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Differential Effect of Nucleotide Substitutions in the 3′ Arm of the Influenza A Virus vRNA Promoter on Transcription/Replication by Avian and Human Polymerase Complexes Is Related to the Nature of PB2 Amino Acid 627

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Using a genetic system that allows the *in vivo* reconstitution of active ribonucleoproteins, the ability to ensure transcription/replication of a viral-like reporter RNA harboring the $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the 3′ arm of the promoter was examined with core proteins from human or avian strains of influenza A viruses. The efficiency of transcription/replication of the viral-like RNA with the triple 3-5-8 mutations in COS-1 cells was found to be slightly decreased as compared to the wild-type RNA when the polymerase was derived from a human virus. In contrast, it was found to be considerably increased when the polymerase was derived from an avian virus, in agreement with published observations using the avian A/FPV/Bratislava virus (G. Neumann and G. Hobom, 1995, *J. Gen. Virol.* 76, 1709–1717). This increase could be attributed to the compensation of the defect in transcription/replication activity in the COS-1 mammalian cell line due to the presence of a glutamic acid at PB2 residue 627, characteristic of avian strains of influenza viruses. Our results thus suggest that PB2 and/or cellular proteins interacting with PB2 could be involved in RNA conformational changes during the process of transcription/replication.

INTRODUCTION

The genome of influenza A viruses consists of eight molecules of single-stranded RNA of negative polarity. The viral RNAs (vRNAs) are packed with the nucleoprotein (NP) and associated with the three subunits of the polymerase complex (PB1, PB2, and PA) to form ribonucleoproteins (RNPs). Once the RNPs have entered the nucleus of the infected cells, they undergo transcription and replication (for a review, see Lamb and Krug, 2001). The three subunits of the polymerase and the NP are the minimum set of proteins required for both processes, although the molecular mechanisms involved are clearly different. The initiation of transcription involves a cap-stealing mechanism, by which 5′-capped oligonucleotides derived from cellular mRNA are used as primers and elongated by the viral polymerase (Bouloy *et al.,* 1978; Plotch *et al.,* 1981). Termination and polyadenylation occur at a stretch of five to seven U residues close to the 5′ end of the template (Luo *et al.,* 1991; Poon *et al.,* 1999). In the replication process, full-length positive-stranded RNAs complementary to the vRNAs (cRNAs) are produced, which in turn serve as templates for amplification of the vRNAs. Initiation of the synthesis of cRNAs and vRNAs is primer-independent, and antitermination occurs at the poly(U) sequence during cRNA synthesis (Hay, 1998). Although there is a recent article about cRNA-promoted transcription of mRNAs (Azzeh *et al.,* 2001), many experimental results indicate that it is very inefficient as compared to vRNA-promoted transcription (Cianci *et al.,* 1995; Honda *et al.,* 2001; Leahy *et al.,* 2002).

Genomic RNA segments of influenza A viruses are characterized by highly conserved sequences of 13 nt at their 5′ end and 12 nt at their 3′ end. Nucleotides 1′–16′ at the 5′ end and 1–15 at the 3′ end (i.e., the conserved sequences plus three additional nucleotides specific of the various segments) have been shown to contain the necessary signals for transcription, replication, and packaging of the genome segments (Fodor *et al.,* 1994; Luytjes *et al.,* 1989; Neumann *et al.,* 1994). Both the 5′ and the 3′ end are required for initiation of transcription in a stepwise interaction with the PB1 subunit of the polymerase complex (Li *et al.,* 1998; Tiley *et al.,* 1994), and thus they constitute the 5′ and 3′ arm of the vRNA promoter, respectively. The corresponding complementary sequences of the cRNA control vRNA synthesis and together form the cRNA promoter (Azzeh *et al.,* 2001). In addition, nonconserved sequences of the noncoding regions (NCR) of the genomic segments were found to modulate the efficiency of transcription/replication (Bergmann and Muster, 1996; Zheng *et al.,* 1996).

Several models have been proposed for the secondary structure of the influenza virus vRNA and cRNA promot-

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ers. The 5′ and 3′ sequences show partial inverted complementarity to each other. Chemical RNA modification experiments of native RNPs have indicated that in the presence of the polymerase complex, the vRNA 5′ and 3′ ends form a double-stranded "panhandle" structure; however, if not stabilized by the polymerase, base-pairing is not observed (Klumpp *et al.,* 1997). *In vivo* mutational analysis of viral-like reporter RNAs have led to the model of a "corkscrew" conformation for the vRNA promoter (Flick *et al.,* 1996). The features of the corkscrew conformation are two short proximal stem-loop structures, followed by a distal double-stranded RNA stem four to seven base pairs in length depending on the genomic segment. The proximal stem-loop elements and the distal stem are separated by a flexible hinge formed by a single unpaired nucleotide on the 5′ arm. In *in vitro* experiments, the presence of a stem-loop at both the 5′ and the 3′ end of the vRNA appears to be required for endonuclease activity (Leahy *et al.,* 2001a,b), while polyadenylation of the mRNAs requires the presence of a stem-loop at the 5′ but not at the 3′ end (Pritlove *et al.,* 1999). Detailed mutational analysis of the 9 nt involved in the formation of stem-loops at the 3′ and 5′ ends of the vRNA indicated that at some positions (e.g., nt 3 and 8 of either end) base-pairing within the promoter is critical with regard to polymerase activity rather than the identity of the nucleotides themselves; while at other positions (e.g., nt 2 and 9 of either end, nt 5 of the 5′ end), the nature of the nucleotide is important, probably because they represent positions of direct RNA–protein interactions (Flick and Hobom, 1999; Leahy *et al.,* 2001b).

A puzzling observation by Neumann and Hobom is that, in cells infected with the A/FPV/Bratislava virus, a viral-like RNA harboring combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the stem-loop structure of the 3′ end undergoes transcription/replication with considerably enhanced efficiency as compared to the wild-type viral-like RNA (Neumann and Hobom, 1995). The aim of the present study was to examine whether the observation of Neumann and Hobom could be extended to other influenza A viruses or whether it was dependent on the avian vs human origin of the virus. We made use of a plasmid-based system described by Pleschka *et al.* (1996) for the *in vivo* reconstitution of functional RNPs, upon expression of wild-type or mutant viral-like RNAs together with the four core proteins (PB1, PB2, PA, and NP) derived from five different strains of human or avian viruses. We found that in COS-1 cells the triple 3-5-8 mutations increased transcription/replication of the vRNA only in the presence of polymerase complexes of avian origin. This increase was shown to correspond to the compensation of the defect in transcription/replication activity due to the presence of a glutamic acid at PB2 residue 627, characteristic of avian strains of influenza viruses (Naffakh *et al.,* 2000; Subbarao *et al.,* 1993). Our results are discussed with regard to the current models for molecular interactions and conformational changes that may take place within the RNP during the process of transcription/replication.

RESULTS

Variable effects of combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations in the 3′ end of a viral-like RNA on the levels of transcription/replication in COS-1 cells, depending on the origin of the polymerase complex

The effects of combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the proximal 3′ NCR of a type A viral-like CAT reporter RNA were studied in the presence of various polymerase complexes. The three mutations were introduced into the previously described pA/PRCAT(−) plasmid (Crescenzo-Chaigne *et al.,* 1999) to generate pA/PRCATmu3-5-8(−), as described under Materials and Methods. Plasmids pA/PRCAT(−) and pA/PRCATmu3-5-8(−) direct the synthesis of a wild-type (wt) and a mutated viral-like RNA, respectively (Fig. 1). Both RNAs are likely to adopt a corkscrew conformation (Flick and Hobom, 1999; Flick *et al.,* 1996) with a six base-pair double-stranded distal element (Fig. 1). They are analogous to the viral-like RNAs derived from the pHL926 and pHL1104 plasmids described by Neumann and Hobom (1995), except for the fact that the latter are predicted to form a four base-pair double-stranded distal element. To allow for direct comparison of our experiments with those from Neumann and Hobom, plasmids pA/PRCAT/NH(−) and pA/PRCAT/NHmu3-5-8(−) were generated by introducing both the $A_{14} \rightarrow C_{14}$ and the $C_{15} \rightarrow U_{15}$ mutations in pA/PRCAT(−) and pA/PRCATmu3-5-8(−), respectively. Thus, in the corresponding vRNAs, the length of the double-stranded distal element was reduced to 4 bp according to the corkscrew conformation model (Fig. 1). In the panhandle conformation model, the terminal nucleotides of the 5′ and 3′ NCRs are predicted to be only partially base-paired in the wt vRNAs derived from pA/PRCAT(−) or pA/PRCAT/NH(−) and to form a nine base-pair double strand in the mutant vRNAs derived from pA/PRCATmu3-5-8(−) or pA/PRCAT/NHmu3-5-8(−) (Fig. 1).

