Comparative Analysis of the Ability of the Polymerase Complexes of Influenza Viruses Type A, B and C to Assemble into Functional RNPs that Allow Expression and Replication of Heterotypic Model RNA Templates *In Vivo*

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Influenza viruses type A, B, and C are human pathogens that share common structural and functional features, yet they do not form natural reassortants. To determine to what extent type-specific interactions of the polymerase complex with template RNA contribute to this lack of genotypic mixing, we investigated whether homotypic or heterotypic polymerase complexes support the expression and replication of model type A, B, or C RNA templates *in vivo*. A plasmid-based expression system, as initially described by Pleschka *et al.* [(1996) *J. Virol.* 70, 4188–4192] for influenza A virus, was developed for influenza viruses B/Harbin/7/94 and C/Johannesburg/1/66. The type A core proteins expressed heterotypic model RNAs with similar efficiencies as the homotypic RNA. The influenza B virus model RNA was efficiently expressed by all three types of polymerase complexes. Although no functional polymerase complex could be reconstituted with heterotypic P protein subunits, when the influenza A virus P proteins were expressed together with heterotypic nucleoproteins, significant, albeit limited, expression of RNA templates of all influenza virus types was detected. Taken together, our results suggest that less strict type-specific interactions are involved for the polymerase complex of influenza A compared with influenza B or C viruses.   © 1999 Academic Press

INTRODUCTION

Influenza viruses type A, B, and C belong to the family of Orthomyxoviridae. They are characterized by a single-stranded RNA genome of negative sense polarity that consists of eight RNA segments for type A and B viruses and of seven segments for type C viruses (Lamb and Krug, 1996). The genomic RNAs of all three types share conserved sequence elements at their 3′ and 5′ ends (Desselberger *et al.,* 1980; Stoeckle *et al.,* 1987), and the various RNA segments harbor highly conserved sequences of 9–12 nucleotides (nt) at the 3′ terminus and 11–13 nt at the 5′ terminus (Robertson, 1979). These sequences are partially complementary to each other and have the ability to form a partially double-stranded panhandle, RNA fork, or hairpin loop structure (Flick *et al.,* 1996; Fodor *et al.,* 1994; Hsu *et al.,* 1987; Pritlove *et al.,* 1999).

The virion RNAs are complexed with the nucleoprotein (NP) and associated with the three P proteins (PB1, PB2, and PA for influenza A or B viruses, or PB1, PB2, and P3 for influenza C viruses) to form viral ribonucleoproteins (RNPs). On infection, the RNPs are released in the cytoplasm of the infected cells and migrate into the nucleus where the viral RNAs (vRNA) are transcribed into mRNA and replicated into full-length complementary (cRNA) molecules, which in turn serve as template for vRNA synthesis. Most of our knowledge on the transcription and replication processes of the influenza virus genome has been derived from studies carried out on type A viruses (Lamb and Krug, 1996). Transcription involves a cap-snatching mechanism by which mRNA synthesis is initiated by capped RNA primers which are cleaved from host cell mRNA by an endonuclease associated with the viral polymerase complex (Bouloy *et al.,* 1978; Plotch *et al.,* 1981). Transcription of mRNA is terminated at a stretch of five to seven uridine residues located 15–17 nt from the 5′end of the vRNA templates that serves for polyadenylation (Luo *et al.,* 1991; Poon *et al.,* 1999). In contrast, cRNA synthesis is initiated by primer-independent transcription, giving rise to a complete copy of vRNA (Hay, 1998).

The transcription and replication processes of influenza A have been analyzed by a number of studies *in vitro* and *in vivo* that allowed for the characterization of the *cis*-acting signals and *trans*-acting factors involved (reviewed in Mena *et al.,* 1995). Functional RNP complexes were assembled *in vitro* from synthetic RNA templates and from purified NP and P proteins (Kobayashi *et al.,* 1992; Martin *et al.,* 1992; Seong and Brownlee, 1992). Moreover, functional RNPs have been reconstituted in mammalian cells by expressing the four core proteins (PB1, PB2, PA, and NP) from cloned cDNAs in the presence of artificial RNA templates that retained only the

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extremities of the genomic RNAs (de la Luna et al., 1993; Huang et al., 1990; Kimura et al., 1992; Mena et al., 1994; Pleschka et al., 1996; Zobel et al., 1993). Such in vivo reconstituted RNPs were shown to be functional for both transcription and replication of the RNA template (Huang et al., 1990; Kimura et al., 1993; Perales and Ortin, 1997; Pleschka et al., 1996). It was thus demonstrated that the promoter sequences for the initiation of both transcription and replication are located within the extremities of the viral RNA and that the three subunits of the polymerase and the NP are the minimum set of viral proteins required for these processes. Biochemical and genetic evidence has shown that within the heterotrimer that constitutes the virus polymerase, the PB1 subunit harbors the RNA-dependent RNA polymerase activity, and the PB2 subunit binds to the 5′ cap structure and is responsible for the endonucleolytic cleavage of host cell mRNAs, whereas the role of the PA subunit is still unclear. The PB1 subunit was found to be required for the transcription of mRNA as well as for the replication of cRNA or vRNA. The PB2 subunit is clearly involved in transcription initiation; however, its requirement for cRNA and vRNA replication is controversial (Nakagawa et al., 1995; Perales and Ortin, 1997). The PA subunit is involved in vRNA synthesis, but its relationship with the induction of proteolysis, the only biochemical activity found to be associated with this protein, is still unclear. Finally, besides its structural role in the RNPs, the NP is required for efficient elongation of RNA chains and is thought to be involved in the switch from transcription to replication (Beaton and Krug, 1986; Honda et al., 1988).

Much less is known about the cis- and trans-acting elements involved in transcription and replication of the genome of influenza B virus. As for influenza A virus, functional RNPs can be isolated from influenza B virions, and the sequences that serve as promoters for the viral polymerase are located at the termini of the viral RNA segments (Lee and Seong, 1996). The NP and P proteins are the minimum set of influenza B virus proteins required for the expression of a model RNA template. Furthermore, using RNPs reconstituted in vitro or in vivo, the polymerases from both type A and B influenza viruses were found to be active in transcribing heterotypic RNA templates (Jambrina et al., 1997; Lee and Seong, 1996; Lee and Seong, 1998; Stevens and Barclay, 1998). Moreover, a chimeric influenza A/B transfectant virus in which the neuraminidase gene of influenza A was flanked by the 5′ and 3′ noncoding sequences from the NS segment of influenza B virus was found to be viable, although its replication in vitro and in vivo was impaired (Muster et al., 1991).

