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In vivo analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA

(viral vector/RNA transcription)

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ABSTRACT **A system for the expression of a foreign gene derived from negative polarity RNA was developed using influenza virus, a negative-stranded RNA virus. From cDNA for the influenza virus RNA genome segment 8, the region coding for the nonstructural protein was deleted and replaced by the chloramphenicol acetyltransferase (CAT) gene. The resulting DNA sequence was placed under the control of the promoter of T7 RNA polymerase such that the antisense RNA to CAT mRNA was produced when transcribed by T7 RNA polymerase. Transfection of HeLa cells with this antisense CAT RNA in the presence of the helper ribonucleoprotein cores led to no significant production of the CAT. In contrast, when the RNA was covered with purified nucleoprotein prior to transfection, the CAT gene was efficiently expressed. This indicated that the viral RNA polymerase transcribed the RNA transfected as the RNA–nucleoprotein complexes. In addition, this system was used for analysis of the cis-acting region in transcription and the promoter structure of the viral RNA genome.**

The genome of influenza virus consists of eight single-stranded RNA segments of negative polarity (1, 2). In the virion, RNA is associated with RNA-dependent RNA polymerase and nucleoprotein (NP), which form ribonucleoprotein (RNP) cores (3, 4). Both RNA polymerase and NP play essential roles as trans-acting factors in the transcription and replication of the viral genome (5, 6). RNA polymerase, which is composed of the three polymerase proteins PB1, PB2, and PA (7), is tightly bound to the double-stranded stem region of the panhandle formed by 3′ and 5′ termini of each RNA segment (8, 9). NP is cooperatively bound to RNA segments every 15–20 nucleotides (10) and is required for the elongation of RNA chains (11).

In contrast to our knowledge of these trans-acting factors, cis-acting elements involved in transcription and replication are presently unknown. Twelve nucleotides at the 5′ terminus of each of the eight segments are identical as are the 13 nucleotides at each 3′ terminus (2). In addition, these terminal sequences are conserved in RNA genomes of defective interfering particles (12, 13). These facts suggest that the transcription promoter and the replication origin may be located either at one end or in the double-stranded stem region of the panhandle structure. *In vitro* studies with isolated RNA polymerase and RNA templates containing mutations suggested that the 3′ terminus is needed for promoter activity (14). Recently, Luytjes *et al.* (15) have reported an *in vivo* system for expression of a recombinant influenza RNA. They suggested that both the 22 nucleotides at the 5′ terminus and 26 nucleotides at the 3′ terminus of influenza virus RNA were sufficient to provide the signals for transcription, replication, and packaging.

Recently, we succeeded in reconstituting RNA–NP complexes structurally similar to native RNP cores with isolated components (10). Here, we describe an *in vivo* transcription system based on the transfection of negatively polarized RNA assembled with NP and discuss the cis-acting elements involved in transcription.

MATERIALS AND METHODS

Reconstitution and Transfection of RNP Complexes Containing the Antisense Chloramphenicol Acetyltransferase (CAT) RNA. Influenza virus A/PR/8/34 (H1N1) was used throughout this study. The purification of virions and the isolation of RNP cores and NP were as described (10). Reconstitution of RNP complexes was carried out by a two-step procedure as follows: step I, to form NP–RNA complexes, the antisense CAT RNA (1 pmol) (see Fig. 1) was mixed with 0.5 ng of NP purified from virions and incubated at 30°C for 10 min in a reaction mixture that contained 10 mM Hepes·NaOH (pH 7.0), 125 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 20% (wt/vol) glycerol, and 2.5 mM dithiothreitol; step II, RNA–NP complexes and native RNP cores (NP content, 4 ng) were mixed and incubated at 0°C for 10 min in 10 mM Hepes·NaOH (pH 7.0), 1 M NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 20% glycerol, and 2.5 mM dithiothreitol. Next, the NaCl concentration was reduced to 125 mM and the incubation was carried out at 30°C for 10 min. The resultant reconstituted complexes were used for transfection. HeLa cells were inoculated into eight-chambered slide glass (Nunc) or 20-mm dishes. After incubation at 37°C for 24 h, cells were washed with serum-free medium and then transfected by the liposome (BRL)-mediated transfection method (16). After incubation at 37°C for 2 h, fresh medium was added and cells were further incubated for 22 h.