We compared the efficiency with which the four types of RNA templates underwent transcription/replication in the presence of *in vivo* reconstituted polymerase complexes derived from several human (A/PR/8/34, A/Victoria/3/75, and A/Paris/908/97) or avian (A/Mallard/NY/6758/78 and A/FPV/Rostock/34) type A influenza viruses. In COS-1 cells transfected with the pA/PRCAT(−) plasmid, the highest levels of CAT expression (in the range of 200-1000 ng per milliliter of cell extract prepared 24 h posttransfection), were achieved when A/PR/8/34 (PR8), A/Paris/908/97 (P908), or A/Victoria/3/75 (VIC) polymerase complexes were reconstituted, whereas reduced levels were observed for the A/FPV/Rostock/34 (FPV)

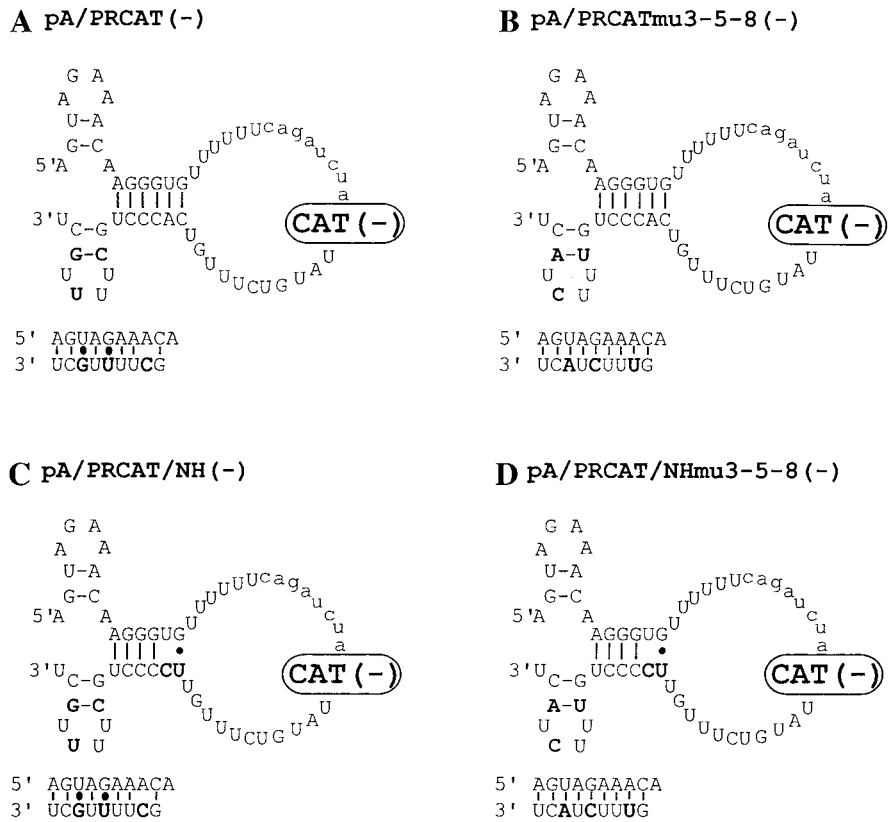

FIG. 1. Nucleotide sequence and predicted "corkscrew" and "panhandle" conformation of the minus (−)-sense model RNA templates. The nucleotide sequence of the extremities of the model RNA transcribed from pA/PRCAT(−) is the same as in the NS genomic segment of A/WSN/33 (uppercase). Unrelated nonviral sequences are shown in lowercase. The nucleotides mutated in pA/PRCAT(−)-derived plasmids are shown in bold. The predicted structures are as suggested by Flick *et al.* (1996).

and A/Mallard/NY/6758/78 (MAL) complexes (about 30 and 10 ng/ml of CAT, respectively) (Fig. 2A, black bars). The levels of CAT measured with the pA/PRCAT/NH(−)-derived RNA template were reduced two- to fourfold as compared to pA/PRCAT(−) (Fig. 2A, gray bars), in agreement with previously published observations which suggested that the length of the double-stranded distal element was not critical with regard to the efficiency of transcription/replication of type A vRNAs (Lee and Seong, 1998b). To examine the effects of the presence of the triple 3-5-8 mutations on the RNA templates, CAT levels were measured in cells transfected with pA/PRCATmu3-5-8(−) and expressed as a percentage of those measured with pA/PRCAT(−) (Fig. 2B, black bars); similarly, CAT levels measured with pA/PRCAT/NHmu3-5-8(−) and pA/PRCAT/NH(−) were compared (Fig. 2B, gray bars). At 24 h posttransfection, the "promoter-up" effect described by Neumann and Hobom (i.e., increased levels of transcription/replication of vRNAs harboring the triple 3-5-8 mutations; Neumann and Hobom, 1995) was observed only in cells expressing the FPV or the MAL complex (Fig. 2B). Indeed, CAT levels measured with the mutated viral-like RNAs (either with a 4-bp or a 6-bp double-stranded distal element) were about threefold (for FPV) and 30-fold (for MAL) higher than those obtained with the corresponding wt RNAs. In contrast, in cells expressing the PR8, P908, or VIC complexes, CAT levels measured with the pA/PRCATmu3-5-8(−)- and pA/PRCAT/NHmu3-5-8(−)-derived RNAs were either similar (for PR8 and P908) or 5- to 10-fold lower (for VIC) than those measured with the pA/PRCAT(−)- and pA/PRCAT/NH(−)-derived RNAs.

To understand better how the mutations in the 3′ NCR did interfere with the transcription/replication process, a kinetic analysis was performed using the pA/PRCAT(−) or pA/PRCATmu3-5-8(−) plasmids, as well as the corresponding pA/PRCAT(+) and pA/PRCATmu3-5-8(+) plasmids that encode (+) sense model RNA templates with a wt of mutated 5′ NCR, respectively. Each type of RNA template was coexpressed with either the P908-, the PR8-, the MAL-, or the FPV-derived complex, and the levels of CAT were measured in cell extracts at 18, 24, and 48 h posttransfection. As shown in Fig. 3A, in the presence of the P908 complex, the kinetics of accumulation of CAT protein were very similar with all four RNA templates. A rapid increase was observed up to 18 h posttransfection, followed by a slower increase between 18 and 24 h posttransfection and a plateau from 24 to 48 h posttransfection. The levels of CAT measured with the mutated RNA templates (hatched lines) were in the

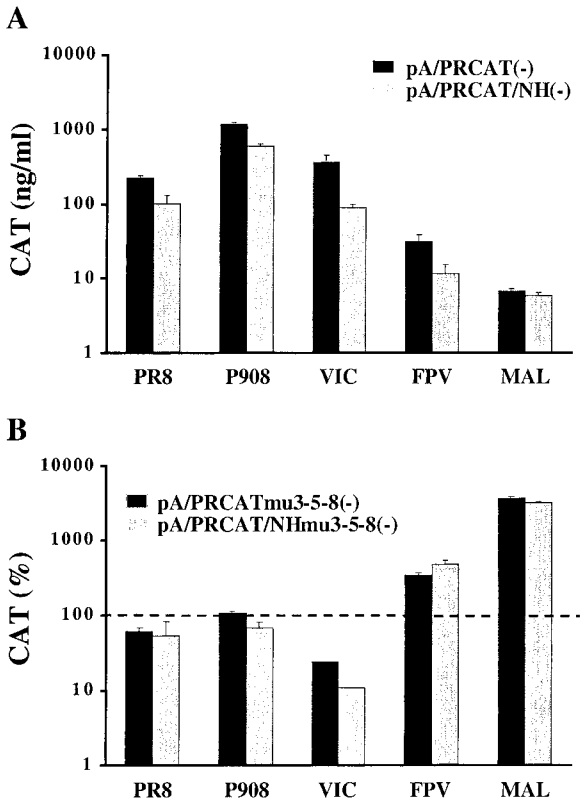

FIG. 2. Efficiency of transcription/replication of the (−)-sense model RNA templates in the presence of polymerase complexes of various origins. COS-1 cells were transfected with plasmids pA/PRCAT(−), pA/PRCATmu3-5-8(−), pA/PRCAT/NH(−), or pA/PRCAT/NHmu3-5-8(−), together with the four pHMG plasmids encoding the core proteins derived from PR8, P908, VIC, FPV, or MAL viruses. At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. (A) For a given polymerase complex, CAT concentrations measured with pA/PRCAT(−) (black bars) were compared to those measured with pA/PRCAT/NH(−) (gray bars). (B) For a given polymerase complex, the CAT levels measured with pA/PRCATmu3-5-8(−) (black bars) were compared to and expressed as a percentage of those measured with pA/PRCAT(−) (100%, dotted line), and the CAT levels measured with pA/PRCAT/NHmu3-5-8(−) (gray bars) were compared to and expressed as a percentage of those measured with pA/PRCAT/NH(−) (100%, dotted line). The results are expressed as the mean ± standard deviation (SD) of duplicate samples from one representative experiment of three independent experiments.