Based on the properties of the RNA-dependent RNA polymerase activity associated with influenza C virions (Nagele and Meier-Ewert, 1984; Yamashita et al., 1989), it is believed that transcription and replication of the influenza C virus genome follow the same strategy as that of influenza A and B viruses. However, the cis- and trans-acting elements involved have not been studied.

Although type A, B, and C viruses share structural and biochemical properties (Lamb and Krug, 1996), they differ in their coding strategies for a number of genome segments (Hay, 1998; Lamb and Horvath, 1991). In addition, they exhibit unique diversity in host range, biological properties, and evolutionary patterns (reviewed in Daly et al., 1998; Webster et al., 1992). Despite the fact that all three influenza virus types infect humans, natural intertypic reassortants have never been observed (Kaverin et al., 1983; Mikheeva and Ghendon, 1982). To obtain further insight into the type-specific characteristics of the transcription and replication processes, we developed for influenza C virus a plasmid-based transient transcription/replication assay, as previously described for influenza A and B viruses (Pleschka et al., 1996; Stevens and Barclay, 1998), and investigated the ability of the polymerase complex of all three types of influenza viruses to transcribe and replicate heterotypic RNA templates.

RESULTS

Cloning of influenza virus type B and C NP and P proteins

To compare the ability of the transcription/replication complexes of all three types of influenza viruses to ensure transcription and replication of homotypic versus heterotypic genomic gene segments, we made use of the transient transcription/replication assay described by Pleschka et al. (1996). To that end, the cDNAs of the NP and P proteins (PB1, PB2, and PA) of the influenza B virus strain B/Harbin/7/94, as well as the cDNAs of the NP and P proteins (PB1, PB2, and P3) of the influenza C virus strain C/Johannesburg/1/66 (C/JHB/1/66), were cloned into the pHMG expression vector as described in Materials and Methods, and their sequence was determined. The sequence of the influenza B NP of B/Harbin/7/94 (GenBank accession number AF170569) was found to differ by 1 nt, resulting in one amino acid change (Ile→Val) at position 322, when compared to the sequence recently reported by Lindstrom et al. (1999). Comparisons of the nucleotide sequences of the P protein genes of B/Harbin/7/94 with the few sequences available from the EMBL–GenBank databases showed that they are closely related to those of the recent isolate B/Panama/45/90 (Table 1). For the NP and P protein genes of influenza C viruses, very few sequences are available from the EMBL–GenBank databases (Table 2). Comparisons of the P genes of strains C/JHB/1/66 and C/JJ/50, the only influenza C virus isolates for which the sequences of all three P genes have been determined, indicated that the percentage of nucleotide differences is similar for the three P genes (1.2%, 0.8%, and 1.8% for the PB1, PB2, and P3 genes, respectively). Comparison of the sequence of the NP gene of C/JHB/1/66 with that of

TABLE 1

Comparison of the Nucleotide and Amino Acid Sequences of Influenza B Virus P-Protein Genes

	PB1			
	B/Lee/40	B/Ann Arbor/1/66	B/Panama/45/90	B/Harbin/7/94
B/Lee/40		12 (1.6%)	14 (1.9%)	17 (2.3%)
B/Ann Arbor/1/66	107 (4.7%)		9 (1.2%)	13 (1.7%)
B/Panama/45/90	150 (6.7%)	77 (3.4%)		4 (0.5%)
B/Harbin/7/94	154 (6.8%)	89 (3.9%)	25 (1.1%)	

	PB2		
	B/Ann Arbor/1/66	B/Panama/45/90	B/Harbin/7/94
B/Ann Arbor/1/66		11 (1.4%)	8 (1%)
B/Panama/45/90	70 (3%)		1 (0.1%)
B/Harbin/7/94	82 (3.5%)	26 (1.2%)	

	PA			
	B/Singapore/222/79	B/Ann Arbor/1/66	B/Panama/45/90	B/Harbin/7/94
B/Singapore/222/79		24 (3.3%)	8 (1%)	10 (1.2%)
B/Ann Arbor/1/66	71 (3.3%)		25 (3.4%)	28 (3.8%)
B/Panama/45/90	44 (2.0%)	91 (4.2%)		4 (0.6%)
B/Harbin/7/94	58 (2.7%)	106 (4.9%)	26 (1.2%)	

Note. The sequences examined were from the following influenza B strains (EMBL/GenBank accession numbers are given in parentheses): B/Lee/40 (PB1: N00004, D00004); B/Ann Arbor/1/66 (PB1: M20479, PB2: M20168, PA: M20478); B/Singapore/222/79 (PA: M16711); B/Panama/45/90 (PB1: AF005736, PB2: AF005737, PA: AF005738); and B/Harbin/7/94 (PB1: AF170571, PB2: AF170572, PA: AF170570). Differences in nucleotides (bottom left) or amino acids (top right) between pairs of strains are given with the percentage in parentheses. For the calculations, the sequence lengths analyzed were 2256 nt (from position 22 to 2278) for the PB1 gene, 2310 nt (from position 23 to 2333) for the PB2 gene, and 2178 nt (from position 30 to 2208) for the PA gene, where the positions are given according to the sequences reported for B/Ann Arbor/1/66.

C/California/78, which is the only other available NP gene sequence, showed 1.9% and 1% divergence at the nucleotide and amino acids level, respectively (Table 2). Interestingly, the NP of C/JHB/1/66 was found to be nine amino acids shorter at its C-terminus, relative to the NP of C/California/78.