Detection of CAT Expression by Immunostaining. Cells were fixed with acetone/methanol (1:1) at room temperature for 2 min and processed for immunostaining according to the manufacturer's instructions (5 Prime → 3 Prime Inc.). A 1:300 dilution of rabbit polyclonal CAT antibody and a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody were used. Cells were examined in a Nikon microscope equipped with phase-contrast and fluorescein optics and equipped for photography.

Detection of CAT mRNA by PCR. Total cellular RNA was prepared from cells transfected with RNP cores containing the antisense CAT RNA–NP complexes by phenol extraction followed by DNase I treatment. Isolated RNA (2×10^3 cell equivalents) was subjected to reverse transcriptase-mediated

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Abbreviations: CAT, chloramphenicol acetyltransferase; RNP, ribonucleoprotein; NP, nucleoprotein.

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PCR (17) with a synthetic oligomer KY9 (5′-ATAGGCCAG-GTTTTCACCGTAACACGCCACA-3′), which is complementary to the CAT coding region [nucleotide sequence at positions 4571–4600 (18)], and KY10 (5′-GGGTGACAAA-GACATAATG-3′), which is identical to the 5′ portion of the viral nonstructural protein mRNA, or KY9 and KY11 (5′-

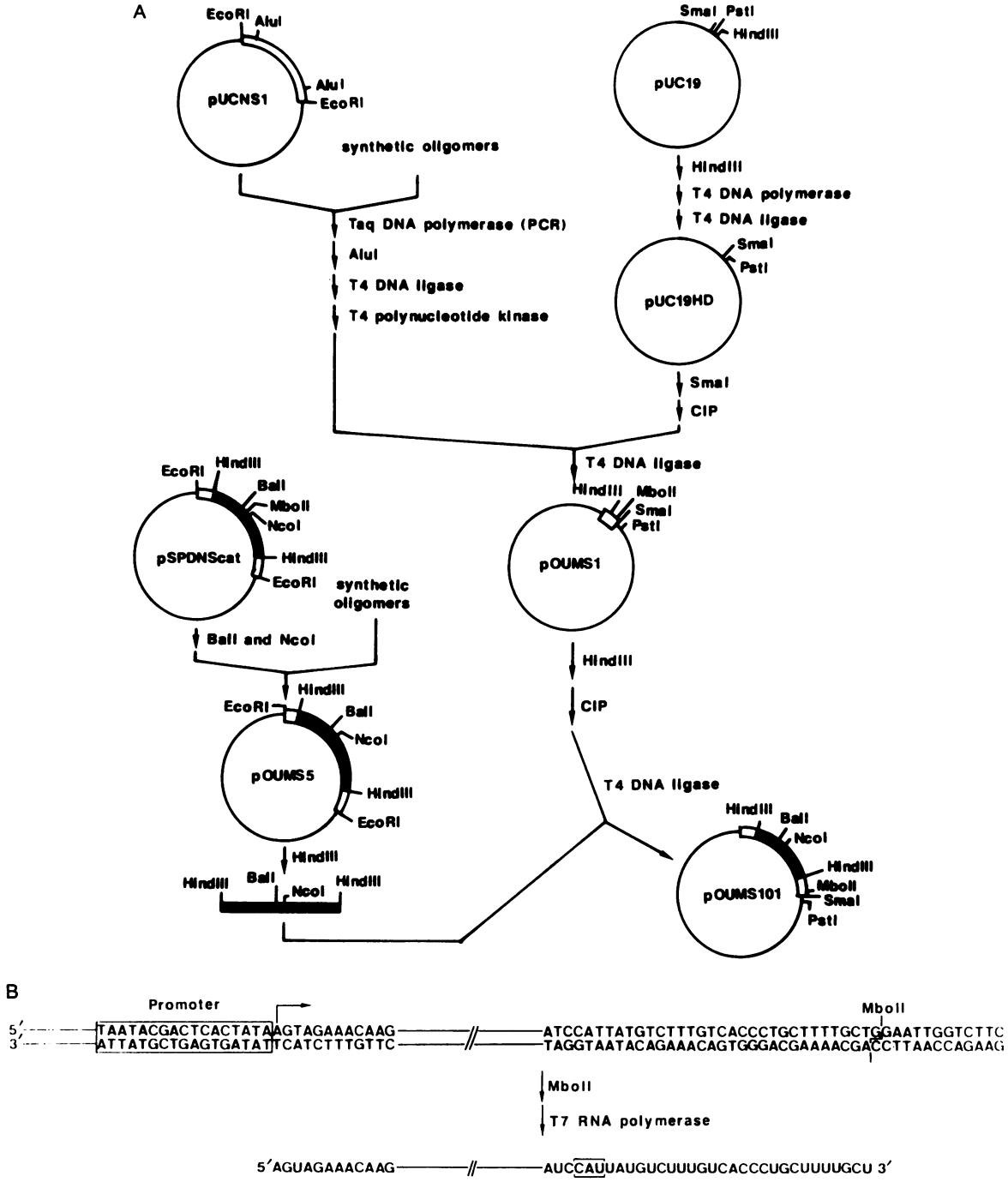

FIG. 1. Construction of the antisense CAT RNA. (*A*) Schematic diagram of the construction of pOUMS101. A cDNA for RNA segment 8 of influenza virus A/PR/8/34 (open boxes) was amplified by PCR using synthetic oligomers KY7 (5′-GGGTAATACGACTCACTATAAG-TAGAAACAAGGGTGTTTTTTA-3′) and KY8 (5′-GGGAAGACCAATTCCAGCAAAAGCAGGGTGACAAAG-3′). This duplex region fragment was digested with *Alu* I. Both terminal fragments were ligated and inserted into pUC19HD vector, a *Hin*dIII site-deficient derivative of pUC19, in the antisense orientation downstream of the promoter of T7 RNA polymerase. The resulting plasmid was designated pOUMS1. To delete the *Mbo* II site from the CAT gene (solid boxes), the *Bal* I/*Nco* I fragment was replaced with the synthetic oligomers KY5 (5′-CCAATATGGACAACTTTTTCGCCCCCGTTTTCAC-3′) and KY6 (5′-CATGGTGAAAACGGGGGCGAAAAAGTTGTCCATATTGG-3′) (18). In the double-stranded