Mutational Analysis of the Conserved Motifs of Influenza A Virus Polymerase Basic Protein 1

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Influenza virus polymerase complex is a heterotrimer consisting of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). Of these, only PB1, which has been implicated in RNA chain elongation, possesses the four conserved motifs (motifs I, II, III, and IV) and the four invariant amino acids (one in each motif) found among all viral RNA-dependent RNA or RNA-dependent DNA polymerases. We have modified an assay system developed by Huang et al. (T.-J. Huang, P. Palese, and M. Krystal, J. Virol. 64:5669–5673, 1990) to reconstitute the functional polymerase activity in vivo. Using this assay, we have examined the requirement of each of these motifs of PB1 in polymerase activity. We find that each of these invariant amino acids is critical for PB1 activity and that mutation in any one of these residues renders the protein nonfunctional. We also find that in motif III, which contains the SSDD sequence, the signature sequence of influenza virus RNA polymerase, SDD is essentially invariant and cannot accommodate sequences found in other RNA viral polymerases. However, conserved changes in the flanking sequences of SDD can be partially tolerated. These results provide the experimental evidence that influenza virus PB1 possesses a similar polymerase module as has been proposed for other RNA viruses and that the core SDD sequence of influenza virus PB1 represents a sequence variant of the GDN in negative-stranded nonsegmented RNA viruses, GDD in positive-stranded RNA virus and double-stranded RNA viruses, or MDD in retroviruses.

Influenza viruses carry an RNA-dependent RNA polymerase, also known as transcriptase or replicase. The viral polymerase, which is a heterocomplex (3P), consists of three proteins, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA), present in a 1:1:1 ratio. By using an in vitro transcription system that involves ribonucleoprotein (RNP)-polymerase complex isolated from either virus or virus-infected cells, much information has been obtained about the mechanism of the transcription reaction, including primer involvement, transcription initiation, and chain elongation (4, 6, 8, 23). However, this in vitro system has limitations since the specific role of individual components cannot be dissected at the molecular level. Data from biochemical as well as genetic studies involving temperature-sensitive mutants have implied that PB2 is involved in the initiation of transcription, including primer recognition and, possibly, cap cleavage, whereas PB1 is involved in mRNA chain elongation (23, 45, 46). However, as yet no specific function of PA in mRNA transcription has been demonstrated (23, 24). These studies have also shown that nucleoprotein (NP) is required for full-length synthesis of plus-strand RNA and subsequent synthesis of minus-strand genomic RNA (4, 5, 40). However, with this in vitro transcription-replication system it has not yet been possible to determine the structure of the polymerase complex or function of the domains of individual polymerase proteins or their role in regulating the switch from transcription (mRNA) to replication (cRNA) or from cRNA synthesis to vRNA synthesis. Over the years we have been interested in defining the structure-function aspect of the influenza virus polymerase proteins. Accordingly, we and others have determined the primary structure of polymerase

proteins from influenza virus types A, B, and C (21, 23, 41, 50). Furthermore, we and others have shown that three polymerase proteins form a heterocomplex in the absence of viral RNP (2, 23) and that they are transported into the cell nucleus (2), the site of viral transcription and replication. We have also shown that PB1 and PB2 possess bipartite nuclear localization signals (28, 30). However, the lack of a suitable system to reconstitute the functional polymerase complex has hampered the detailed structure-function analysis of individual components of the polymerase complex (22, 44).

Recently, novel experimental approaches by reverse genetics have been developed for the rescue of heterologous RNA into infectious virus (9, 10, 26, 29). This system involves in vitro reconstituted RNP with synthetic RNA containing NP and the polymerase complex, which is transfected into cells infected by a helper virus. This system has been successfully used to introduce defined mutations into the viral genome and viral proteins and to study their role in viral pathogenesis (9, 10, 29). Similarly, by using either a reporter gene activity in transfected cells or a synthetic template in an in vitro transcription system, it has also been possible to perform detailed mutational analysis of the viral promoter including the requirements for cis-acting noncoding sequences of influenza virus RNA in transcription-replication (31, 48, 49). Similar studies with the hybrid NA gene (type A) containing the noncoding sequences from influenza virus type B implied the requirement of homologous cis-acting noncoding sequences for efficient transcription-replication by type A virus polymerase (14, 29). Recently, Huang et al. (17) have extended this system by using the vaccinia virus expression system and have demonstrated that only three polymerase proteins (PB1, PB2, and PA) and NP can perform the transcription-replication of a reporter gene containing the promoter sequence of influenza virus RNA in vivo, thus making it possible to study the function of individual polymerase proteins. In another negative-strand RNA virus system (vesicular stomatitis virus), similar vaccinia virus-ex-

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pressed proteins have been used to study the transcription-replication as well as encapsidation and budding of the viral nucleocapsids (32, 33).