Construction of influenza A, B, and C model RNA templates

For the generation of artificial influenza A, B, or C RNA segments, the CAT gene sequences in antisense orientation, flanked by the cDNA sequences corresponding to the extremities of the nonstructural (NS) gene segments of A/WSN/33, B/Lee/40, or C/JHB/66 viruses (Barclay and Palese, 1995; Desselberger *et al.,* 1980; Pleschka *et al.,* 1996), were cloned in plasmid pPR between the *Pol*I promoter and the hepatitis delta ribozyme sequences, as described in Materials and Methods, giving rise to plasmids pA/PRCAT(−), pB/PRCAT(−), and pC/PRCAT(−), respectively (Fig. 1). On transfection of these plasmids into COS-1 cells, transcription from the *Pol*I promoter results in the synthesis of artificial A, B, or C viral RNA-like templates of minus-sense (genomic) polarity, with exact 5′ and 3′ extremities. The accuracy of the 5′ and 3′ ends

is determined by the way in which the cDNAs have been inserted relative to the *Pol*I promoter initiation site and hepatitis delta ribozyme sequence, respectively. Similarly, insertion of the same cDNA sequences in reverse orientation into plasmid pPR gave rise to plasmids pA/PRCAT(+), pB/PRCAT(+), and pC/PRCAT(+), which direct the synthesis of artificial A, B, or C RNA templates of plus-sense (antigenomic) polarity.

Comparison of the homotypic transcription/replication systems

For the transient replication assay, COS-1 cells were transfected with each of the pHMG-PB1, pHMG-PB2, pHMG-PA (or -P3), and pHMG-NP plasmids, together with either of the pPRCAT plasmids, as described in Materials and Methods. The level of transcription/replication of the model RNAs was evaluated by measuring the amount of CAT in cell extracts prepared at various times after transfection. We first compared the level of transcription/replication of each of the (−) sense model RNAs in the presence of the homotypic NP and P proteins. As shown in Fig. 2, detectable levels of CAT were observed 12 h after transfection with the type A or C homotypic transcription/replication complexes but only

TABLE 2

Comparison of the Nucleotide and Amino Acid Sequences of Influenze C Virus Core Protein Genes
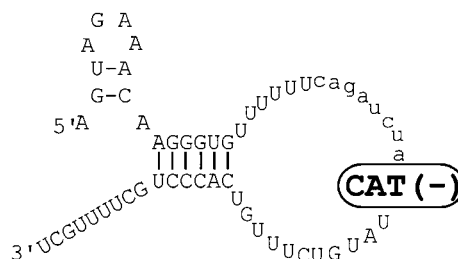
	PB1		P3	
	C/JJ/50	C/JHB/1/66	C/JJ/50	C/JHB/1/66
C/JJ/50		8 (1%)		5 (0.7%)
C/JHB/1/66	27 (1.2%)		38 (1.8%)	

	PB2		
	C/JJ/50	C/JHB/1/66	C/Berlin/1/85
C/JJ/50		5 (0.6%)	8 (1%)
C/JHB/1/66	18 (0.8%)		7 (0.9%)
C/Berlin/1/85	44 (1.9%)	44 (1.9%)	

	NP	
	C/California/78	C/JHB/1/66
C/California/78		6 (1.0%)
C/JHB/1/66	32 (1.9%)	

Note. The sequences examined were from the following influenza C virus strains (EMBL/GenBank accession numbers are given in parentheses): C/JJ/50 (PB1: M28060, PB2: M28061, P3: M28062); C/JHB/1/66 (NP: AF170573, PB1: AF170575, PB2: AF170576, P3: AF170574); C/California/78 (NP: 325338); and C/Berlin/1/85 (PB2: X55992). Differences in nucleotides (bottom left) or amino acids (top right) between pairs of strains are given with the percentage in parentheses. For the calculations, the sequence lengths analyzed for influenza C viruses were 2262 nt (from position 18 to 2280) for the PB1 gene, 2322 nt (from position 22 to 2344) for the PB2 gene, and 2127 nt (from position 22 to 2149) for the P3 gene, where the positions are given according to the sequences reported for C/JJ/50. For the NP gene, a 1668-nt-long sequence (from position 30 to 1697 according to the sequence reported for C/California/78) was analyzed.

fold when the amount of transfected pHMG-NP plasmid was increased from 1 to 4 μg. Optimal levels of CAT were reached when the pHMG-PB1, -PB2, -P3, and -NP plasmids were used at a 1:1:1:4 ratio (data not shown). Using analogous plasmid-based transient transcription/replica-
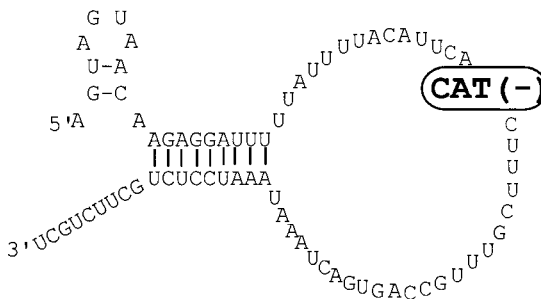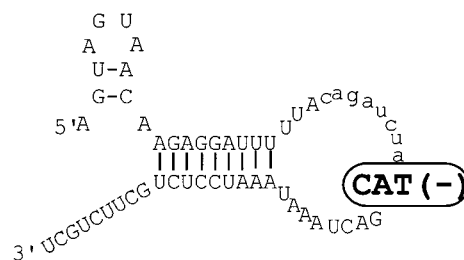
after 18 h with the type B homotypic complex. The level of CAT reached a plateau after 38 h, and comparable amounts of CAT were obtained with either of the type A or C homotypic transcription/replication complexes. The amount of CAT detected at 48 h post-transfection in the case of type B (1471 $\pm$ 395 ng/ml; mean $\pm$ SD of 13 assays) was consistently found to be approximately two-fold lower than that for type A (3418 $\pm$ 1044 ng/ml; mean $\pm$ SD of 35 assays) or type C (3156 $\pm$ 871 ng/ml; mean $\pm$ SD of 23 assays). Omission of any of the components resulted in complete loss of CAT expression (data not shown). This indicated that as previously shown for influenza A (de la Luna *et al.,* 1993; Huang *et al.,* 1990; Kimura *et al.,* 1992; Mena *et al.,* 1994; Pleschka *et al.,* 1996; Zobel *et al.,* 1993) and B (Jambrina *et al.,* 1997; Stevens and Barclay, 1998) viruses, the core proteins NP, PB1, PB2, and P3 of influenza C virus are the minimum set of viral proteins required for transcription and replication of the viral genome. The levels of CAT at 48 h post-transfection were found to be increased up to three-

