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Translational Regulation of Influenza Virus mRNAs

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Abstract

cDNAs for genome RNAs of influenza virus A/PR/8/34 were cloned, and portions containing the ATG for initiation codon of translation were inserted into the 5′ leader sequence of the chloramphenicol acetyltransferase (CAT) gene in a pSV2cat vector. When transfected cells were super-infected with influenza virus, the CAT activity was found to vary in a time-dependent fashion: A construct containing a cDNA segment for the nonstructural (NS) protein directed the highest activity during the early stage of infection, while a construct containing a cDNA segment for the neuraminidase (NA) directed the highest activity during the late stage of infection. This time-dependent variation in the CAT activity is in good agreement with that of the synthesis rate of respective viral proteins in infected cells. We propose that the translational efficiency of viral mRNA is subjected to temporal control following viral infection, although viral protein synthesis itself is regulated primarily at the level of mRNA synthesis.

Introduction

The genome of influenza virus consists of eight different RNA segments of minus polarity with respect to functional mRNA (1,2). Following infection, three species of RNA are synthesized in cell nuclei (3-5): mRNA of positive polarity, which contains a cap structure of cellular origin at the 5′ end and poly(A) tail at the 3′ end; complementary RNA (cRNA) of positive polarity, which is a complete copy of viral RNA (vRNA) without the modifications at the 5′ and 3′ ends; vRNA of negative polarity, which is synthesized by transcribing cRNA. Newly synthesized

mRNAs are rapidly transported from the host cell nuclei into the cytoplasm for translation. In contrast, the eight vRNA molecules are synthesized coordinately at similar rates and accumulate in the nuclei until the late phase of infection (6). The eight segments of the viral genome code for ten species of proteins, PB1, PB2, PA, HA, NP, NA, M1, M2, NS1, and NS2. The synthesis rates of these proteins are regulated with respect to total amount and timing (1,7). The synthesis of each protein is controlled mainly at the transcriptional level (6,8). The synthesis rate and accumulation level of the mRNAs differ considerably among the eight RNA segments and are under temporal control (6,9). Little is known, however, about contribution of post-transcriptional processes to this control.

To elucidate the translational regulation in influenza viral gene expression, we have inserted DNA sequences containing the ATG for initiation codon of translation of influenza viral mRNAs into the cap-proximal leader region of the CAT gene in a vector pSV2cat. After transfection of such recombinants into HeLa cells, the transiently expressed CAT activity was measured. In these recombinants, transcription of the CAT gene is under the control of the SV40 early enhancer-promoter and therefore the level of CAT mRNA could be kept equal in transfected cells. Using this system, we found that temporal control extends into post-transcriptional processes as well.

Materials and Methods

cDNA cloning

Influenza virus A/PR/8/34 (H1N1) was grown in allantoic sacs of 10-day-old embryonated eggs and purified as described previously (10). Viral RNA was extracted with NaDodSO_4-phenol. Full-length cDNAs were synthesized as described previously (11). Reverse transcriptase was prepared from avian myeloblastosis virus using a previously described procedure (12). cDNAs were cloned into a pSP65 vector.

Construction of recombinants

Full-length cDNAs for NS and NA RNAs were excised with *Eco*RI and *Hin*dIII, and with *Eco*RI and *Hin*fI, respectively. Fragments were isolated and *Hin*dIII linkers were added at both termini. These fragments were then inserted into a *Hin*dIII-digested pSV2cat vector. All construction was carried out essentially following general procedures (13). The insertion junctions of the recombinants were then sequenced to confirm the constructs (14).

DNA transfection and transient expression assays

HeLa cells were plated at a density of 3×10^5 cells per 60 mm tissue culture dish and grown in MEM supplemented with 10% fetal calf serum at 37°C. After 24 hr, the dishes received fresh medium. After an additional three hours of incubation, calcium phosphate-DNA precipitates (4 μg plasmid per 60 mm dish) were added. Cells were incubated for 4 hr and then glycerol-shocked for 30 sec. After 24 hr incubation in fresh growth medium, transfected HeLa cells were super-infected with influenza virus at a multiplicity of infection (m.o.i.) of 10. Cells were harvested at 0, 2.5, 5, and 7.5 hr after infection, and extracts were prepared by freezing and thawing 10 times. The CAT activity was measured as described by Gorman (15). Spots corresponding to acetylated and unacetylated [^{14}C]chloramphenicol were excised from TLC plates and quantitated by counting the radioactivity with a liquid scintillator. Protein concentrations were determined by the method of Bradford (16).