DNA consisting of KY5 and KY6, the *Mbo* II site was abolished by a single nucleotide substitution, which did not alter the amino acid when translated. The resultant *Mbo* II-less CAT gene was excised with *Hin*dIII and inserted into *Hin*dIII-digested pOUMS1 plasmid in the antisense orientation. The resulting plasmid was designated pOUMS101. Plasmid DNA was purified through cesium chloride gradients. (*B*) Structure of RNA synthesized from *Mbo* II-linearized pOUMS101 by T7 RNA polymerase. Open box indicates the promoter of T7 RNA polymerase. *In vitro* transcription from *Mbo* II-linearized pOUMS101 was carried out with T7 RNA polymerase (Boehringer Mannheim). The initiation site of transcription is shown by an arrow. Both 5′- and 3′-terminal residues of the synthesized RNA were identical to those of the influenza viral genome RNA segment 8. The triplet representing the initiation codon on RNA, in the antisense orientation, is indicated by brackets.

GATATACCACCGTTGATATATCCCAATGGC-3′), which is identical to the CAT coding region [nucleotide sequence at positions 4921–4950 (18)] (19).

RESULTS

Plasmid Construction for *in Vitro* RNA Synthesis. A plasmid for preparation of negatively polarized RNA containing a reporter gene was constructed as shown in Fig. 1*A*. The resulting plasmid, pOUMS101, contains the CAT gene sandwiched between 5′- and 3′-terminal sequences of the cDNA for RNA segment 8 of influenza virus A/PR/8/34 in the antisense orientation relative to the promoter of T7 RNA polymerase. Transcription of *Mbo* II-digested pOUMS101 by T7 RNA polymerase resulted in synthesis of the antisense CAT RNA in which both 5′ and 3′ termini were identical to the viral RNA sequences (Fig. 1*B*). It should be noted that translation of mRNA with a sequence complementary to this antisense RNA leads to the production of a CAT protein fused with an N-terminal extra sequence of 19 amino acids derived from the viral nonstructural protein encoded by RNA segment 8 (20). The viral mRNA sequence from the 5′ terminus to the translation initiator AUG codon contains the translation regulatory signal involved in efficient expression of viral proteins in virus-infected cells (19, 20).