same range as those measured with the wt templates (solid lines) at 18 and 24 h posttransfection, in agreement with the results shown in Fig. 2B, and appeared to be three- to fivefold lower at 48 h posttransfection. Very similar results were obtained with the PR8 complex (data not shown). With the MAL complex, when the wt RNA templates were used, the kinetics of accumulation of CAT were delayed as compared to that observed with the P908 complex: although CAT levels appeared higher with the (+)-sense than with the (−)-sense wt template, in both cases CAT levels remained low up to 18 h and increased rapidly between 18 and 48 h posttransfection (Fig. 3B, solid lines). Strikingly, when the mutated RNA templates were used, the kinetics of accumulation of CAT in the presence of MAL proteins were similar to that observed in the presence of P908 proteins, although CAT levels remained lower overall (Fig. 3B, hatched lines).

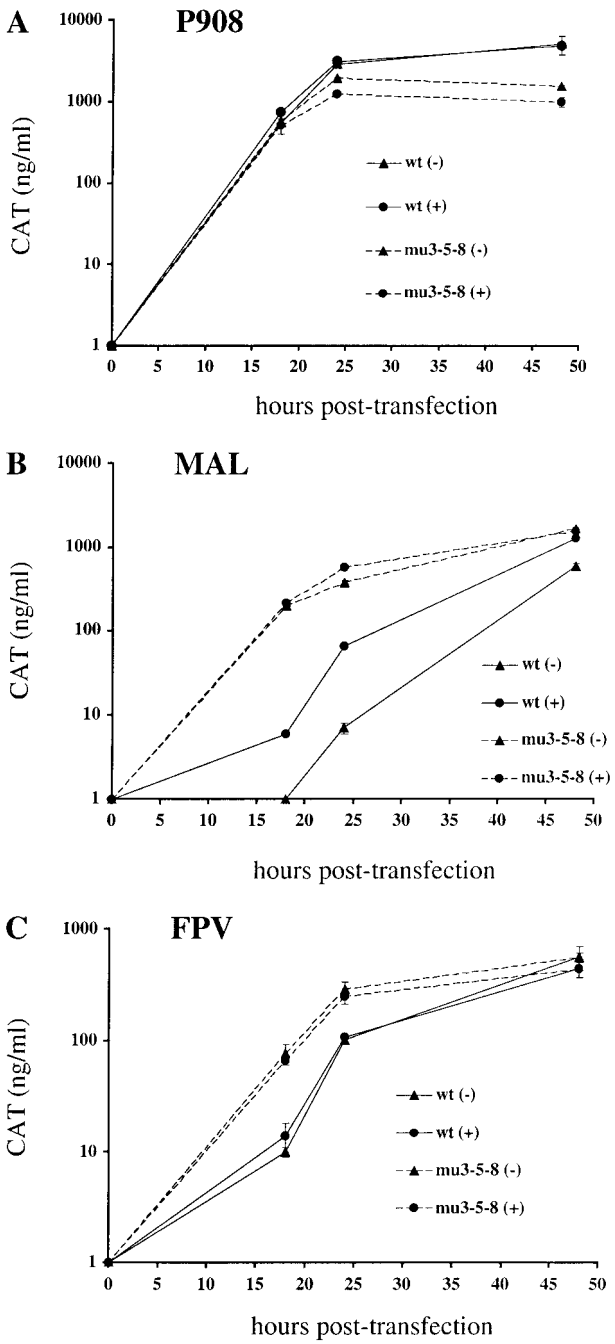

FIG. 3. Effect of the triple 3-5-8 mutations on the kinetics of transcription/replication of (−)- and (+)-sense model RNA templates in the presence of (A) P908-, (B) MAL-, or (C) FPV-derived polymerase complexes. COS-1 cells were transfected with the four pHMG plasmids encoding the core proteins together with either plasmids pA/PRCAT(−) (triangles, solid lines), pA/PRCAT(+) (circles, solid lines), pA/PRCATmu3-5-8(−) (triangles, broken lines), or pA/PRCATmu3-5-8(+) (circles, broken lines). At various times posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. The results are expressed as the mean ± SD of duplicate samples from one representative experiment of two.

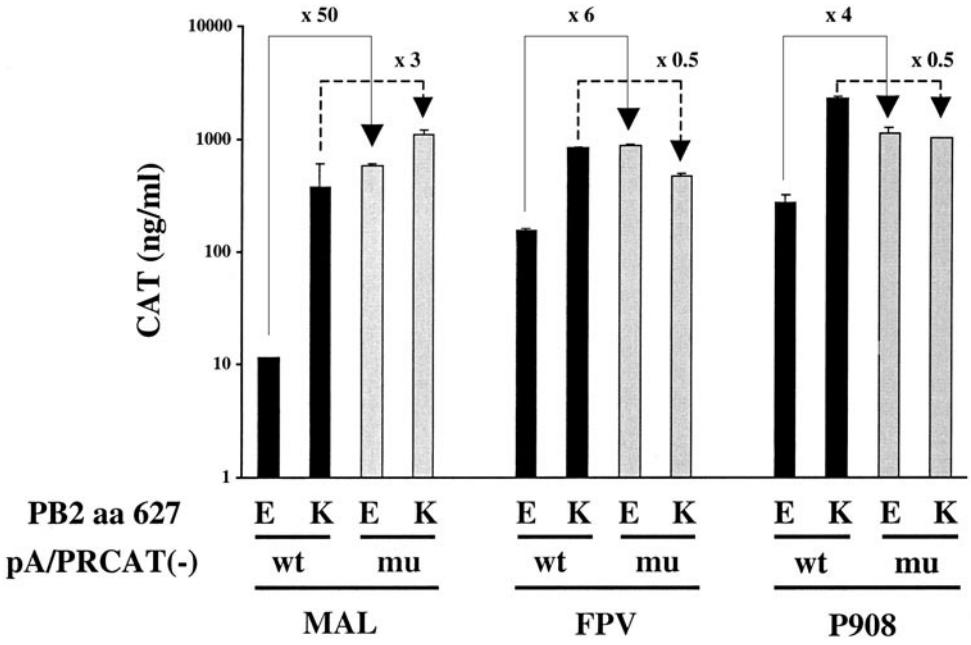

FIG. 4. Effect of the nature of PB2 amino acid 627 on the transcription/replication of wild-type or mutant (−)-sense model RNA templates. COS-1 cells were transfected with either plasmid pA/PRCAT(−) (wt, black bars) or pA/PRCATmu3-5-8(−) (mu, gray bars) together with the four pHMG plasmids encoding the core proteins derived from MAL, FPV, or P908 viruses. The pHMG-PB2 plasmids encoded PB2 proteins with either a glutamic acid (E) at residue 627 (wild-type PB2 for MAL and FPV-PB2 or mutant K627E-PB2 for P908) or a lysine (K) at residue 627 (mutant E627K-PB2 for MAL and FPV or wild-type PB2 for P908). At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. Arrows indicate the effect of the triple 3-5-8 mutations on complexes with a glutamic acid (solid arrows) or a lysine (broken arrows) at residue 627 of PB2. The results are expressed as the mean ± SD of duplicate samples from one experiment representative of two.