FIG. 1. Nucleotide sequences and predicted panhandle structures of the model RNA templates. The nucleotide sequences of the model RNAs transcribed from the indicated plasmids are those of the NS genome segment (uppercase) of A/WSN/33 (pA/PRCAT), B/Lee/40 (pB/PRCAT and pB/PRCAT short), or C/JHB/66 (pC/PRCAT). Unrelated non-viral sequences are shown in lowercase. The predicted structure is as suggested by Pritlove *et al.* (1999).
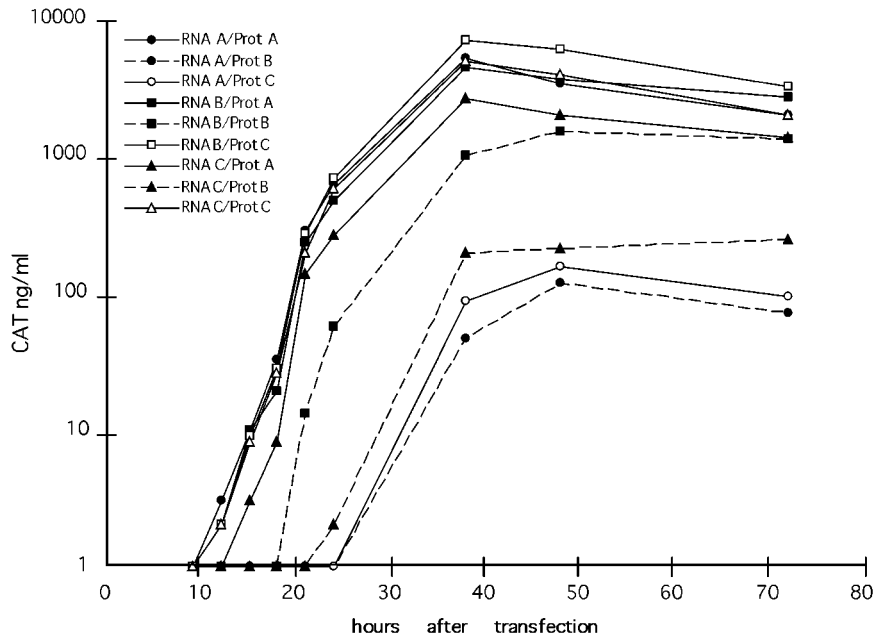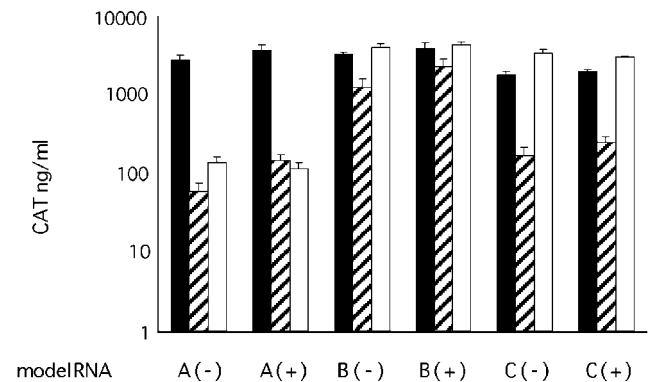
FIG. 2. Expression of (−) sense model RNA templates with homotypic or heterotypic core protein complexes. COS-1 cells were transfected in duplicate with the four pHMG plasmids encoding the core proteins of either the A/PR8/34 (closed symbols; solid lines), B/Harbin/7/94 (open symbols; solid lines), or C/JHB/1/66 (closed symbols; dotted lines), together with 1 μg of either plasmid pA/PRCAT(−) (circles), pB/PRCAT(−) (squares), or pC/PRCAT(−) (triangles) expressing the (−) sense model RNA templates of the indicated influenza virus type (model RNA A, B, C). At various times after transfection, cell extracts were prepared and tested for the levels of CAT as described in Materials and Methods.

tion systems of model RNA templates, the optimal ratios of PB1, PB2, PA, and NP proteins were reported to be 1:1:1:2 or 0.5:0.5:0.5:1 for influenza A or B virus, respectively (Pleschka *et al.,* 1996; Stevens and Barclay, 1998). Thus as shown here for influenza C virus, as for influenza A or B viruses, the optimal ratio of the viral core proteins for the *in vivo* reconstitution of functional RNPs reflects the need for large amounts of NP for the encapsidation of the model RNA template.

Activity of homotypic core protein complexes on heterotypic (−) sense model RNA templates

As mentioned in the introduction, it has been previously shown that the influenza type A polymerase can recognize the influenza B virus promoter *in vivo* (Muster *et al.,* 1991) and that the polymerases from both influenza A and B viruses are able to transcribe type A and B model RNAs *in vitro* (Lee and Seong, 1996). However, although the sequences at the termini of influenza C virus RNA segments share conserved nucleotides with both type A and B influenza viruses, it has not been determined whether they could also serve as a promoter for heterotypic polymerases. We therefore analyzed the ability of homotypic NP and P proteins of all three influenza virus types to transcribe and replicate heterotypic model RNA templates. As shown in Figs. 2 and 3, when type A NP and P proteins were used, heterotypic B (−) and, to a somewhat lesser extent, C (−) RNA templates were transcribed and replicated as efficiently and with

similar kinetics as the homotypic A (−) RNA template. When type C NP and P proteins were used, similar or even higher levels of CAT activity were obtained with the heterotypic B (−) RNA template compared with the homotypic C (−) RNA. However, the maximum level of CAT was found to be reduced 20- to 30-fold, and delayed synthesis was observed with the heterotypic A (−) RNA

FIG. 3. Expression of (−) and (+) sense model RNA templates with homotypic or heterotypic core protein complexes. COS-1 cells were transfected in duplicate with the four pHMG plasmids encoding the core proteins of influenza virus type A (closed bars), type B (hatched bars), or type C (open bars), together with 1 μg of pPRCAT (−) or pPRCAT(+) plasmids expressing the (−) sense or (+) sense model RNA templates of the indicated influenza virus type. At 48 h after transfection, cell extracts were prepared and the levels of CAT were determined as described in Materials and Methods. The results are expressed as the average ± SD of two independent experiments.

template. When type B NP and P proteins were used, wild-type levels of CAT were reached only with the homotypic B model RNA, whereas a 10- or 20-fold reduction in the maximum amount of CAT as well as a delayed synthesis was observed with the heterotypic C or A (−) RNA templates, respectively. Thus the NP and P proteins of influenza A virus were able to transcribe and replicate heterotypic (−) sense model RNAs as efficiently as the homotypic type A RNAs. Furthermore, the type B (−) sense model RNA was efficiently transcribed and replicated by each of the three types of NP and P protein complexes.

