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Inhibition of influenza viral polymerases by minimal viral RNA decoys

Guangxiang Luo, Stephanie Danetz and Mark Krystal

Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492-7660, USA

All gene segments of influenza virus share a common feature at their respective termini. Both the 5′- and 3′-terminal sequences are highly conserved and possess partial inverted complementarity. This allows for the formation of a double-stranded duplex, which plays a major role in transcription, replication and packaging of the viral genome. *In vitro* studies have shown that the viral polymerase binds to short RNA molecules containing these termini. In this study, attempts were made to test whether mini-RNA decoys containing either or both termini can inhibit the activity of the viral polymerase *in vivo*. RNA molecules containing either the 5′ or the 3′ noncoding sequences were unable to inhibit NS–CAT RNA replication, while mini-RNA decoys consisting of both the 5′ and 3′ noncoding sequences of vRNA or cRNA were able to efficiently inhibit the activity of the viral polymerases expressed from vaccinia virus vectors.

The genome of influenza A virus consists of eight single-stranded RNA segments of negative polarity. Sequence analyses of each RNA segment of the influenza A virus genome revealed a short conserved noncoding sequence at both the 5′ and 3′ termini (Skehel & Hay, 1978; Robertson, 1979; Desselberger *et al.*, 1980). The first 13 nucleotides at the 5′ terminus are identical among all eight gene segments, while the first 12 nucleotides at the 3′ terminus exhibit variation at only one position. In addition, the sequences at the 5′ and 3′ termini possess partial inverted complementarity that could allow an RNA duplex to form. The existence of such an RNA duplex was experimentally demonstrated *in vivo* (Hsu *et al.*, 1987). Further work has demonstrated that the structure and/or sequence of these termini function as *cis*-acting signals for transcription, replication and assembly of the viral genome (Luytjes *et al.*, 1989; Luo *et al*, 1991; Luo & Palese, 1992; Li & Palese, 1992; Seong & Brownlee, 1992*b*; Piccone *et al.*, 1993; Hagen *et al.*, 1994; Fodor *et al.*, 1994). This was made possible through the *in vitro* reconstitution of ribonucleoprotein (RNP) using viral polymerase (PB1, PB2 and PA) proteins and nucleoprotein (NP) purified from virions, together with *in vitro* cDNA-derived RNAs (Parvin *et al.*, 1989; Seong & Brownlee, 1992*a*). Genetic analyses of the panhandle structure *in vivo* revealed that the RNA duplex, along with a juxtaposed stretch of uridines about 17–22 nucleotides from the 5′ terminus, is needed for poly(A) addition of the viral mRNA (Luo *et al.*, 1991). Interestingly, it was later suggested that this RNA duplex was also involved in the initiation of *in vitro* transcription of short model RNAs (Hagen *et al.*, 1994; Fodor *et al.*, 1994). Besides functioning as *cis*-acting signals for transcription and replication, the 5′- and 3′-terminal sequences have been found to be required for differential activation of the viral polymerase complex (PB1, PB2 and PA) activities (Hagen *et al.*, 1994; Cianci *et al.*, 1995).

On the other hand, most of our knowledge about the *trans* elements required for transcription and replication of the viral genome has been derived through genetic analyses of the viral RNAs synthesized *in vivo* and from studies using extracts prepared from wild-type and mutant virus-infected cells (Krug *et al.*, 1989). The recent development of an influenza virus-free replication system using recombinant vaccinia virus vectors has facilitated our understanding of the *trans* functions required for virus replication (Huang *et al.*, 1990). Using the recombinant vaccinia virus expression system, it was found that the viral polymerase complex composed of PB1, PB2 and PA proteins is inactive until it specifically binds to the 5′ and 3′ termini of either vRNA or cRNA (Hagen *et al.*, 1994; Tiley *et al.*, 1994; Cianci *et al.*, 1995).