The promoter-up effect of the mutations in pA/PRCATmu3-5-8(−) in the presence of the MAL complex was much more pronounced at 18 h (about 200-fold) than at 24 h (about 50-fold), or at 48 h posttransfection (threefold). Similarly, the promoter-up effect of the mutations in pA/PRCATmu3-5-8(+) was the highest at 18 h posttransfection (40-fold). The results obtained with the FPV complex were very similar to those observed with the MAL complex, although the differences between the CAT levels measured with the wt and mutated templates were lower (about sevenfold at 18 h, and threefold at 24 h posttransfection) (Fig. 3C). On the whole, these observations suggested that the presence of the triple 3-5-8 mutations in the 3′ NCR of the vRNA and/or the presence of the corresponding mutations in the 5′ NCR of the cRNA compensated for a functional defect of the MAL or FPV complex in transcription/replication of the wt viral-like RNA in COS-1 cells. This compensatory effect could operate at the level of recognition, amplification, and/or transcription of the RNA.

The triple 3-5-8 mutations in the 3′ end of a viral-like RNA compensate the defect in transcription/replication in COS-1 cells due to the presence of a glutamic acid at residue 627 of PB2

We have previously published that the efficiency with which the MAL or FPV complexes ensure transcription/replication of a wt viral-like RNA in COS-1 cells is enhanced if a MAL-PB2 or FPV-PB2 protein with a Glu → Lys mutation at residue 627 is expressed instead of the wt PB2 (Naffakh *et al.,* 2000). Here we asked whether the defect linked to residue Glu627 of PB2-MAL was compensated by the presence of the triple 3-5-8 mutations in the 3′ end of the vRNA. Thus we examined whether the efficiency with which the pA/PRCATmu3-5-8(−)-derived RNA underwent transcription/replication in COS-1 cells was still determined by the nature of PB2 residue 627. In addition to the plasmids encoding the E627K-MAL-PB2 and E627K-FPV-PB2 mutant proteins, which have been previously described (Naffakh *et al.,* 2000), we generated a plasmid encoding a P908-PB2 protein with a Lys → Glu substitution at position 627 (K627E-P908-PB2). The transcription/replication activity of MAL, FPV, and P908 complexes reconstituted with either a wild-type or a mutant PB2 protein was tested, using pA/PRCAT(−) or pA/PRCATmu3-5-8(−) as a template for synthesis of the viral-like RNA. The CAT levels measured at 24 h posttransfection are shown in Fig. 4. Using pA/PRCAT(−) as a template (black bars), CAT levels were 32-fold higher in cells expressing E627K-MAL-PB2 (385 ng/ml) than cells expressing the wt MAL-PB2 (12 ng/ml), fivefold higher in cells expressing E627K-FPV-PB2 (852 ng/ml) than cells expressing the wt FPV-PB2 (157 ng/ml), and fivefold lower in cells expressing K627E-P908-PB2 (327 ng/ml)

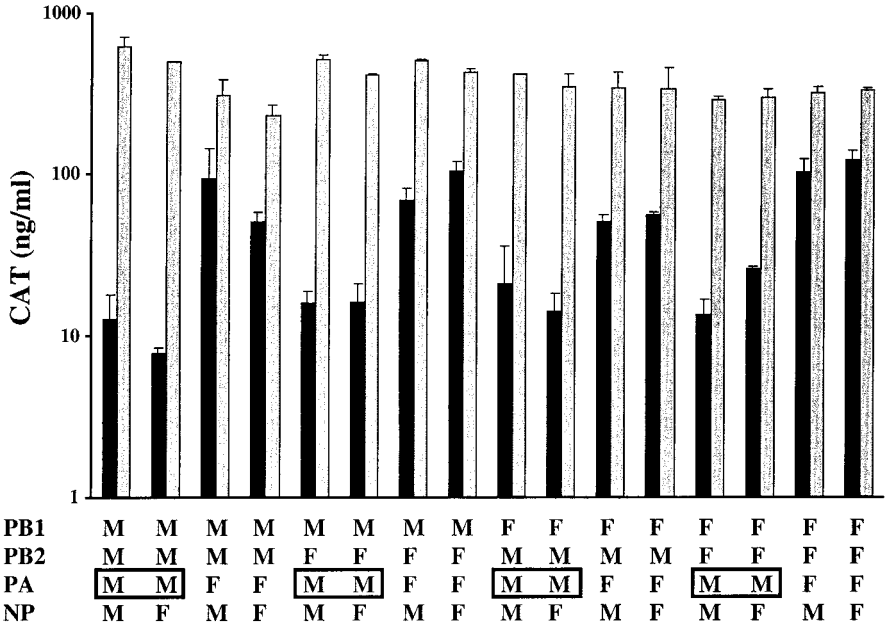

FIG. 5. Efficiency of transcription/replication of the wild-type or mutant (−)-sense model RNA templates in the presence of various combinations or MAL and FPV core proteins. COS-1 cells were transfected with plasmids pA/PRCAT(−) or pA/PRCATmu3-5-8(−), together with all 16 possible combinations between plasmids encoding the four MAL- and FPV-derived core proteins. At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. For a given combination of core proteins, CAT concentrations measured with pA/PRCAT(−) (black bars) were compared to those measured with pA/PRCATmu3-5-8(−) (gray bars). The results are expressed as the mean ± SD of duplicate samples.

than cells expressing the wt P908-PB2 (1624 ng/ml). These observations were in agreement with our previously published data (Naffakh *et al.,* 2000) and confirmed that the nature of PB2 residue 627 is essential for the efficiency with which a polymerase complex ensures transcription/replication of a wt viral-like RNA in COS-1 cells. In contrast, when pA/PRCATmu3-5-8(−) was used as a template (gray bars), expressing the mutant instead of the wt PB2 proteins had little effect on CAT levels, which were in the range of 500-1000 ng/ml, whatever the origin of the polymerase complex and the nature of PB2 amino acid 627. As a consequence, the 50-fold promoter-up effect of the triple 3-5-8 mutations observed in the presence of the wild-type MAL complex (Fig. 4, solid arrow) was reduced to threefold when E627K-MAL-PB2 was expressed (Fig. 4, broken arrow). In the case of the FPV complex, a sixfold promoter-up effect was observed at 24 h with the wt (E627) FPV-PB2 but not with the E627K-FPV-PB2; conversely, a fourfold promoter-up effect was observed with the K627E-P908-PB2 protein but not with the wt (K627) P908-PB2 protein with which a twofold reduction was observed (Fig. 4).

The effects of the nature of PB2 amino acid 627 and of the triple 3-5-8 mutations were much more pronounced with the MAL than with the FPV complex, suggesting that they were modulated by some other molecular characteristics of each of the polymerase complexes. All 16 possible combinations between the core proteins of MAL and FPV were analyzed for the efficiency of CAT production in the presence of either the pA/PRCAT(−) or the pA/PRCATmu3-5-8(−) template. Whatever the combination of proteins, the levels of CAT measured in the presence of pA/PRCATmu3-5-8(−) were in the same range (250–650 ng/ml) (Fig. 5, gray bars). In contrast, the levels of CAT measured in the presence of pA/PRCAT(−) varied depending on the combination of core proteins: they were systematically lower when the PA subunit derived from MAL (10–20 ng/ml, instead of 50–120 ng/ml when PA derived from FPV) (Fig. 5, black bars), which resulted in a higher promoter-up effect of the 3-5-8 triple mutations. These results suggested that the difference observed between the FPV and MAL complexes was linked to the nature of the PA subunit, FPV-PA allowing a better efficiency of transcription/replication of the wt viral-like RNA in COS-1 cells than MAL-PA.