RNA analysis

Plasmid-transfected cells were harvested at 0, 2.5 and 5 hr following virus infection. Cytoplasmic and nuclear RNAs were prepared essentially as described previously (17) except that 1000 units/ml of RNasin were used instead of 2 mM vanadylribonucleoside complexes. Primer extension analysis was performed essentially as described previously (13). For the detection of CAT-specific mRNA, a synthetic 19 nucleotide single-stranded DNA fragment, 5'CAACGGTGGTATATCCAGT3' (nucleotide sequence at positions 4936–4954) (15) was radiolabeled with [γ-^{32}P]ATP and used as a primer. The extended cDNA products were separated from the primer by electrophoresis on an 8% polyacrylamide gel containing 50% urea. Labeled products were identified by autoradiography. The relative amounts of CAT specific RNA were determined by densitometric scanning of the autoradiograms.

Chemicals and enzymes

All enzymes used were purchased from Takara Shuzo, Japan. Reverse transcriptase was prepared from avian myeloblastosis virus in this laboratory as described previously (12). [γ-^{32}P]ATP (>7000Ci/mM) was obtained from ICN Radio-chemicals, USA; D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol (54 mCi/mM) from Amersham, England; unlabeled nucleotides from P-L Biochemicals; and chloramphenicol acetyltransferase from Boehringer. All reagents used were analytical grade.

Results

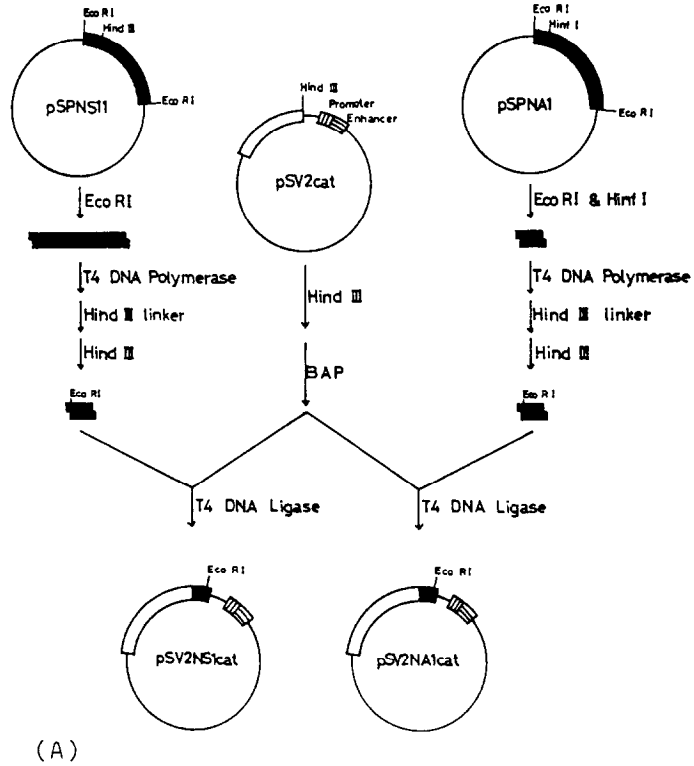
Construction of recombinant plasmids containing cDNAs for influenza virus RNA fused to the CAT sequence

In order to study translational regulation in the expression of influenza virus genes, recombinant plasmids were constructed, each carrying a viral cDNA sequence containing the ATG for translation of the initiation codon within the cap-proximal leader sequence of the chloramphenicol acetyltransferase (CAT) gene of plasmid pSV2cat. To accomplish this, double-stranded cDNAs of all eight segments of influenza virus RNA were synthesized and cloned into a pSP vector as described previously (11). The subcloning into pSV2cat was designed to lead to a production of fused proteins consisting of short N-terminal fragments of influenza viral proteins and complete CAT. Following transfection, the level of expression of the fused CAT gene was compared among the various recombinant plasmids. Plasmids containing cDNAs for segments 6 and 8, coding for NA and NS, respectively, were examined first. Analyses of these two segments were thought to be particularly meaningful since NA and NS are known to be representative proteins synthesized during the late and early phases of infection, respectively (1). Furthermore the level of mRNAs for both proteins are known not to differ significantly in influenza virus infected cells (6).

