 5' end of the vRNAs (Fig.1). Replication of vRNAs involves synthesis of full length complementary RNAs (cRNA), which in turn are the templates for the synthesis of vRNA molecules (Fig.1). Thus there are three types of virus specific RNAs, i.e. vRNA, cRNA, and mRNA. Their synthesis takes place in the nucleus of infected cells and it is dependent on the presence of viral polymerase (Krug *et al.*, 1989; Lamb and Choppin, 1983).

Sequence analysis indicates that the coding region of each segment of the influenza virus is flanked by noncoding sequences at both the 3' and the 5' ends. The first 12 nucleotides at the 3' terminus and the first 13 nucleotides at the 5' terminus are highly conserved among the RNA segments. In addition, the 3' and the 5' noncoding sequences show partial inverted complementarity, and vRNAs form panhandle structures (Fig.2; Skehel and Hay, 1978; Robertson, 1979; Desselberger *et al.*, 1980; Hsu *et al.*, 1987). It was also suggested that the noncoding sequences and the panhandle structure might serve as regulatory elements for transcription, replication and possibly also for the packaging of vRNAs into virions. The establishment of reverse genetics techniques

for influenza viruses made it possible to study the functions of these cis-acting elements.

A. Proteins required for viral RNA transcription and RNA replication

The segmented genome of influenza A viruses codes for at least ten proteins. Each of the two smallest genomic fragments encodes two proteins. The M1 and M2 and NS1 and NS2 proteins are encoded by segment 7 and segment 8, respectively (Ritchey *et al.*, 1976; Lamb, 1989). The integral membrane protein M2 and the nonstructural protein NS2, are synthesized from spliced mRNAs. All the other viral proteins are translated from unspliced viral mRNAs (Lamb, 1989).

The polymerase complex consists of three proteins-PB1, PB2, and PA which are encoded by the three largest virion RNA segments (1-3) (Palese *et al.*, 1977). The function of the polymerase proteins has been extensively studied. It was shown that the three polymerase proteins form a complex when infected cell proteins were labelled with [^{35}S]-methionine and subsequently subjected to immunoprecipitation analysis using an anti-PB1 antiserum (Krug *et al.*, 1987). It was found that all three polymerase proteins were precipitated. Immunoelectron microscopy studies using monospecific antisera against the individual proteins and indirect immunogold labeling has shown that each of the polymerase proteins is essentially located at only one end of each virion nucleocapsid (Murti *et al.*, 1988). The formation of the polymerase protein complex appears to occur in the absence of other influenza virus gene products. For example, viral polymerase proteins which were expressed in Xenopus oocytes were shown to form complexes (Digard *et al.*, 1989).

When all three polymerase proteins were expressed in insect cells by baculovirus vectors, they were precipitated by both anti-PB1 and anti-PB2 antisera (Krug *et al.*, 1987; St. Angelo *et al.*, 1987).

The function of the polymerase complex in mRNA synthesis was studied using ultraviolet (UV) light-induced crosslinking and *ts* mutants (Braam *et al.*, 1983). It is likely that the complex of the three polymerase proteins, rather than any particular one of the polymerase proteins, recognizes and binds to the 3' ends of the vRNAs to initiate mRNA synthesis (Ulmanen *et al.*, 1981; Blaas *et al.*, 1982; Braam *et al.*, 1983). It was found that the PB2 protein in this complex recognizes and binds to the cap of the primer RNAs (Ulmanen *et al.*, 1981; Blaas *et al.*, 1982; Braam *et al.*, 1983). Nucleocapsids isolated from mutants which have temperature-sensitive (ts) lesions in the PB2 lose the cap-dependent endonuclease activity and fail to bind to capped primer fragments at high temperatures (Ulmanen *et al.*, 1983). PB1 was shown to catalyze the addition of each nucleotide during transcription (Braam *et al.*, 1983). The specific role of PA in the polymerase complex is not known. However, there is some evidence indicating that PA is involved in viral RNA replication (Krug *et al.*, 1975; Mahy *et al.*, 1981).

The fourth protein which plays an important role in viral RNA replication is the NP which is encoded by segment 5. The function of NP has been studied *in vitro* and by using *ts* mutants. An *in vitro* RNA replication system was obtained by preparing nuclear extracts from virus-infected cells (Beaton and Krug, 1984; 1986). The nuclear extracts contained both polymerase complex and endogenous viral RNA templates. These extracts could be further fractionated by centrifugation. Little activity of either viral mRNA or cRNA synthesis was displayed when

the supernatant fraction alone was assayed. The pellet fraction containing nucleocapsids synthesized mRNA, but little or no cRNA. When the supernatant fraction was added back to the nucleocapsid fraction, cRNA synthesis was restored. Antibody depletion experiments were performed to identify that the NP in the supernatant was responsible for the restoration of cRNA synthesis activity. This was confirmed by studies using a mutant with defects in the NP protein. In cells infected with this mutant at nonpermissive temperature the synthesis of cRNA and vRNA was inhibited, but not that of viral mRNA (Krug *et al.*, 1975; Mahy *et al.*, 1981; Shapiro *et al.*, 1988). Nuclear extracts isolated from the cells infected with this *ts* mutant also provided evidence for a role of the NP in viral RNA replication (Shapiro *et al.*, 1988).

The protein requirement for genome replication was also investigated by expressing viral proteins using different vectors. Huang et al. (1990) employed a vaccinia virus-driven replication system, in which the viral proteins were supplied through infection with chimeric vaccinia viruses. It was determined that the minimum subset of influenza virus proteins needed for specific replication and expression of viral RNAs are the three polymerase proteins and the NP. Vectors expressing either the NS1 or the NS2 proteins were not needed for RNA replication (Huang *et al.*, 1990). A similar system was developed using SV40 expression vectors, and identical results were obtained (de la Luna *et al.*, 1993). Further confirmation of this work was recently obtained by using a cell line which expressed all three polymerase proteins and the NP (Kimura *et al.*, 1992).

B. Establishment of reverse genetics methods for influenza viruses

The establishment of reverse genetics techniques for studying influenza viruses was dependent on the successful isolation of biologically active viral polymerase complexes (Honda *et al.*, 1988; Parvin *et al.*, 1989). The first method was based on disrupting viral particles and isolating ribonucleoprotein (RNP) cores through discontinous glycerol gradient centrifugation. Viral cores were subsequently subjected to CsCl-glycerol gradient centrifugation to remove associated RNA molecules. Fractions containing viral polymerase and nucleoprotein (NP) were collected. The purified polymerase complex was active in both *in vitro* and *in vivo* systems (Fig.3, 4; Parvin *et al.*, 1989; Luytjes *et al.*, 1989; Luo *et al.*, 1991; Li and Palese, 1992, 1993). Seong and Brownlee (1992a) reported a second method of isolating viral polymerase from virions. Viral cores were prepared from viral particles using a similar glycerol gradient centrifugation as described above (Honda *et al.*, 1988; Parvin *et al.*, 1989). RNAs in RNP cores were then digested by micrococcal nuclease in the presence of Ca^{++}. EGTA was added to inactivate micrococcal nuclease after incubation. The resulting RNP cores were active in viral RNA synthesis *in vitro* (Fig.3) and *in vivo* (Fig.4).

Luytjes *et al.* (1989) developed an RNP transfection system (Fig.4) using purified viral polymerase. A recombinant RNA molecule (IVACAT1) was constructed which contained the chloramphenicol acetyltransferase (CAT) gene in negative sense flanked by the 26 nucleotides of the 3' and the 22 nucleotides of the 5' noncoding sequences of the WSN virus segment encoding the nonstructural (NS) protein. The RNP was reconstituted by mixing the viral polymerase and the synthetic RNA

molecules, and was transfected into Madin-Darby bovine kidney (MDBK) cells which were infected with influenza A/WSN/33 virus as a helper virus. CAT activity was detected in RNP-transfected cells. In addition, the recombinant RNA was packaged into virus particles. The results demonstrated that the 26 nucleotides of the 3' and the 22 nucleotides of the 5' noncoding sequences are sufficient to provide the signals for RNA transcription, RNA replication, and packaging of RNA into influenza virus particles. However, since CAT expression is the result of multiple cycles of viral RNA replication and involves the transcription and translation of mRNAs, *in vitro* systems were required to study specific promoters of influenza virus RNAs.

C. The promoter required for cRNA synthesis

Parvin *et al.* (1989) established the first *in vitro* influenza RNA synthesis system using the purified polymerase complex. Short, synthetic RNA molecules containing, at their ends, sequences corresponding to the 3' and the 5' terminal nucleotides of the influenza virus WSN NS gene, were used as templates. When the templates were mixed with the polymerase complex, they were copied into complementary strands. A schematic diagram illustrating the strategy for this *in vitro* viral RNA synthesis system is shown in Fig.3. It was shown that the first 15 nucleotides of the 3' noncoding sequences contain the promoter for cRNA synthesis, and that the 5' noncoding sequences are not necessary for this activity. The addition of extra nucleotides at the 3' end of the templates reduced promoter activity (Parvin *et al.*, 1989).

A similar *in vitro* mutational analysis of the promoter for cRNA synthesis was performed using short, synthetic RNA templates and

micrococcal nuclease-treated viral cores. RNA molecules containing only the first 12 nucleotides of the 3' noncoding sequence were active as templates in RNA synthesis. Based on this observation, it was concluded that the promoter for cRNA includes the first 12 conserved nucleotides of the 3' end of vRNA. Subsequently, single substitution mutations were introduced into each position and changes at position 9-11 were shown to significantly reduce promoter activity, whereas a mutation at position 12 only slightly reduced the template activity (Seong and Brownlee, 1992b). The specific binding of the polymerase complex to the vRNA promoter was demonstrated using a photochemical cross-linking assay. The effect of mutations in all 12 positions on the binding activity was studied and it was demonstrated that nucleotides 9 to 12 are crucial for the effective binding of the polymerase complex to the vRNA promoter and that they represent a binding site (Fodor *et al.*, 1993). To study the promoter *in vivo*, an RNP transfection system without the use of a helper virus was developed, and the mRNA level in transfected cells instead of CAT activity was directly measured (Yamanaka *et al.*, 1991). A limited number of mutants was examined, and it was concluded that the promoter required for transcription resides at positions 6-14 with respect to the 3' end of the genomic segment.

To precisely define the promoter for cRNA synthesis, Piccone *et al.* (1993) performed extensive analyses of the promoter sequence. A long RNA template (IVACAT1) was used in this study, which may better resemble the vRNA segments in size (Luytjes *et al.*, 1989). A complete set of mutants containing single substitution mutations in the first 15 positions of the 3' noncoding sequence was prepared and tested in the *in vitro* and the *in vivo* system (Fig.3, 4; Parvin *et al*, 1989; Enami and

Palese, 1991). The results suggested that nucleotides 1 to 14 at the 3' end represent the promoter on the vRNA. It was also confirmed that the addition or the deletion of nucleotides at the 3' end of the promoter reduced the *in vitro*, and the *in vivo* activity, indicating that exact ends are required for optimal promoter activity (Piccone *et al.*, 1993).

D. The promoter required for vRNA synthesis

The cRNA is a complete copy of the vRNA, and serves as template for vRNA synthesis. The promoter required for vRNA synthesis was investigated extensively. An *in vitro* system was established by reconstituting viral polymerase purified from a CSCl-glycerol gradient and synthetic plus-sense RNA templates (Fig.3; Li and Palese, 1992). The model RNA used in the study contained the CAT coding sequence in the plus sense and the noncoding sequences derived from the cRNA of the NS segment of WSN virus. Since the first 13 nucleotides are conserved among all 8 segments, mutations were introduced into each of the 13 positions. Mutants were then examined using the *in vitro* system. It was found that the first 11 nucleotides contain the minimum promoter required for vRNA synthesis *in vitro*. Some of the mutations decreased and some increased the template activity whereas others had no significant effect. Extra nucleotides added at the 3' end decreased vRNA synthesis, indicating that the viral polymerase does not recognize an internal promoter sequence efficiently. The mutants were also evaluated using an *in vivo* RNP transfection system (Fig.4). It was found that the majority of substitution mutations abolished CAT expression. The results suggested that the cRNA promoter sequence most likely overlaps other essential cis-acting elements involved in viral gene expression (Li and

Palese, 1992). A more detailed description of these experiments will follow in Chaper 2.

Seong and Brownlee (1992) did similar experiments using viral RNP cores digested by micrococcal nuclease. They found that RNAs containing the first 13 nucleotides of the 3' noncoding sequence of a cRNA were active templates for vRNA synthesis *in vitro*. Mutations at positions 12 and 13 decreased the *in vitro* synthesis of vRNA. Based on these experiments, it was concluded that the first 13 nucleotides are the promoter for vRNA synthesis. An *in vivo* replication system was also developed based on a cell line expressing three viral polymerase proteins and the NP (Kimura et al., 1993). Transfection of the model cRNA (NSCAT) into the cell line led to expression of CAT activity in the absence of helper virus. A mutational analysis of the promoter required for replication was performed by measuring the CAT expression of the mutants in the cell line. In general the pattern of the effects of the mutations was consistent with what had been observed previously (Li and Palese, 1992).

E. Viral mRNA synthesis

Influenza virus mRNAs are incomplete copies of the individual genome segments, lacking sequences complementary to the 5'-terminal nucleotides of the virion RNA (Hay *et al.*, 1978). Initiation of viral mRNA synthesis involves a cap-snatching process (Bouloy, *et al.*, 1978; Krug *et al.*, 1989). The viral transcriptase has endonuclease activity which cleaves cap-containing fragments from host mRNAs and uses them as primers for viral mRNA synthesis. Viral mRNAs terminate in a poly A tail. Although the same templates are used, viral mRNAs are different from

cRNAs not only in structure, but also in the way they are synthesized. First, mRNA synthesis needs the presence of primers, cRNA synthesis does not. mRNA synthesis terminates at the polyadenylation site whereas cRNA synthesis continues to the 5' terminus. Finally, mRNAs and cRNAs are synthesized at different times of infection. The molecular mechanisms underlying these differences are not well understood.

It has been shown that the conserved tract of uridine residues, approximately 17 to 22 nucleotides from the 5' end of each segment, is the site of polyadenylation of influenza virus mRNA (Robertson *et al.*, 1981). However, for polymerase to avoid premature polyadenylation at other tracts of uridine residues elsewhere within the genome, additional features must be involved in signaling polyadenylation. There is no conserved nucleotide sequence upstream of the specific uridine tracts which are involved in signaling polyadenylation. However, it has been hypothesized that the panhandle structure of the genome adjacent to the common uridine tracts might provide a physical barrier to the movement of the polymerase (Fig.2). This hindrance results in "stuttering" or "chattering" of the polymerase which would allow the repetitive copying of the uridine tract (Robertson *et al.*, 1981).

There is as yet no *in vitro* system which allows viral mRNA synthesis to be reconstituted from purified viral polymerase and synthetic vRNA templates. However, the cis-acting elements required for mRNA polyadenylation, were investigated *in vivo* using an RNP-transfection system (Luo *et al.*, 1991). A series of mutants was made with changes in the 3' and the 5' noncoding region of IVACAT1 RNA. It was found that a mutation (U to G) in the middle of the U stretch abolished CAT expression, confirming the role of the U stretch in polyadenylation. The

importance of the duplex structure adjacent to the U stretch was shown by studying two kinds of mutants. The first group of mutants contained changes resulting in the gradual opening of the duplex structure. Destroying one or two base pairs adjacent to the U stretch did not affect CAT expression. However, disruption of the dsRNA structure by three or four mismatches significantly decreased CAT expression. In the second set of mutants, the duplex structure was inverted by swapping the 3' and 5' sequences which form the base pairs. Even swapping of nucleotides of four base pairs of the duplex structure adjacent to the U stretch did not affect polyadenylation. In this study it was demonstrated that CAT expression correlated with mRNA levels in transfected cells. These data suggested that the duplex structure made up of the 3' and the 5' terminal sequences of the RNA is involved in the polyadenylation of influenza viral mRNAs (Luo *et al.*, 1991).