These studies lead to the assumption that RNAs containing the conserved termini of influenza virus may be efficient inhibitors of viral replication *in vivo*, through sequestering of viral polymerase. This hypothesis was examined through the use of an *in vivo* RNA expression system (Pattnaik *et al.*, 1992). Firstly, DNA vectors were constructed from which RNA molecules with precise 5′ and 3′ ends of influenza virus genome could be produced, as these are required for full recognition of the RNA template by the viral polymerase complex (Li & Palese, 1992; Piccone *et al.*, 1993; Tiley *et al.*,

Author for correspondence: Guangxiang Luo.
Fax +1 203 284 6088. e-mail Guangxiang_Luo©ccmail.bms.com

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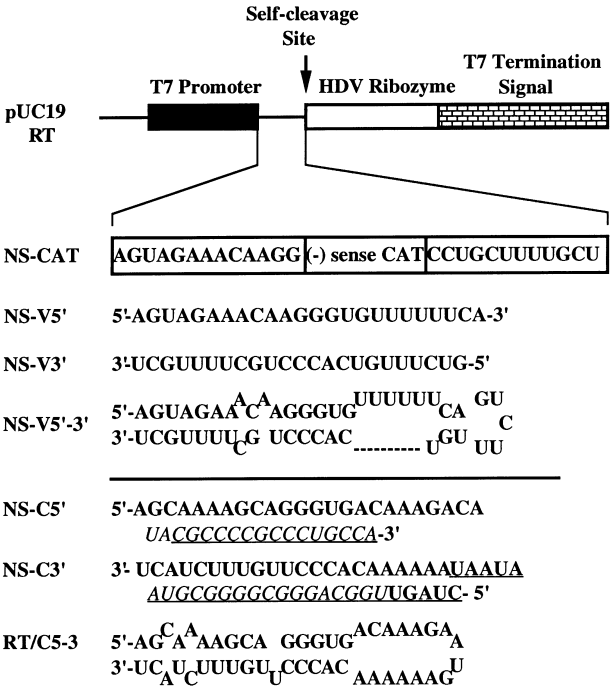

Fig. 1. Expression clones for the NS–CAT reporter plasmid (Luytjes *et al*., 1989) and decoy RNAs. RNA transcription is driven by a T7 promoter and terminated by a T7 termination signal sequence (Pattnaik *et al*., 1992), while the 3′ end of the RNA is processed by the transcribed HDV genomic ribozyme (Perrotta & Been, 1991). The sequences of the minimal viral RNA decoys are shown. The extra nucleotides introduced into the NS-C5′ and NS-C3′ RNAs are underlined. The complementary sequences added in the NS-C5′ and NS-C3′ RNAs are indicated in italic.

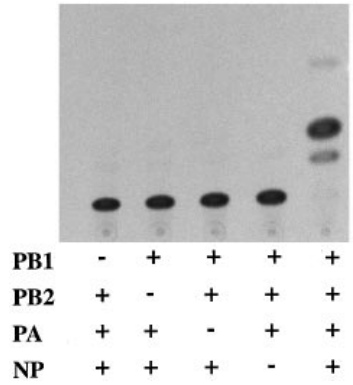

Fig. 2. Replication and expression of the NS–CAT RNA by viral polymerases expressed from vaccinia virus vectors. 293 Cells were infected with (+) or without (−) recombinant vaccinia virus vectors in different combinations. The viral polymerase (PB1, PB2 and PA) proteins and nucleoprotein (NP) expressed from the recombinant vaccinia virus are indicated.

