Journal of General Virology (2000), **81**, 2843–2847. Printed in Great Britain SHORT COMMUNICATION

Unidirectional RNA polymerase I–polymerase II transcription system for the generation of influenza A virus from eight plasmids

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Recently, we developed a system for the generation of influenza A virus by cotransfecting only eight plasmids from which negative-sense vRNA and positive-sense mRNA are expressed (Hoffmann *et al.*, *Proceedings of the National Academy of Sciences, USA* 97, 6108–6113, 2000). Here we report the establishment of a different transcription system for the expression of virus-like RNAs, allowing the intracellular synthesis of noncapped positive-sense cRNA and 5′-capped mRNA from one template. Cotransfection of eight RNA pol I–pol II tandem promoter plasmids containing the cDNA of A/WSN/33 (H1N1) resulted in the generation of infectious influenza A virus, albeit with a lower yield than the bidirectional system. Our approach of producing either vRNA and mRNA or cRNA and mRNA intracellularly from a minimum set of plasmids should be useful for the establishment or optimization of reverse genetics systems for other RNA viruses.

Influenza A viruses are enveloped RNA viruses belonging to the family *Orthomyxoviridae* (Lamb & Krug, 1996). Influenza A virions contain eight ribonucleoproteins (vRNPs), which are negative-sense viral RNA (vRNA) segments associated with nucleoproteins (NP) and complexed with polymerase proteins PB1, PB2 and PA (Hsu *et al.*, 1987). Manipulation of the influenza A virus genome by genetic engineering has allowed molecular analysis of the virus life-cycle. This methodology contributes to our understanding of the molecular mechanisms which underlie influenza A virus–host cell interactions, including the molecular basis for pathogenicity of certain influenza A virus strains (Lamb & Krug, 1996). Viruses generated by these methods can be used for the production of inactivated or live attenuated vaccines. Two reverse genetics systems for influenza virus that have been developed are based on the principle that only vRNAs complexed with NP, PB1, PB2 and PA form a replication unit. One system involves the *in vitro* transcription of cDNA constructs by T3 or T7 RNA polymerase and the subsequent *in vitro* reconstitution of RNPs and transfection into host cells (Luytjes *et al.*, 1989; Enami *et al.*, 1990; Li *et al.*, 1999). The second involves transfection of cDNA constructs and the *in vivo* transcription of the cDNAs by RNA polymerase I (pol I) (Zobel *et al.*, 1993; Neumann *et al.*, 1994; Pleschka *et al.*, 1996). In both systems, infection with influenza helper virus results in the generation of viruses possessing genes derived from cloned cDNA. However, the utility of these methods is limited, because with the use of a helper virus, a selection system is needed to obtain the desired virus from a vast background of wild-type virus. To overcome these technical limitations, plasmid-based systems were established that do not require helper virus infection (Neumann *et al.*, 1999; Fodor *et al.*, 1999). In these systems, eight negative-sense vRNA transcripts are synthesized *in vivo* by RNA pol I. Cotransfection of eight pol I-plasmids together with plasmids encoding the viral polymerase complex genes (PB1, PB2, PA and NP) into cells results in the production of vRNP, and initiation of a virus transcription–replication cycle results in the formation of infectious influenza A virus. Recently, we generated infectious influenza A virus by using a bidirectional RNA pol I–pol II system in which eight plasmids contained all of the viral cDNAs (Hoffmann *et al.*, 2000*a*, *b*). In this system, negative-sense vRNAs and mRNAs derived from the same cDNA are synthesized intracellularly.