The recombinant plasmids, designated pSV2NS1cat and pSV2NA1cat, consisted of the CAT gene fused to cDNA sequences for the NS and NA genes, respectively (Fig. 1a). Sequence analysis indicated that the most 5′-proximal AUG codons in transcripts of the fused CAT genes were the functional initiation AUG in influenza viral mRNA for either the NS or NA genes (Fig. 1b). Transcription of the fused CAT genes was expected to start at the same cap site as that of the authentic CAT gene in pSV2cat. Translation of mRNAs derived from either pSV2NS1cat or pSV2NA1cat was, however, expected to commence from the influenza virus AUG, leading to production of fused CAT proteins with N-terminal extra sequences of 19 and 15 amino acids, respectively (Fig. 1b).

Level of CAT activities and CAT transcripts in cells transfected with recombinant plasmids

pSVcat and the recombinants were introduced into HeLa cells by transfection using the calcium-phosphate coprecipitation method (15). Cells were harvested 24 hr after transfection, and the CAT activity in cell extracts was determined. As shown in Table 1, the CAT activity was slightly higher for extracts prepared from cells transfected with pSV2cat than that from either pSV2NS1cat- or pSV2NA1cat-transfected cells. Between the two recombinants, the CAT activity was higher for pSV2NS1cat than for pSV2NA1cat. Qualitative and quantitative analyses of CAT-specific transcripts from the recombinants were then conducted. Cytoplasmic



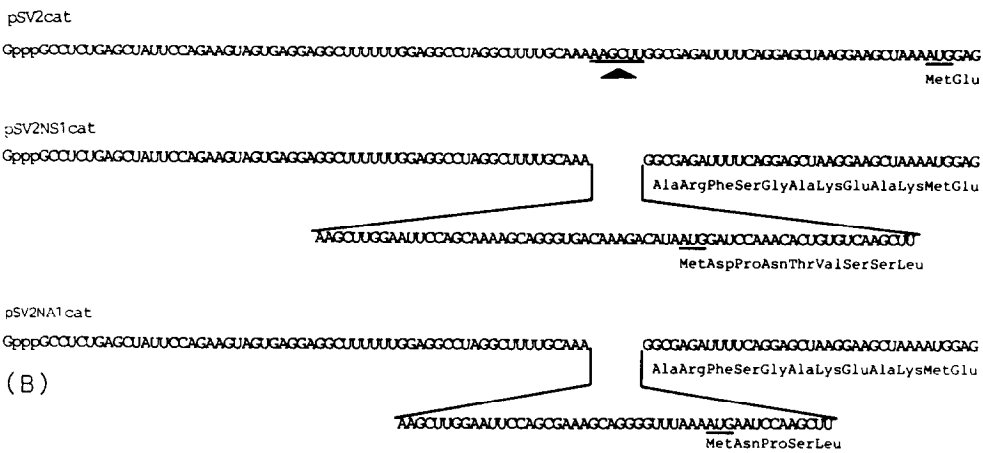


Fig. 1. (A) Construction of pSV2NS1cat and pSV2NA1cat. Open boxes: CAT sequences. Solid boxes: cDNA sequences of influenza virus genome RNAs. (B) Sequences of mRNAs synthesized from pSV2cat, pSV2NS1cat, and pSV2NA1cat and deduced amino acid sequences. The closed triangle represents the *Hind*III site into which portions of influenza virus cDNA were inserted. Initiator AUG codons for translation are indicated by the underline.

Table 1. Expression of CAT activity from pSV2cat, pSV2NS1 cat, and pSV2NA1cat in HeLa cells

	CAT activity*	CAT-specific mRNA**	Specific activity***
pSV2cat	7.4	1	7.4
pSV2NS1cat	3.4	0.66	5.2
pSV2NA1cat	2.1	0.41	5.1

*2.5×10^4 cell equivalent-extracts prepared from HeLa cells transfected with either pSV2cat, pSV2NS1cat, or pSV2NA1cat were subjected to the CAT assay. CAT activity is represented as percent of acetylated chloramphenicol.
**CAT-specific RNA was determined by primer extension method as described in the legend to Fig. 3, and is shown as the amount relative to that from pSV2cat.
***CAT activity was normalized against the relative amount of CAT-specific RNA.