The polyadenylation signal of the influenza virus RNA was further characterized by experiments described in Chapter 3. It was found that the optimal length of the U stretch is 5 to 7 uridine residues and that the optimal distance of the U stretch to the 5' terminus is 16 nucleotides. It was also found that the upstream sequences (3' to the U stretch) do not affect polyadenylation.

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Figure 1

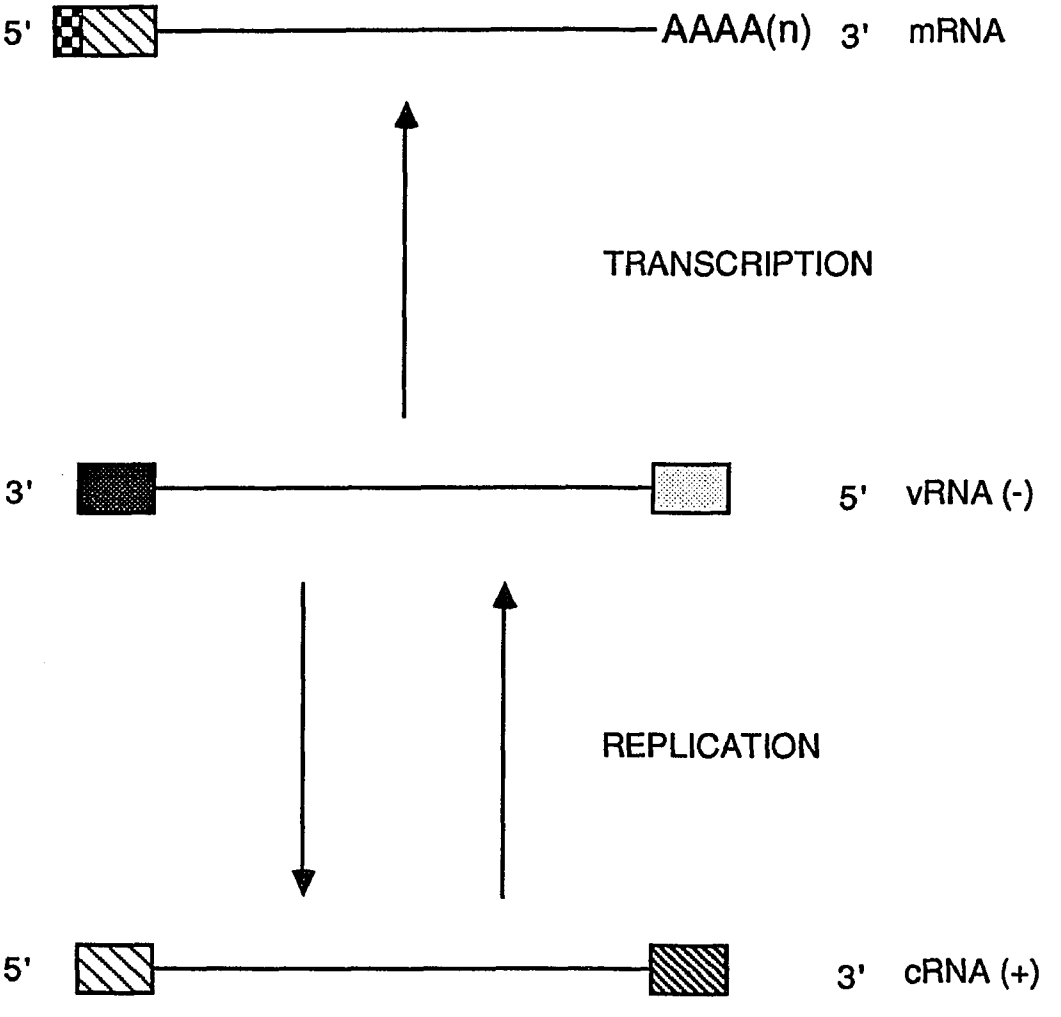

FIG. 1. Schematic diagram showing the synthesis of influenza virus RNAs . Influenza virion RNAs (vRNAs, minus sense) are the templates for both messenger RNA (mRNA) and complementary RNA (cRNA) synthesis. mRNA synthesis is primed by 5' capped fragments of 10-13 nucleotides in length which are derived from host cell mRNAs. Termination of mRNAs occurs at a stretch of U residues 17-22 nucleotides away from the 5' end of vRNAs. cRNAs are complete copies of vRNAs, and in turn serve as templates for vRNA synthesis. The dark stippled box and the light stippled box represent the 3' noncoding sequence and the 5' noncoding sequence of the vRNA, respectively; the light hatched box and the dark hatched box represent the 3' and the 5' noncoding sequences of the cRNA, respectively. The box with black squares at the 5' end of the mRNA represents the 5' capped fragment derived from host-cell mRNA.

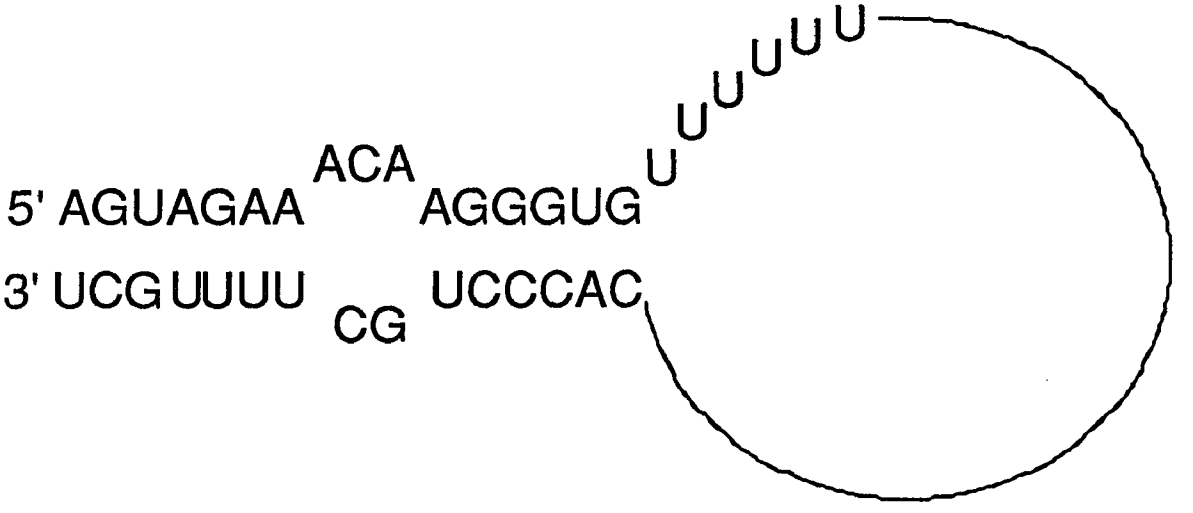

FIG.2. Panhandle structure of the NS segment of influenza WSN virus. The partially complementary sequences of the 5' and 3' ends and the U stretch near the 5' end of the NS segment of WSN virus are shown. It has been demonstrated that genomic RNAs of influenza virus may form a panhandle structure in virions and in infected cells.

Figure 3

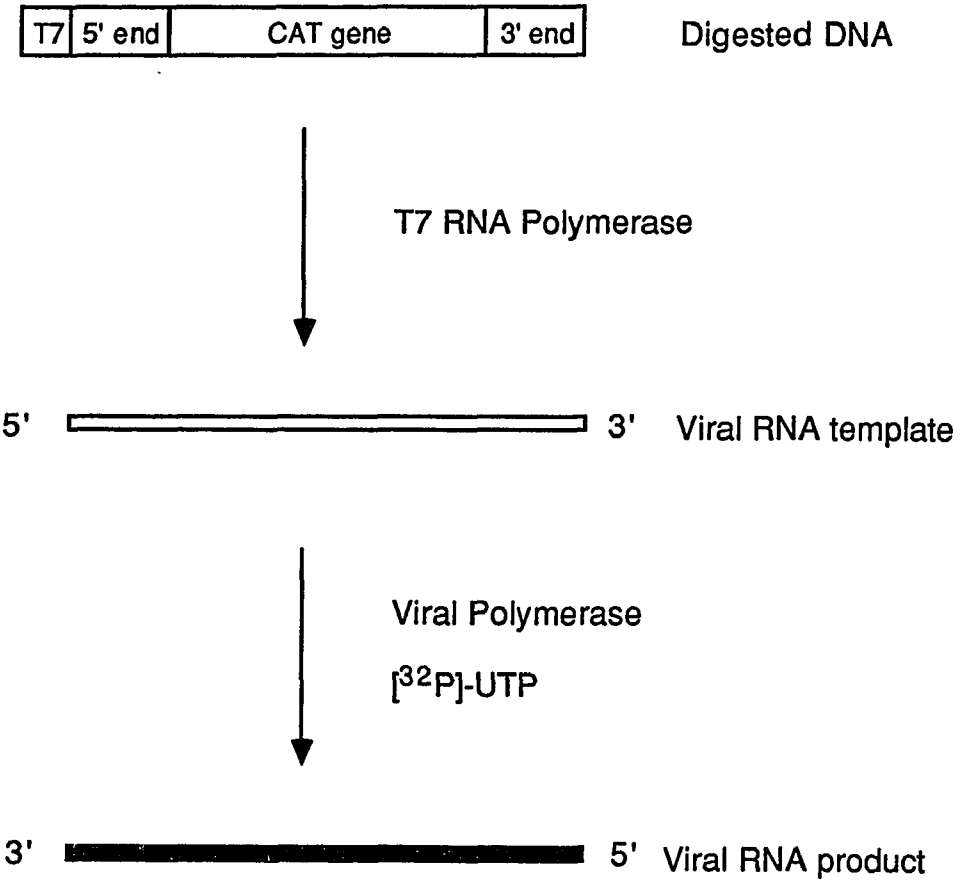
T7
5' end
CAT gene
3' end
Digested DNA
T7 RNA Polymerase
5'
3'
Viral RNA template
Viral Polymerase
[32P]-UTP
3'
5'
Viral RNA product

FIG. 3. *In vitro* system used to study viral RNA synthesis. Plasmids contain a T7 promoter, the 3' and the 5' noncoding sequences of the NS gene of influenza virus as well as the coding sequence of a reporter gene (CAT). A variety of mutants can be generated which contain mutations in the promoter or other regions of the 3' and the 5' noncoding sequences. The plasmids are linearized with appropriate restriction enzymes and used for generation of template RNAs by T7 RNA polymerase transcription. The synthetic viral (template) RNAs are then mixed with purified viral polymerase in the presence of [^{32}P]-UTP and other three NTPs. The labeled products are analyzed on polyacryamide gels. The abbreviations T7: T7 RNA promoter; 5' and 3' end: the 5' and 3' noncoding sequences derived from the either the vRNAs or the cRNAs of influenza viruses; CAT gene: CAT coding sequence either in minus or in plus sense, representing vRNA or cRNA, respectively.

Figure 4

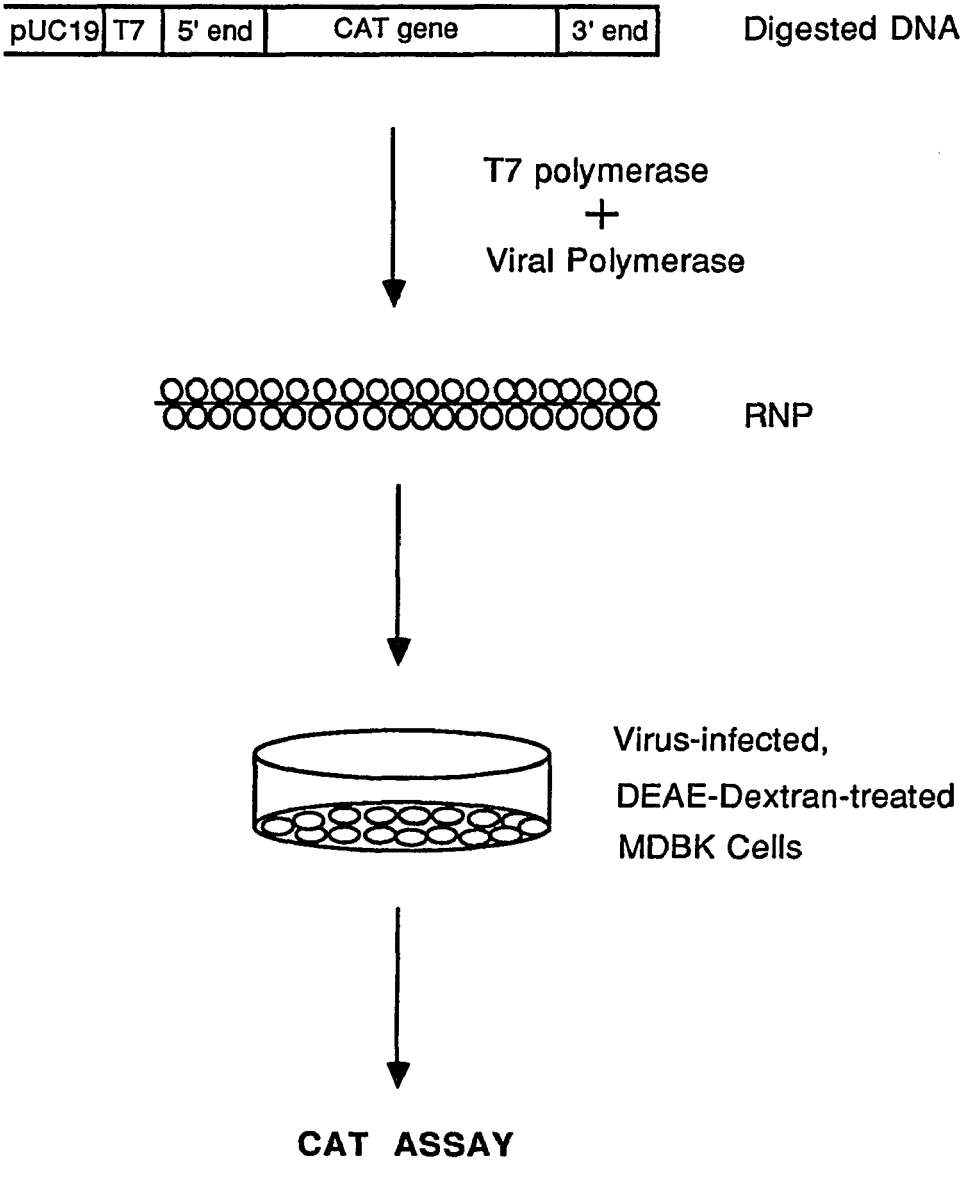
pUC19
T7
5' end
CAT gene
3' end
Digested DNA
T7 polymerase
+
Viral Polymerase
RNP
Virus-infected,
DEAE-Dextran-treated
MDBK Cells
CAT ASSAY

FIG. 4. Ribonucleoprotein (RNP) transfection system used to study RNA synthesis *in vivo*. Appropriate (linearized) plasmids are transcribed by T7 RNA polymerase in the presence of purified influenza viral polymerase complex. The newly synthesized RNAs and the viral polymerase complex form RNPs which are transfected into helper virus-infected MDBK cells by the DEAE-Dextran method. CAT assays are used to quantitate CAT gene expression which is dependent on the presence of viral noncoding sequences (i.e. promoter sequences and other cis-acting signals). The abbreviations are the same as shown in Fig.3.

CHAPTER 2

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Mutational Analysis of the Promoter Required for Influenza Virus Virion RNA Synthesis

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ABSTRACT

An *in vitro* RNA synthesis system was established in which the influenza virus vRNA (virion RNA) was made from the synthetic plus sense RNA (cRNA) template by purified viral polymerase complex. The cRNA promoter was studied by mutational analysis using the *in vitro* system and based on these experiments, the first 11 nucleotides of the 3' noncoding sequence were found to contain the minimum promoter required for vRNA synthesis. Addition of extra nucleotides at the 3' end decreased promoter activity of the templates, indicating that the viral polymerase does not recognize an internal promoter efficiently. The wild type and mutated RNA templates were also tested *in vivo* using the ribonucleoprotein transfection system. In contrast to the *in vitro* system, it was found that the majority of mutations at the 3' terminal sequence significantly decreased or abolished chloramphenicol acetyltransferase (CAT) expression. These results suggest that the cRNA promoter overlaps other essential cis elements required for CAT expression *in vivo*.