For the generation of negative-sense RNA virus, either negative-sense vRNA or positive-sense cRNA can serve as a template. To reduce the number of plasmids needed for the recovery of virus, we reasoned that it might be possible for cellular RNA pol I and pol II to synthesize cRNA and mRNA from one template. Therefore we attempted to develop a

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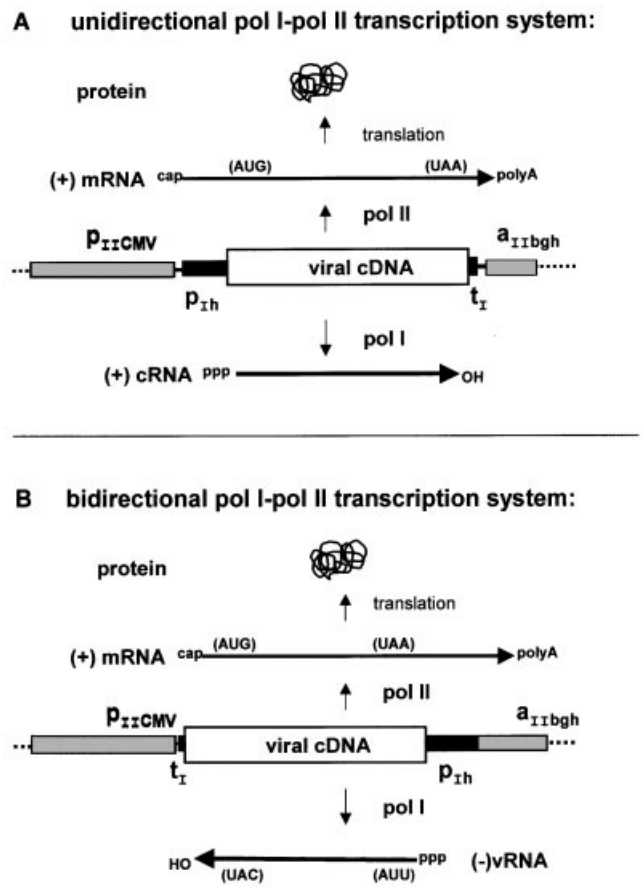

Fig. 1. Unidirectional and bidirectional RNA pol I–pol II transcription systems. In the unidirectional pol I–pol II transcription system (A), viral cDNA is inserted in the positive-sense orientation between a human pol I promoter (p_{Ih}) and terminator sequence (t_I). This entire pol I transcription unit is flanked by a pol II promoter (p_{IICMV}: immediate early promoter of human cytomegalovirus) and the polyadenylation site of the gene encoding bovine growth hormone (a_{IIbgh}). After transfection, two types of RNA transcripts are expected to be synthesized. Positive-sense cRNA with a triphosphate group at its 5′ end synthesized by pol I, and positive-sense mRNA synthesized by pol II with a 5′ cap structure and a poly(A) tail at its 3′ end. Both elements of the mRNA are required for efficient translation. In the bidirectional transcription system (B), viral cDNA is inserted in the negative-sense orientation between the pol I promoter and terminator sequence, but is in the positive-sense orientation with regard to the pol II promoter.