Of the three polymerase proteins, PB1 has been implicated in catalytic activity including nucleotide polymerization and chain elongation (8, 23). Comparative sequence analysis has demonstrated that PB1 possesses four conserved motifs that are present among all viral RNA-dependent RNA polymerases and RNA-dependent DNA polymerases (38). In the work reported in this paper we have modified the protocol used by Huang et al. (17) to reconstitute the function of the influenza virus type A polymerase complex in vivo. Using this system we have performed mutational analyses of each of the four conserved motifs of PB1 to define their role in polymerase activity. Our data demonstrate that all the conserved motifs in PB1 play critical roles in influenza virus RNA transcription-replication.

MATERIALS AND METHODS

Viruses and cells. Influenza A/PR/8/34 and A/WSN/33 viruses were grown in embryonated chicken eggs and MDCK cells, respectively. Egg-grown influenza A/PR/8/34 virus was concentrated by polyethylene glycol precipitation and purified by gradient centrifugation (15). Viral RNP cores were prepared from the whole virus as described by Parvin et al. (31). Recombinant vaccinia viruses expressing PB1 (PB1-Vac), PB2 (PB2-Vac), PA (PA-Vac), NP (NP-Vac), and T7 RNA polymerase (VTF7.3) were obtained from Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (43). Stocks of recombinant vaccinia viruses were propagated in HeLa cells, and infectivity titers were determined by plaque assay on CV1 cells.

Plasmids and site-directed mutagenesis of the PB1 gene. Plasmid pGEM PB1 was constructed by cloning the entire PB1 cDNA of A/WSN/33 (30) into the HindIII site of pGEM4 (Promega Inc., Madison, Wis.) under T7 RNA polymerase promoter control. The conserved I, II, and IV motifs were mutated by site-directed mutagenesis (Amersham, Arlington Heights, Ill.). Mutations in the conserved motif III (DD motif) were introduced by PCR. PB1 cDNA between the AccI site (nt 778) and the EcoRI site (nt 1495) was used as the template for PCR amplification with different oligonucleotide primers spanning the BsmI site (nt 1377) site at one end but the same oligonucleotide primer spanning the PstI site (nt 1130) site at the other end. The PCR product was double digested with BsmI and PstI, and the 247-bp DNA fragment was ligated into pGEM PB1, digested with BsmI and PstI, by three-way ligation. Therefore all wild-type or mutated PB1 cDNAs were under the control of the T7 promoter in pGEM 4. Individual pGEM PB1 clones containing different mutant PB1 sequences were identified by sequencing the entire PCR-amplified DNA (247 nt) to ensure that additional mutations were not introduced by amplification. All plasmids were isolated through a column for transfection (Qiagen Inc., Chatsworth, Calif.). The plasmid IVACAT1, which directs an NS-like transcript containing the chloramphenicol acetyltransferase (CAT) gene (26), was kindly provided by P. Palese, Mt. Sinai School of Medicine, New York, N.Y.

RNP complex formation and transfection. Plasmid IVACAT1 was digested with HgaI, filled with Klenow enzyme, and transcribed with T7 RNA polymerase to produce IVACAT1 RNA containing the precise ends of the NS gene (26). The RNP complex was made by the procedure of Luytjes et al. (26). Briefly, CV1 cells were infected at a multiplicity of infection (MOI) of 5 for 1 h with each of the different recombinant vaccinia viruses expressing PB2, PA, and NP proteins as well as VTF7.3 expressing T7 RNA polymerase. Unadsorbed viruses were removed by washing with phosphate-buffered saline containing 0.01% Ca^{2+} and Mg^{2+} (PBS^+), and vaccinia virus-infected cells were transfected together with the mixture of RNP complex (1 μg of IVACAT1 RNA plus 2 μg of RNP polymerase complex) and pGEM PB1 DNA by the lipofectin-mediated transfection method (11, 27). After incubation for 1 h at 37°C, fresh medium was added and cells were further incubated for 16 h, at which time they were used for preparing the lysate and for the CAT assay.