Activity of homotypic core protein complexes on heterotypic (+) sense model RNA templates

The same approach was used to compare the ability of the NP and P proteins of the three influenza virus types to initiate the replication of homotypic versus heterotypic model RNA templates of (+) sense polarity. As shown in Fig. 3, for all three virus types, at 48 h post-transfection, comparable levels of CAT were reached whether (+) or (−) sense model RNAs of the same type were used. No CAT expression could be detected when the pPRCAT(+) plasmids were transfected alone (data not shown), indicating that the RNA transcripts expressed from these plasmids were either not directly translated, as they do not represent *bona fide* mRNAs, or were not present at a sufficient level to yield detectable amounts of CAT at 48 h post-transfection. These results indicated that the level of CAT observed with the (+) sense RNA templates was the result of vRNA synthesis and subsequent mRNA transcription. They also suggested that as reported previously in the case of influenza virus type A (Pleschka *et al.,* 1996), the level of CAT observed with the (−) sense RNA templates did not simply reflect transcription but was rather the result of both replication and subsequent transcription of the RNAs.

When the ability of NP and P proteins to replicate heterotypic (+) sense RNA templates was analyzed, the same results were essentially obtained as with the (−) sense RNAs. As judged from the level of CAT, the type A NP and P proteins were found to replicate heterotypic B or C (+) RNA templates to the same extent as the homotypic A model RNA (Fig. 3). Furthermore, the type B (+) RNA template was found to be replicated in the presence of either type of NP and P proteins, whereas the (+) model RNA of influenza A was not efficiently replicated in the presence of either the type B or C proteins. In the case of influenza C virus, for the (+) sense, as for the (−) sense RNA template, the level of CAT was reduced more than 10-fold in the presence of type B proteins compared with type A or C proteins.

Taken together, these observations suggest (1) that the type A proteins are able to recognize the promoter sequences located at the termini of the genomic and
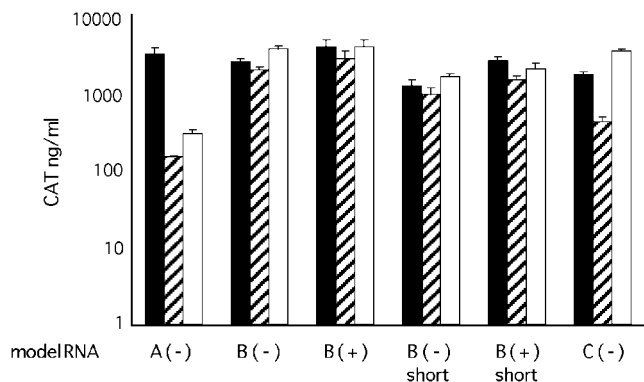

FIG. 4. Comparison of influenza B virus model RNA templates with "long" and "short" noncoding sequences. The legend is as in Fig. 3, except that plasmids pB/PRCAT(−)short [B(−)short] or pB/PRCAT(+)short [B(+)short] were used as indicated. These plasmids direct the expression of influenza B virus (−) sense or (+) sense model RNA templates that comprised only 22 and 26 nt from the 5′ and 3′ ends of the influenza B virus NS genome segment, respectively.

antigenomic RNAs of all types of influenza virus and (2) that the promoter sequences of influenza virus type B are efficiently recognized by the transcription/replication machinery of any type of influenza virus.

Expression of influenza B model RNA templates with "long" and "short" noncoding sequences

Although the influenza virus A and C model RNAs used in this study both contained the 22 and 26 nt from the 5′ and 3′ termini of the viral NS segment, the type B RNA expressed from pB/PRCAT retained 33 and 42 nt from the 5′ and 3′ termini, respectively (Fig. 1). To determine whether the relative lack of type specificity of the type B model RNA template toward the polymerase could be attributed to the presence of longer noncoding sequences, plasmids pB/PRCAT-short (+) and (−) were constructed. Model RNA templates expressed from pB/PRCAT-short comprised only the 22 and 26 nt from the 5′ and 3′ termini of the NS viral segment, as for the type A and C RNA templates. As shown in Fig. 4, both the (−) and (+) B-short model RNAs were replicated slightly less efficiently than the corresponding B RNAs that retained longer noncoding sequences. This was observed irrespective of the type of NP and P proteins used. However, the level of CAT reached in the presence of heterotypic A or C proteins was in the same range as that observed in the presence of the homotypic B proteins. These results therefore showed that minimal type B promoter sequences were sufficient to allow heterotypic recognition by the transcription/replication machinery of any type of influenza virus, although additional noncoding sequences seemed to contribute to the efficiency of recognition.
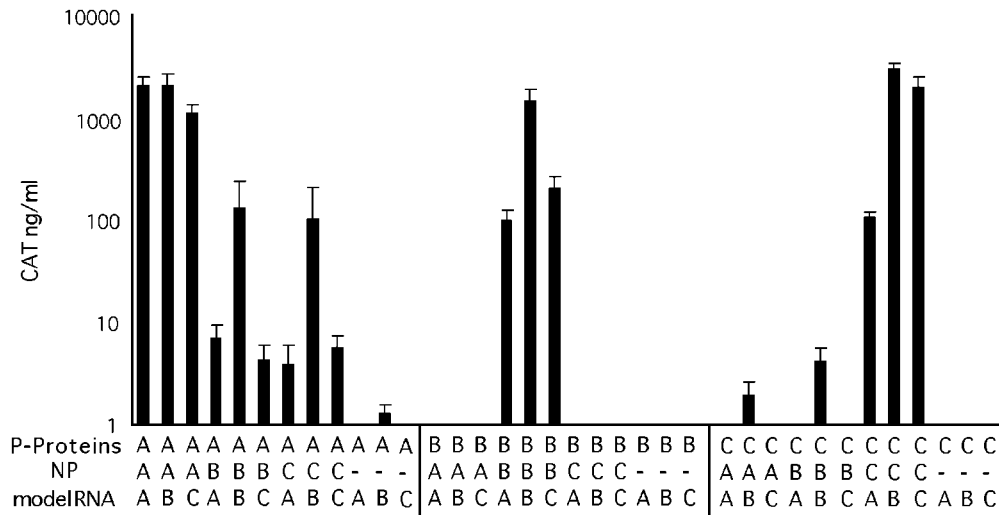
FIG. 5. Expression of (−) sense model RNA templates with homotypic P protein complexes in the presence of homotypic or heterotypic NPs. COS-1 cells were transfected in duplicate with 1 μg of each of the three pHMG plasmids encoding the P proteins of the indicated influenza virus type (P proteins), together with 4 μg of pHMG-NP of the indicated influenza virus type (NP) and with 1 μg of plasmid pPRCAT(−) expressing the (−) sense model RNA template of the indicated influenza virus type (model RNA). At 48 h after transfection, cell extracts were prepared and the levels of CAT were determined as described in Materials and Methods. The results are expressed as the average ± SD of three independent experiments.