To examine whether the effects linked to the nature of PB2 amino acid 627 and to the nature of nucleotides 3, 5, and 8 at the 3′ end of the vRNA were similarly related to transcription and/or replication, we analyzed the RNA species involved these processes. COS-1 cells were transfected with plasmids encoding either MAL- or P908-derived core proteins together with the pA/PRCAT(−) or pA/PRCATmu3-5-8(−) plasmids. Total RNA was prepared at 24 h posttransfection, and 10 μg were used for the purification of polyadenylated RNAs. To detect vRNAs, total RNA (2 μg) was analyzed by Northern blotting and hybridization using a positive-sense CAT-specific ^{32}P-labeled riboprobe, as described under Materials and

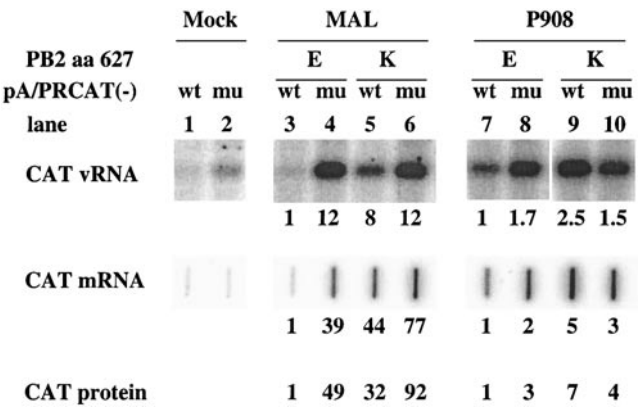

FIG. 6. Detection of CAT-specific RNAs in the transient transcription/replication assay. COS-1 cells were transfected with either plasmid pA/PRCAT(−) (wt) or pA/PRCATmu3-5-8 (−) (mu) together with the four pHMG plasmids encoding the core proteins derived from MAL (Lanes 3–6) or P908 viruses (Lanes 7–10), or with the empty pHMG plasmid (Mock, Lanes 1–2). The pHMG-PB2 plasmids encoded PB2 proteins with either a glutamic acid (E) or a lysine (K) at residue 627 as in Fig. 4. At 24 h posttransfection, total RNAs and poly(A)$^+$ RNAs were prepared and analyzed by Northern blotting followed by hybridization with a ^{32}P-labeled (+)-sense riboprobe, or by slot blotting followed by hybridization with a ^{32}P-labeled (−)-sense riboprobe, respectively, as described under Materials and Methods. The CAT-specific signals obtained by scanning the blots with a STORM820 optical scanner are shown. The amounts of CAT vRNA and mRNA were evaluated by using the Image Quant software and the corresponding amounts of CAT protein were measured by ELISA. After substraction of the values obtained in the mock samples, the values were expressed relative to those obtained with pA/PRCAT(−) (wt) in the presence of the wild-type PB2 for MAL (Lane 3), or mutant K627E-PB2 for P908 (Lane 7). The ratios are indicated below the corresponding CAT-specific RNA signals.

Methods. To detect mRNAs, polyadenylated RNAs were analyzed by slot blotting and hybridization using a negative-sense CAT-specific ^{32}P-labeled riboprobe, as described under Materials and Methods. Quantification of the hybridization signals indicated a good correlation between the levels of CAT mRNA and the protein (Fig. 6). Different levels of CAT vRNA were observed between the mock-transfected samples, the amount of CAT vRNA detected with the mutated template being repeatedly two- to threefold higher than the wt template (Fig. 6, Lane 2 compared to Lane 1). This difference could be due to an enhancement of the efficiency of ribozyme cleavage and/or to a higher stability of the CAT vRNAs synthesized from pA/PRCATmu3-5-8(−), because of the presence of the 3-5-8 triple mutations in the 3′ NCR. It is unlikely that it interfered with our analysis, because dose-response experiments indicated that CAT vRNA synthesized from pA/PRCAT (−) was in large excess (data not shown). In the presence of the MAL or P908 core proteins, significant variations of CAT mRNA and to a lesser extent of CAT vRNA were observed, when comparing the wt and mutant RNA templates, or the wt and mutant PB2 proteins. A Glu → Lys substitution in PB2 resulted in increased levels of vRNA and mRNA synthesized from the pA/PRCAT(−) template, albeit to a less significant extent with the P908 complex as compared to the MAL complex (Fig. 6, Lane 5 compared to Lane 3, and Lane 9 compared to Lane 7). Moreover, in the presence of a complex with a Glu627 residue in PB2, when pA/PRCATmu3-5-8(−) was used instead of pA/PRCAT(−), the levels of vRNA and mRNA were found to be increased, again to a much less significant extent with the P908 complex than the MAL complex (Fig. 6, Lane 4 compared to Lane 3, and Lane 8 compared to Lane 7). Consequently, when pA/PRCATmu3-5-8(−) was used as a template, a Glu → Lys substitution in PB2 had no significant effect on the levels of vRNA and mRNA synthesized (Fig. 6, Lane 6 compared to Lane 4, and Lane 10 compared to Lane 8). On the whole, these observations indicated that the transcription, and to a lesser extent the replication activity, was impaired when residue 627 of PB2 was a Glu, and that both defects were compensated by the presence of A_3, C_5, and U_8 nucleotides at the 3′ end of the vRNA. Furthermore, both the effects of residue 627 of PB2 and the effects of the triple 3-5-8 mutations were much more pronounced with the MAL than with the P908 complex.

The nature of nucleotides 3 and 8 at the 3′ end of a viral-like RNA is essential for the promoter-up effect of the triple 3-5-8 mutations observed in the presence of the MAL or FPV complex

To evaluate the respective contribution of mutations at nucleotides 3, 5, and 8 of the 3′ NCR to the promoter-up effect observed in the presence of the MAL or FPV complexes, two additional pA/PRCAT(−)-derived plasmids were generated, which contained either the $G_3 \rightarrow A_3$ and $C_8 \rightarrow U_8$ mutations (pA/PRCATmu3–8(−)) or the $U_5 \rightarrow C_5$ mutation alone (pA/PRCATmu5(−)). These plasmids were tested in the presence of P908-, MAL-, or FPV-derived complexes, in parallel with pA/PRCAT(−) and pA/PRCATmu3-5-8(−). The levels of CAT measured in cell extracts at 24 and 48 h posttransfection are shown in Fig. 7. Whatever the polymerase complex which was used, similar CAT levels were observed with plasmids pA/PRCATmu3-5-8(−) and pA/PRCATmu3–8(−) on the one hand (hatched lines), and with plasmids pA/PRCAT(−) and pA/PRCATmu5(−) on the other hand (solid lines). Similar results were obtained using the PR8-derived complex (data not shown). These observations indicated that the mutations at nt 3 and 8, but not nt 5 at the 3′ end of the viral-like RNA, were responsible for the promoter-up effect observed in the presence of the MAL or FPV complex. In agreement with the data from Flick *et al.* (1996), any single mutation at nucleotide 3 or 8, which disrupted the G_3-C_8 base pair predicted by the corkscrew model of vRNA, completely abolished transcription/replication of the RNA template (data not shown). When a U_3-A_8 or a C_3-G_8 base pair was restored