INTRODUCTION

It has recently become possible to study functions of the cis-acting elements of influenza viruses. We developed a ribonucleoprotein (RNP) transfection system in which a recombinant RNA (CAT1 RNA) molecule containing the chloramphenicol acetyltransferase (CAT) gene in the negative sense and influenza virus RNA specific noncoding sequences was transfected into Madin-Darby bovine kidney (MDBK) cells. Following infection with influenza A/WSN/33 virus, CAT activity was detected in the RNP-transfected cells (Luytjes *et al.*, 1989). In addition, the recombinant RNA was packaged into virus particles. These results demonstrated that the 3' and 5' noncoding sequences are sufficient to provide the signals for RNA transcription, RNA replication, and packaging of RNA into influenza virus particles (Luytjes *et al.*, 1989). A similar *in vivo* RNP transfection system was also recently reported and it was confirmed that regions of the 3' end of vRNAs act in cis to promote transcription (Yamanaka *et al.*, 1991). We then proceeded to further investigate the signals for mRNA polyadenylation using our *in vivo* RNP-transfection system (Luo *et al.*, 1991). In this study, a series of mutants was made with changes in the 3' and the 5' noncoding region of CAT1 RNA. It was found that the stretch of uridine residues and the panhandle structure of vRNAs, which is formed by the 3' and the 5' termini, are essential for polyadenylation of viral mRNAs (Luo *et al.*, 1991).

We had also established an *in vitro* influenza RNA transcription system which utilizes purified influenza virus polymerase complexes (Parvin *et al.*, 1989). Short RNA molecules containing, at their ends, sequences corresponding to the 3' and the 5' terminal nucleotides of the influenza virus A/PR8/34 NS gene, were transcribed. It was shown that the promoters for cRNA and vRNA synthesis lay within the 3' noncoding sequence of the respective templates (Parvin *et al.*, 1989; Piccone *et al.*, 1993). Recently, a similar *in vitro* approach was reported using micrococcal nuclease-treated viral cores and synthetic cRNA or vRNA templates (Seong and Brownlee, 1992).

In the present paper, we expand on such *in vitro* studies and attempt to precisely define the promoter required for influenza vRNA synthesis. Conditions were established to quantitatively measure the synthesis of vRNA *in vitro* using purified viral polymerase and synthetic cRNAs as template. A complete set of mutants with substitutions in the first 13 nucleotides of the 3' noncoding sequence of cRNA was made and the effects of substitution mutations on vRNA synthesis were assayed. Our results indicate that the first 11 nucleotides at the 3' end represent the minimum promoter for vRNA synthesis. An additional feature of this promoter is that it must be located at the very 3' end of the cRNA because the viral polymerase did not efficiently recognize these sequences when they were followed by extra nucleotides at the 3' end. The RNP transfection system was used to evaluate the same mutants *in vivo*. It was found that the majority of substitution mutations abolished CAT expression. Since several mutations which show no loss of promoter activity *in vitro* have diminished CAT expression *in vivo*, the cRNA promoter sequence most

likely overlaps other essential cis elements involved in viral gene expression.

MATERIALS AND METHODS

Purification of the viral RNA polymerase. Influenza virus nucleoprotein (NP) and the three polymerase proteins were purified from A/PR8/34 virus essentially following a previously published protocol (Enami and Palese, 1991; Parvin *et al.*, 1989). Briefly, ten milligrams of influenza A/PR8/34 virus were purified by sucrose cushion centrifugation and were disrupted in 2 ml of disruption buffer containing 1.5% Triton N-101, 10 mg of lysolecithin at 31°C for 25 minutes. Disrupted virus was fractionated by centrifugation on a 30-70% glycerol (w/v) step gradient. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were identified by silver staining. The fractions containing the RNP were pooled and were then subjected to CsCl glycerol gradient centrifugation. Polymerase fractions were again identified by SDS-PAGE and silver staining. Polymerase fractions containing the NP and three polymerase proteins were collected. These fractions were pooled and dialyzed first against 50 mM Tris hydrochloride (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol for 3 h at 4 °C. Subsequently, the dialysis was continued for another 3 h against the same solution containing 50% glycerol.

Construction of plasmids. Plasmid IVACAT200 was generated using the polymerase chain reaction (PCR) with the following primers: 5'-GATTACGCCAAGCTTCTCGAGTAATACGACTCAC-3' and 5'-GGGATCCT CTAGACGCCCTGCAGTAGAAACAAGGGTGTTTTTTGATCATTACGCCCC GCC-3' (Applied Biosystems, Inc.) and pIVACAT9 (Huang et al., 1990) as

template (Luytjes *et al.*, 1989). One insertion (G, underlined) and one mutation (T to C, underlined) were included in the latter primer which led to the generation of a new BclI site (Fig.1). The PCR product was digested by restriction enzymes XbaI and HindIII and inserted into the appropriate sites of pUC19 (Fig.1). Thus the plasmid pIVACAT200 contains the sequences which would direct transcription of a plus sense RNA (CAT200 RNA). The BclI site was introduced to facilitate the construction of the mutant plasmids. The short, complementary oligonucleotides containing the 3' noncoding sequences with a single substitution mutation were synthesized using a DNA synthesizer (Applied Biosystems, Inc.) and were phosphorylated and annealed. Either PstI and BclI or XbaI and BclI overhangs were formed upon annealing of the two oligonucleotides. Meanwhile, the pIVACAT200 was digested with either PstI and BclI or XbaI and BclI and the larger DNA fragments were isolated by using a geneclean kit (Bio 101, Inc.). The annealed oligonucleotides and the purified DNA fragments were ligated and were transformed into competent GM161 cells (Fig.1). The presence of substitutions in the plasmids was confirmed by DNA sequencing. A similar strategy was used to obtain constructs which resulted in RNAs with an extra G, an extra GCGAG or a deletion of the first five nucleotides at the 3' terminus. The RNAs with 13 extra nucleotides and with a deletion of the first nucleotide at the 3' end were obtained by T7 RNA polymerase transcription from pIVACAT200 digested with XbaI and PstI respectively. In the latter case, DNA digested with PstI was rendered blunt with T4 DNA polymerase (Sambrook *et al.*, 1989).

Preparation of RNA templates. Plasmid DNAs were digested with HgaI or other appropriate restriction enzymes. The T7 RNA polymerase reaction was then performed using the standard protocol

(Promega Corporation). 100 units of T7 RNA polymerase (Stratagene, Inc.) were combined with 10 mM DTT, 0.5 mM NTPs, 100 units of RNasin (Promega Corporation), 0.08 μCi (~ 80,000 CPM) [^{3}H]UTP (Du Pont-NEN, Inc.) and 4 μg DNA template in a final volume of 100 μl of 1x transcription buffer (5x transcription buffer was provided by Stratagene, Inc.). Reactions were incubated at 37^{0}C for 2 h, DNA template was degraded with RQ1 DNase I (4 U, Promega Corporation). Run-off RNA transcripts were phenol/chloroform extracted and free nucleotides were removed by using Quick-Spin G-50 columns (Boehringer Mannheim Biochemicals). Following precipitation in ethanol, purified RNAs were resuspended in water. Quantitation of RNA templates was achieved by measuring ^{3}H incorporation. Under these conditions, about 1160 CPM of [^{3}H]UTP was incorporated into one microgram RNA template. The RNA was analyzed by PAGE (7.7 M urea) and visualized by silver staining.

Influenza virus polymerase reaction. The *in vitro* RNA synthesis was performed using conditions described previously (Parvin *et al.*, 1989). Briefly, in a total volume of 25 μl, about 0.5 μg of protein (three polymerase proteins and NP) were mixed with 1 μg cRNA template (unless stated otherwise). 0.4 mM dinucleotide ApG, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 20 μM UTP, and about 0.53 μM [α-^{32}P] UTP (40 μCi at 3,000 Ci/mmol; Du Pont-NEN, Inc.) were included. The reaction mixtures were incubated at 30^{0}C for 2h and terminated by adding 420 μl of ice-cold solution containing 0.3 M sodium acetate and 10 mM EDTA. The mixtures were extracted twice with phenol/chloroform (1:1). 10 μg of yeast tRNA was added as carrier for precipitation in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and resuspended in a dye

mix containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF.

Viral polymerase products labeled by [a-^{32}P]UTP were analyzed on 4% denaturing PAGE. Gels were exposed to X-ray films and the appropriate bands were cut out from the gels. SOLVABLE (Du Pont-NEN, Inc.) was used to elute RNA from the gel slices. The counts of ^{3}H and ^{32}P were measured in the corresponding channels in a Beckman LS 5000TD counter and corrections for spillover were made. The ratio of ^{32}P and ^{3}H counts was established and was used to measure template activity of synthetic mutant RNAs. The CAT200 RNA was included in every experiment to serve as control.

RNase T1 digestion. The reaction products of viral polymerase were analyzed by 4% polyacrylamide gel electrophoresis. The wet gel was exposed to an X-ray film, and the appropriate gel slices were excised. The gel slices were crushed in 300 µl elution buffer (provided in the ribonuclease protection assay kit by Ambion, Inc.) and incubated at 37°C overnight. The gel pieces were pelleted and the supernatant was collected. RNA was precipitated by adding ethanol in the presence of 10 µg tRNA as carrier. The RNA pellet was resuspended in 10 µl of formamide, denatured in boiling water for 2 min, and chilled on ice. Then 200 µl of RNase digestion buffer (Ambion, Inc.) was added along with 400 U of RNAse T1 (Boehringer Mannheim Biochemicals). The samples were incubated at 37°C for 30 minutes. Control RNAs identical to CAT1(vRNA) and CAT200 (cRNA) were obtained by T7 RNA polymerase transcription in the presence of [a-^{32}P]UTP, similarly gel purified, and then digested with RNase T1. Reaction products were extracted in phenol-chloroform,

precipitated in ethanol, and then analyzed on 20% polyacrylamide gel containing 7.7M urea.

RNP transfection and CAT assay. The RNP transfection protocol was essentially as described previously (Enami and Palese, 1991). Briefly, $1x10^6$ MDBK cells in a 35 mm dish were first infected with A/WSN/33 seed virus (moi ~ 1-2), then treated with 1 ml of a solution containing 330 μg/ml DEAE-dextran, 0.5% dimethyl sulfoxide in PBS-gelatin (0.1 mg/ml gelatin) for 30 min. 0.5 μg HgaI-digested DNA and 2 μl of T7 RNA polymerase (Stratagene, Inc.) were added in the standard T7 polymerase transcription reaction in the presence of 10 μl of viral polymerase complex (1 μg of NP and 3P). After incubation at 37°C for 15 minutes, 2 μl of RQ1 DNase I was added to digest the DNA template for three additional minutes. The reaction mixture was diluted with 200 μl of cold PBS-gelatin and then added to DEAE-dextran treated cells. After incubation at room temperature for 1 h, 2 ml of REM was added to each dish. Cells were further incubated at 37°C for 14-16 h and were harvested by using a rubber policeman and spun down. The cell pellet was resuspended in 100 μl of 0.25 M Tris HCl buffer (pH 7.5) and was disrupted by freeze-thawing three times. Cell debris was discarded and the supernatant was used for CAT assays.

CAT assays were performed according to the standard protocol (Luytjes *et al.*, 1989). One assay contained, in a volume of 150 μl, 2 μl of [^{14}C] chloramphenicol (0.1 μCi, 11μM, Du Pont-NEN, Inc.), 20 μl of 10 mM acetyl-CoA (Pharmacia P-L Biochemicals Inc.), 25 μl of 1M Tris HCl (pH 7.5), and 50 μl of cell extract in 0.25 M Tris-buffer (pH 7.5). In some cases, the cell extracts were diluted with 0.25 M Tris HCl buffer (pH 7.5) to obtain

CAT conversion rates in a linear range. The mixtures were incubated at 37°C for 2 h.

RESULTS

Constructs. The design of pIVACAT200 is shown in Fig.1. The plasmid contains the truncated T7 RNA polymerase promoter, the 26 nt of the 5' noncoding sequence and the 22 nt of the 3' noncoding sequence of the plus sense copy of the NS gene of the influenza A/PR8/34 virus (as indicated in the corresponding boxes). The CAT coding sequence in the plus sense is inserted between the 5' and the 3' noncoding sequences. The plasmid contains convenient restriction enzyme sites which allow mutagenesis of the 3' noncoding region (see Materials and Methods, Fig.1). The CAT200 RNA and mutated RNA derivatives result from T7 RNA polymerase transcription of HgaI-digested plasmid DNAs (Fig. 1).

***In vitro* vRNA synthesis by viral polymerase complex.** The CAT200 RNA contains the coding sequence of the CAT gene and is comparable in size (713 nt) to the smallest influenza virus gene (890 nt). When CAT200 RNA was mixed with purified viral polymerase complex in the presence of dinucleotide adenylyl-(3'-5')-guanosine (ApG), a product was obtained which comigrated with the CAT1 RNA marker transcribed from pIVACAT1 digested with HgaI (Luytjes *et al.*, 1989; Fig.2A, lane 3). In addition to the template specific product, a general background of bands (Fig. 2A) is seen, which most likely corresponds to products transcribed from truncated viral genomic RNA and DI RNA not removed from RNP complexes during the CsCl-glycerol centrifugation step, since they are also present in lane 1 of Fig.2A, in which no synthetic RNA template is added. Under the same condition, the vRNA synthesis activity of the viral

polymerase complex was 20% of the cRNA synthesis activity using CAT1 RNA as template (Fig.2A, lane 4). It should also be noted that the reaction is absolutely dependent on the presence of ApG (Fig.2A, lane 2), which is a unique feature of influenza virus associated polymerase (Parvin *et al.*, 1989; Ploth and Krug, 1977). The vRNA synthesis increases linearly up to 2h (Fig.2B). We also determined the linearity of the vRNA synthesis with respect to the concentration of input RNA template. In the range from 0.5 to 2 μg RNA template used, the product of the viral polymerase reaction was proportional to the amount of the input RNA template (Fig.2C).

In order to determine whether or not the plus sense RNA template is completely copied, a short RNA template was used so that a minor difference in size between template and product could be detected. The plasmid pM-wt was digested with MboII (Parvin *et al.*, 1989) and rendered blunt by T4 DNA polymerase (Sambrook *et al.*, 1989). A 53 nt long RNA resulted from the T7 RNA polymerase transcription and it contained the 5' and the 3' noncoding sequences derived from the plus sense RNA of the PR8 NS gene (Parvin *et al.*, 1989). This RNA molecule was then used as template in an *in vitro* viral polymerase reaction. As shown in Fig.2D, the product of the viral polymerase reaction (lane 3) comigrates with the marker prepared by T7 RNA polymerase transcription on a sequencing gel. This result suggests that vRNA synthesis primed by ApG starts at the third nt of the template and terminates at the very 5' end of the template.

An RNase T1 digestion of the *in vitro* products was also performed in order to confirm that the viral polymerase products were vRNA molecules. The products of the viral polymerase reaction using CAT200 RNA and CAT200/3C RNA molecules were isolated on 4% PAGE, eluted from gel slices, and then digested with RNase T1. The digestion products

were analyzed by electrophoresis, and the patterns of RNase T1 digestion (Fig. 2E, lanes 3 and 4) were compared with that generated by RNase T1 digestion of labeled CAT1 and CAT200 RNA molecules (Fig.2E, lanes 1 and 2). The CAT200/3C RNA containing an A to C mutation in the third position of the 3' noncoding sequence was used because of its high template activity (see below). The RNase T1 digestion patterns of the *in vitro* reaction products were essentially identical to that of CAT1 RNA (minus sense) and were different from the digestion pattern of CAT200 RNA (positive sense). The predicted lengths of the large T1 oligonucleotides of CAT1 RNA and CAT200 RNA are 28 and 21 and 18 and 15 nucleotides, respectively, in accordance with the patterns seen in Fig.2E. The results clearly show that the viral polymerase complex copied the CAT200 and CAT200/3C RNA templates to generate minus sense RNAs.