mRNA was isolated from cells transfected with pSV2cat or its derivatives, and CAT-specific mRNA was analyzed by the primer-extension method using the 19 nucleotide single-stranded DNA fragment from the CAT coding region as a primer (see Materials and Methods). cDNA products of 131, 191, and 173 nucleotides in length, as expected from the known DNA sequences, were observed for pSV2cat, pSV2NS1cat, and pSV2NA1cat, respectively (date not shown). The results indicate that the recombinant CAT genes were transcribed from the authentic cap site of CAT gene in pSV2cat. The level of CAT-specific RNA transcripts appears to be slightly different among the three samples, but the specific CAT activity normalized against the level of CAT-specific RNA is essentially the same between pSV2NS1cat and pSV2NA1cat. Thus, the translational efficiency of CAT-specific RNA from these plasmids eventually appears to reach the same level, suggesting that the chimeric CAT proteins possess the same specific enzyme activity. However, their specific CAT activities are slightly lower than those of pSV2cat (Table 1). This might be due to the presence of pSV2NS1cat and pSV2NA1cat.

Translational regulation of recombinant CAT gene expression in infected cells

In influenza virus-infected cells, the genes on RNA segments 1, 2, 3, 5, and 8 encoding the PB2, PB1, PA, NP, and NS proteins, respectively, are expressed immediately following infection, while the genes on segments 4, 6, and 7 encoding the HA, NA, and M proteins, respectively, are expressed during the late phase of infection (1). Although regulation of differential gene expression takes place at the transcription step, "late gene" mRNAs seem to be predominantly translated during the late stage of infection (7). This suggests that translational regulation also operates in infected cells. To test this possibility and to identify a signal responsible for translational control, translation levels of the recombinant CAT mRNAs in influenza virus-infected cells were examined. In the system utilized here, the

synthesis of chimeric mRNAs was catalyzed by cellular RNA polymerase II, and thus could be shut off by super-infecting with influenza virus (18). Therefore, factors influencing pre-existing mRNA can be examined in this experimental system.

The CAT activity from pSV2NS1cat and pSV2NA1cat was increased up to 36 hr in time-dependent fashion as was that from the authentic pSV2cat (data not shown). HeLa cells were transfected with either pSV2NS1cat or pSV2NA1cat, and after 24 hr, super-infected with influenza virus. At various times, the cells were harvested, and the cell extracts were subjected to the CAT assay. The CAT activity in cells transfected with pSV2NS1cat reached a maximum during the early phase of infection (2.5 hr postinfection (p.i.)) and thereafter decreased gradually (Fig. 2). In contrast, the maximal CAT activity from pSV2NA1cat was obtained in extracts prepared from cells during the late phase of infection (5 hr p.i.). These variations in the pattern of CAT activity are consistent with the known order of gene expression for segments 8 (for NS) and 6 (for NA). The CAT activity from the control pSV2cat simply decreased gradually over the course of the infection period (data not shown). From these results, it appears that a temporal control mechanism exists for regulating the translation efficiency of viral mRNAs, and that a signal(s) for this translational control exists near the initiation codon.

Finally, the metabolic stability of CAT mRNA and its transport from nuclei to cytoplasm in virus-infected cells was examined. For this purpose, RNA was isolated from both nuclear and cytoplasmic fractions of CAT plasmid-transfected and virus-infected cells, and quantitatively analyzed by the primer-extension method using a 19 nucleotide single-stranded DNA fragment from the CAT coding region as primer. When the primer was hybridized to mRNA and extended with reverse transcriptase, 191 and 173 nucleotide cDNA products were obtained for RNAs from pSV2NS1cat and pSV2NA1cat-transfected cells, respectively (Fig. 3), as was expected from the known DNA sequences (see Fig. 1). The level of CAT mRNA prepared from cytoplasmic fraction of cells transfected with pSV2NS1cat remained unchanged (lanes 1, 3, and 5), and that from nuclear fraction was also unchanged during the early stage of infection (lanes 2 and 4), but decreased during the late stage (lane 6). In the case of pSV2NA1cat, the level of CAT mRNA from either cytoplasmic (lanes 7, 9, and 11) or nuclear fraction (lanes 8, 10, and 12) was essentially the same. The additional bands (about 195 bases) appearing in lanes 5, 6, 11, and 12 were unidentified as yet. The values for CAT activity shown in Fig. 2 were normalized against the relative amounts of cytoplasmic CAT RNA, where the amount of cytoplasmic CAT RNA for pSV2NS1cat at 0 hr p.i. (lane 1) was set to be 1. In the case of pSV2NS1cat, the specific CAT activity was 2.26, 15.4, and 14.1 for 0, 2.5, and 5 hr p.i., respectively, whereas in the case of pSV2NA1cat, it was 1.58, 0.79, and 5.18, respectively (where the amount of CAT RNA for pSV2NS1cat at 0 hr p.i. was set to be 1). This result is in agreement with the variation pattern shown in Fig. 2. Taken together, these observations support the notion that translational control operates during influenza virus infection and that the signal involved in this control is located near the translation initiation site.