CAT assays. Cells were lysed and assayed for CAT activity by a standard method as described by Gorman et al. (13). The reaction mixture for the CAT assay contained 1 to 1.5 μl of [^{14}C]chloramphenicol (0.025 mCi/ml; Amersham), 10 μl of n-butyryl coenzyme A (5 mg/ml; Sigma Chemical Co., St. Louis, Mo.), and 25 μg of cell lysate in a 125-μl final volume of 0.25 M Tris-HCl (pH 7.5). The incubation was done at 37°C for 4 h, which was in the linear range of the reaction. Finally, the product was extracted with ethyl acetate and analyzed by thin-layer chromatography for the acetyl form of [^{14}C]chloramphenicol. Each assay was done at least from duplicate plates and from two independent transfection experiments. The same amount of cellular protein was used in all assays, and results of all CAT assays were based on the linear range of acetylation with substrate in excess. For quantification, spots of acetylated forms were marked on the thin-layer chromatography plate and the material on each spot was scraped and counted in a scintillation counter. The percent activity was based on the amount of the product obtained by using four recombinant vaccinia viruses (each at an MOI of 5) as a positive control (100%) in each experiment. All other conditions for infection and assay were kept constant.

Radiolabeling, immunoprecipitation, and Western blotting of the polymerase complex. For radiolabeling of proteins, cells at 14 h posttransfection were washed in PBS^+, incubated in methionine-free medium for 1 h at 37°C, and labeled for 1 h in 1 ml of methionine-free medium containing 50 μCi of Trans-^{35}S-label (ICN Biomedicals Inc., Irvine, Calif.) per ml. At the end of labeling, the cell monolayer was washed twice in cold PBS^+, scraped into cold PBS^+, and pelleted by centrifugation. For immunoprecipitation of the polymerase complex, the cell pellet was resuspended in RIPA buffer (10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% aprotinin) and immunoprecipitated with monospecific anti-PB1 antibodies as described previously (2). The polymerase complex was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide). For Western immunoblotting, the cell pellet was directly lysed in sample buffer by boiling and protein samples were electrophoresed by SDS-PAGE (8% polyacrylamide) transferred to and Nytran membrane (Schleicher & Schuell Inc., Keene, N.H.). Polymerase proteins were detected by standard procedures with monospecific rabbit anti-PB1 antibodies and alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (2).

RESULTS

In vivo reconstitution of influenza virus transcription-replication by component expression of the polymerase complex. To define the functional domains of PB1 in transcription-replication, we needed an assay system in which individual components of the polymerase complex could be used to reconstitute the functional polymerase complex. For this, we modified the system described by Huang et al. (17). First, we

CV1 cells

↓

**Infect with PB2-Vac, PA-Vac,
NP-Vac and VTF7.3 at m.o.i. 5 (each)**

↓ 37 °C, 1 hour

**Transfect with mixture of IVACAT1 RNA,
RNP-polymerase complex and pGEM PB1
DNA by lipofectin method**

↓ 37°C, 16 hours
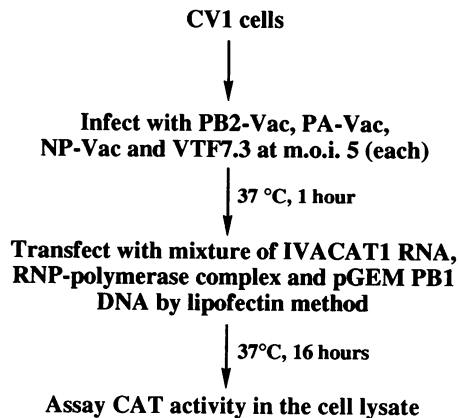
Assay CAT activity in the cell lysate