Mutational analysis of the promoter sequence required for vRNA (minus sense RNA) synthesis. In order to identify precisely those elements important in promoter function, a set of mutated RNA molecules was made which contained single nt substitutions within the 3' terminal sequence. The template activity of each mutated RNA was determined in independent experiments. Two or more experiments were used to calculate the promoter activity of each mutated RNA. In each experiment the template was ^{3}H-labeled and the product was ^{32}P-labeled to facilitate easy quantitation of the activity of each mutant template. In addition, each separate experiment contained a CAT200 RNA control. The results of the mutational analysis are shown in Fig.3. Substitution mutations in positions 12 and 13 showed no effect on template activity.

However, mutations in the 11th nt position reduced the template activity. We thus conclude that the 11 3' terminal nts of cRNA represent the minimum promoter sequence required for vRNA synthesis as determined in this system. The substitution mutations in the first 11 nts could be divided into three groups according to the effect on vRNA synthesis. Group one consists of mutations in positions 5, 8, 9 and 11. The common feature of these positions was that they showed little flexibility for mutations. Substitutions in these positions decreased vRNA synthesis *in vitro*. Therefore these positions appear essential for recognition by the viral polymerase complex. Positions 3, 4 and 6 belong to a second group. Some substitutions in these positions resulted in an increase of template activity. Of particular note, were substitutions 3A to 3C (3.4 fold), 4U to 4C (2.6 fold), and 6U to 6G (3.1 fold) which led to a substantial increase in template activity. A last group includes positions 2 and 10. These two positions could tolerate substitutions without much effect on promoter strength.

The mutational analysis of the first two nucleotides was complicated by the fact that ApG was used as primer. Thus two strategies were employed to address the role of terminal nucleotides in promoter function. Different dinucleotides were tested for their ability to stimulate viral polymerase, and RNA templates containing additional nucleotides at the 3' end and deletions of the terminal nucleotides were made to determine their effects on promoter activity (see below).

Priming activity of dinucleotides in vRNA synthesis *in vitro*. It has been speculated that dinucleotide primers had to be complementary to the 3' terminus of vRNA templates to be functional

(Ploth and Krug, 1977). The main evidence for this assumption was that ApG, the most active primer, is complementary to the first two nucleotides (UC) of vRNA (Honda *et al.*, 1986; Ploth and Krug, 1977). However, in our study, the promoter activity of RNA templates containing mutations at the second position of the 3' terminus of cRNA was not changed in the presence of the ApG primer (Fig.3). To further address this question, we tested three other available dinucleotides CpG, ApA and ApU in the *in vitro* vRNA synthesis system using RNA templates which contained mutations at the first two positions as template. The results are shown in Fig.4. CpG efficiently primes the vRNA synthesis when CAT200/1G was used as template in which the first nucleotide U was mutated to G. In this case, CpG becomes complementary to the first two nucleotides (GC) of the 3' terminus. When RNA molecules CAT200/1A and CAT200/1C were used as template, viral polymerase is not efficiently primed by CpG. On the other hand, ApA and ApU both possess a low priming activity for the wild type RNA. These results indicate that in the case of vRNA synthesis, the priming activity of dinucleotides is helped by complementarity at the first nucleotide position. Thus the decreased transcription of mutants in position 1 (Fig.3) is dependent on the presence of ApG as primer and might be artifactual if a different primer is used by the virus *in vivo*. The data also suggest that the G in the second position of ApG is important since its presence increases activity, but this G does not have to be complementary to the template in position 2 (see Fig.4). It should also be noted that ApA and ApU do not show an increase in primer activity when the complementary templates CAT200/2U and CAT200/2A were used, respectively (data not shown).

The majority of mutations at the 3' noncoding sequence of the plus sense RNA abolish CAT expression. The RNP transfection system has been used to elucidate cis elements of the influenza virus genome (Luytjes *et al.*, 1989; Luo *et al.*, 1991; Yamanaka *et al.*, 1991). The model RNAs used in most previous studies were negative sense RNA molecules similar to the genomic RNA of influenza virus (Luytjes *et al.*, 1989; Luo *et al.*, 1991). But it was also shown that the plus sense RNA molecule CAT 9 could be replicated in cells containing viral proteins provided by vaccinia virus vectors (Huang *et al.*, 1990). We first tested whether CAT200 RNA could be amplified to the same level as CAT9 RNA following transfection into MDBK cells. A new efficient transfection protocol was employed (Enami and Palese, 1991), in which CAT200 RNA and CAT9 RNA were transcribed by T7 RNA polymerase in the presence of viral polymerase complex and were transfected into MDBK cells infected with WSN helper virus. 14-16 hours after transfection, cells were harvested. The same level of CAT activity was detected (data not shown) from the cell extracts derived from these two transfections. Usually, 100 μl of cell extract were harvested from each transfection and 50 μl of 1:100 diluted cell extract generated a 15-30% conversion of chloramphenicol. Under the same condition, CAT activity detected from CAT 1 RNA transfections was about two times higher than that of CAT200 transfection (data not shown).

All RNA molecules which contained single substitution mutations in the first 13 nucleotides of the 3' noncoding sequence of cRNA were examined using this system along with CAT200 RNA as positive control. The CAT expression of the mutant RNAs is normalized against that of the CAT200 RNA. The results are shown in Fig.5. Mutations at positions 3, 4,

5, 8 and 9 abolished CAT expression completely and two of the three mutations at positions 2, 7, 12 and 13 led to a complete loss of CAT expression. The third mutation in these positions significantly decreased CAT expression. CAT expression was however detected for all RNAs with mutations in position 1, 6 and 10.

This dramatic decrease and loss of CAT expression are in contrast with results of the *in vitro* template activity of these RNAs. It suggests that mutations at the 3' noncoding sequence also affect other important cis elements necessary for RNA replication, viral mRNA synthesis and/or protein synthesis *in vivo*.

Analysis of the effect of additions and deletions of nt at the 3' noncoding sequence on vRNA synthesis and CAT expression. RNAs with an extra G, an extra GCGAG or 13 extra nts and a deletion of one or five nts at the 3' terminus were tested for promoter activity. The results are shown in Fig.6. When the length of additional nt at the 3' terminus of the cRNA is increased, the promoter activity of the RNA templates decreases rapidly. Deletion of the first nt or the first five nt also decreases the template activity. These results indicate that the promoter required for vRNA synthesis is located in the 3' terminus of the cRNA segment and that the viral polymerase complex can not efficiently recognize an internal promoter. All mutant RNAs were also tested in the RNP transfection system. Although the results confirm the observations made *in vitro* , the level of activity *in vivo* was consistently lower than that *in vitro*.

DISCUSSION

We have described conditions under which vRNA molecules were copied *in vitro* from synthetic cRNA templates by purified viral polymerase. The reaction was shown to be sequence-specific and to be dependent on the presence of ApG as primer. This system enabled us to analyze the promoter sequence required for vRNA synthesis. A series of mutants was made which contained single nt substitutions at the first 13 nt of the 3' noncoding sequence of the cRNA and these mutants were investigated using the *in vitro* vRNA synthesis system. Only the first 11 nt of the 3' noncoding sequence of the cRNA were found to contain the minimum promoter required for vRNA synthesis. This finding is partially supported by the evidence from *in vivo* studies using RNP transfection of CAT18 RNA which contains a mutation in position 13 and is amplified to the same level as the wild type RNA *in vivo* (Luo *et al.*, 1991). Although position 13 had been shown to be conserved at the 3' end of the cRNAs of all influenza A viruses, the above result suggested that this position would not be part of the cRNA promoter. In contrast, Seong and Brownlee (1992) have shown that in an *in vitro* replication system positions 13 and 12 are required for promoter activity. The difference between those and our results could be due to the difference of RNA templates used in the two studies. In our system, genome-size RNA templates, rather than short RNA molecules, were employed in the mutational analysis. It is possible that if the RNA template is too short, the proper nucleocapsid structure does not form. Thus, the vRNA synthesis activity of different length templates could be subject to variation. In this context, it should be mentioned that our earlier polymerase preparations were different from the ones used at present since the current polymerase preparations allow

synthesis of vRNA using segment length cRNA templates while the previous assay condition did not result in the synthesis of genome size vRNA segments (Parvin *et al.*, 1989). Alternatively our *in vitro* system may be less sensitive or the polymerase preparations may have different properties from that employed by Seong and Brownlee (1992). Finally, the fact that the first 13 nts (and not only the first 11 nts) at the 3' end of cRNAs among influenza virus RNA segments are conserved may be explained by requirements imposed by other cis acting sequences, such as packaging signals and not solely by those defined by the cRNA promoter.

Surprisingly, mutations in the second position of the templates do not alter priming by ApG, suggesting that the polymerase does not require a complementary base pair in this position. This is in contrast to the findings made with primers using influenza virus associated polymerase (Mcgeoch and Kitron, 1975; Ploth and Krug, 1977) or isolated polymerase complexes (Piccone *et al.*, 1993) leading to plus RNA synthesis. In this respect, recognition of the vRNA promoter by viral polymerase leading to plus sense RNA synthesis differs from recognition of the cRNA promoter leading to minus sense RNA synthesis (Piccone *et al.*, 1993).

CAT activity was detected upon transfection into influenza virus-infected cells of reconstituted RNP formed by synthetic CAT200 RNA or mutant RNAs and purified viral polymerase complex. Surprisingly, the majority of mutations abolished CAT expression. This difference of *in vitro* and *in vivo* data can be explained by the fact that the *in vitro* system measures only the step involved in vRNA synthesis whereas CAT expression is the result of multiple steps of replication of vRNA and cRNA followed by transcription and translation of viral mRNAs. Apparently,

single mutations in the 3' end affect not only vRNA synthesis in infected cells, but also disrupt the other overlapping cis elements that are essential for CAT expression. For example, the panhandle structure of vRNA segments which is formed by both the 5' and the 3' noncoding sequences of vRNAs was shown to play an important role in the polyadenylation of viral mRNA (Luo *et al.*, 1991). In addition, it is possible that the viral polymerase complex prepared from virus particles is different from the viral replicase in infected cells (Shapiro and Krug, 1998). For example, the polymerase complex used in this and an earlier (Seong and Brownlee, 1992) study can be primed for vRNA synthesis by a globin mRNA primer (data not shown), but this is most likely not the mechanism by which vRNA synthesis is primed *in vivo*. In fact, Shapiro and Krug (1988) have shown that an *in vitro* replication system using nuclear extracts from infected cells is independent of any primers. However, this system did not allow the study of synthetic RNA templates. Work is now in progress to isolate the viral replicase (free of endogenous cRNA) from virus-infected cells, to characterize it, and to compare it with that obtained from purified virus.

The addition of extra nt at the 3' noncoding sequence decreases the promoter activity of cRNA templates *in vitro* as well as CAT expression *in vivo*. The template activity of these RNA molecules *in vitro* decreased with the number of extra nt at the 3' end. When the RNA molecule with one extra nucleotide was transfected into virus-infected cells, the CAT activity was less than twenty percent of that of the wild type RNA. The addition of five extra nt at the 3' end of the RNA abolished CAT expression. These results indicate that the viral polymerase complex cannot efficiently recognize an internal promoter, and that a free 3' end is

important for the promoter *in vivo*. Deletion of one or five nt also markedly affected template activity *in vitro* and CAT expression *in vivo* (Fig.6). These results confirm that the promoter required for vRNA synthesis is located at the 3' terminus of the 3' noncoding sequence of cRNAs. A similar pattern had been observed earlier for the interaction between the vRNA promoter and the viral polymerase complex *in vitro* and *in vivo*. Parvin *et al.* (1989) showed that an RNA template with five extra nucleotides at the 3' noncoding sequence of the vRNA promoter was recognized and copied at approximately one-third the efficiency of the wild type template, and the addition of 13 or 38 extra nucleotides at the 3' end abolished the template activity. Recently Collins et al. reported that addition of 11 heterogeneous nts at the 3' terminus of an analog of the respiratory syncytial virus (RSV) genomic RNA abolished CAT expression in a similar transfection system (Collins *et al.*, 1991). Our results are, however, different from those obtained with the RSV system, in which deletion of the first three nucleotides at the 3' end did not affect CAT expression in a similar *in vivo* replication system (Collins *et al.*, 1991).

The study of viral promoters may have important practical implications in the light of recent developments to rescue transfectant viruses (Enami *et al.*, 1990; Enami and Palese, 1991; Li *et al.*, 1992; Muster *et al.*, 1991). Influenza viral RNA segments transcribed from plasmids were introduced into virus particles, and infectious viruses (transfectants) were obtained. Some of these transfectants have novel biological properties. For example, a chimeric neuraminidase gene which contained the coding region of the influenza A/WSN neuraminidase and the 3' and the 5' noncoding sequences of the NS gene of the influenza B/Lee virus was introduced into the genome of the A/WSN virus (Muster *et*

al., 1991). The rescued chimeric virus was attenuated in mice, and was highly immunogenic and protected mice against challenge virus. The results demonstrated that a change in the 3' and the 5' noncoding sequences of the influenza viral RNA segment can alter expression of the gene and thus confers new characteristics to the rescued virus. The detailed analysis of regulatory cis elements will enhance our ability to control expression of the introduced genes, as well as further our understanding of how influenza viruses replicate. On a practical level, these studies may result in the development of appropriate attenuated influenza virus vaccine strains.