Effect of type dissociation of NP and P proteins

To investigate the contribution of the NP to the type specificity of the transcription/replication process, the replication of A, B, or C (−) model RNA templates was analyzed in the presence of all possible combinations of the three types of NP on the one hand and the three types of P proteins on the other. As shown in Fig. 5, when the P proteins of influenza B were combined with a heterotypic A or C NP, the level of CAT was below detectable levels irrespective of the type of RNA. When the P proteins of influenza C were combined with heterotypic A or B NP, a detectable, albeit greatly reduced, level of CAT was observed for the type B model RNA but not for the homotypic type C or the type A RNA templates. In contrast, when the P proteins of influenza A virus were combined with heterotypic B or C NPs, a significant level of CAT could be detected with all types of model RNA. However, although the level of CAT was found to be dramatically reduced in the case of the type A or C RNA templates, only an approximately 10-fold reduction was observed when the type B model RNA was used.

DISCUSSION

It was previously demonstrated that functional RNPs can be reconstituted *in vivo* by expressing the four core proteins (PB1, PB2, PA, and NP) from cloned cDNAs in the presence of artificial vRNA templates in the case of influenza type A and B viruses (Jambrina *et al.,* 1997; Pleschka *et al.,* 1996; Stevens and Barclay, 1998) as well as for the tick-borne Thogoto virus, another member of the Orthomyxoviridae family (Weber *et al.,* 1998). In this report, we have extended this observation to influenza

virus type C, indicating that the three subunits of the polymerase and the NP are the minimum set of viral proteins required for transcription and replication of the viral genome for representatives of the three genera of the Orthomyxoviridae family. Indeed, in the case of influenza C virus, as is the case for the other Orthomyxoviridae studied so far, no transcription/replication of the model RNA template was observed in the absence of any of the core proteins. As judged by the levels of CAT expressed from the respective RNA templates, the influenza C virus polymerase complex was found to be at least as efficient as that of influenza A for the transcription and replication of homotypic vRNA or cRNA templates, whereas the influenza B polymerase complex was consistently found to be somewhat less efficient, which is in agreement with previous observations (Jambrina *et al.,* 1997). We have shown here that for influenza B virus, as for influenza A or C virus, the 22 and 26 nt from the 5′ and 3′ termini of the viral NS segment are sufficient to ensure transcription and replication of the vRNA or cRNA templates. However, as shown for influenza A RNAs (Zheng *et al.,* 1996), additional nonconserved nucleotides at the 3′ and 5′ ends clearly contributed to the efficiency of recognition of the promoter sequences.

When examining the ability of the polymerase complex of the three types of influenza virus to transcribe and replicate heterotypic RNA templates, the type A core proteins were found to be able to recognize the promoter sequences located at the termini of the vRNA and cRNA templates of all types of influenza virus. This finding was in contrast to previous reports that indicated when using the RNP-transfection system, that the influenza A virus expressed polymerase amplified the model type B RNA

10-fold less efficiently than the cognate type A RNA (Muster *et al.,* 1991), whereas when using a vaccinia virus-based system to express the influenza A core proteins, amplification of the type B RNA template was found to be reduced twofold to fivefold compared with the homotypic template (Jambrina *et al.,* 1997; Weber *et al.,* 1998). However, our results are in agreement with *in vitro* studies that showed that the influenza A polymerase could transcribe A or B RNA templates with similar efficiencies (Lee and Seong, 1996). This observation suggests that differences in the levels of expression and mode of delivery of the NP and P proteins may result in significant differences in model RNA amplification and expression *in vivo*. In contrast to influenza A, the influenza B polymerase complex was only able to use the homotypic B RNA template efficiently. A similar lack of transcription/replication *in vivo* of type A model RNA in the presence of the type B polymerase has been previously reported (Jambrina *et al.,* 1997; Lee and Seong, 1998), although efficient transcription has been documented *in vitro* (Lee and Seong, 1996). A possible interpretation for the discrepancy between the results observed *in vitro* and *in vivo* may be that the type B polymerase complex is able to transcribe, but not replicate, heterotypic RNAs. Similar levels of CAT expression were observed when cRNA, rather than vRNA, templates were used. This observation suggests that the synthesis of vRNA, which is the required template for mRNA synthesis, was not significantly impaired. However, detailed analysis of the molecular RNA species synthesized will be needed to determine precisely which replication and/or transcription step is impaired *in vivo* when the type B polymerase complex is used in the presence of heterotypic RNAs. Finally, our results show that the type C polymerase complex amplified the heterotypic type B model RNA as efficiently as the homotypic type C RNA; whereas CAT expression from the heterotypic type A model RNA was found to be reduced 25- to 30-fold.