FIG. 1. Schematic representation of the protocol for analysis of influenza virus RNA transcription-replication in cells expressing influenza virus proteins. m.o.i., multiplicity of infection.
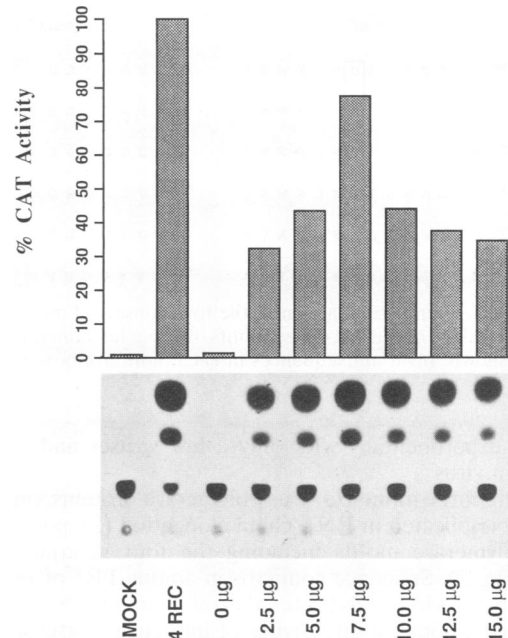

FIG. 2. Optimization of influenza virus RNA transcription-replication by PB1 transfection. CV1 cells in 60-mm plates were infected with PB2-Vac, PA-Vac, NP-Vac, and VTF7.3 at an MOI of 5 (each) for 1 h and subsequently transfected with a mixture of 1 μg of IVACAT1 RNA, 2 μg of RNP-polymerase complex, and different amounts of pGEM PB1 DNA as shown. Cells were incubated for 16 h, harvested, and assayed for CAT activity as described in Materials and Methods. The numbers under the lanes indicate the amount of transfected pGEM PB1 DNA. 4 REC indicates four recombinant vaccinia viruses expressing PB1, PB2, PA, and NP, used as the positive control (100%).

confirmed that PB1, PB2, PA, and NP under the control of vaccinia virus can efficiently transcribe and replicate the IVACAT1 RNA as reported previously (17, 26). In our system, we expressed PB2, PA, and NP through recombinant vaccinia viruses but provided PB1 (wild type or mutated) expression via a vaccinia virus-T7 RNA polymerase-based transfection system (12). In this system, bacteriophage T7 RNA polymerase, expressed in the cytoplasm of cells infected with a recombinant vaccinia virus (VTF7.3), was used to transcribe the transfected plasmid DNA which contained the PB1 gene under the control of the T7 RNA polymerase promoter. The basic protocol for analyzing the influenza virus RNA replication is outlined in Fig. 1. Briefly, CV1 cells were infected with VTF7.3 along with recombinant vaccinia viruses expressing PB2, PA, and NP (each at an MOI of 5) and then transfected with a mixture of IVACAT1 RNP and pGEM PB1 DNA (11, 27). The efficiency of transcription-replication of the recombinant CAT RNA was measured by analyzing the CAT activity present in the cell lysate at 16 h posttransfection. In this system, CAT activity measures the amount of CAT protein, which in turn depends on the relative amount of CAT mRNA. The level of the CAT mRNA is dependent on the function of the active influenza virus polymerase complex since the influenza virus promoter controls the transcription-replication of the CAT RNA. Initial results demonstrated that CAT activity was present in cells in which PB1 was expressed either via infection with a recombinant vaccinia virus or by using a vaccinia virus T7-RNA polymerase system (data not shown). In addition, omission of CAT RNP or pGEM PB1 DNA abolished the CAT activity completely, indicating that functional PB1 was necessary for the transcription-replication of the synthetic influenza virus RNA. We also found that under these conditions transfection with 1 μg of RNA and 2 μg of RNP produced the maximum level of CAT activity, as was reported before (26).

To optimize the T7 transfection system for CAT expression, we examined the quantitative relationship between the amount of CAT activity and the amount of the transfected pGEM PB1 DNA. Accordingly, CV1 cells were infected with PB2-Vac, PA-Vac, NP-Vac, and VTF7.3 (each at an MOI of 5) and subsequently transfected with various amounts of pGEM PB1 DNA mixed with the fixed amount of CAT RNP. The cells were lysed at 16 h posttransfection and assayed for CAT activity. For quantification, the amount of radioactive [14C]chloramphenicol converted to acetylated forms was

counted and compared with the positive control by using all four vaccinia viruses. The results showed that CAT activity increased with increasing DNA concentrations and reached its maximum at 7.5 μg of DNA, which was essentially same as the positive control (Fig. 2). However, further increasing the amount of plasmid DNA for transfection resulted in the decrease of CAT activity. The reduced activity may be due to the use of two components in the transfection system in which both IVACAT1 RNA and pGEM PB1 DNA were transfected together. This might have resulted in competitive inhibition of RNA transfection at higher concentrations of DNA. Therefore we used 7.5 μg of DNA for transfection in all our experiments.