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Figure 1

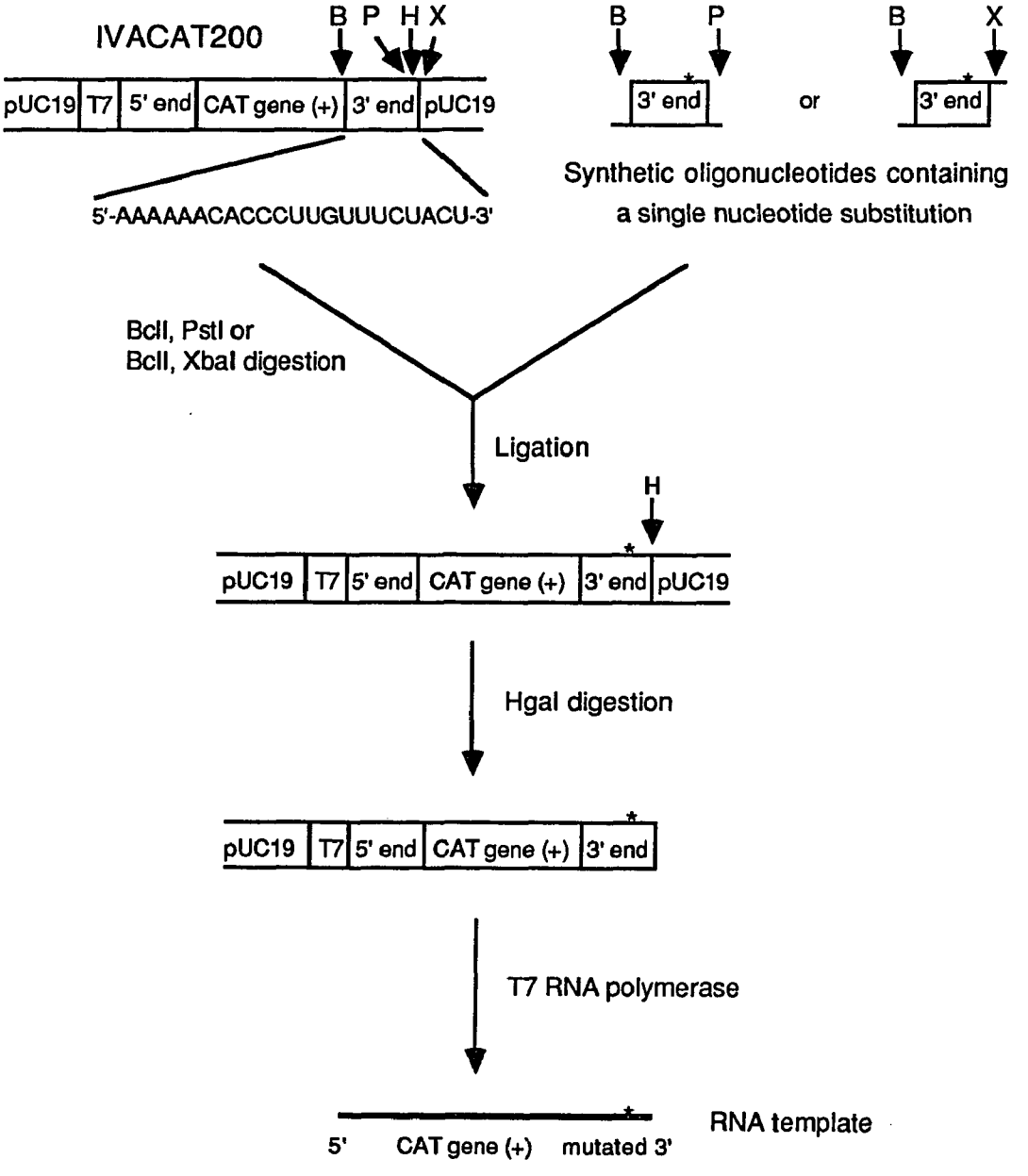
IVACAT200
B P H X
pUC19 T7 5' end CAT gene (+) 3' end pUC19
5'-AAAAAACACCCUUGUUUCUACU-3'
B P
3' end
or
B X
3' end
Synthetic oligonucleotides containing a single nucleotide substitution
BclI, PstI or
BclI, XbaI digestion
Ligation
H
pUC19 T7 5' end CAT gene (+) 3' end pUC19
HgaI digestion
pUC19 T7 5' end CAT gene (+) 3' end
T7 RNA polymerase
RNA template
5' CAT gene (+) mutated 3'

Fig.1. Preparation of the wild type and mutated plus sense RNA templates. The plasmid pIVACAT200 was digested with either BclI and XbaI or BclI and PstI. Synthetic oligonucleotides were obtained by using a DNA synthesizer (Applied Biosystems, Inc.) and inserted into the enzyme digested pIVACAT200. For example, an A to G change at the third position of the 3' terminal sequence of pIVACAT200 was achieved by inserting the following oligonucleotides: 5'-CTAGACGCCCTGCAG CAGAAACAAGGGTGTTTTTT-3' and 5'-GATCAAAAAACACCCTTG TTTC TGCTGCAGGGCGT-3' between the BclI and XbaI site of pIVACAT200. The presence of all mutations was confirmed by DNA sequencing . The resulting plasmids were digested with HgaI and then used in an *in vitro* T7 RNA polymerase transcription. Run-off transcripts were used as RNA templates for subsequent experiments. The various domains of pIVACAT200 are indicated: T7, truncated T7 promoter; 5' end and 3' end, the 5' and the 3' noncoding sequences of the cRNA (plus sense) of the influenza A/PR/8/34 NS gene; CAT (+), entire CAT gene coding sequence in the plus sense. Restriction enzyme sites are indicated: B, BclI; P, PstI; H, HgaI; X, XbaI. * represents a single nucleotide mutation in the 3' terminal 13 nucleotides. The 3' noncoding sequence of the CAT200 RNA is shown below the corresponding box. For construction of pIVACAT200 see Materials and Methods section.

Figure 2

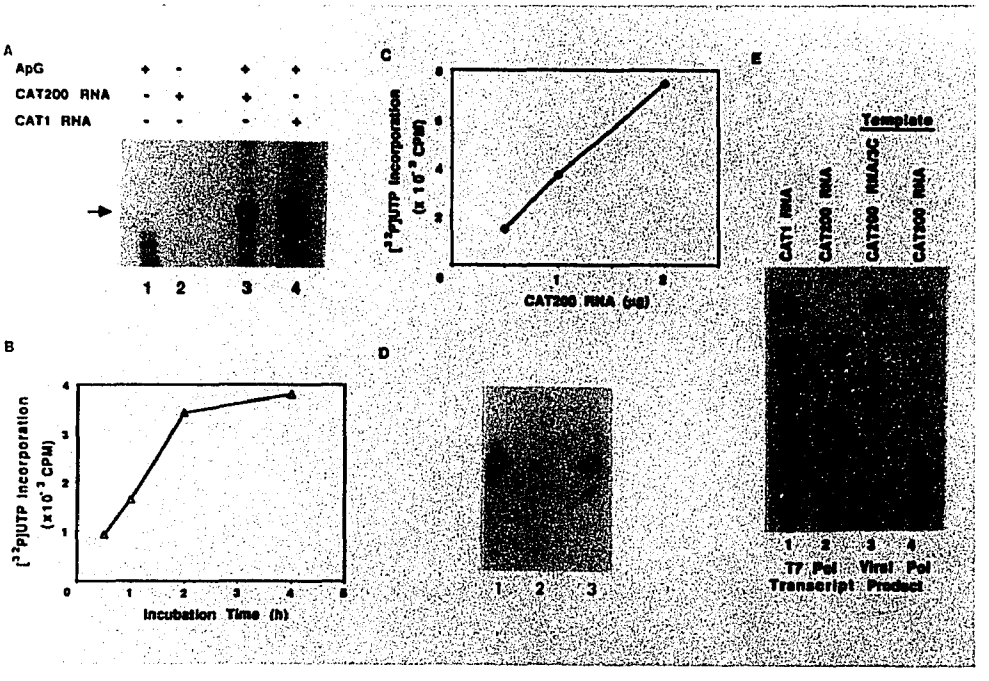
A
ApG
CAT200 RNA
CAT1 RNA
1 2 3 4
B
[32P]UTP Incorporation
(x10-3 CPM)
Incubation Time (h)
C
[32P]UTP Incorporation
(x 10-3 CPM)
CAT200 RNA (ng)
D
1 2 3
E
Template
CAT1 RNA
CAT200 RNA
CAT200 RNA/3C
CAT200 RNA
1 2 3 4
T7 Pol Transcript
Viral Pol Product

Fig.2. *In vitro* Synthesis of vRNA. (A) CAT200 RNA was used as template for vRNA synthesis and CAT1 RNA was used as template for cRNA synthesis. The reactions took place under conditions described in Materials and Methods. Lane 1, no input RNA; lane 2, CAT200 RNA; lane 3, CAT200 RNA; lane 4, CAT1 RNA. 0.4 mM ApG was used in lanes 1, 3 and 4. (B) 1 μg CAT 200 RNA was reconstituted with purified viral polymerase complex and incubated at 30°C for different time periods as indicated. The vRNA synthesized was quantitated by measuring incorporation of [^{32}P]UTP. (C) The different amounts of CAT200 RNA as indicated were reconstituted with purified viral polymerase complex and incubated at 30°C for 2h. The incorporation of [^{32}P]UTP is used to represent the amount of vRNA synthesized. (D) pM-wt was digested with MboII and transcribed using T7 RNA polymerase. The resulting RNA which contains the 5' and the 3' noncoding sequences of the cRNA of PR8 NS gene is 53 nt long in size and is used as template in viral polymerase reaction. Lane 1, marker RNA prepared from MboII digested and T7 RNA polymerase transcribed pM-wt; lane 2, no input RNA; lane 3, M-wt RNA. (E) The products of the viral polymerase reactions were analyzed on denaturing polyacrylamide gels, eluted from the gels, and subjected to RNase T1 digestion. Lanes 3 and lane 4 show the RNase T1 digestion patterns of viral polymerase products using CAT200/3C RNA and CAT200 RNA as templates. The CAT200/3C RNA contains an A to C mutation in the third position of the 3' noncoding sequence. The RNase T1 digestion patterns of CAT1 RNA and CAT200 RNA transcribed from pIVACAT1 and pIVACAT200 by T7 RNA polymerase are shown for comparison in lanes 1 and 2.

Figure 3

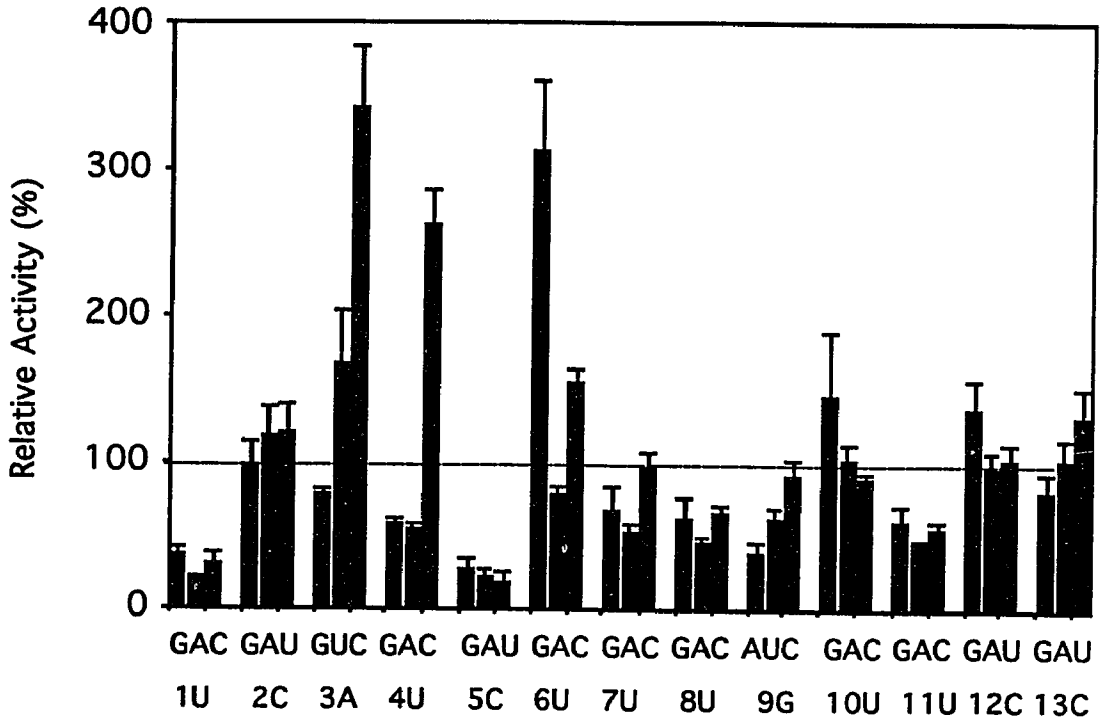
400
300
200
100
0
Relative Activity (%)
GAC 1U
GAU 2C
GUC 3A
GAC 4U
GAU 5C
GAC 6U
GAC 7U
GAC 8U
AUC 9G
GAC 10U
GAC 11U
GAU 12C
GAU 13C

Fig.3. Mutational analysis of the promoter sequence required for vRNA synthesis *in vitro*. The wild type nucleotide sequence of the 3' terminal 13 nucleotides of CAT200 RNA is indicated (from left to right). The three substitution mutations of each position are shown above each nucleotide. All RNA templates containing a single point mutations were examined using reaction conditions as described in Materials and Methods. The CAT200 RNA was used as control in each experiment. The vRNA synthesis activity of mutated RNA templates is represented as relative activity compared with that of the wild type template given as 100%. The relative activity is the mean value of two or more separate experiments using RNA templates which were synthesized independently by T7 RNA polymerase transcription. Error bars were calculated as standard deviation of two or more samples of independent experiments.

Figure 4

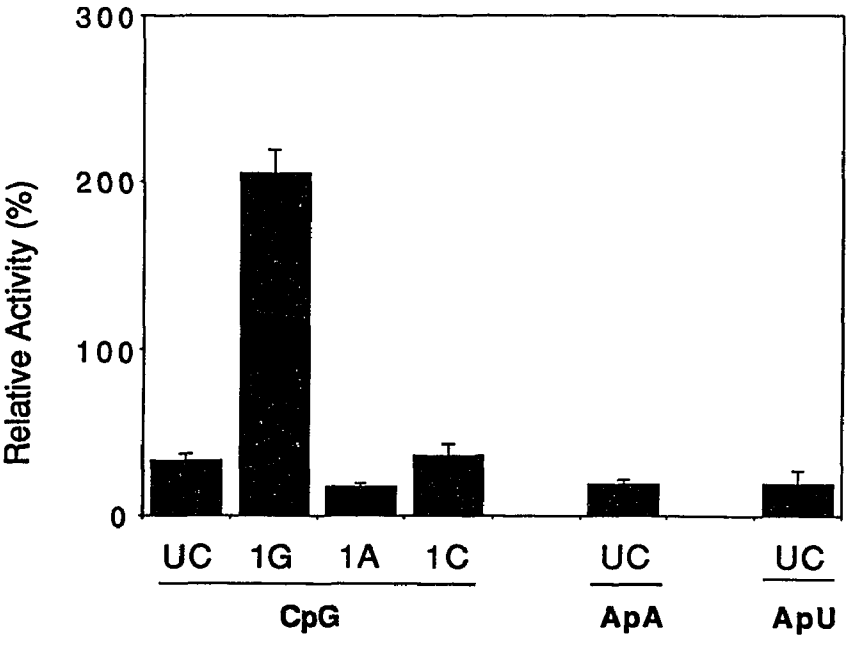

Fig.4. Priming activity of different dinucleotides on vRNA synthesis *in vitro*. Three different dinucleotides CpG, ApA and ApU were tested in the polymerase reaction to determine their ability to stimulate vRNA synthesis *in vitro* . Above each dinucleotide, the template RNA molecules examined are indicated. UC, CAT200 RNA; 1G, 1A, and 1C, RNA templates containing a U to G, U to A or U to C mutation in the first position. The vRNA synthesis activity is represented as relative to that of the reaction in which CAT200 RNA is used as template and ApG was used as primer. Error bars were calculated as standard deviation of two or more independent experiments.

Figure 5

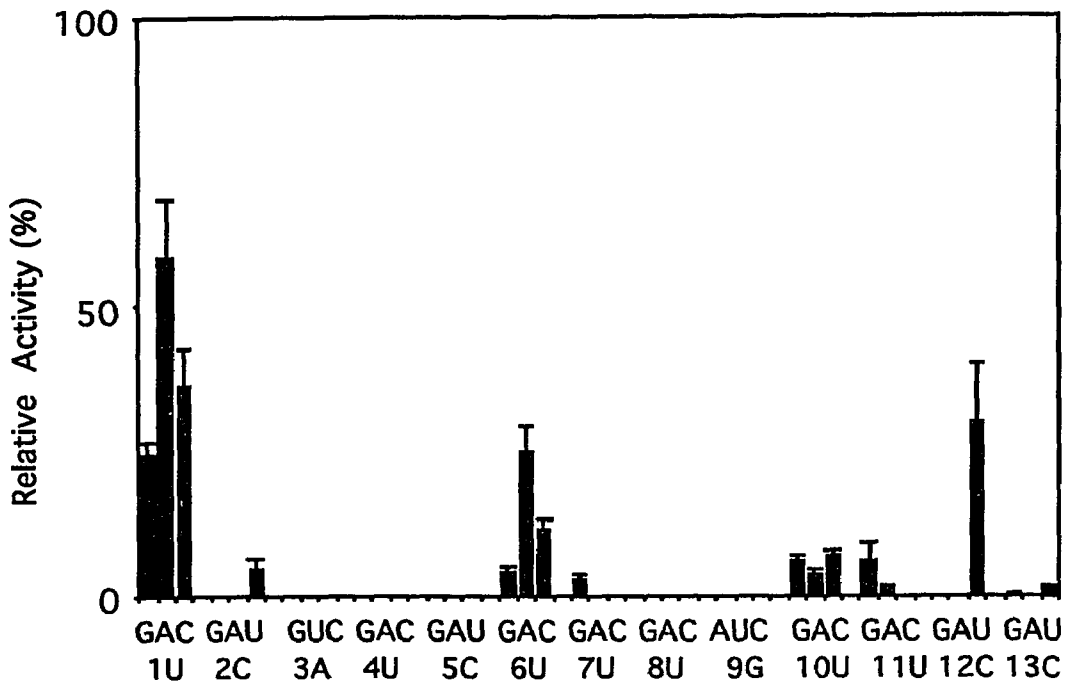
100
50
0
Relative Activity (%)
GAC 1U
GAU 2C
GUC 3A
GAC 4U
GAU 5C
GAC 6U
GAC 7U
GAC 8U
AUC 9G
GAC 10U
GAC 11U
GAU 12C
GAU 13C

Fig.5. Effect of mutations on CAT expression. The wild type sequence of the 3' terminal 13 nucleotides is indicated (from left to right). The three mutations at each position are shown above each nucleotide. The RNP complexes reconstituted from mutated RNA molecules and purified viral polymerase complex were transfected into WSN virus-infected MDBK cells. After 14-16 h incubation, the cells were harvested and disrupted. The CAT activity of the cell extracts were detected. In some cases, the cell extracts were diluted with 0.25 M Tris HCl, pH 7.5 to obtain CAT assays in the linear range. The CAT expression is presented relative to that produced by transfection of the CAT200 RNA (100%). Error bars were calculated as standard deviation of two or more independent experiments.