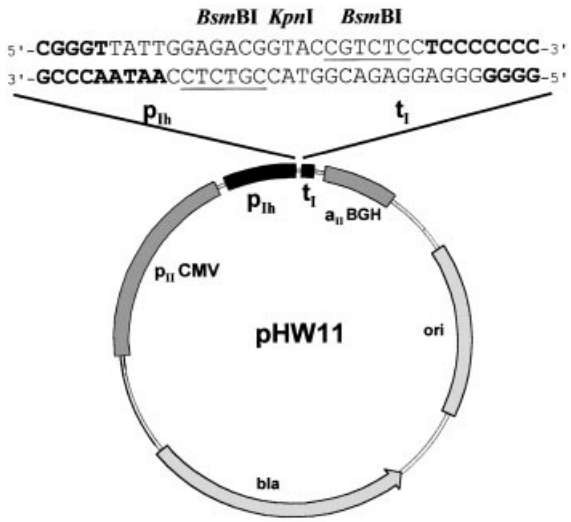

Fig. 2. Cloning vector pHW11 with a pol I and a pol II promoter arranged in tandem. The plasmid contains the 225 bp human RNA pol I promoter (p_{Ih}) and the 33 bp murine terminator (t_I). The pol I promoter and terminator sequences are flanked by the RNA polymerase II promoter (p_{II}CMV) of human cytomegalovirus and the polyadenylation signal (a_{II}BGH) of the gene encoding bovine growth hormone. For insertion of viral cDNA between the pol I promoter and terminator, two *Bsm*BI restriction sites (indicated by underlining) were introduced. Digestion of the vector with *Bsm*BI created a vector fragment with sticky but noncomplementary protruding ends. The design of this vector allows the precise fusion of viral cDNA in the positive-sense orientation with respect to the pol I promoter and terminator sequence. For propagation in *E. coli*, the plasmid has an origin of replication (ori), and for selection in ampicillin-containing medium, the plasmid contains a β-lactamase gene (bla).

unidirectional pol I-pol II transcription system (Fig. 1). Viral cDNA is inserted in the positive-sense orientation between an RNA pol I promoter and a terminator sequence. This whole pol I transcription unit is inserted in the positive-sense orientation between an RNA pol II promoter and a polyadenylation site (Fig. 1A). Unlike the negative-sense vRNA and positive-sense mRNA generated in our bidirectional transcription system (Fig. 1B), two types of positive-sense RNAs were expected to be synthesized. From the pol II promoter, an mRNA with a 5′-cap structure should be transcribed in the nucleoplasm. This transcript should be translated into protein. In the nucleolus, cellular pol I is expected to synthesize full-length, positive-sense influenza virus cRNA with a triphosphate group at the 5′ end (Fig. 1). To have a cloning vector that can be used for insertion of arbitrary cDNA fragments, we constructed pHW11 (Fig. 2). This plasmid contains the pol II promoter (immediate early promoter of the human cytomegalovirus) and the human pol I promoter that are upstream of a pol I terminator sequence and a poly(A) site.

To test whether infectious influenza A virus can be generated by synthesizing cRNA and mRNA from a single template, we constructed eight plasmids. These plasmids, pHW171-PB2, pHW172-PB1, pHW173-PA, pHW174-HA, pHW175-NP, pHW176-NA, pHW177-M and pHW178-NS, contain the cDNAs representing the eight gene segments of influenza A strain A/WSN/33 (H1N1). All of these cDNAs are in the positive-sense orientation with regard to the pol I and pol II promoters. The eight plasmids (1 μg of each plasmid) were transfected into 293T or COS-1 cells with or without coculturing with MDCK cells as described previously (Hoffmann *et al.*, 2000*b*). The virus yield in the supernatant of transfected cells at different times was determined by plaque assay after passage on MDCK cells. Forty-eight hours after transfection $2–5 \times 10^3$ infectious virions were produced (Table 1). Seventy-two hours after transfection the supernatant contained 4×10^4 p.f.u./ml after transfection of 293T or

Table 1. Plasmid sets used for the production of A/WSN/33 (H1N1)