Sequence comparison of the conserved motifs of RNA-dependent RNA polymerases. Comparison of the amino acid sequences of the RNA-dependent DNA polymerases and RNA-dependent RNA polymerases revealed four highly conserved motifs conforming a large domain of 120 to 210 amino acids (35). Each of these conserved motifs is present in all retroviruses and in positive-strand RNA viruses, double-stranded RNA viruses (including plant, yeast, and animal viruses), and segmented and nonsegmented negative-strand RNA viruses (Fig. 3). This domain possesses at least 4 invariant amino acids (one in each motif) and 18 conservatively maintained amino acids among the polymerase proteins of all RNA viruses analyzed to date. This domain has been proposed to constitute the polymerase module (3, 20, 35). In particular, the invariant DD (or DN) sequence in motif III is flanked by hydrophobic residues in all viruses and has been implicated in metal binding, template recognition, and catalytic activity (3, 20). However, the functions of individual motifs have been
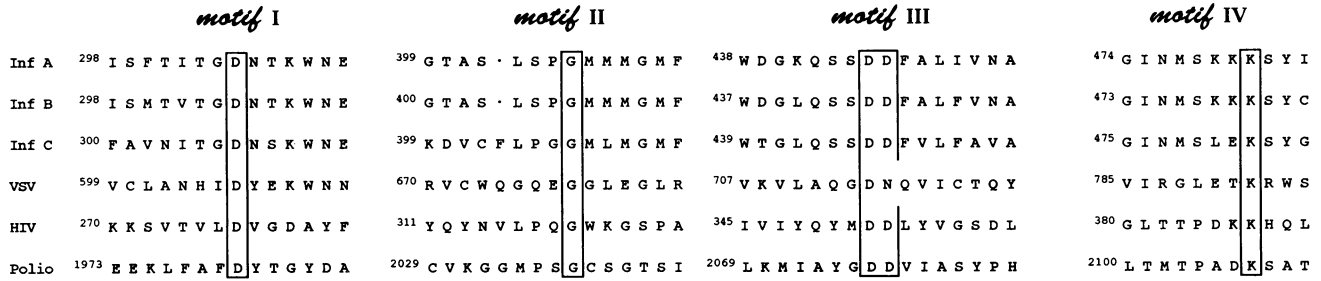
FIG. 3. Sequence comparison of the four conserved motifs of PB1 of influenza A/WSN/33 (41), B/Lee/40 (21), and C/JJ/50 (50) viruses. The relative positions of the conserved motifs of vesicular stomatitis virus (VSV) (34), Human immunodeficiency virus (HIV) (38), and polio virus (37) are also shown. Invariant sequences in each motif are boxed.

studied experimentally with only a few viruses and not with influenza virus.

Among three influenza virus polymerase proteins, only PB1, which is implicated in RNA chain elongation (8), possesses all four polymerase motifs including the four invariant amino acids (Fig. 3). Sequence comparison among PB1 of influenza A, B, and C viruses indicates that all four motifs are highly conserved among all three types of influenza viruses and that the observed changes are very rare and conservative in nature. Since there is no information available regarding the structural requirement of any influenza virus polymerase proteins, we decided to examine the role of invariant amino acids in each of the four motifs. In particular, we wanted to determine the role of SDD in motif III, which is different from that present in nonsegmented negative-strand RNA viruses (GDN), retroviruses (MDD), or positive-strand RNA viruses (GDD). These three amino acids are invariant within each group of viruses, suggesting that they may provide similar functions.