Figure 6

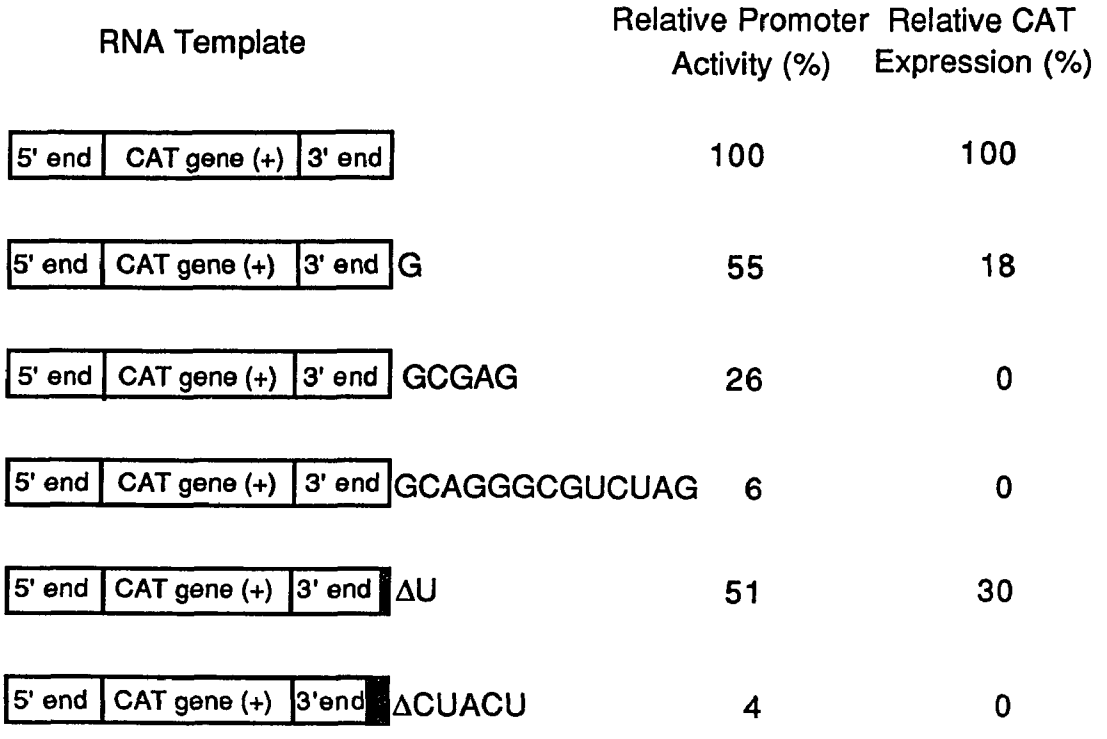
RNA Template
Relative Promoter Activity (%)
Relative CAT Expression (%)
5' end
CAT gene (+)
3' end
100
100
G
55
18
GCGAG
26
0
GCAGGGCGUCUAG
6
0
ΔU
51
30
ΔCUACU
4
0

Fig.6. Additions and deletions of nucleotides at the 3' end of cRNA reduce promoter activity and CAT expression. The RNA templates were examined for promoter activity and CAT expression using conditions described in Materials and Methods. CAT expression is indicated as activity relative to that obtained by transfection using CAT200 RNA. The values are averages of two or three experiments. The variation of each experiment is within 20% of that value.

CHAPTER 3

Journal of Virology

submitted

Characterization of the Polyadenylation Signal of Influenza Virus RNA

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ABSTRACT

It has been shown that a stretch of Us near the 5' end of the virion RNA of influenza A virus is the polyadenylation site for viral messenger RNA synthesis. In addition, the RNA duplex made up of the 3' and 5' terminal sequences adjacent to the U stretch is also involved in polyadenylation. We have further characterized the polyadenylation signal of influenza virus RNA using a ribonucleoprotein transfection system. We found that the optimal length of the U stretch is 5 to 7 uridine residues. We also showed that the upstream sequence at the 5' end is not involved in polyadenylation and that the optimal distance between the 5' end and the U stretch is 16 nucleotides. The combination of these features defines the polyadenylation site and differentiates this signal from other U stretches scattered throughout the genome of influenza viruses.

The genome of influenza A virus contains eight single-stranded RNA segments of negative polarity (Palese, 1977) and an RNA-dependent RNA polymerase is responsible for the replication and transcription of the viral RNAs. The genomic RNAs (vRNA) are templates for the synthesis of viral messenger RNA (mRNA) and complementary RNA (cRNA) in infected cells. The cRNA is a complete copy of the vRNA. In contrast, viral mRNAs are incomplete copies of vRNAs: mRNA synthesis initiates at the 3' end of the vRNA; cap-containing fragments of 10-13 nucleotides derived from host mRNAs are used as primers; mRNA synthesis terminates at a stretch of uridine residues 17-22 nucleotides away from the 5' end of the vRNA; and finally a poly A tail is added (Krug et al., 1989; Lamb and Choppin et al., 1983; Mcgeoch and Kitron, 1975; Plotch and Krug, 1977).

The polyadenylation site for influenza virus mRNA has been mapped to the U stretch near the 5' end of vRNA (Robertson et al., 1981). However, the fact that U stretches of varying lengths are present throughout the genomic RNA of influenza viruses raises the question of why it is only this particular U stretch which serves as the polyadenylation site (Lamb and Choppin et al., 1983; Robertson et al., 1981). In other negative stranded RNA viruses, e.g. vesicular stomatitis virus (VSV), the consensus tetranucleotides AUAC or AUUC were found preceding the U stretch at which a poly A tail is added (Gupta and Kingsbury, 1982; Schubert et al., 1980). No such conserved sequences preceding the U stretch have been detected in influenza virus RNAs. Instead, sequence

analysis of influenza virus genomic RNAs shows that the 3' and 5' terminal sequences form a panhandle structure (Desselberger et al., 1980; Hsu et al., 1987; Robertson et al., 1979; Skehel and Hay, 1978), and it has been postulated that this duplex structure adjacent to the U stretch is required for polyadenylation (Hsu et al., 1987; Robertson et al., 1981; Skehel and Hay, 1978). However, in the past it was not possible to examine this hypothesis because of the absence of an *in vitro* or an *in vivo* system that allowed a mutational analysis of the template RNA. Recently a ribonucleoprotein (RNP) transfection system involving the use of synthetic RNA was established. This system has allowed us to demonstrate that the RNA duplex of the panhandle structure--in juxtaposition to the U stretch--is indeed involved in polyadenylation of viral mRNA (Enami et al., 1990; Enami and Palese, 1991; Li and Palese, 1992; Luo et al., 1991; Luytjes et al., 1989). Disruption of the duplex structure and a mutation in the U stretch abolished expression of the reporter gene encoding chloramphenicol acetyltransferase (CAT) (Luo et al., 1991). We have now used this same system to further examine the features of this polyadenylation signal.

We prepared several mutants (Fig.1) which contained either shortened or elongated stretches of Us and tested them using the RNP transfection system. The results are shown in Fig.2A. The wild type construct BXPCAT1 has 6 uridines. The reduction or elongation of the U stretch by one uridine residue has no significant effect on CAT expression (87% and 80%, respectively). This is consistent with sequence data showing that the length of the U stretches at the 5' end is highly conserved (five to seven) among genomic RNAs of influenza A viruses isolated from humans or animals. The deletion of two uridines in

the model RNA dramatically decreased CAT expression (4%, Fig.2A), suggesting that the signal requires at least 5 uridines adjacent to the panhandle structure. Increasing the length of the U stretch to eight uridines also decreased CAT expression (15%) and the construct with 12 uridines showed less than 0.5% CAT activity as compared to that of the wild type RNA (Fig.2A). This result was surprising since the panhandle structure in the two mutant constructs was unaltered and at least six uridines were in juxtaposition to the RNA duplex. One hypothesis to explain these unexpected results is that the polymerase falls off its template when it encounters eight or more uridine residues. This notion is supported by an analysis of GenBank sequences which reveals that influenza A virus genomes do not appear to contain a stretch of eight or more uridine residues in either the plus or the minus sense RNA. Alternatively, sequence changes upstream (i.e. toward the 3' end) of the stretch of 5 to 7 uridines might affect the polyadenylation signal. We believe that this is not the case since the CAT activity of HACAT1 and HACAT2 (86%) are comparable (Fig.2B). These two constructs differ by 28 nucleotides 3' to the uridine stretch. We have chosen the latter pair of constructs since the hemagglutinin (HA) gene contains 28 noncoding nucleotides between the uridines and the stop codon of the open reading frame whereas the stop codon of the NS gene overlaps with the stretch of uridines of the polyadenylation signal. It thus appears that in the HA construct the upstream sequence (3' to the uridine stretch) does not affect the polyadenylation signal.

We then proceeded to determine CAT-specific mRNA levels in RNP-transfected cells in order to prove that the expression of CAT correlates with mRNA levels. The decreased CAT expression of

BXPCAT2 and BXPCAT6 which contain 4 and 12 uridine residues, respectively, correlates with a reduced (abolished) CAT-specific mRNA level (Fig.3, lane 4 and 9). In our mutants, no changes were made in the 3' end of the RNAs and thus the promoter(s) for mRNA and cRNA synthesis was (were) not affected. The ribonuclease protection assays in Fig.3 show that the cRNA levels of the wild type and the mutant RNAs in RNP-transfected cells are unchanged. Thus mutations at the 5' end are likely to influence the termination (polyadenylation) step. We hypothesize that non- (or under-) polyadenylated mRNAs are less stable than mature mRNA species and that this is the cause for the reduced mRNA levels of the mutated constructs. It should be noted that only a fraction (less than 5%) of all cells is RNP-transfected (unpublished results) and that this low efficiency of expression prevents a further biochemical characterization of the CAT-specific RNAs in RNP-transfected cells.

We then studied the effect of insertions into the panhandle structure of BXPCAT1. The insertion of a single A residue into the 5' end (BXPCAT1/I-1A) resulting in an extended RNA duplex structure led to a dramatic decrease of CAT activity (Fig.4A) as well as of the mRNA level (Fig.3, lane 5). Additional constructs with insertions of two, five and ten nucleotides confirmed this finding (Fig.4A). Since the changes at the 5' end gave rise to an extended panhandle structure, the RNA duplex structure may have become more stable and could not be opened by the viral polymerase (or its putative "unwinding activity"). This explanation, however, is unlikely since the mutated RNA BXPCAT1/I-1A is also an excellent template for cRNA synthesis (Fig.3, lane 5). We thus hypothesize that shifting the U stretch away from the 5' end results in an altered polyadenylation signal with concomitant lower levels of mRNAs.

To further examine this possibility, we constructed two mutant RNAs which contained one (C) or two nucleotides (UG) inserted between position 16 (counting from the 5' terminus) and the U stretch. The inserted nucleotides were not complementary to corresponding nucleotides at the 3' end and they did not extend the length of the panhandle structure. The mutants did not produce CAT activity when transfected into Madin-Darby bovine kidney (MDBK) cells (Fig.4B). These results suggest that changing the position of the U stretch relative to the 5' end is responsible for ablation of CAT expression.

To provide further support for this notion, we extended our study using constructs containing noncoding sequences derived from the neuraminidase (NA) segment. We noticed that there are only 15 nucleotides between the 5' end and the U stretch in the NA segment. One (G) or two nucleotides (UG) were inserted into the region between position 15 and the U stretch. Interestingly, insertion of one nucleotide increased CAT expression by a factor of nearly two, while insertion of two nucleotides (UG) radically diminished CAT expression (Fig.5). These results confirm the observations made with NS gene-like (BXPCAT) constructs and suggest that the optimal distance between the 5' end and the U stretch is 16 nucleotides.

It is not clear what the mechanism is which leads to such a stringent positional requirement of the U stretch relative to the 5' end of the RNA. It is possible that the polymerase (auxiliary factor) interacts with both the U stretch and the 5' terminal sequence. Such a model may explain the results obtained in a previous study (Luo et al., 1991). Two mutants (IVACAT12, IVACAT13) contained long sequences (a bubble) between the 5' end and the RNA duplex structure with the adjacent U

stretch. When transfected into helper virus-infected cells, they produced low level of CAT expression (Luo et al., 1991). It is possible that the inserted sequences are long enough to allow bending of the RNA structure so that the polymerase (auxiliary factor) interacts with the U stretch and the 5' terminal sequence resulting in low level of polyadenylation.

We also moved the U stretch closer to the 5' end by eliminating one, two, or three nucleotides between the 5' end and the U stretch (Fig.6). In order to shift the U stretch towards the 5' end by only one nucleotide, we deleted the U in position 15 because deletion of the G in position 16 would add the U in position 15 to the U stretch, thus complicating the analysis (Fig.6). When examined in the RNP transfection system, all mutants showed significantly decreased CAT expression levels (Fig.6) but, as shown for the mutant BXPCAT1/D-15U, the cRNA level is identical to that of cells transfected with the wild type construct (Fig.3, lane 7 and 8). Thus it is likely that the reduction of CAT expression is caused by an altered polyadenylation signal rather than by changes in the promoter(s) involved in full length RNA synthesis.

In conclusion, the polyadenylation signal of influenza virus RNAs has the following features: (1) the U stretch is 5-7 uridine residues long; (2) the optimal distance between the U stretch and the 5' terminus of vRNA is 16 nucleotides; (3) the RNA duplex structure adjacent to the U stretch is required for polyadenylation; (4) specific sequences upstream of the U stretch in the 5' noncoding sequence are not required for polyadenylation. Attempts will now be made to define the precise sequences which are involved in the differential regulation of transcription of specific influenza virus RNA segments.

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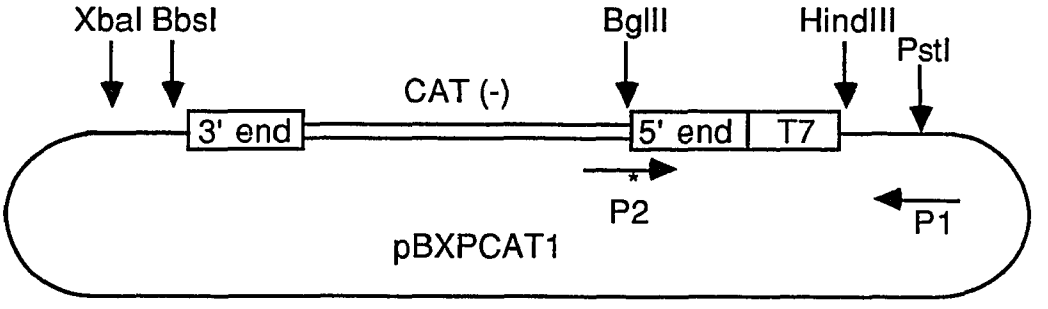
Figure 1

A.