Virus gene segment	Plasmid*							
	Unidirectional system				Bidirectional system			
1	pHW171-PB2	pHW171-PB2	pHW171-PB2	pHW171-PB2	pHW181-PB2	pHW181-PB2	pHW181-PB2	pHW181-PB2
2	pHW172-PB1	pHW172-PB1	pHW172-PB1	pHW172-PB1	pHW182-PB1	pHW182-PB1	pHW182-PB1	pHW182-PB1
3	pHW173-PA	pHW173-PA	pHW173-PA	pHW173-PA	pHW183-PA	pHW183-PA	pHW183-PA	pHW183-PA
4	pHW174-HA	pHW174-HA	pHW174-HA	pHW174-HA	pHW184-HA	pHW184-HA	pHW184-HA	pHW184-HA
5	pHW175-NP	pHW175-NP	pHW175-NP	pHW175-NP	pHW185-NP	pHW185-NP	pHW185-NP	pHW185-NP
6	pHW176-NA	pHW176-NA	pHW176-NA	pHW176-NA	pHW186-NA	pHW186-NA	pHW186-NA	pHW186-NA
7	pHW177-M	pHW177-M	pHW177-M	pHW177-M	pHW187-M	pHW187-M	pHW187-M	pHW187-M
8	pHW178-NS	pHW178-NS	pHW178-NS	pHW178-NS	pHW188-NS	pHW188-NS	pHW188-NS	pHW188-NS
	Virus titre (p.f.u./ml)†							
Transfected cells‡…	293T	293T + MDCK	COS-1	COS-1 + MDCK	293T	293T + MDCK	COS-1	COS-1 + MDCK
Transcripts§	cRNA and mRNA				RNA and mRNA			
$t = 24$ h	0	0	0	0	5×10^2	4×10^2	1×10^3	1×10^3
$t = 48$ h	4×10^3	5×10^3	2×10^3	5×10^3	8×10^6	1×10^7	6×10^6	1×10^7
$t = 72$ h	4×10^4	2×10^5	2×10^4	4×10^5	1×10^7	2×10^8	1×10^7	3×10^8

* The plasmids with the unidirectional transcription units and the plasmids with bidirectional transcription units (Fig. 1) contain cDNAs representing the eight gene segments of A/WSN/33 (H1N1).
† Virus titre of the supernatant was determined at the indicated times (24, 48, 72 h) after transfection by plaque assay on MDCK cells.
‡ 293T or COS-1 cells were transfected either without or with cocultured MDCK cells.
§ RNA transcripts synthesized by pol I or pol II.

2×10^4 p.f.u./ml after transfection of COS-1 cells. The virus yield after 72 h could be increased by coculturing 293T cells or COS-1 cells with MDCK cells (Table 1). The generation of virus proves that after transfection of the eight plasmids, RNA pol I synthesized the eight noncapped, positive-sense cRNAs. The four viral polymerase proteins translated from cellular RNA pol II-synthesized transcripts bound to the naked virus-like cRNAs to form cRNPs. The polymerase subunit PB1 is important for recognition of the terminal structure and binding of the virus-like cRNAs (González & Ortín, 1999*a*, *b*; Li *et al.*, 1998). The interaction with other polymerase proteins started the replication–transcription cycle, which resulted in the synthesis of vRNPs and viral mRNAs (Toyoda *et al.*, 1996; González *et al.*, 1996). In the pol I–pol II transcription system, two different mRNA types are synthesized. One is directly transcribed from the plasmid DNA by RNA pol II and contains the 225 nt pol I promoter sequence in the 5′ end and the pol I terminator sequence in the 3′ end. Another mRNA is synthesized by viral polymerase complex proteins that use the vRNA as template. The 5′ cap structure of this mRNA is acquired by the cap-snatching mechanism in which the polymerase subunit PB2 takes the cap from cellular RNAs (Ulmanen *et al.*, 1981). Although both mRNA types differ in their 5′ and 3′ noncoding regions, they contain the same open reading frames for all viral proteins. The translated structural proteins together with the vRNPs assemble to create infectious influenza A virus.

Although the generation of WSN virus from cells transfected with eight tandem-promoter plasmids proved to be very reliable, the virus yield from this cRNA–mRNA approach was lower than that of the bidirectional system that produces vRNA and mRNA transcripts. Seventy-two hours after 293T or COS-1 cells had been transfected with the eight plasmids containing the bidirectional pol I–pol II transcription system (Fig. 1B; Hoffmann *et al.*, 2000*b*; pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M and pHW188-NS), the virus titre was 1×10^7 p.f.u./ml (Table 1). Twenty-four hours after transfection of COS-1 or 293T cells $0{\cdot}4–1 \times 10^3$ p.f.u./ml were found in the supernatant. These data show that the eight-plasmid bidirectional system has the same efficiency for virus generation with a similar kinetic as the multi-plasmid system requiring cotransfection of 12 or 17 plasmids (Neumann *et al.*, 1999). No infectious virus was found 24 h post-transfection with eight tandem promoter plasmids (Table 1). These results suggest that the differences in virus yields between the