Mutational analyses of PB1 sequences. All mutations were done either by site-directed mutagenesis or by PCR amplifications as described in Materials and Methods. Since few experimental data are available on the function of motifs I, II, and

IV for any polymerase protein, we mutated the invariant amino acids D-305 (motif I), G-406 (motif II) and K-481 (motif IV) to Q, R, and Q, respectively, to determine whether these motifs are functionally involved in replicase-transcriptase activity (Fig. 4). In addition, another mutation, T-400→I adjacent to the invariant G residue in motif II was examined. All mutant proteins were expressed in CV1 cells by using the T7 RNA polymerase promoter in pGEM4 and VTF7.3 vaccinia virus expressing T7 polymerase. The CAT activity was assayed and quantified as described in Materials and Methods. Results (Fig. 5) show that mutation of each invariant amino acid in motifs I, II, and IV caused a drastic reduction in CAT activity, ranging from 1 to 7% of the wild-type PB1 activity, supporting the notion that these motifs and the invariant amino acids in the motif are important in replicase-transcriptase activity of PB1 (Fig. 5). Clearly, many more mutations will be required to delineate the function of each motif and of the individual amino acids in the motif.

Motif III containing the SSDD sequence was studied more extensively. All viral polymerases contain the central DD, except in motif III of rhabdoviruses and paramyxoviruses, which contains DN instead (35). In addition, there is consid-

	Motif I	Motif II	Motif III	Motif IV
Wildtype:	T G D N	E G T A S L S P G M	W D G L Q S S D D F A L I V N	K K K S
305DQ	- - Q -	- - - - - - - - - -	- - - - - - - - - - - - - - -	- - - -
400TI	- - - -	- - I - - - - - - -	- - - - - - - - - - - - - - -	- - - -
406GR	- - - -	- - - - - - - - R -	- - - - - - - - - - - - - - -	- - - -
440GS	- - - -	- - - - - - - - - -	- - S - - - - - - - - - - - -	- - - -
440GR	- - - -	- - - - - - - - - -	- - R - - - - - - - - - - - -	- - - -
443SA	- - - -	- - - - - - - - - -	- - - - - A - - - - - - - - -	- - - -
443ST	- - - -	- - - - - - - - - -	- - - - - T - - - - - - - - -	- - - -
444SG	- - - -	- - - - - - - - - -	- - - - - - G - - - - - - - -	- - - -
445DH	- - - -	- - - - - - - - - -	- - - - - - - H - - - - - - -	- - - -
445DE	- - - -	- - - - - - - - - -	- - - - - - - E - - - - - - -	- - - -
446DY	- - - -	- - - - - - - - - -	- - - - - - - - Y - - - - - -	- - - -
446DN	- - - -	- - - - - - - - - -	- - - - - - - - N - - - - - -	- - - -
446DE	- - - -	- - - - - - - - - -	- - - - - - - - E - - - - - -	- - - -
447FY	- - - -	- - - - - - - - - -	- - - - - - - - - Y - - - - -	- - - -
447FS	- - - -	- - - - - - - - - -	- - - - - - - - - S - - - - -	- - - -
449LP	- - - -	- - - - - - - - - -	- - - - - - - - - - - P - - -	- - - -
449LR	- - - -	- - - - - - - - - -	- - - - - - - - - - - R - - -	- - - -
451VA	- - - -	- - - - - - - - - -	- - - - - - - - - - - - - A -	- - - -
451VE	- - - -	- - - - - - - - - -	- - - - - - - - - - - - - E -	- - - -
481KQ	- - - -	- - - - - - - - - -	- - - - - - - - - - - - - - -	- - Q -