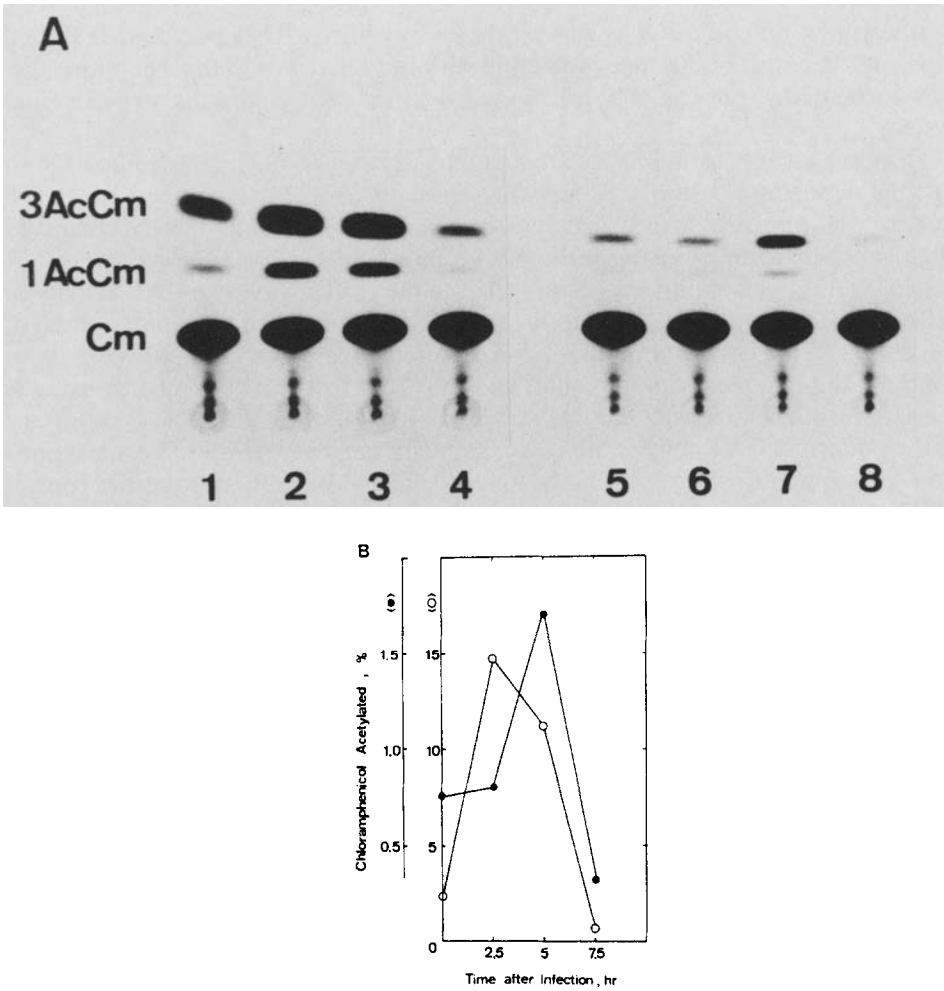


Fig. 2. Effect of influenza virus infection on pSV2NS1cat- and pSV2NA1cat-directed CAT activity. HeLa cells were transfected with either pSV2NS1cat (lanes 1–4) or pSV2NA1cat (lanes 5–8), incubated for 24 hr before super-infection with influenza virus at an m.o.i. of 10, and harvested at 0 (lanes 1 and 5), 2.5 (lanes 2 and 6), 5 (lanes 3 and 7), and 7.5 (lanes 4 and 8) hr after infection. (A) Extracts (4μg of protein/assay) were subjected to the CAT assay. (B) The CAT activity was measured as described below.