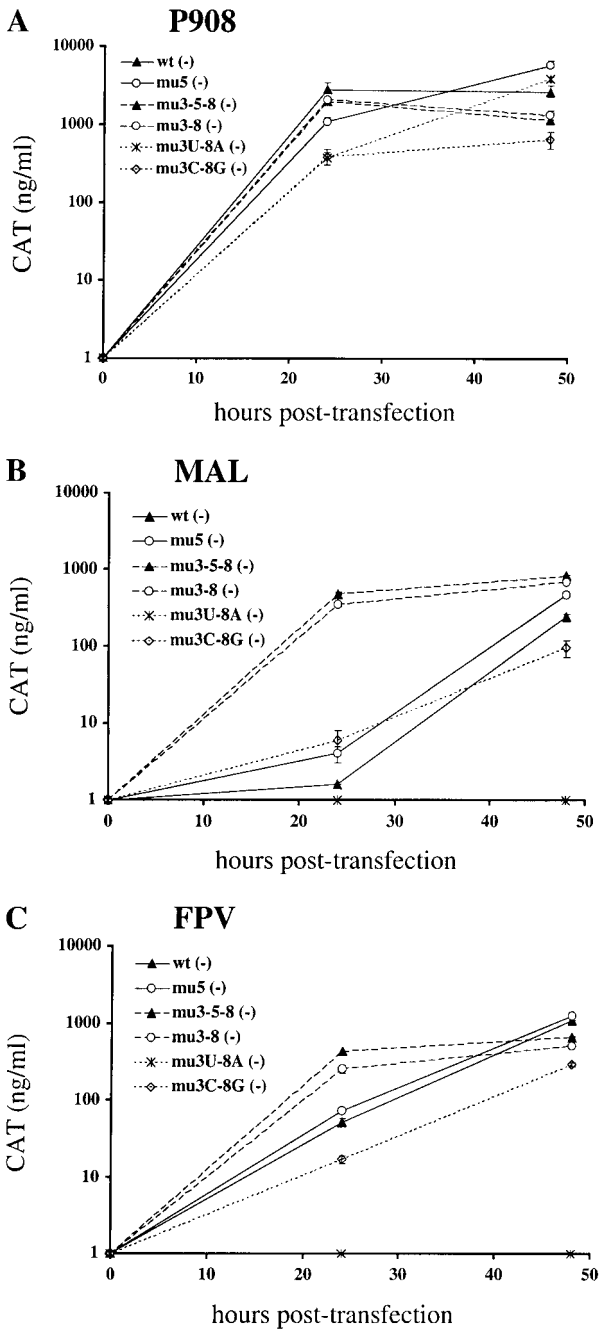

FIG. 7. Effect of single or double mutations as compared to the triple 3-5-8 mutations in the 3′ end of the (−)-sense model RNA template on transcription/replication in the presence of P908, MAL, or FPV-derived polymerase complexes. COS-1 cells were transfected with the four pHMG plasmids encoding the core proteins derived from (A) P908, (B) MAL, or (C) FPV viruses as indicated, together with either plasmid pA/PRCAT(−) (solid lines, closed triangles), pA/PRCATmu3-5-8(−) (broken lines, closed triangles), pA/PRCATmu5(−) (solid lines, open circles), pA/PRCATmu3-8(−) (broken lines, open circles), pA/PRCATmu3C-8G(−) (dotted lines, open squares), or pA/PRCATmu3U-8A(−) (dotted line, crosses). At 24 and 48 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. The results are expressed as the mean ± SD of duplicate samples from one representative experiment of two.

in the stem-loop (plasmids pA/PRCATmu3C-8G(−) and pA/PRCATmu3U-8A(−)), the levels of CAT measured in the presence of the P908 complex at 24 h posttransfection were about 10-fold lower than those observed with pA/PRCAT(−) or pA/PRCATmu3-5-8 (−) (Fig. 7A, dotted lines). In the presence of the MAL or FPV complexes, the levels of CAT measured at 24 h posttransfection with pA/PRCATmu3C-8G(−) were respectively about 100- and 25-fold lower than those measured with pA/PRCATmu3-5-8(−) (Figs. 7B and 7C, dotted lines, square symbols), while the levels of CAT measured with pA/PRCATmu3U-8A(−) were at background levels (Figs. 7B and 7C, dotted line, cross symbols). These very different levels of CAT observed depending on the nature of the predicted base pair in the 3′ stem-loop of the RNA templates, i.e., A_3-U_8 (mu3-5-8), U_3-A_8 (mu3U-8A), or C_3-G_8 (mu3C-8G), indicated that not only the base pairing between nt 3 and 8 but also the nature of nt 3 and 8 was essential for the promoter-up effect.

No sequence variations are found at nt 3, 5, or 8 in the 3′ and 5′ ends of the eight genomic RNA segments derived from the MAL and PR8 viruses upon sequencing

Based on all available sequences, the 12 nt at the 3′ end and the 13 nt at the 5′ end of influenza A genomic RNAs are highly conserved. However, the observation that a viral-like RNA with A_3, C_5, and U_8 nucleotides at the 3′ end could undergo efficient transcription/replication prompted us to sequence the 3′ and 5′ ends of the eight genomic segments derived from the MAL and PR8 viruses, to determine whether nt 3, 5, and 8 were conserved, or whether some sequence variation could occur and possibly be involved in controlling the segment-specific transcription/replication level. The 3′ and 5′ terminal nucleotides of PR8 segments had been determined by Desselberger *et al.* in 1980 (Desselberger *et al.,* 1980), but might have varied along the many subsequent passages of the virus. Data on the 3′ and 5′ terminal nucleotides of MAL segments were not available, and vRNA sequencing was performed on a batch of virus with the same passage history as for cloning of the MAL-PB1, -PB2, -PA, and -NP cDNAs used in our experiments. We optimized PCR-based protocols for direct sequencing of the 3′ and 5′ NCR, as described under Materials and Methods. Genomic RNA was extracted from the MAL and PR8 viruses and the sequence of the extremities of the eight genomic segments was determined. The 12 nt at the 3′ terminus and 13 nt at the 5′ terminus were found to be wild-type in all segments (data no shown). In agreement with published data (Desselberger *et al.,* 1980; Robertson, 1979), a unique variation was observed within these conserved sequences, U or C at position 4 of the 3′ end of the vRNA (Table 1). The distal double-stranded element in the corkscrew conformation model

TABLE 1

Sequence Characteristics of the Extremities of the Genomic Segments Derived from the MAL and PR8 Viruses as Compared to All Sequences Available in GenBank

	nt 4 of the 3′ end			Length of the ds distal element (bp)		
Segment	MAL	PR8	Other[a]	MAL	PR8	Other[a]
PB1	C	C	U/C	5	5	5
PB2	C	C	U/C	5	5	5
PA	C	C	U/C	7	7	7/6
HA (H1)	—	U	U	—	4	4
HA (H2)	U	—	U	4	—	4
NP	U	U	U	6	6	6
NA (N1)	—	C	U	—	3//4[b]	8
NA (N2)	U	—	U	6	—	6
M	U	C	U	6	6	6
NS	U	U	U	6	6	6

[a] All sequences available in GenBank for influenza A viruses other than MAL and PR8 were aligned, and the sequence consensus is indicated. The number of sequences available for the extremities was variable depending on the segment: 20 for PB1, 24 for PB2, 16 for PA, 11 for H1, 11 for H2, 88 for NP, 10 for N1, 9 for N2, 14 for M, and 21 for NS.

[b] Two elements of three and four base-paired nucleotides separated by a 1-nt gap.

(starting with base-pairing of nt 10 of the 3′ end and nt 11′ of the 5′ end) was found to be 4 to 8 bp long depending on the segments (Table 1). For PR8, the terminal sequences were found identical to those described by Desselberger *et al.* (1980), and the features that appear characteristic of PR8 as compared to all available sequences in the databases, i.e., a C at nt 4 in the 3′ end of segments NA and M, and a discontinuity in the distal stem of the NA segment, were confirmed (Table 1). On the whole, our data confirm the high conservation and stability of the terminal sequences of influenza A virus genomic segments.

DISCUSSION

Effect of the triple 3-5-8 mutations in the 3′ end of vRNA

It was previously observed by Neumann and Hobom that in murine B82 cells infected with the influenza virus A/FPV/Bratislava, a viral-like reporter RNA harboring triple 3-5-8 mutations in the 3′ end underwent transcription/replication more efficiently than a wild-type viral-like RNA, i.e., the mutations showed a promoter-up effect (Neumann and Hobom, 1995). Here, we report that this observation cannot be extended to all influenza A viruses. Indeed, using a plasmid-driven system for the *in vivo* reconstitution of functional ribonucleoproteins in COS-1 cells, we have observed that the triple mutations had a promoter-up effect when the polymerase complex was derived from the MAL or FPV viruses (avian viruses, as A/FPV/Bratislava), but not from the human PR8, VIC, or P908 viruses. In the presence of human-derived polymerase complexes, the levels of transcription/replication achieved with the mutated viral-like RNA were even lower (at least twofold) than the wild-type RNA, i.e., the mutations had a slight "promoter-down" effect. Similar results were obtained whether the RNA templates were designed to form a 6-bp-long (NS segment-like templates) or a 4-bp-long (HA segment-like templates) double-stranded distal element in the corkscrew conformation, which indicated that the effects of the triple 3-5-8 mutations were independent of the length of the distal stem. Moreover, experiments using RNA templates harboring various double or single mutations at nt 3, 5, 8 of the 3′ end indicated that the promoter-up and promoter-down effects observed with the RNA harboring the triple 3-5-8 mutations in the presence of avian- and human-derived complexes, respectively, were determined by the nature of nt 3 and 8, but not nt 5. Our finding, that not only base-pairing between nt 3 and 8 but also the nature of the nucleotide at these positions was critical for promoter activity, is contradictory with data from Flick and Hobom, which suggested that only base-pairing was critical in an HA segment-like vRNA (Flick and Hobom, 1999). Our observation that a viral-like RNA with a single U5 → C5 mutation in the 3′ end underwent transcription/replication as efficiently as the wild-type RNA is again in contradiction with data from Neumann and Hobom, who reported that this nucleotide exchange at position 5 completely abolished the promoter activity (Neumann and Hobom, 1995). Interestingly, Lee and Seong (1998a) as well as Bergmann and Muster (1995) reported that they were unable to rescue transfectant WSN viruses carrying stably the U5 → C5 mutation in the 3′ end of the NA vRNA. These apparent contradictions may result from the fact that the effects of the mutations were analyzed in the context of the noncoding sequences from different viral segments (i.e., NS in this article, HA (Neumann and Hobom, 1995; Flick and Hobom, 1999) or NA (Lee and Seong, 1998a; Bergmann and Muster, 1995) in the other studies). In addition, the discrepancies may be linked to the fact that in our exclusively plasmid-based experimental system, there is no expression of viral genes other than PB1, PB2, PA, and NP, whereas the results of the three teams cited above were obtained in the context of a viral infection.