FIG. 4. Single-amino-acid mutations in the conserved motifs of influenza A/WSN/33 virus PB1. 305DQ represents the D-305→Q mutation, and so on. All mutations are underlined in the text.
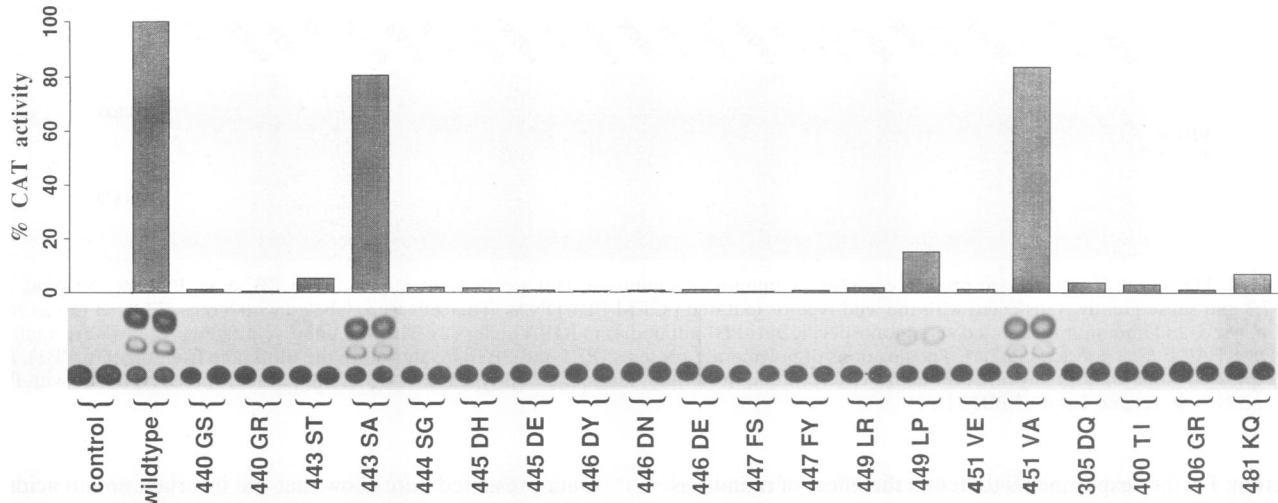
FIG. 5. Transcriptase-replicase activity of mutant PB1s. CV1 cells in 60-mm plates were infected with PB2-Vac, PA-Vac, NP-Vac, and VTF7.3 as described in the legend to Fig. 2. At 1 h after infection, cells were transfected with a mixture of 1 μg of IVACAT1 RNA, 2 μg of RNP, and 7.5 μg of the wild-type or different mutated pGEM PB1 DNA. At 16 h posttransfection, cells were lysed and assayed for CAT activity. The results were quantified as described in Materials and Methods. The bar diagram represents the percent CAT activity, using the activity of the wild-type pGEM PB1 DNA as 100%. Each experiment was done in duplicate. The labels under the lanes indicate the names of the mutants.

erable sequence conservation around the DD sequence. We therefore undertook two approaches for systematic mutational analysis of this region: (i) we introduced either the conserved or nonconserved amino acid changes in the flanking region of DD motif; and (ii) we introduced mutations that altered the PB1 sequence in such a way that it resembled the sequence of other viral RNA-dependent RNA polymerases. In total, we introduced 16 independent mutations in this motif (Fig. 4). These mutations were introduced by PCR as described in Materials and Methods, and their effects were analyzed by assaying CAT activity. Results (Fig. 5) show that DD was absolutely critical for influenza virus polymerase activity. When either of these DD residues were changed, D-445→H or E, or D-446→Y, N, or E, the mutated PB1 became essentially nonfunctional. It should be noted that in nonsegmented negative-strand RNA viruses, DD is replaced by DN but some activity is retained when DN of the vesicular stomatitis virus L protein is mutated to DD (42). When the S-444 was changed to G, making the sequence GDD as is found in positive-strand RNA viruses, polymerase activity was drastically reduced (1.9%), suggesting that influenza virus PB1 cannot accommodate G in this position even though all plus-strand and unsegmented negative-strand RNA viruses have a G in this position. The next preceding residue, S-443, could be mutated to A without affecting the activity whereas S→T greatly reduced the activity drastically.

When G-440 (5 amino acids upstream of the DD residues) was mutated to either S or R, PB1 was inactive (~1% of the wild-type activity), although some negative-strand RNA viruses (measles virus, tacaribe virus) possess S in this position (35). Downstream of DD, mutation of F-447 to either Y or S inactivated PB1, although positive-strand plant RNA viruses (e.g., tobacco mosaic virus, brome mosaic virus) possess S in this position (35). PB1 was inactive when L-449 was mutated to R but partially active (15%) when it was changed to P. Finally, when V-451 was changed to A, PB1 remained active but V-451→E rendered PB1 inactive.