vRNA–mRNA and cRNA–mRNA approaches are due to the different polarities of the primary pol I transcripts. The bidirectional system starts with the intracellular synthesis of vRNA, a situation resembling the natural influenza A virus infection in which vRNPs are transported to the nucleus and vRNAs initially serve as templates for mRNA and cRNA synthesis. In the unidirectional system, cRNPs are the first replication-competent units that are produced. To produce mRNAs, the cRNAs have to be replicated into vRNAs, and the vRNPs are ultimately packaged into progeny virus particles. (Hsu *et al*., 1987). Because of the additional reactions required for the generation of vRNPs from cRNPs, the formation of virus in the unidirectional system occurs at a later time than does virus formation by the bidirectional system. Other possible reasons for the differences in virus yields from the two systems are that sequence elements in the cDNA decrease the efficiency of transcription by terminating transcription, or sequences in the RNA transcripts reduce the steady-state level of the pol I or pol II transcripts. A lower concentration of only one of the eight virus-like cRNAs or mRNAs reduces the overall efficiency of this system because all vRNPs and structural proteins have to be synthesized in concentrations that are optimal for virus replication and virus assembly.

The high efficiency of the eight-plasmid system for the generation of influenza A virus indicates that this system should be applicable to other orthomyxoviruses, e.g. influenza B virus, influenza C virus and Thogoto virus. The results in this study suggest that the vRNA–mRNA system will be the most efficient way for generating these viruses entirely from plasmids. A different challenge is the establishment of reverse genetics-based systems for the generation of RNA viruses other than members of the family *Orthomyxoviridae*, e.g. members of the *Paramyxoviridae*, *Arenaviridae* or *Bunyaviridae* (Roberts & Rose, 1998; Bridgen & Elliott, 1996; Lee *et al*., 2000). Unlike orthomyxoviruses, most RNA viruses replicate in the cytoplasm of infected cells. During their evolution the RNAs of these viruses have not been subjected to selection pressures found in the nucleus, e.g. splicing. Generally, reverse genetics systems for nonsegmented negative-strand RNA viruses are based on intracellular transcription from a T7 promoter, as pioneered by K. K. Conzelmann and colleagues for the rescue of rabies virus (Schnell *et al*., 1994). The expression of virus-like RNAs is driven by T7 RNA polymerase provided either by infection with a recombinant vaccinia virus or by using cell lines constitutively expressing T7 RNA polymerase. Unlike pol I transcription, which occurs in the nucleus, transcription by T7 RNA polymerase takes place in the cytoplasm. Use of the pol I transcription system for cytoplasmic RNA viruses would require that the RNA transcripts have to be transported out of the nucleus. That pol I transcripts are indeed transported out of the nucleus is supported by the detection of protein production in cells containing pol I transcripts that had an internal ribosomal entry site inserted into the 5′ noncoding region (Palmer *et al*., 1993; Lachmann *et al*., 1996). Because information about the sequences crucial for export or retention of pol I transcripts is limited, synthesis of negative-sense or positive-sense RNAs may result in different efficiencies of nuclear export. In addition, the export of a large pol II-generated coronavirus-like transcript ($>$ 30 000 nt) from the nucleus (Almazán *et al*., 2000) indicates that specific RNA sequences rather than the length of a transcript may be crucial for export. The pol I–pol II cloning vectors that we have developed and the efficient cloning method based on the use of type IIs restriction endonucleases will allow researchers to test whether the positive- or negative-sense RNA synthesized in the nucleus is suitable for the generation of cytoplasmic RNA viruses at reasonable costs and within a reasonable period of time.

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