Transcription of the Antisense CAT RNA in Transfected Cells. To examine transcription from the antisense RNA, RNA synthesized *in vitro* by T7 RNA polymerase was introduced into HeLa cells by liposome-mediated transfection and the expression of the CAT gene was monitored by staining the transfected cells with anti-CAT antibody and fluorescence-conjugated secondary antibody. Transfection of naked RNA resulted in no expression of the CAT gene, since the introduced RNA was of negative polarity. Even when naked RNA was introduced into influenza virus-infected HeLa cells that could supply the trans-acting factors, no significant amount of CAT was produced (data not shown). These results suggested that RNA must be complexed with viral RNA polymerase and NP prior to transfection. By characterizing the interaction between RNA and NP, we could reconstitute RNA–NP complexes structurally similar to native RNP cores (10). For this reason, we have developed a two-step procedure to form active templates. First, the *in vitro* synthesized RNA was assembled with NP purified from virions under the optimum assembly conditions (step I). Second, the resulting RNA–NP complexes were mixed with helper RNP cores, which were purified from virions and treated with high salt concentration, and the mixture was subjected to the reconstitution protocol (step II). Cells transfected with RNP cores alone did not crossreact with the anti-CAT antibody (Fig. 2*A*). In contrast, when cells were transfected with reconstituted RNP cores containing the antisense CAT RNA–NP complexes, significant amounts of fluorescence-positive cells were detected (Fig. 2*B*). This suggested that the transfected CAT RNA of negative polarity was transcribed into mRNA in transfected cells. This was verified by the detection of CAT mRNA by PCR (Fig. 3). Total cellular RNA prepared from cells transfected with reconstituted RNP cores containing the antisense CAT RNA–NP complexes was reverse transcribed in the presence of the first primer complementary to the CAT coding region. cDNA thus synthesized was subjected to PCR in the presence of both the first primer and the second primer, which was identical to the 5′ portion of the viral nonstructural protein mRNA. The expected DNA fragment (470 nucleotides long) was formed by reverse transcription carried through 20 PCR cycles. No PCR products were detected when RNA from cells transfected with RNP cores alone was used (Fig. 4, lanes 3 and 4). These observations indicate that

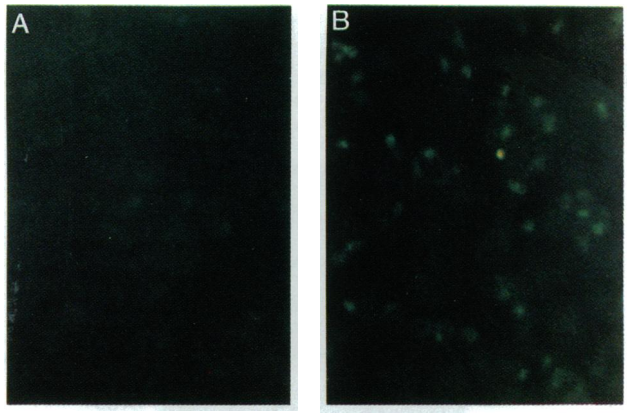

FIG. 2. Expression of the CAT gene from the antisense CAT RNA. Purified native RNP cores (*A*) and reconstituted RNP complexes containing the antisense CAT RNA (*B*) were used for transfection. Reconstitution of RNP complexes containing the antisense CAT RNA was carried out as described, and expression of CAT was monitored by staining the transfected cells with anti-CAT antibody.

transcription of negative polarity RNA takes place in transfected cells. Based on the structure of the template RNA, it is assumed that the promoter activity resides in the 49 nucleotides at the 3′ terminus and the 35 nucleotides at the 5′ terminus of viral genome RNA.

Requirements for Reconstitution. In developing this system, we found that the reconstitution step was critical (Table 1). Omission of either step I or step II resulted in no significant expression of the CAT. When RNA–NP complexes were cotransfected with virus particles instead of step II, the expression of CAT was not detected (data not shown).