XbaI BbsI
BglII
HindIII
PstI
CAT (-)
3' end
5' end
T7
P2
P1
pBXPCAT1

B.

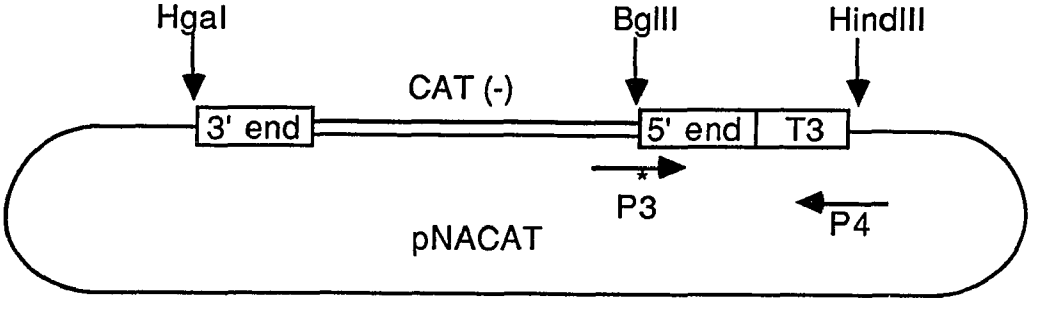

HgaI
BglII
HindIII
CAT (-)
3' end
5' end
T3
P3
P4
pNACAT

FIG.1. Construction of plasmids. (A) pBXPCAT1 is a modified form of pIVACAT1 (10). pBXPCAT1 was made by polymerase chain reaction (PCR) using specific primers and pIVACAT1 as template. New restriction enzyme sites were created to facilitate the introduction of mutations at the 5' end and to allow usage of a cheap restriction enzyme (BbsI) resulting in the linearization of the plasmids at the 3' end. The primers have the following sequences P0: 5'-GGATCCTCTAGAAGACGCAGCAAAAGCA GGGTGACTCGAGACATAATGG-3' and P1: 5'-GGCACCCTGCAGGCTTT ACAC-3'. The PCR product using primers P0 and P1 was digested with XbaI and PstI and was inserted into pUC19. Two Us in the 3' end of IVACAT1 were mutated into AGC (underlined in primer P0) to generate a XhoI site (not shown). A PstI site was introduced into the pUC19 sequence so that a PCR strategy could be used to create mutations in the 5' end of the noncoding sequence. BXPCAT1 RNA was examined in the ribonucleoprotein (RNP) transfection system, and it produced CAT levels similar to that of IVACAT 1 RNA (Li and Palese, 1992; Luo et al., 1991; Luytjes et al., 1989) after transfection into WSN virus-infected cells (data not shown). Mutated constructs derived from pBXPCAT1 were made using a PCR strategy in which pBXPCAT1 was used as template. P2 represents a set of primers which contained mutations in the 5' noncoding sequence. The asterisk indicates the site of the mutations. For example, for the construction of pBXPCAT5 (Fig.2A), the primer has the following sequence: 5'-CGTAATAGATCTGAAAAAAAACAC-3'. (B) pNACAT has a structure similar to that of pIVACAT1 except that the

noncoding sequences flanking the CAT coding sequence were derived from the neuraminidase (NA) segment of WSN virus. Also, position 4 at the 3' end is a C instead of a U. The same PCR strategy as described above was used to create mutations at the 5' noncoding sequence of NACAT. P3 represents a set of primers which contained mutations. The asterisk indicates the site of the mutations. For example, for the construction of pNACAT/16G (Fig.5), the two primers have the following sequences: 5'-CGTAATAGATCTAGTTTGTTCAAAAAACCTCC TTGT-3' and P4: 5'-CGCCTTAAGCTTCTGCAGAATTAAC-3'. T7: T7 RNA promoter; T3: T3 RNA promoter; 5' end: 5' noncoding sequence; 3' end: 3' noncoding sequence.

Figure 2A

		Relative CAT Expression
BXPCAT1	5' AGUA GAA ACA AGG GUG UUUUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGUAU	100%
BXPCAT2	5' AGUA GAA ACA AGG GUG UUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGUAU	4%
BXPCAT3	5' AGUA GAA ACA AGG GUG UUUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGUAU	87%
BXPCAT4	5' AGUA GAA ACA AGG GUG UUUUUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGUAU	80%
BXPCAT5	5' AGUA GAA ACA AGG GUG UUUUUUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGAU	15%
BXPCAT6	5' AGUA GAA ACA AGG GUG UUUUUUUUUUUU 3' UCGUUUU CG UCC CAC UGAGCUCUGUAU	<0.5%

Figure 2B

					Relative CAT Expression
HACAT1	5' AGUA GAA 3' UCGUUUU	ACA CG	AGGG UCCC	UGUUUUU CCUCAUAUUUCUGAAAUUCUAAUCAAUC CUUUUAUUUUUGUUGGUUU	100%
HACAT2	5' AGUA GAA 3' UCGUUUU	ACA CG	AGGG UCCC	UGUUUUU CUUUUAUUUUUGUUGGUUU	86%

FIG.2. Effects of changes in the length of the U stretch and in the upstream sequence on CAT expression. (A) The U stretch in the constructs was either shortened or elongated as indicated. The mutant constructs were examined using the RNP transfection system (Li and Palese, 1992; Luo et al., 1991). The level of CAT expression of the mutant constructs is presented relative to that produced by transfection of the BXPCAT1 construct (100%). (B) HACAT1 RNA has a structure similar to that of IVACAT1 and NACAT RNAs (Fig.1) except that the noncoding sequences were derived from the hemagglutinin (HA) segment of WSN virus. The construction of pHACAT1 will be described elsewhere (Bergmann and Palese, unpublished data). pHACAT2 was prepared by digestion of pHACAT1 and pBXPCAT3 with HindIII and BglII. Subsequently the fragment containing the T7 RNA promoter and the 5' end noncoding sequence from pBXPCAT3, and the fragment containing the CAT coding sequence and the 3' noncoding sequence of HACAT1 were isolated and ligated. The level of CAT expression of HACAT2 is relative to that of HACAT1. The values are averages of two or more experiments.

Figure 3

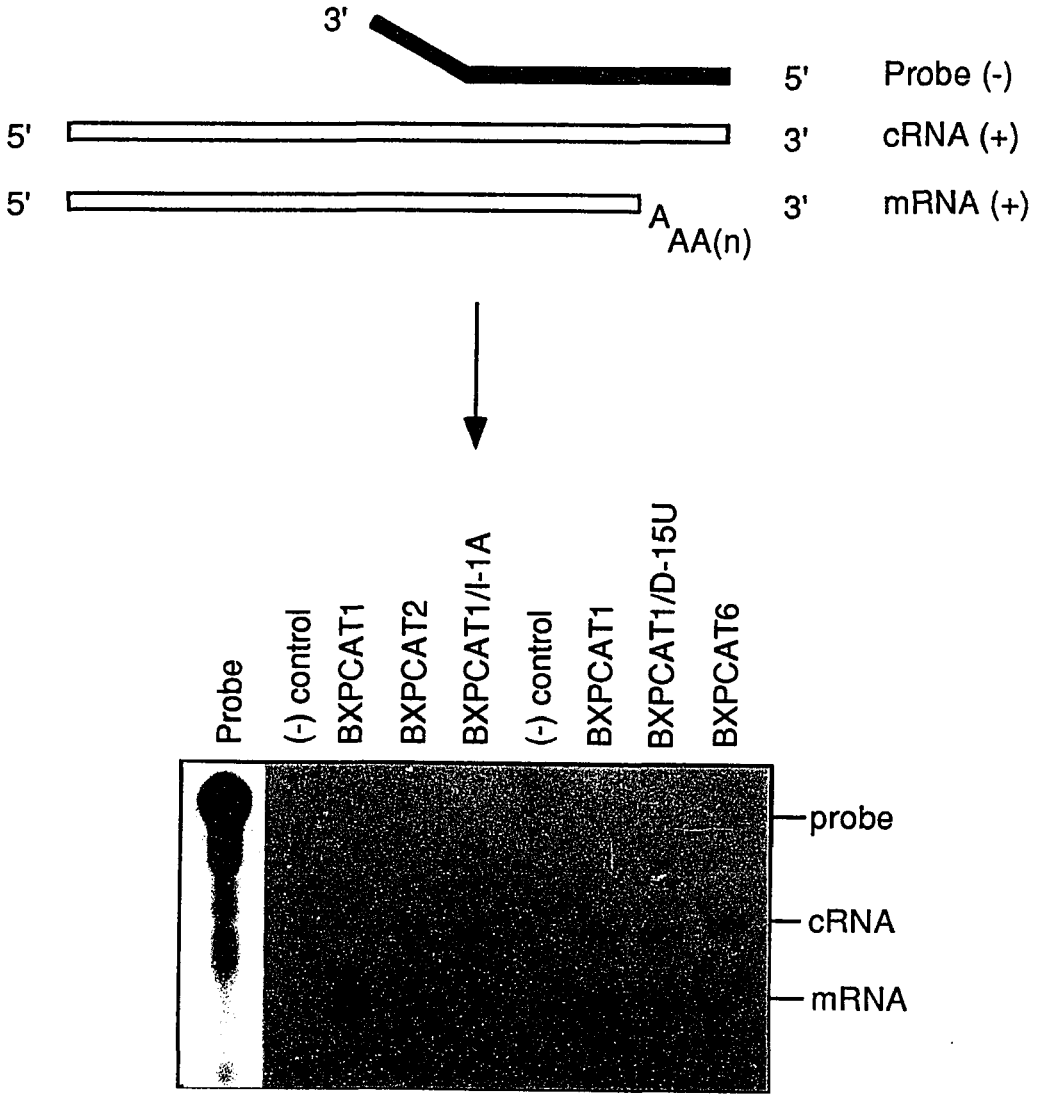

3'
5'
Probe (-)
5'
3'
cRNA (+)
5'
3'
mRNA (+)
A
AA(n)
Probe
(-) control
BXPCAT1
BXPCAT2
BXPCAT1/I-1A
(-) control
BXPCAT1
BXPCAT1/D-15U
BXPCAT6
probe
cRNA
mRNA

FIG.3. Detection of CAT-specific mRNA and cRNA in RNP-transfected cells. The upper panel illustrates the ribonuclease protection assay (RPA). The probes are in minus sense and contain 53 extra non-viral nucleotides. cRNA and mRNA molecules are in plus sense. Fragments protected by cRNA will be 53 nucleotides shorter than the probes, and fragments protected by mRNAs are even shorter. Specific probes were prepared for each of the mutants. The probe plasmids were constructed by digesting each mutant plasmid with HindIII and BglII (Fig.1). The fragments containing the 5' end sequences were isolated and inserted into the plasmid pIVACAT1-BalI/SalI (Luo et al., 1991). The lower panel shows the levels of CAT-specific mRNA and cRNA in RNP-transfected cells as determined by the RPA assay (Luo et al., 1991). The positions of probes and protected fragments by cRNAs and mRNAs are indicated. (-) controls represent BXPCAT1 (lane 2) and BXPCAT1/D-15U (lane 6) probes treated with RNase only.

Figure 4A

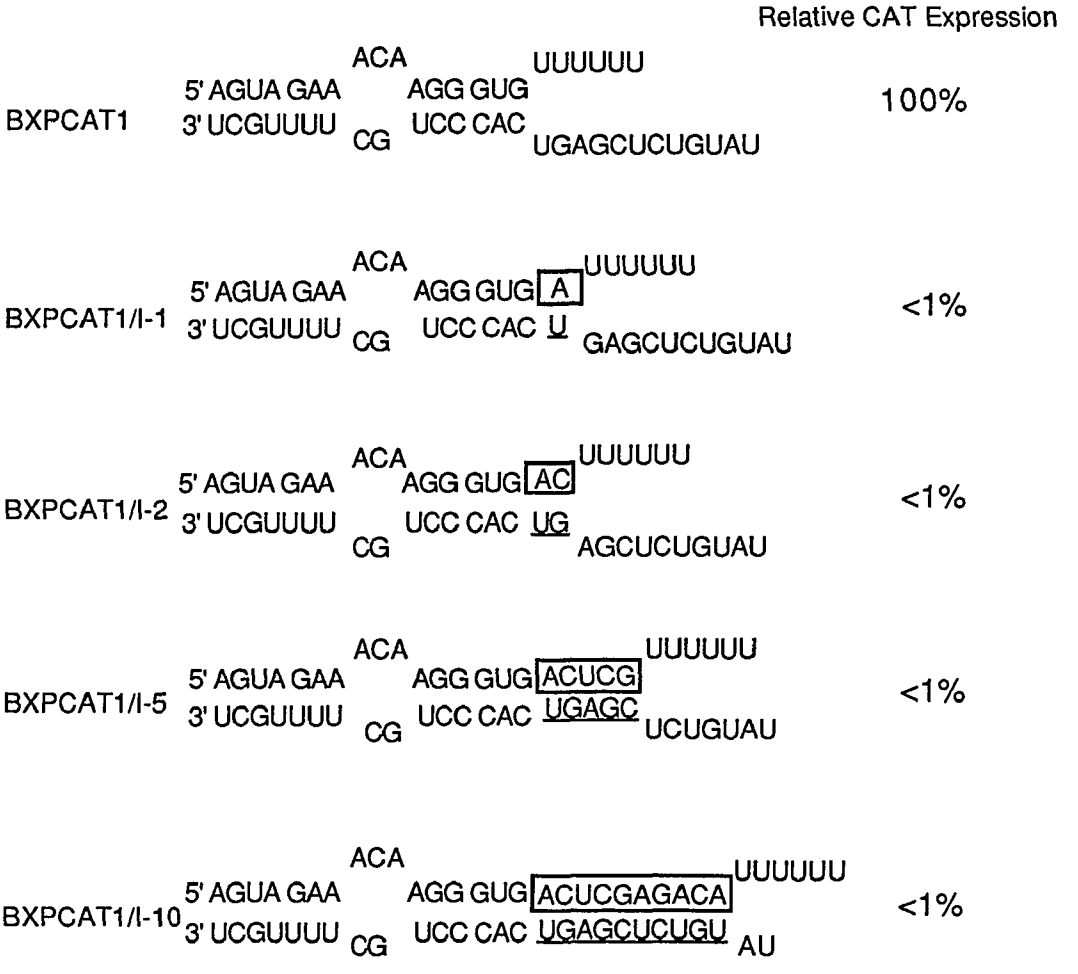

Relative CAT Expression
BXPCAT1
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG
CG
UCC CAC
UUUUUU
UGAGCUCUGUAU
100%
BXPCAT1/I-1
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG A
CG
UCC CAC U
UUUUUU
GAGCUCUGUAU
<1%
BXPCAT1/I-2
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG AC
CG
UCC CAC UG
UUUUUU
AGCUCUGUAU
<1%
BXPCAT1/I-5
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG ACUCG
CG
UCC CAC UGAGC
UUUUUU
UCUGUAU
<1%
BXPCAT1/I-10
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG ACUCGAGACA
CG
UCC CAC UGAGCUCUGU
UUUUUU
AU
<1%