In the course of a mutagenic analysis of the 3′ terminal sequence of the vRNA with regard to endonuclease activity of the polymerase complex of A/PR/8/34 virus, Leahy *et al.* observed that the identity of residue 5 did not affect endonuclease activity, whereas replacing the G-C base pair at position 3–8 in the stem-loop structure with an A-U base pair reduced endonuclease activity by about 50% (Leahy *et al.,* 2001a, 2002). This decrease in endonuclease activity could very well contribute to the two- to threefold decrease in the levels of CAT that we measured

with RNA templates harboring the double 3–8 or triple 3-5-8 mutations in the presence of PR8 or P908 complexes, as compared to the wild-type RNA.

Influence of the nature of residue 627 of PB2 on the effect of the triple 3-5-8 mutations

Our results indicate that the promoter-up effect of the triple 3-5-8 mutations observed with avian-derived complexes corresponds to the compensation of a defect of the transcription/replication activity of these complexes in COS-1 cells as compared to human-derived complexes. Indeed, the levels of transcription/replication measured at 24 h posttransfection with the MAL or FPV complex were 10- to 100-fold lower than those obtained with human-derived complexes when a wt viral-like RNA was used, whereas they were in the same range when the mutant viral-like RNA was used. Experiments using MAL-PB2 and FPV-PB2 mutants with a Glu627Lys substitution and a P908-PB2 mutant with a Lys627Glu substitution indicated that the transcription, and to a lesser extent the replication activity, was impaired when residue 627 of PB2 was a Glu (typical of avian viruses), and that both defects were compensated by the presence of the triple mutations at the 3′ end of the vRNA. Noticeably, both the effects of the nature of PB2 amino acid 627 and the effects of the triple 3-5-8 mutations were much more pronounced with the MAL than with the P908 complex and appeared intermediate with the FPV complex, which was probably linked to some other molecular characteristics of the FPV and P908 polymerase complexes, allowing a better transcription/replication activity in COS-1 cells than the MAL complex. Analysis of all 16 possible combinations between the core proteins of MAL and FPV indicated that the difference observed between the two complexes was linked to the PA subunit. The alignment of MAL- and FPV-PA with all other sequences available for PA reveals that several residues differ between MAL- and FPV-PA, but that none of them correspond to a position that differs between the human and avian lineages of influenza A viruses, which could have accounted for the intermediate behavior of the FPV complex as compared to MAL and P908 complexes.

It has been shown previously that PB2 and more specifically amino acid 627, as well as to a lesser extent PA, were determinants of the temperature sensitivity of the polymerase complex derived from avian viruses (Massin *et al.,* 2001), suggesting that the effect of the triple 3-5-8 mutations might be temperature-dependent. However, when the transcription/replication activities of avian (MAL, FPV) and human (P908) complexes were compared at 33, 37, and 40°C, the same differential effect of the triple mutations was observed at all temperatures (data not shown). Thus the hypothesis of a temperature-dependent effect seems unlikely.

Amino acid 627 of PB2 has recently been shown by Hatta *et al.* to be an essential determinant of the pathogenicity in mice of A(H5N1) influenza viruses which were transmitted from birds to humans in Hong Kong in 1997 (Hatta *et al.,* 2001). This was in agreement with previous studies on human/avian reassortant viruses which indicated that PB2 residue 627 was a host-range determinant of influenza A viruses (Subbarao *et al.,* 1993). Some of our previous data suggested that the mechanism by which the nature of PB2 amino acid 627 alters the efficiency of transcription/replication in mammalian (COS-1) cells is probably complex, relying on interactions of PB2 with viral and most importantly cellular proteins (Naffakh *et al.,* 2000). Our present data suggest that RNA–protein interactions are also involved, but the exact nature of these interactions and the mechanism by which they may be influenced by the presence of the triple 3-5-8 mutations in the 3′ end of the vRNA are still unclear, as little is known on the structure/function relationships for vRNA.

Structure dynamics of viral RNA and functional interactions with the polymerase complex

Flick *et al.* have suggested that during the process of transcription/replication, the vRNA could be in the panhandle conformation for initial interaction with the polymerase and then switch to a corkscrew conformation, the equivalent of an open complex structure (Flick *et al.,* 1996). The cRNA is also likely to adopt a panhandle and/or corkscrew conformation while interacting with the polymerase complex (Azzeh *et al.,* 2001). Leahy *et al.* have established that the identity of nt 5 in the 5′ arm of the cRNA promoter, together with the absence of a "hinge" A nt in the 5′ arm and the presence of a hinge U nt in the 3′ arm of the cRNA promoter in contrast to the vRNA promoter is the basis for the lack of activation of endonuclease activity of the complex upon binding to the cRNA (Leahy *et al.,* 2002). Finally, melting of the 3′ end of the vRNA and cRNA is probably required for the initiation of synthesis of (+)- and (−)-sense RNAs, respectively.

The triple 3-5-8 mutations in the 3′ end of the vRNA are likely to alter the structure of both the vRNAs and the cRNAs. First they are predicted to increase the stability of the terminal RNA duplex in the panhandle conformation model by restoring a complete Watson–Crick base pairing between the nine terminal nucleotides of the 3′ and 5′ ends. Interestingly, Bae *et al.* have recently described the structure of the vRNA promoter in the panhandle conformation using magnetic resonance spectroscopy and showed that there is a bending of the RNA duplex at position 4, which could lower the energy for unwinding and opening the helix (Bae *et al.,* 2001). They demonstrated that mutations in the 3′ end that restore a complete Watson–Crick base pairing within the RNA duplex suppress the bending of the helix, which could stabilize the panhandle structure. The triple 3-5-8 muta-

tions in the 3′ end of the vRNA are also predicted to lead to (i) a decreased stability of the 3′ arm of the vRNA promoter and of the 5′ arm of the cRNA promoter in the corkscrew conformation; and (ii) a sequence identity between the vRNA and cRNA promoters, except for the difference in the hinge localization which is maintained.

The most simple interpretation of our data is that the triple mutations compensate the inability of a polymerase complex with a Glu at PB2 amino acid 627 to stabilize the panhandle conformation and/or to melt stem-loop structures in the corkscrew conformation in COS-1 cells. This would suggest that PB2 and/or cellular proteins interacting with PB2 could be involved in RNA conformational changes during the process of transcription/replication. Some of the cellular proteins implicated could be helicases. Indeed, no helicase activity has been found associated with the polymerase complex of influenza A viruses, but Huarte *et al.* recently reported the detection of an interaction between the PA subunit of the complex and a protein containing an helicase domain (Huarte *et al.,* 2001). Further experiments involving both mammalian and avian cell lines could help in testing these hypotheses.