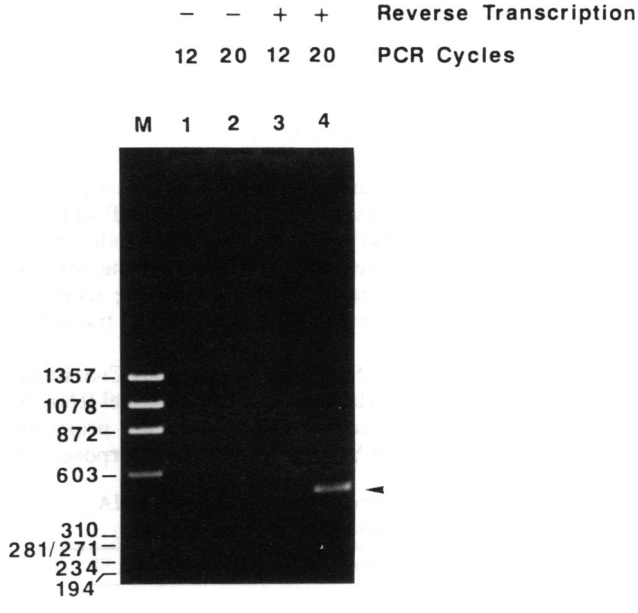

FIG. 3. Detection of CAT mRNA by PCR. Isolated RNA (from 2×10^3 cells) was reverse transcribed into cDNA in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of synthetic oligomer KY9. After reverse transcription, PCR was carried out with synthetic oligomers KY9 and KY10. Twelve cycles (lanes 1 and 3) or 20 cycles (lanes 2 and 4) of amplification involved denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 3 min. The PCR product was subjected to 1.5% agarose gel electrophoresis and was detected by staining with EtdBr. The position of the DNA fragment 470 nucleotides long is indicated by an arrowhead. Lane M, *Hae* III fragments of ϕX174 DNA used as size markers (in nucleotides).

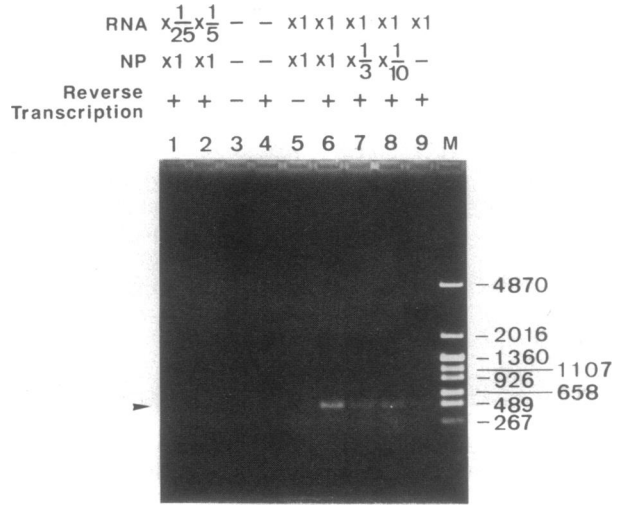

FIG. 4. Effects of reconstitution condition on the expression of the antisense CAT gene. Reconstitution step I was carried out as described without (lanes 3 and 4) or with 0.04 (lane 1), 0.2 (lane 2), and 1 (lanes 5–9) pmol of the antisense CAT RNA in the absence (lanes 3, 4, and 9) or presence of 0.5 (lanes 1, 2, 5, and 6), 0.17 (lane 7), and 0.05 (lane 8) ng of NP. RNA isolated from 2×10^3 transfected cells (lanes 1, 2, 4, and 6–9) was reverse transcribed into cDNA. Eighteen cycles of PCR were carried out with synthetic oligomers KY9 and KY10. The PCR product was subjected to 1.5% agarose gel electrophoresis and detected by staining with EtdBr. The position of the DNA fragment 470 nucleotides long is indicated by an arrowhead. Lane M, pHY DNA standard size markers (in nucleotides) (Takara Shuzo, Japan).

These results suggested that the reconstituted CAT RNA–NP complexes, but not CAT RNA nonspecifically associated with RNP cores, were the active templates. We also examined the protein specificity in step I (Table 1). Adenovirus DNA binding protein or calf thymus histones, both of which can bind to RNA (21, 22), substituted for NP to a limited extent. This suggests that the specific complex formed by NP and RNA is recognized by RNA polymerase.