Figure 4B

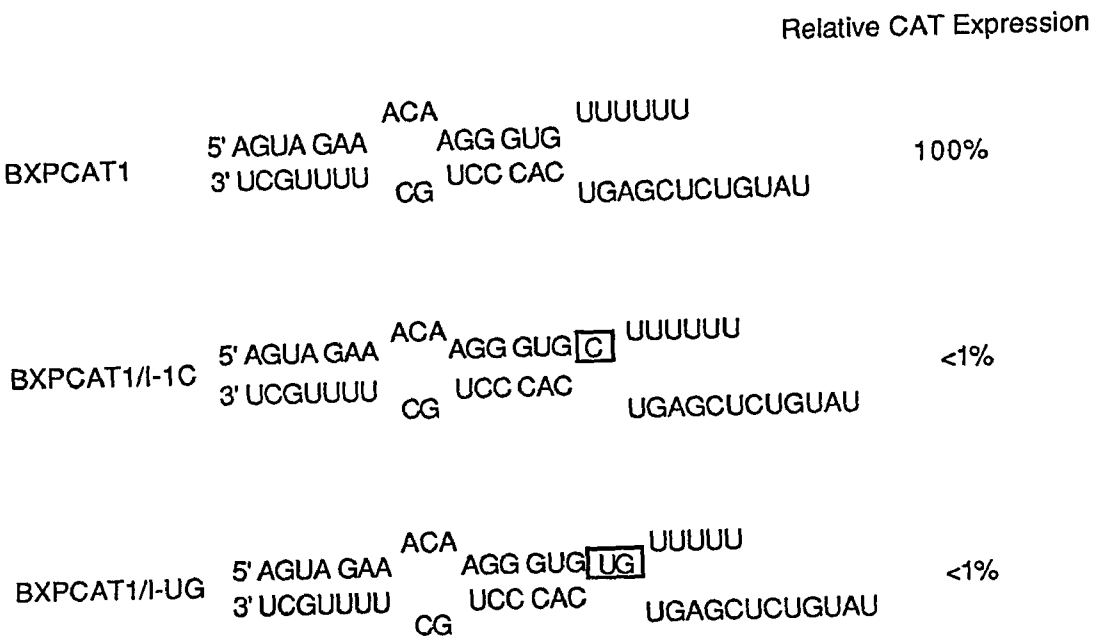

Relative CAT Expression
BXPCAT1
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG
CG
UCC CAC
UUUUUU
UGAGCUCUGUAU
100%
BXPCAT1/I-1C
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG C
CG
UCC CAC
UUUUUU
UGAGCUCUGUAU
<1%
BXPCAT1/I-UG
5' AGUA GAA
3' UCGUUUU
ACA
AGG GUG UG
CG
UCC CAC
UUUUU
UGAGCUCUGUAU
<1%

FIG.4. Effect of shifting the U stretch away from the 5' end on CAT expression. (A) Nucleotides (boxed) were inserted between position 16 and the U stretch at the 5' end of vRNA. The inserted nucleotides are complementary to the corresponding sequences (underlined) at the 3' end and have the potential to form base pairs and thus to extend the panhandle structures. (B) The inserted nucleotides (C and UG in the boxes) are not complementary to the 3' noncoding sequences, and the panhandle structures are not affected. These mutant constructs were examined along with BXPCAT1 using the RNP transfection system. The CAT expression level is represented relative to that of BXPCAT1 (100%). The values are averages of two or more experiments.

Figure 5

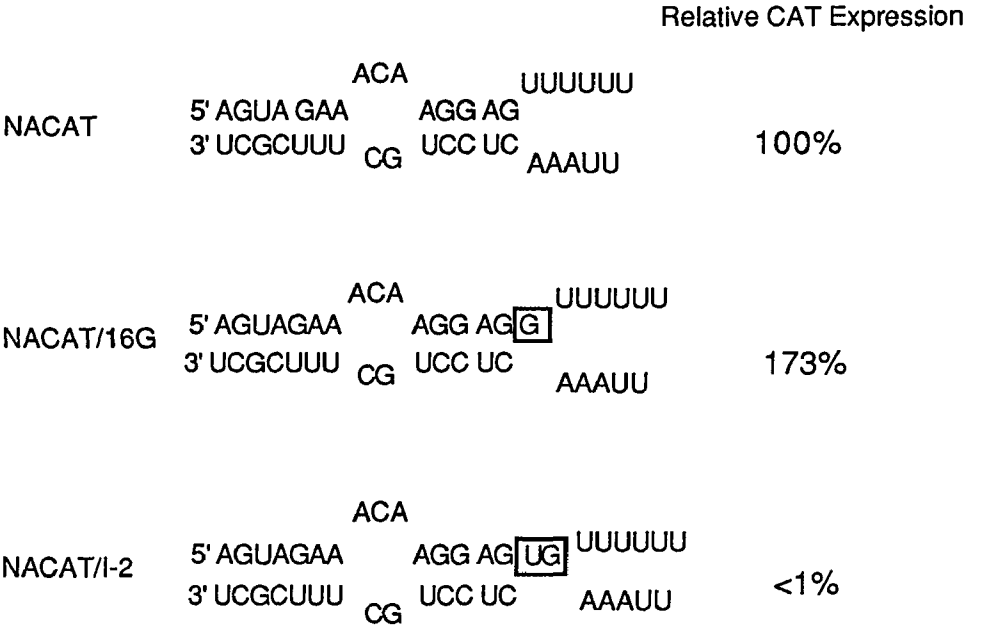

Relative CAT Expression
NACAT
5' AGUA GAA
3' UCGCUUU
ACA
CG
AGG AG
UCC UC
UUUUUU
AAAUU
100%
NACAT/16G
5' AGUAGAA
3' UCGCUUU
ACA
CG
AGG AG G
UCC UC
UUUUUU
AAAUU
173%
NACAT/I-2
5' AGUAGAA
3' UCGCUUU
ACA
CG
AGG AG UG
UCC UC
UUUUUU
AAAUU
<1%

FIG.5. Effect of insertions of nucleotides into NACAT on CAT expression. There are 15 nucleotides between the 5' end and the U stretch in the 5' noncoding sequence of the specific NA vRNA. One (G) or two (UG) nucleotides (boxed) were inserted between position 15 and the U stretch of the model RNA NACAT. Mutants were tested using the RNP transfection system and CAT activity is expressed relative to that of NACAT (100%). The values are averages of two or more experiments.

Figure 6

		Relative CAT Expression
BXPCAT1	5' AGUA GAA ACA AGGGUG UUUUUU 3' UCGUUUU CG UCCCAC UGAGCUCUGUAU	100%
BXPCAT1/D-15U	5' AGUA GAA ACA AGGGΔG UUUUUU 3' UCGUUUU CG UCCCA C UGAGCUCUGUAU	8%
BXPCAT1/D-2	5' AGUA GAA ACA AGGGΔΔ UUUUUU 3' UCGUUUU CG UCCC A C UGAGCUCUGUAU	2%
BXPCAT1/D-3	5' AGUA GAA ACA AGGΔΔΔ UUUUUU 3' UCGUUUU CG UCCC A C UGAGCUCUGAU	1%

FIG. 6. Effect of shortening the distance between the 5' end and the U stretch on CAT expression. Nucleotides in the 5' noncoding sequence of BXPCAT1 were deleted and the positions were represented as Δ. Mutants were examined using the RNP transfection system and CAT activity is expressed relative to that produced by transfection of BXPCAT1 (100%). The values are averages of two or more experiments.

DISCUSSION AND OUTLOOK

The cis-acting elements of influenza A virus RNAs have been investigated by using *in vitro* reconstitution systems in which purified viral polymerase and synthetic RNA templates were mixed. The resulting ribonucleoprotein (RNP) complexes exhibited polymerase activity (Li and Palese, 1992; Piccone et al., 1993; Parvin et al., 1989; Seong and Brownlee, 1992a, 1992b). These systems allowed the identifications of the minimum promoters required for cRNA and vRNA synthesis *in vitro* and permitted the detailed mutational analysis of the nucleotides involved in promoter functions. The RNP complexes were also transfected into helper virus-infected cells and the expression of the reporter (CAT) gene present in the synthetic templates was measured (Li and Palese, 1992; Kimura et al., 1993; Piccone et al., 1993). Analysis of the mutant constructs in this *in vivo* system revealed that the majority of mutations in the promoters decreased or abolished CAT expression, indicating that these mutations have more significant effects on CAT expression *in vivo* than on RNA synthesis *in vitro* (Li and Palese, 1992; Piccone et al., 1993).

In comparing the *in vitro* and *in vivo* data, two situations were observed. First, several mutations significantly reduced CAT expression *in vivo*. However, these same mutations did not decrease *in vitro* activity (for example, substitution mutations in positions 2, 10, 12 and 13), or they actually increased vRNA synthesis *in vitro* (for example, substitutions 3A to 3C, 4U to 4C, and 6U to 6G). This may be explained by the fact that the

in vitro system measures only one step of RNA synthesis. CAT expression in the RNP transfection system, however, requires viral RNA replication, RNA transcription and protein synthesis. Thus it is likely that the promoter required for vRNA synthesis overlaps with other cis-acting elements which are involved in those steps. For instance, it has been demonstrated that the panhandle structure of vRNAs is required for viral mRNA synthesis *in vivo* (Luo et al., 1991). Many of the above mentioned mutations would lead to a disruption of the panhandle structure of vRNAs by either opening up base pairs in the stem structure or the formation of new base pairs resulting in the closing of the bubble in the panhandle structure (Chapter 1-Fig.2). These mutations were tolerated *in vitro* since the panhandle structure is not required for viral RNA synthesis *in vitro* (Parvin et al., 1989; Seong and Brownlee, 1992a, 1992b).

There is a second set of mutations which show the same trend in the *in vitro* and the *in vivo* experiments. Mutations in position 5, 8, 9, and 11 of the 3' end, for example, revealed a decrease of *in vitro* activity, and also led to a reduction of CAT expression in the RNP transfection system. In these cases, the effects of mutations in the *in vitro* experiments are consistent with those observed *in vivo*, although there is no linear relationship between the *in vitro* and the *in vivo* data. One possible explanation is that in the RNP transfection system, CAT expression requires multiple cycles of amplification of CAT-specific RNAs. On the other hand, the *in vitro* system may only allow the (inefficient) one step replication of vRNAs from synthetic cRNA templates. The RNAs are amplified many times *in vivo* thus resulting in a significant reduction of CAT expression. Another factor which may also attribute to this lower level of activity *in vivo* is the competition between viral RNAs derived from

helper viruses and the transfected CAT-specific RNAs. Because all these RNAs have viral promoters, they will compete for binding to viral polymerase. Mutant RNAs may not interact with viral polymerase efficiently, and thus could not compete with wild type viral RNAs resulting in low or no CAT expression. Again, this problem did not exist in the *in vitro* system. One possible way to examine this hypothesis is to compare *in vitro* the kinetics of vRNA synthesis using mutant RNAs as templates with that of using the wild type RNA as template.

Another difference between the *in vitro* and the *in vivo* system is that a dinucleotide (ApG) primer was utilized to enhance the activity of the viral polymerase in the *in vitro* system (Li and Palese, 1992; Piccone et al., 1993; Parvin et al., 1989; Seong and Brownlee, 1992a, 1992b). In contrast, it was shown that vRNA synthesis most likely does not involve the use of a dinucleotide primer in infected cells (Shapiro and Krug, 1988). In my study, the decreased vRNA synthesis activity of mutant RNAs containing mutations in position 1 of the 3' noncoding sequence may have been caused by the use of ApG as the primer (Chapter 2-Fig.3 and Fig.4). With respect to the other positions (beyond the first two), it is unlikely that the use of ApG dramatically influences the outcome of my experiments. This notion is supported by the fact that the use of ApG did not significantly affect the results of the *in vitro* activity of the promoter required for cRNA synthesis (Piccone et al., 1993; Seong and Brownlee, 1992a and 1992b). It is known that in infected cells the viral polymerase cleaves the cap-containing fragments from cellular mRNA and uses them as primer for viral mRNA synthesis (Krug et al., 1989). Seong and Brownlee (1992a,1992b) and Piccone et al. (1993) compared the template activity of mutant RNAs in the absence, or presence of either

ApG or globin mRNAs (as cellular mRNAs) as primer. It was found that the relative effects of mutations on template activity were similar under these conditions (Piccone et al., 1993; Seong and Brownlee, 1992a and 1992b).

Another approach to address the differences between the *in vitro* and the *in vivo* systems is to purify viral polymerase from infected cells and use that preparation in *in vitro* study. Recently Martin et al. (1992) reported a procedure to isolate viral polymerase and NP fractions from influenza virus infected cells. The fractions were active in *in vitro* synthesis of cRNA using a synthetic, short vRNA template and they were also used in the RNP transfection system. Purification of the protein components and their characterization should help in further defining the requirements for virus specific RNA synthesis.

It is also possible that the viral synthetic machinery is modified during the purification of the viral polymerase from virus, which results in nonspecific effects. So far, three different procedures have been reported to isolate viral polymerase-NP complexes from virions or infected cells (Martin et al., 1992; Parvin et al., 1989; Seong and Brownlee, 1992a). Attempts have been made to compare the two methods used to isolate viral polymerase from virions (Seong et al., 1992c). Careful comparison of these three polymerase preparations might shed light on this question.

The *in vivo* RNP transfection system also allowed me to study and define the polyadenylation signal required for the termination of viral mRNAs and the addition of poly A tails to these molecules. It is now clear that the polyadenylation signal contains specific features which differentiates this sequence from other U stretches scattered throughout

the genomes of influenza viruses. It is not known, however, why there is such a stringent requirement of the position of the U stretch relative to the terminus of the 5' noncoding sequence. One possible explanation is that the viral polymerase is a complex of proteins which interacts at the same time with both the U stretch and the 5' end of the RNAs. The part of the polymerase complex which contacts the U stretch most likely contains the enzymatic domain catalyzing the synthesis of poly A using the U stretch as template. The other end of the complex contacts the 5' terminus ensuring that the correct U stretch (one near the 5' end) is copied.

Several basic questions remain concerning the regulation of viral gene expression. For example, it is not understood how viral gene expression is differentially regulated at the transcription level (Inglis and Mahy, 1979; Shapiro et al., 1987). For instance, the NP and NS gene-specific mRNAs are expressed at high levels while the polymerase genes are expressed at much lower levels in infected cells (Shapiro et al., 1987). It is possible that some segment-specific sequences or combination of segment-specific nucleotides acting in cis are involved in differential regulation of viral gene expression. As demonstrated before, the RNP transfection system which allows the introduction of specific mutations into viral genomes, should help in answering some of these questions. Another possibility is that specific viral protein(s) differentially regulate expression of viral genes.

It should be noted, however, that there is little information about the trans-acting factors (cellular or viral) which are involved in regulating viral gene expression. For instance, it is still not clear what determines the switch from mRNA to cRNA synthesis. Although the vRNA is the template for both viral mRNA and cRNA synthesis, the two processes are

completely different. Viral mRNAs at the 5' end contain cap-containing fragments derived from cellular mRNAs, while cRNA initiates with the exact 5' end copied from vRNAs. The termination process is also different for the synthesis of cRNA and of mRNA. The cRNA is a complete copy of the vRNA while the mRNA is not and has a polyA tail. Also, cRNA is encapsidated as an RNP, mRNA is not (Krug et al., 1989). Furthermore the kinetics of viral mRNA and cRNA synthesis are different (Shapiro and Krug, 1987). Some evidence suggested that free NPs (those not associated with RNP) were required for cRNA synthesis (Beaton and Krug et al., 1986). It is not known, however, how this is accomplished and if other proteins (viral or cellular) are involved in this transition.

Future work should also be directed at the identification of the cellular proteins that are involved (associated) with the viral replication machinery of influenza viruses. One of the approaches which can be used to identify such cellular proteins is dependent on the binding of cellular protein(s) with viral proteins (Luban et al., 1993). This approach which can also be used to study interactions between viral proteins (Luban et al., 1992). Other approaches involve the binding of viral RNAs to cellular proteins using different crosslinking strategies (Luz and Beck, 1991). Finally, with the development of more efficient selection methods for transfectant viruses, it will become possible to readily (site specifically) mutate any segment of the influenza virus genome. Careful analysis of transfectant viruses which contain mutations in putative regulatory proteins should provide further insight into these questions.

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Chapter 1

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ABSTRACTS

1. **Li, X.**, and P. Palese. 1991. Mutational analysis of the promoter sequence required for influenza virus genomic RNA replication. Eighth International Conference on Negative Strand Viruses. Charleston, SC, USA. p63.