MATERIALS AND METHODS

Plasmids for the expression of viral proteins

Plasmids pHMG-PR8-PB1, -PB2, -PA, and -NP, which express the PB1, PB2, PA, and NP proteins, respectively, of influenza virus A/Puerto Rico/8/34 (PR8) under the control of the hydroxymethylglutaryl coenzyme A reductase (HMG) promoter were kindly provided by J. Pavlovic (Institut für Medizinische Virologie, Zurich, Switzerland). The analogous pHMG-derived plasmids encoding the PB1, PB2, PA, and NP proteins of A/Victoria/3/75, A/Mallard/NY/6758/78, and A/FPV/Rostock/34 viruses have been described previously (Naffakh *et al.,* 2000). Virus isolate A/Paris/908/97 (P908) was grown on MDCK cells. After four passages, viral genomic RNA was extracted from MDCK supernatant using the Trizol reagent (Gibco). Molecular cloning and sequencing of the cDNAs encoding P908-PB1, -PB2, -PA, and -NP were performed as described previously (Naffakh *et al.,* 2000). The corresponding sequences have been submitted to GenBank under the following Accession Nos.: AF483601, AF483602, AF483603, AF483604. The pHMG-MAL-PB2-E627K and pHMG-FPV-PB2-E627K plasmids, which express MAL and FPV-derived PB2 proteins mutated at residue 627, have been described in Naffakh *et al.,* 2000. Similarly, a plasmid allowing the expression of a P908-derived PB2 protein with a K627E mutation was generated using an overlap extension PCR protocol (Pogulis *et al.,* 1996). The pHMG-P908-PB2 plasmid was used as a template and oligonucleotides 5′-GCCGCTCCACCAGAACAAAGCAGGATG-3′ and 5′-CATCCTGCTTTGTTCTGGTGGAGCGGC-3′ were used as mutagenic primers. The sequence of the additional primers used for mutagenesis can be obtained from the authors upon request. The conditions of amplification were as described in Naffakh *et al.,* 2001. The fusion PCR product was digested with the *Hpa*I and *Kpn*I enzymes and subcloned into the pHMG-P908-PB2 plasmid at the *Hpa*I and *Kpn*I sites. Positive clones were sequenced using a Big Dye terminator sequencing kit (Perkin–Elmer) and analysis on an ABI prism 377 automated sequencer to assess the presence of the site-directed mutation and the absence of unanticipated nucleotide changes.

Plasmids for the expression of viral-like RNAs

The pA/PRCAT(−) and pA/PRCAT(+) plasmids, which direct the expression of an influenza virus-like RNA derived from the nonstructural (NS) segment of the A/WSN/33 virus, were described previously (Crescenzo-Chaigne *et al.,* 1999). They contain, inserted at the *Bbs*I site of vector plasmid pPR (Crescenzo-Chaigne *et al.,* 1999), the CAT gene sequences in an antisense (pA/PRCAT(−)) or sense (pA/PRCAT(+)) orientation flanked by the 5′ and 3′ extremities of the NS gene segment. Expression of the viral-like RNA is driven by a truncated human RNA *Pol*I promoter. The correct 3′ end is ensured by the use of the hepatitis delta virus ribozyme sequence.

The PCR-based protocol used to introduce mutations in the sequences of pA/PRCAT(−) corresponding to the 3′ end of the viral-like RNA has been described previously (Crescenzo-Chaigne and van der Werf, 2001). Primers 5′-AG*TAGAAA*CAGGGTGACAAAGAC-3′, 5′-AGCAAAAGCAGGG*GA*ACAAAGACATAATG-3′, and 5′-AG*TAG*A*A*CAGGG*GA*ACAAAGACATAATG-3′ were used with the primer 5′-AGTAGAAACAAGGGTGTTTTTTCAG-3′ for amplification of the CAT gene and NS noncoding sequences using pA/PRCAT(−) as a template, to generate the pA/PRCATmu3-5-8(−) and (+), pA/PRCAT/NH(−), and pA/PRCAT/NHmu3-5-8(−) plasmids, respectively. Similarly, primers 5′-AG*T*AAAA*A*CAGGGTGACAAAGAC-3′, 5′-AGCA*G*AAGCAGGGTGACAAAGAC-3′, 5′-AG*A*AAAA*T*CAGGGTGACAAAGAC-3′ and 5′-AG*GAAAAC*CAGGGTGACAAAGAC-3′ were used to generate additional mutant plasmids pA/PRCATmu3-8(−), pA/PRCATmu5(−), pA/PRCATmu3U-8A(−), and pA/PRCATmu3C-8G(−). The amplified products were cloned at the *Bbs*I sites of pPR. The presence of the mutations in the positive clones was assessed by sequence determination as described above.

Transfections and CAT assays

COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Subconfluent monolayers (1.2×10^5 cells in 21-mm dishes) were transfected by using 5 μl FuGENE 6 (Roche) with 0.5 μg of either pA/PRCAT(−) or one of the pA/PRCAT(−)-de-

rived plasmids together with pHMG-PB1 (0.5 μg), pHMG-PB2 (0.5 μg), pHMG-PA (0.5 μg), and pHMG-NP (1 μg) plasmids. Cells were incubated at 37°C until harvest at 18, 24, or 48 h posttransfection. Using the CAT ELISA Kit (Roche), cell extracts were prepared in 250 μl of the lysis buffer provided in the kit and tested for CAT levels. This procedure allowed detection of 0.05 ng/ml CAT. All experiments were done in duplicate.

RNA analysis

Total RNAs were prepared from COS-1 cells at 24 h posttransfection using the Trizol reagent (Gibco-BRL) and analyzed by Northern blot. The RNA samples were run on a 1.2% agarose, MOPS 1×, and 0.6 M formaldehyde gel, blotted onto a nylon membrane (Hybond N, Amersham), and fixed by UV irradiation. Membranes were hybridized with a CAT-specific ^{32}P-labeled riboprobe, washed three times in 0.1× SSC, 0.1% SDS at 75°C for 15 min, dried, and exposed on a STORM820. Quantification was obtained using the Image Quant program (Molecular Dynamics). For detection of the viral-like mRNAs, poly(A)$^+$ RNAs were purified from total RNAs using the Gen-Elute mRNA Miniprep Kit (Sigma) and were analyzed by slot blotting as described previously (Naffakh *et al.,* 2001).

Sequencing of the 3′ end of genomic RNA segments

Virus isolates PR8 and MAL were grown in 11-day-old embryonated chicken eggs. Viral genomic RNA was extracted using the Trizol reagent (Gibco-BRL) and submitted to a polyadenylation reaction for 30 min at 30°C using the yeast poly(A) polymerase (Amersham Pharmacia Biotech) in the presence of 10 mM ATP. Complementary DNAs were prepared by reverse transcription of the polyadenylated RNAs using a $(dT)_{15}$ oligonucleotide as a primer and 20 U AMV reverse transcriptase (Promega) with incubation at 42°C for 1 h followed by incubation at 55°C for 15 min. Amplification was performed using an anchored $(dT)_{15}$ oligonucleotide (5′-AGATGAATTCGGTACC $(T)_{15}$-3′) together with a primer of negative polarity specific for the coding sequences of each of the eight segments of vRNA located about 300 bp upstream of the 3′ end. The AmpliTaq DNA Polymerase (Perkin–Elmer) was used in the presence of 10 mM $MgCl_2$. Thirty-five cycles were performed, each consisting of 10 s at 94°C, 30 s at 40°C, and 30 s at 72°C. The PCR products were purified using a PCR Purification Kit (Qiagen) and sequenced with an internal oligonucleotide, using the protocol described above. The exact sequences of the primers used for amplification and sequencing of the 3′ end of each segment can be obtained from the authors upon request.

Sequencing of the 5′ end of genomic RNA segments

Viral genomic RNA was submitted to reverse transcription as described above, using as primers oligonucleotides of positive polarity specific of the coding sequences of each of the eight segments and located around 400 bp upstream of the 5′ end. The cDNAs were purified using a PCR Purification Kit (Qiagen) and submitted to an elongation reaction for 45 min at 37°C with the *Escherichia coli* Terminal Deoxynucleotide Transferase (Amersham Pharmacia Biotech) in the presence of 5 mM dATP. A first round of amplification was performed using the anchored $(dT)_{15}$ oligonucleotide described above, together with an oligonucleotide of positive polarity specific for the coding sequence of each of the eight segments of vRNA, located about 300 bp upstream of the 5′ end. A second round of that "half-nested" reaction was performed after a 1:25 dilution step, using more proximal segment-specific primers together again with the anchored $(dT)_{15}$ primer. The conditions used for amplification were the same as described above for determination of the vRNA 3′ ends. The PCR products were purified using a PCR Purification Kit (Qiagen) and sequenced with an internal oligonucleotide, using the protocol described above. The exact sequences of the primers used for amplification and sequencing of the 5′ end of each segment can be obtained from the authors upon request.

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