The effects of the reconstitution conditions on CAT expression were investigated (Fig. 4). The level of transcription from the transfected antisense CAT RNA was proportional to the amount of antisense CAT RNA and NP added in reconstitution of RNA–NP complexes. PCR products depended on the input antisense CAT RNA and the reverse transcription step. These results indicate that the reconstitution of the RNP structure is critical for maximal transcription.

Analysis of the Promoter Structure Using RNAs Containing Mutations at the 3′ Ends. The expression of a model RNA of negative polarity can be used to determine the promoter structure of the virus RNA genome. For this purpose, we

Table 1. Requirement for reconstitution of protein–RNA complexes containing the antisense CAT gene

Step I	Step II	Positive cells, %
None	–	2.2
None	+	3.9
NP	–	3.9
NP	+	61.9
AdDBP	+	6.9
Core histones	+	12.7

Reconstitution step I was carried out with all samples in the absence (None) or presence of 0.5 ng of NP, 0.4 ng of adenovirus DNA binding protein (AdDBP), or 0.1 ng of calf thymus core histones. –, RNA–NP complexes and RNP cores were simply combined before transfection. Positive cells are expressed as the number of fluorescence-positive cells per total cells × 100.

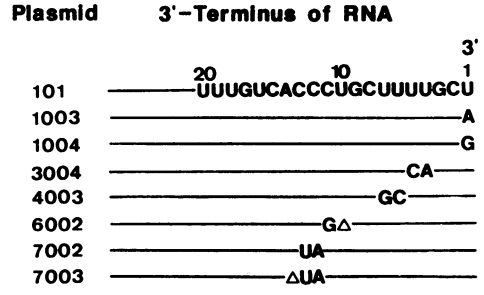

FIG. 5. Structure of 3′ termini of base-substituted mutants. Plasmids for preparation of the antisense CAT RNA containing base-substituted mutations in the 3′-terminal sequence were constructed. A *Bam*HI (between *Hin*dIII and *Mbo* II sites)/*Pst* I fragment in pOUMS101 was replaced with double-stranded synthetic oligonucleotides containing given mutations. The resulting plasmids were designated pOUMS1003, pOUMS1004, pOUMS3004, pOUMS4003, pOUMS6002, pOUMS7002, and pOUMS7003. pOUMS101 is a wild-type plasmid. DNA sequences replaced with mutated oligonucleotides were verified by dideoxynucleotide sequencing using Sequenase (United States Biochemical). *Mbo* II-linearized plasmids were transcribed *in vitro* by T7 RNA polymerase. The 3′-terminal RNA sequences derived from these plasmids are shown. Bases substituted and deleted (Δ) are indicated.

carried out *in vitro* mutagenesis on cloned cDNA and prepared a set of mutated RNAs, each of which contains base-substituted mutation(s) at the 3′-terminal sequence of the RNA, the putative promoter region (Fig. 5). These mutant RNAs were subjected to the reconstitution and transfection procedure and the level of transcription was monitored by PCR (Fig. 6). The relative amounts of the CAT mRNAs formed are summarized in Table 2. Mutations of the 3′-terminal base hardly affected transcription, while mutations at positions 6–14 from the 3′ end significantly reduced transcription. These observations suggest that important

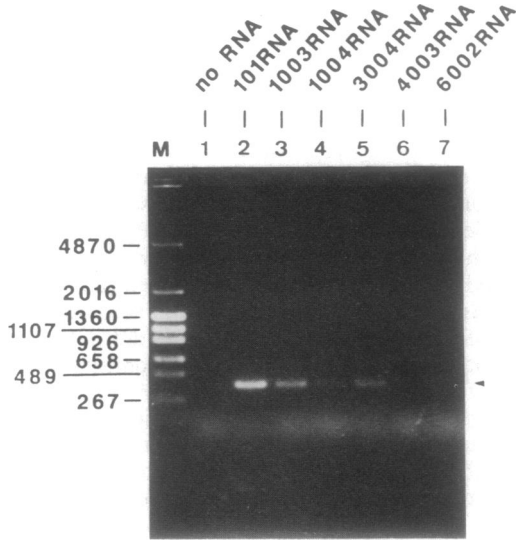

FIG. 6. Transcription promoter activities of wild-type RNA and mutant RNAs. Reconstitution of RNA–NP complexes was carried out without (lane 1) or with RNAs derived from pOUMS101 (lane 2), pOUMS1003 (lane 3), pOUMS1004 (lane 4), pOUMS3004 (lane 5), pOUMS4003 (lane 6), and pOUMS6002 (lane 7). RNA isolated from 2×10^3 cells transfected with these RNA–NP complexes was reverse transcribed into cDNA. Seventeen cycles of PCR were carried out with synthetic oligomers KY9 and KY11. The PCR product was subjected to 1.5% agarose gel electrophoresis and detected by staining with EtdBr. The position of the DNA fragment 380 nucleotides long is indicated by an arrowhead. Lane M, pHY DNA standard size markers (in nucleotides).