In contrast to the A or B vRNA or cRNA templates, the influenza C RNA templates were amplified by the influenza A polymerase with a somewhat reduced efficiency (60% of the A RNA template). The influenza C vRNA template differs from the type A vRNA template by a number of changes. The U5→C5 and A6′→U6′ substitutions at positions 5 and 6′ from the 3′ and 5′ ends, respectively, are also found in the type B RNA template. These changes are not likely to account for the reduced level of amplification of the C RNA template, as it has been shown that substitutions at these positions do not affect the ability of an influenza A model RNA to be transcribed and replicated (Flick *et al.,* 1996). Likewise, the U3′:A8′→C3′:G8′ base pair substitution in the proposed hook structure at the 5′ end, which has no effect on the amplification of an influenza A model RNA (Flick *et al.,* 1996), probably does not account for the reduced level of amplification of the C RNA template by the type

A or B polymerase complexes. The most striking differences between the three types of model RNAs reside in the distal base paired element of the promoter sequence. Although natural variation between segments, as well as mutagenesis studies, have shown that the nature of the base pairs between positions 12–15 and 13′-16′ is not essential (Desselberger *et al.,* 1980; Flick *et al.,* 1996; Fodor *et al.,* 1998; Kim *et al.,* 1997), the number of base paired nucleotides was shown to be critical for efficient use of the promoter sequence. The distal promoter element consists of 6, 9, and 5 bp for influenza A, B, and C RNAs, respectively, whereas the optimal length has been determined to be 8 and 11 bp for the type A and B polymerases, respectively (Lee and Seong, 1998). Therefore, the length of the base paired distal element might be an essential determinant of type specificity for efficient transcription and replication. This accounts for the fact that as shown here, the influenza type B RNA is efficiently recognized by the polymerase of any type of influenza virus and was also shown to be used to some extent by the polymerase from Thogoto virus (Weber *et al.,* 1998); whereas the influenza B polymerase complex is only functional on the homotypic RNA template. However, differences in the length of the base paired distal element do not account for the fact that type B and C, but not type A, RNA templates were efficiently amplified by the influenza C polymerase, thus suggesting that other determinants of the promoter sequences are specifically required for influenza C. Site-directed mutagenesis is in progress to identify those type C-specific determinants.

It was of interest to determine whether functional RNPs could be assembled *in vivo* in the presence of heterotypic mixtures of the four core proteins and, in particular, whether homotypic P protein complexes could assemble with heterotypic NP. Therefore, all possible heterotypic combinations of the four core proteins were analyzed against all three types of RNA templates. No significant CAT expression from any type of model RNA could be detected when every possible heterotypic combination of P proteins from type A and B, type A and C, or type B and C viruses were used (data not shown). This indicated that the three P proteins should derive from the same virus type to reconstitute a functional polymerase complex. Moreover, in agreement with previous reports (Jambrina *et al.,* 1997; Stevens and Barclay, 1998), the influenza virus B NP in combination with the three type B P proteins could not be functionally replaced by either the type A or C virus NP. However, when influenza A virus NP was substituted by the NP of type B or C virus, a limited (10%) CAT expression could be observed in the presence of the type B vRNA template, whereas residual (1%) but significant CAT expression was detected in the presence of the type A or C vRNA templates. The level of CAT expression was reduced 10-fold when the type B cRNA template was substituted for the vRNA (data not shown), suggesting that a homotypic NP is essential for

vRNA synthesis, although it may not be absolutely required for transcription of mRNA and/or synthesis of cRNA. Significant amplification of either type A or B model vRNAs was not detected in the presence of heterotypic NP when using vaccinia virus-driven expression of the influenza core proteins (Jambrina et al., 1997). This discrepancy may be due to an overall lower level of expression and amplification of the model RNA when using the vaccinia virus-driven, compared with the plasmid-based, expression system used here. Indeed, significant CAT expression in the presence of heterotypic NP was more readily detected for those combinations of P proteins and model RNA template that gave the highest levels of CAT expression in the presence of homotypic NP. Taken together, our results point to the existence of type-specific interactions between the NP and the P proteins that are essential for replication of the viral genome, which is in agreement with the role of NP in the synthesis of cRNA and vRNA molecules and its implication in the switch from mRNA to cRNA synthesis (Beaton and Krug, 1986; Honda et al., 1988). Both direct and indirect evidence for interactions between NP and P proteins has been recently provided (Biswas et al., 1998; Mena et al., 1999). Analysis of the ability of heterotypic chimeric NP proteins to support transcription and replication of model RNAs in vivo should be of interest to further characterize these interactions.

MATERIALS AND METHODS

Plasmids

Plasmids pHMG-PB1, pHMG-PB2, pHMG-PA, and pHMG-NP that were used to express the PB1, PB2, PA, and NP of influenza A/PR8/34 virus under the control of a mouse hydroxymethylglutaryl-coenzyme A reductase (HMG) promoter (Gautier et al., 1989) were kindly provided by J. Pavlovic (Institut für Medizinische Virologie, Zurich, Switzerland). Plasmids pPOLI-CAT-RT (Pleschka et al., 1996) and pNS/B/CAT (Barclay and Palese, 1995) were kindly provided by P. Palese (Mount Sinai Medical Center, New York, NY) and W. S. Barclay (School of Animal and Microbial Sciences, United Kingdom). Plasmids pPOLI-CAT-RT and pNS/B/CAT contain, respectively, a truncated human RNA Pol I promoter or the T7 promoter, and the CAT gene coding sequence (in negative-sense polarity), which itself is flanked by the 5′ and 3′ nontranslated sequences of the virus RNA segment encoding, respectively, the influenza A/NS or B/NS proteins. In addition, pPOLI-CAT-RT contains the hepatitis delta ribozyme sequence downstream of the 3′ nontranslated sequences.

Cloning of influenza B and C virus genes

Virus genomic RNA was extracted from virions of influenza virus strains B/Harbin/7/94 or C/Johannesburg/ 1/66 (C/JHB/1/66) and reverse transcribed into cDNA in 50 mM Tris–HCl (pH 8.3), 50 mM KCl, 10 mM MgCl$_2$, 10 mM DTT, 0.5 mM spermidine, 0.4 U/μl ribonuclease inhibitor, the four dNTPs (0.5 mM each), 0.4 U/μl avian myeloblastosis virus reverse transcriptase, and 80 nM oligonucleotide uni 1 (5′ AGC AAA AGC AGG) complementary to the nucleotides conserved at the 3′ ends of the RNA segments of all three types of influenza viruses (Robertson, 1979). The cDNA was next amplified by PCR (30 cycles) using Expand High Fidelity (EHF) polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of primers specific for each of the four core protein segments of influenza virus type B or C (Table 3). The amplified products were purified from LMP-agarose gels by β agarase (Biolabs) treatment. The PB1, PB2, PA, and NP gene fragments from B/Harbin/7/94 were directly inserted at the EcoRV site of expression vector pHMG. The amplified products from C/JHB/1/66 were first digested with XhoI and inserted between the NcoI and SalI sites of pGEM5Z(f+). Next, the core protein gene fragments from C/JHB/1/66 were excised by digestion with ApaI and SacI for PB1, NdeI and SphI for PB2, ApaI and SacI for P3, and SacI and NciI for NP; treated with Klenow enzyme (Amersham); and inserted into the EcoRV site of expression vector pHMG.