Discussion

In influenza virus-infected cells, ten kinds of viral proteins are synthesized and their synthesis is regulated with respect to total amount and timing (1,7). Recent studies revealed that transcription is the key step for gene expression of influenza virus (6,8). On the other hand, the contribution of post-transcriptional control is not well known. This communication described the quantitative examination of

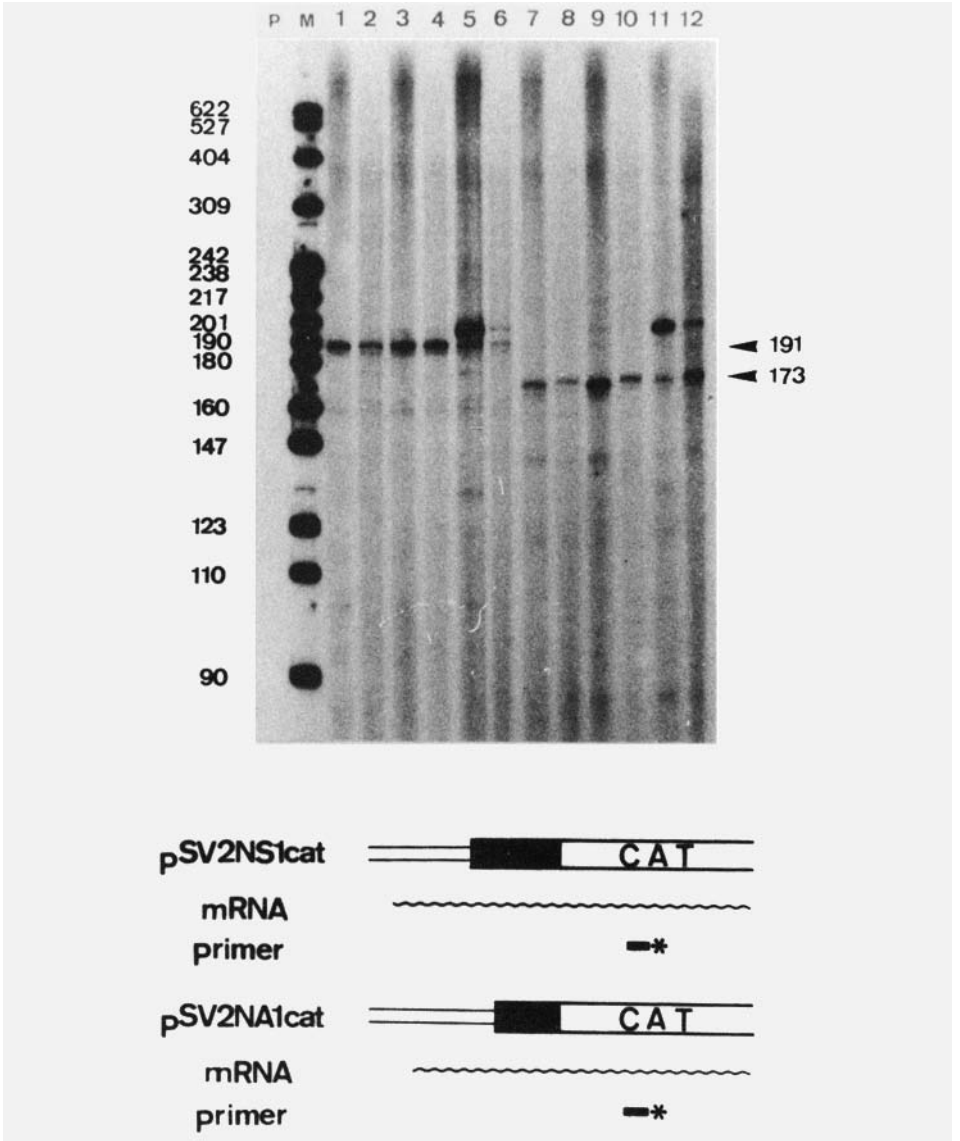


Fig. 3. Primer extension analysis of CAT mRNA. HeLa cells were transfected with either pSV2NS1cat (lanes 1-6) or pSV2NA1cat (lanes 7-12), then infected with influenza virus at moi of 10 and harvested at 0 hr (lanes 1, 2, 7 and 8), 2.5 hr (lanes 3, 4, 9 and 10) and 5 hr (lanes 5, 6, 11 and 12) after infection. Cytoplasmic (lanes 1, 3, 5, 7, 9 and 11) and nuclear RNAs (lanes 2, 4, 6, 8, 10 and 12) were prepared, and each subjected to primer extension analysis, with the control experiment in the absence of any cellular RNA (lane P). Numbers on the left indicate DNA length in nucleotides determined by *Hin*fI-digested pBR322 fragments (lane M). Symbols are as shown in the legend for Fig. 1. ~~~ : ^{32}P-5′-labeled primer. ▬*: mRNA.

the translational control of the viral mRNAs in virus-infected cells using the transient CAT expression system.