1994) (Fig. 1). The 5′ end of the RNA molecule initiates through the use of a T7 promoter, while the 3′ end of the transcript is created through autocatalytic processing by the genomic ribozyme of hepatitis delta virus (HDV) (Perrotta & Been, 1991). A T7 termination signal sequence is placed downstream of the HDV ribozyme to eliminate further transcription by the T7 RNA polymerase. The construct, NS–CAT, was designed to express a functional vRNA encoding chloramphenicol acetyltransferase (Luytjes *et al*., 1989). Four additional DNA constructs expressed short RNAs containing either the 5′ or 3′ terminus of vRNA or cRNA, while two other clones expressed both termini of minus sense (NS-V5′–3′) or plus sense (NS-C5′–3′) viral RNA (Fig. 1). The expression of authentic NS–CAT RNA was initially examined *in vitro* after T7 transcription. Most of the RNA transcripts terminated at the engineered T7 terminator, with more than 70% of the transcripts processed by the genomic HDV ribozyme to produce RNA of the correct size (data not shown). The authenticity of the NS–CAT RNA generated from this DNA vector was then validated *in vivo* (Fig. 2) using viral polymerases expressed from vaccinia virus vectors (Huang *et al*., 1990). Only if the 5′ and 3′ end sequences were precise would viral polymerase efficiently replicate and transcribe the NS–CAT RNA *in vivo*. 293 Cells were first infected with recombinant vaccinia viruses individually expressing PB1, PB2, PA, NP and T7 RNA polymerase proteins. At 30 min post-infection, the NS–CAT DNA was transfected into cells using lipofectin. At 16–18 h post-transfection, cells were harvested and lysed three times by freeze–thawing. The level of protein expression was determined by CAT assays (Luytjes *et al*., 1989). As observed in Fig. 2, CAT activity is expressed *in vivo* from the transfected DNA, and is dependent on the expression of all viral polymerase proteins and the NP protein.

The level of CAT expression using the DNA vector producing NS–CAT RNA was then used to monitor the activity of the viral polymerases in the presence of the minimal RNA decoys (Fig. 1). 293 Cells were first co-infected with five recombinant vaccinia viruses expressing the three viral polymerases, NP and T7 RNA polymerase. Subsequently, these cells were lipofectin-transfected with 0·5 μg of pNS–CAT DNA plus 2·5 μg of either NS-V5′, NS-V3′ or NS-V5′–3′, which produce 5′, 3′ or 5′ and 3′ noncoding sequences of the NS gene, respectively (Fig. 1). To correct for DNA concentrations, the total amount of DNA transfected into each dish of 293 cells was kept constant by the addition of the parental pUC19/RT vector. At 16–18 h post-transfection, cells were collected and the level of CAT expression was determined. Expression of mini-RNA containing either the 5′ or 3′ ends of vRNA had no significant effect on the expression of functional CAT enzyme. However, the mini-RNA genome containing both 5′ and 3′ noncoding sequences of the NS vRNA was able to inhibit most of the CAT expression (Fig. 3A). This is presumably due to the sequestering of viral polymerase through binding to the decoy RNA. Interestingly, the viral polymerase activity was not affected even if the DNAs expressing RNAs with the 5′ or 3′ noncoding sequences were co-transfected together (Fig. 3A). This differs from *in vitro* experiments, which showed that the endonuclease activity of viral polymerase could be rescued through the addition of 5′ and 3′ ends from single or separate RNAs (Hagen *et al*., 1994). To examine the potency of the NS-V5′–3′ RNA plasmid DNA