Table 2. Effects of base substitutions on influenza virus transcription promoter activity *in vivo*

Plasmid	mRNA synthesis, %
pOUMS101	100
pOUMS1003	87
pOUMS1004	60
pOUMS3004	54
pOUMS4003	41
pOUMS6002	17
pOUMS7002	48
pOUMS7003	31

Expression of CAT mRNAs from wild-type and mutant antisense CAT RNA was assayed as described in the legend to Fig. 6. The amount of PCR product was determined after electrophoresis on a 1.5% agarose gel by densitometric scanning of the EtdBr-stained gel. The results are represented relative to the amount obtained with wild-type RNA derived from pOUMS101 (100%). A mean value from five independent experiments is shown.

signals for transcription exist near the 3′ terminus but not at the 3′-terminal nucleotide.

DISCUSSION

We have described an expression system dependent on the transcription of an RNA of negative polarity by the influenza virus RNA polymerase. Ahlquist *et al.* (23, 24) observed that the positive-stranded RNA prepared from full-length cDNA of brome mosaic virus genome RNA was infectious in tobacco leaf. Recently, infectious RNAs were also constructed from cDNAs of positive-stranded RNA genomes of poliovirus (25), human rhinovirus (26), sindbis virus (27), and tobacco mosaic virus (28). Additional factors (proteins) were not required for transcription and replication of these RNAs when they were transfected into cells. In contrast, RNA of negative polarity should first be transcribed into mRNA by viral-specific RNA polymerases. We have overcome this difficulty in the case of influenza virus by reconstituting RNA–protein complexes in the presence of helper RNP cores. In positive-stranded RNA viruses, when the infectious RNAs derived from cDNAs were transfected, progeny viruses were produced (29). Recently, Luytjes *et al.* (15) reported that transfection of cells with RNA and purified influenza viral proteins in the presence of helper virus amplified and expressed the recombinant RNA that was packaged into virus particles. Preliminary experiments with PCR revealed the amplification of the antisense CAT RNA in transfected cells, suggesting that not only transcription but also replication takes place (data not shown).

Several lines of evidence suggest that the transcription promoter may be located either at the 3′ termini or within the panhandle structure formed by 3′ and 5′ termini of each RNA segment (2, 12, 13). The *in vitro* mutagenesis of cloned cDNA enabled us to analyze cis-acting regions involved in transcription (Fig. 6 and Table 2). It was shown that important promoter signals resided at positions 6–14 with respect to the 3′ end but not at the 3′-terminal base. In the case of positive-stranded infectious RNAs, the terminal sequences of RNAs exactly similar to viral genomes are required for their template activities (29). In contrast, a significant level of the transcription was observed even when 20–30 more nucleotides were added to the 3′ terminus of our model RNA template (data not shown). These observations support the notion that the promoter signals are not located at the 3′-terminal base.

It is not known whether the primary sequence of the 3′ terminus or the double-stranded structure formed by the interaction between the complementary regions at the 3′ and 5′ termini supports the promoter activity. The free energy of the maximally stable double-stranded structures formed between the 3′-terminal sequences of wild-type and mutated RNAs and the 5′-terminal sequence was calculated. However, there was no apparent correlation between the value of the free energy and the promoter activity (data not shown). This favors the concept that the promoter does not lie in the double-stranded structure but on the single-stranded primary sequence. This is also in good agreement with the observation that only the 3′-terminal sequence is required for transcription by influenza viral RNA polymerase *in vitro* (14).

Using *in vitro* mutagenesis of cloned cDNA, the cis-acting regions involved in transcription as well as replication can be examined. In addition, if the recombinant RNA is recovered in the progeny virus particles, influenza virus could be used as a viral vector.

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