Eukaryotic messenger RNAs are translated with different efficiencies in vitro (19,20) and in vivo (21–23). Enhancing effects of the leader sequences from some viral mRNAs have been described recently. Gallie et al. (24) have reported that the 5′ non-coding sequence of tobacco mosaic virus RNA enhances the translation of contiguous foreign gene mRNA both in vitro and in vivo. Jobling and Gehrke (25) have shown that the untranslated leader sequence of mRNA for the coat protein of alfalfa mosaic virus enhances the translation of chimeric mRNAs. They have suggested that the chimeric mRNAs have either a higher affinity or a diminished requirement for a limiting component(s) of the translational machinery. Several sequences within the 5′ leader region of mRNA have been suggested to affect the level of translation. In prokaryotes, the Shine-Dalgarno sequence, 5′AGGAGG3′, complementary to the consensus sequence in the 3′-terminal region of 16S rRNA, 3′UCCUCC5′ plays a major role in translation (21,26). In eukaryotes, 40S ribosomal subunits are known to bind to the capped 5′ end and scan the mRNA sequence until reaching an AUG codon (27). The lack of interaction between ribosomes and circular mRNA supports the end-dependent binding mechanism. The fact that eukaryotic ribosomes usually translate only the 5′-proximal cistron in polycistronic viral mRNAs is also rationalized by the scanning model. It is therefore likely that the ribosomal binding signal is located near the AUG initiator codon. Search of the 5′-noncoding sequences from a number of eukaryotic mRNAs shows (GCC)GCCA/GCC*AUG*G emerging as the consensus sequence for the initiation of translation (28). However, this consensus sequence is not found in mRNAs for all vRNA segments of influenza virus strain A/PR/8/34 used in this study. Only the A residue positioned at −3 relative to the A residue in the AUG is conserved in all segments other than segment 2. This suggests that influenza viral mRNAs cannot be translated efficiently.

Examination of the CAT activity in extracts prepared from virus-infected cells provided an indication of a novel aspect for translational control. The CAT activity derived from pSV2NS1cat reached a maximum during the early phase of infection, whereas that from pSV2NA1cat was maximum during the late phase (Fig. 2). Since the relative level of CAT mRNA from pSV2NS1- and pSV2NA1-transfected cells was virtually unaffected by virus infection, the temporal control of CAT gene expression must have been due to differential translation in infected cells. Essentially the same results were obtained for recombinant plasmids prepared from cDNA for segments 4 and 7, which encode two late proteins, hemagglutinin (HA) and matrix protein (M) (data not shown). These observations suggest that the cDNA fragments examined contained the signal for temporal control of translation.

Recently, Levis et al. (29) reported that two polypeptides were produced from a sindbis virus nonstructural gene-CAT chimeric construct. One of the two polypeptides reacted with both anti-CAT and anti-sindbis NS protein antibodies, indicating that this was a fused protein. The other polypeptide reacted with only anti-

CAT antibodies and was therefore a product translated from the authentic initiation codon of the CAT gene. Translation initiation from internal AUG codons sometimes occur in animal, yeast, and plant cells (30,31). Preliminary results of an in vitro translation experiment revealed that only the chimeric CAT proteins were produced from pSV2NS1cat and pSV2NA1cat RNA synthesized in vitro. However, it could not be ruled out that both the chimeric CAT and authentic CAT were simultaneously produced in vitro from a single species of fused CAT mRNA at various ratios depending on the time after infection. To address this possibility, in vitro translation experiments are currently ongoing using extracts from infected cells.

The point to be made from this study is that the expression of viral genes is controlled, not only at the transcriptional level but also, at the translational level as well. A systematic measurement of individual mRNAs and viral proteins at various times after infection will provide a clearer view on involvement of post-transcriptional mechanisms for the control of gene expression.

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