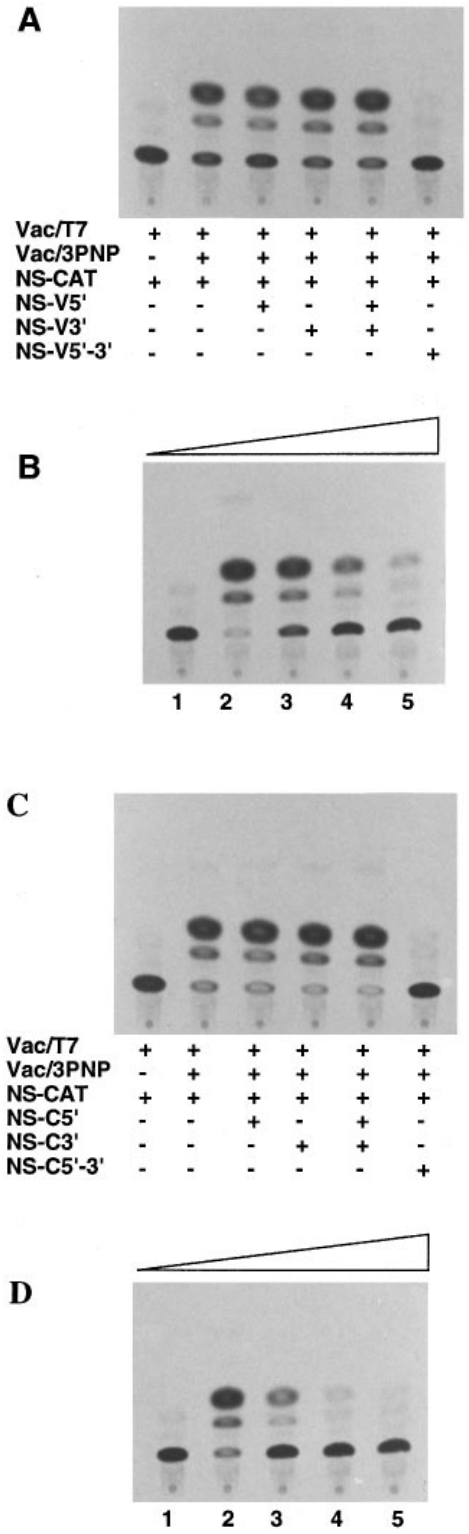

Fig. 3. (A) Inhibition of NS–CAT RNA replication by minimal vRNA decoys. 293 Cells were first infected with vaccinia virus vectors individually expressing PB1, PB2, PA, NP and T7 RNA polymerase proteins. At 30 min post-infection, cells in 35 mm^2 dishes were transfected with 0·5 μg of NS–CAT DNA and 2·5 μg of either NS-V5′, NS-V3′, NS-V5′ and NS-V3′, or NS-V5′–3′ DNA, as indicated by (+). pUC19/RT vector DNA was added to keep the total amount of DNA transfected constant for each transfection. At 16–18 h post-transfection, cells were harvested and lysed, and one tenth of each cell extract was used for CAT assay, as described previously by Luytjes *et al.* (1989). (B) Dose-dependent effect of NS-V5′–3′ RNA on inhibition of CAT expression. 293 Cells were transfected with 0·5 μg of NS–CAT DNA and 0 μg (lane 2), 0·1 μg (lane 3), 0·5 μg (lane 4) and 2·0 μg (lane 5) of NS-V5′–3′ DNA. A control was included without DNA transfection (lane 1). pUC19/RT vector DNA was used to keep the amount of DNA for each transfection constant at 2·5 μg. The level of CAT activity in the presence of 0, 0·1, 0·5 or 2·0 μg is 100, 77·4, 20 and 4·4%, respectively. (C) Inhibitory activity of the minimal cRNA decoys. This experiment differs from (A) only in that the minimal RNA decoys expressed *in vivo* are cRNA sense, as indicated in NS-C5′, NS-C3′ and NS-C5′–3′ (Fig. 1). (D) Titration of the inhibitory activity of NS-C5′–3′ RNA. Cells were transfected with 0 μg (lane 2), 0·1 μg (lane 3), 0·5 μg (lane 4) and 2·0 μg (lane 5) of NS-C5′–3′ DNA. Lane 1 is a control with only vaccinia virus vector infection. The level of CAT activity in the presence of 0, 0·1, 0·5 or 2·0 μg of NS-C5′–3′ is 100, 19, 2·2 and 0·59 %, respectively.

was titrated, keeping the total DNA concentration constant. As shown in Fig. 3 (B), the inhibitory activity of this mini-RNA genome consisting of only 5′ and 3′ noncoding sequences of the NS gene is concentration-dependent. By adding equal amounts of NS–CAT and NS-V5′–3′ DNA (approximately 1:1·2 molar ratio) the level of CAT expression was inhibited by 80% compared to the control (Fig. 3 B).