Cloning of A, B, and C model RNA templates

A plasmid vector pPR, in which BbsI restriction sites are flanked by the Pol I promoter and hepatitis delta ribozyme sequences, was constructed by PCR using pPOLI-CAT-RT as a template in the presence of specific primers for the amplification of the Pol I and ribozyme sequences (Table 3). The amplified products were purified; digested with HindIII and SacI, respectively; and cloned into the corresponding sites of pUC19 giving rise to plasmid pPR. For the construction of plasmids in which the CAT gene sequence, in negative (−) or positive (+) sense orientation, was flanked by the noncoding sequences of the NS gene segment of A/WSN/33, B/Lee/ 40, or C/JHB/66 (Fig. 1), the CAT gene and NS noncoding sequences were amplified by PCR in the presence of appropriate primers (Table 3) using as a template pPOLI-CAT-RT for the construction of pA/PRCAT, pC/PRCAT, and pB/PRCATshort or pNS/B/CAT for the construction of pB/ PRCAT. The amplified products were purified and inserted between the BbsI sites of pPR that had been treated with Klenow enzyme (Amersham).

Sequencing

Nucleotide sequences were determined using the dRhod or Big Dye terminator sequencing kit (Perkin–Elmer) according to the supplier's instructions and analyzed on an ABI Prism automatic sequencer (Perkin–Elmer).

TABLE 3

Sequences of Oligonucleotide Primers

Template	Sequence	Resulting plasmid	Oligonucleotide primer[a] Forward	Reverse
B/Harbin/7/94 cDNA	PB1	pHMG/B-PB1	agc gtc agc ATG AAT ATA AAT CCT TAT TTT C	ctc gtc gac GAT GAC CAA TAA CCC CAT AGA C
	PB2	pHMG/B-PB2	atc ggt acc GGA GCG TTT TCA AGA TGA CAT TGG C	atc gtc gac TTA GCT CAA GGC CCA CCC CAT G
	PA	pHMG/B-PA	atc ggt acc ATG GAT ACT TTT ATT ACA AG	atc gtc gac TCA TTC ATC CAT TAT TTC ATC
	NP	pHMG/B-NP	atc ggt acc ATG TCC AAC ATG GAT ATT GAC	atc gtc gac TTA ATA ATC GAG GTC ATC ATA ATC C
C/JHB/1/66 cDNA	PB1	pGem5Z/C-PB1[b]	GAA ATC AAC CCA TAT TTG ATG TTT CTA AAC AAC GAC G	gga cgt cga ctc GAG GAA AGG GTT TAA CAG
	PB2	pGem5Z/C-PB2[b]	TCT CTT CTA TTG ACA ATA GCA AAG GAA TAC AAA AGA C	gga cgt cga ctc GAG GAT TTT TAG TTA GAC ATC
	P3	pGem5Z/C-P3[b]	TCG AAA ACT TTT GCC GAA ATA GCA GAG ACT TTT CTA GAG CC	gga cgt cga ctc gAG CAG TAG CAA GGG GAT TTT TTC
	NP	pGem5Z/C-NP[b]	TCT GAC AGA CGT CAA AAC AG	gga cgt cga ctc gAG CAG TAG CAA G
pPOLI-CAT-RT	PolI	pPR	cgc caa gct tAC GGG CCG GCC CCT G	cag ata tcg aag acc taa taA CCC GGC GGC CCA AAA TGC
pPOLI-CAT-RT	ribozyme	pPR	cag ata tcg aag acc tGG CCG GCA TGG TCC CAG C	gtg agc tcG TCC CAT TCG CCA TTA CCG
pPOLI-CAT-RT	A/WSN/33 NS noncoding	pA/PRCAT	AGT AGA AAC AAG GGT GTT TTT Tca g	AGC AAA AGC AGG GTG ACA AAG AC
pNS/B/CAT	B/Lee/40 NS noncoding	pB/PRCAT	AGT AGT AAC AAG AGG ATT TTT ATT TTA CAT TC	AGC AGA AGC AGA GGA TTT ATT TAG TCA CTG GC
pPOLI-CAT-RT	B/Lee/40 NS noncoding	pB/PRCATshort	AGT AGT AAC AAG AGG ATT TTT Aca gat	AGC AGA AGC AGA GGA TTT ATT TAG Tca tgg ag
pPOLI-CAT-RT	C/JHB/1/66 NS noncoding	pC/PRCAT	AGC AGT AGC AAG GGG ATT TTT Aca gat c	AGC AGA AGC AGG GGT ACT TTT CCA AAA TGG AG

[a] All the oligonucleotide sequences are given in the 5' to 3' orientation; the sequences corresponding to the template are indicated in uppercase, and the unrelated sequences that include restriction sites are shown in lowercase.

[b] The core protein gene fragments from C/JHB/1/66 were finally inserted into plasmid pHMG as described in Materials and Methods.

Transfections and CAT detection assays

COS-1 cells were grown in DMEM containing 10% FCS. Subconfluent monolayers of COS-1 cells (3×10^5 cells on 35-mm dishes) were transfected by using 10 μl of Fugene-6 (Boehringer Mannheim) with 1 μg of the pPRCAT plasmids, together with plasmids pHMG-PB1 (1 μg), pHMG-PB2 (1 μg), pHMG-PA/P3 (1 μg), and pHMG-NP (4 μg) expressing the core proteins from influenza A, B, or C viruses as indicated in the text. After incubation at 37°C in DMEM supplemented with 10% FCS, cell extracts were prepared at 48 h post-transfection, unless otherwise stated, and tested for the level of CAT, using the CAT ELISA kit (Boehringer Mannheim). The lower limit of CAT detection was 0.05 ng/ml.

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