As the termini of vRNA were able to inhibit CAT expression, the system was then used to evaluate the cRNA sense mini-RNA decoys for their inhibitory activity. The RNA sequence of the mini-cRNA decoy is exactly complementary to that of the mini-vRNA decoy. However, the mini-RNAs containing either 5′ or 3′ noncoding sequences of the NS cRNA were engineered to be slightly longer than their vRNA counterparts. The length of the NS-C5′ or NS-C3′ RNA (Fig. 1) was extended beyond the noncoding region by the addition of extra nucleotides in order to match the size of the mini-cRNA NS-C5′–3′. In addition, the nucleotides (Fig. 1, underlined) introduced into the NS-C5′ and NS-C3′ RNAs in order to increase the length were also engineered to be complementary to each other, to enable a more stable RNA duplex structure to be formed when the two RNAs are co-expressed. The results derived from the experiments on these RNAs are shown in Fig. 3 (C, D). The observations are the same as those obtained from vRNA sense mini-RNA. Only the mini-cRNA decoy has potent inhibitory activity against the viral polymerase (Fig. 3 C). Neither the constructs expressing one of the termini, nor the co-expression of NS-C5′ and NS-C3′ RNAs, significantly inhibited viral polymerase activity. In addition, the inhibitory activity of NS-C5′–3′ RNA is also proportional to the amount of transfected DNA (Fig. 3 D). It appears that the cRNA sense NS-C5′–3′ RNA has about five to ten times better inhibitory activity than that of the vRNA sense NS-V5′–3′ RNA (Fig. 3 B, D). At this time, it is not known why the cRNA termini are better inhibitors than vRNA termini. It was previously reported that the copy number of vRNA is much greater than that of cRNA in the virus-infected cell (Mukaigawa *et al.*, 1991). Therefore, it could be that the promoter in the cRNA decoy is stronger than that in the

vRNA decoy, and it would sequester the polymerase with greater efficiency. Also, if the viral polymerase can replicate the decoy RNAs, a better promoter *in vivo* would produce more progeny decoys.

The results described in this study suggest that the mini-RNA decoys containing both 5′ and 3′ noncoding sequences of either vRNA or cRNA of influenza virus genome can be competitive inhibitors of the viral polymerases *in vivo*. Presumably, this is due to the ability of the decoys to bind to and sequester polymerase. An alternative mechanism of inhibition is that these decoy RNAs are acting as antisense molecules and binding to the NS–CAT replicative RNAs. It is unlikely that this is a significant factor in the inhibition, as the RNAs expressing only one terminus do not exhibit inhibitory effects on viral polymerase-dependent CAT activity (Fig. 3C). The inhibition described for the panhandle decoy RNA is directed against an *in vivo* recombinant replication/transcription system. It is not known whether these mini-RNA decoys would be active against influenza virus growth *in vivo*. It is not feasible to use this vaccinia virus-based expression system in this context, as vaccinia virus infection by itself depresses influenza virus growth (unpublished observations). Alternative expression systems are required to address this question.

These *in vivo* studies differ from published *in vitro* analyses in one regard. *In vitro*, polymerase has been shown to bind tightly to the isolated 5′ sequences and less avidly to isolated 3′ end sequences (Tiley *et al*., 1994; Fodor *et al*., 1993). In fact, binding to the 5′ end sequence *in vitro* could prevent polymerase from binding to the duplex RNA consisting of both 5′ and 3′ ends (Tiley *et al*., 1994). Polymerase has even been shown to bind to 5′ end sequences in the context of mRNA, where this sequence is offset from the end by 9–15 bases of host sequence (Shih *et al*., 1995). However, the mini-RNAs containing either 5′ or 3′ noncoding sequences alone did not inhibit the viral polymerase activity *in vivo*. The reason for this is not yet known. One possible explanation may be that the level of RNA containing either 5′- or 3′-terminal sequences, which is transcribed from T7 polymerase, is not high enough to compete out binding by the viral polymerase, as the level of RNA decoy determines its activity *in vivo* (Sullenger *et al*., 1990). However, the mini-vRNA and -cRNA decoys may be replicated by the viral polymerase complex as they contain all the viral sequences necessary for replication (Luytjes *et al*., 1989; Huang *et al*., 1991), thereby amplifying their inhibitory activity. In this regard, they may be working through a mechanism similar to that thought to be used by defective-interfering RNAs (Nayak *et al*., 1989). Further studies are needed to illustrate their mechanism of action and to determine whether these molecules can be developed as possible antiviral agents.

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