The phenotypic behavior caused by the mutation in PB1 residues could be due to a number of factors. Therefore we

wanted to determine whether the mutated protein was expressed and stable in CV1 cells. The steady-state level of the proteins was examined by analyzing a fraction of the cell lysate used for CAT assay by SDS-PAGE and Western blot analysis with monospecific rabbit antibodies against PB1. The results show that equivalent amounts of the wild-type and mutated PB1 proteins were made in transfected cells (data not shown). Two other factors are important in influenza virus polymerase activity: (i) PB1 must form a complex with PB2 and PA, and (ii) PB1 should migrate into the nucleus, where transcription-replication of the influenza virus RNP takes place. Previously, we have shown that the bipartite nuclear localization sequence of PB1 is present within residues 180 to 217 (30), whereas the polymerase motifs are located between residues 298 and 485 (Fig. 3). Therefore mutations in the polymerase motif are unlikely to affect the nuclear localization of PB1. Furthermore, Western blot analysis of the nuclear extract showed that all PB1 proteins are transported into the nucleus (data not shown). To examine whether the polymerase complex formation was affected by the mutated PB1, cells expressing PA, PB2, NP, and the mutated PB1 proteins were pulse-labeled for 1 h and immunoprecipitated with anti-PB1 or anti-PB2 antibodies. As can be seen (Fig. 6), anti-PB1 antibodies coprecipitated PB2, indicating that polymerase complex formation did take place with all mutated PB1s. Association of PA appears to be less stable in the complex and often dissociates during immunoprecipitation (2). These results show that both the wild-type and mutated PB1 proteins were stable and formed a complex with other polymerase proteins. Similar results were obtained when immunoprecipitated with anti-PB2 antibody (data not shown). A second, smaller PB1 band was found in all lanes (Fig. 6). A similar band was observed in influenza virus-infected cells (1). The significance of the second band remains unknown at present. Since mutated PB1s were stable, migrated to the nucleus, and formed complexes with PB2 and PA, the phenotypic behavior of the mutants might be due to the difference in either recognizing and binding the vRNA (or vRNP) template or initiating and continuing the polymerase
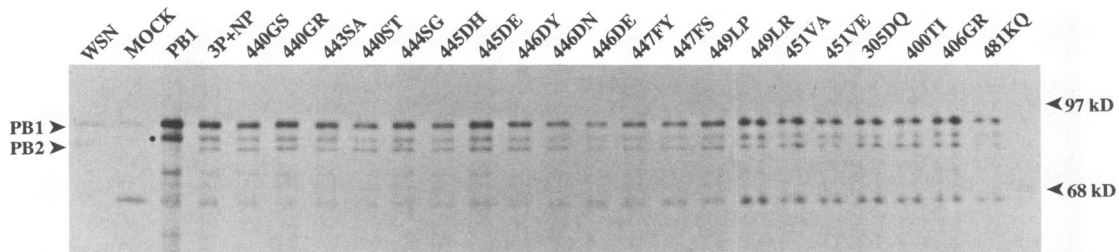
FIG. 6. Detection of polymerase protein complex by immunoprecipitation. HeLa cells were infected with PB2-Vac, PA-Vac, NP-Vac, and VTF7.3 and subsequently transfected with the wild-type or mutated pGEM PB1 DNAs. The cells were labeled with Trans-^{35}S-label (50 μCi/ml) for 1 h, lysed, and immunoprecipitated with monospecific anti-PB1 antibodies in RIPA buffer (2). The immunoprecipitated proteins were analyzed by SDS-8 PAGE (8% polyacrylamide). The positions of polymerase proteins (PB1 and PB2) are shown. In the third lane from the left (PB1), PB1 was expressed alone without any other influenza virus protein. In all other lanes, the wild-type (3P+NP) or mutant PB1 was coexpressed with PB2, PA, and NP. ●, second band of PB1 (1).

reaction. Further experiments to define the effect of mutations are in progress.

DISCUSSION

Genetic and biochemical studies strongly argue for the involvement of three polymerase proteins (PB1, PB2, and PA) in the transcription and replication of influenza virus RNA (23). However, the lack of a suitable reconstitution system has been the major block in defining the function of individual polymerase proteins in viral transcription-replication and delineating specific domains within the protein for specific functions. Using a vaccinia virus expression system and a reporter gene (CAT) containing the influenza virus promoter, Huang et al. (17) demonstrated that three polymerase proteins and NP were sufficient for transcription-replication of influenza virus RNA. In the present study, we have modified this system to reconstitute an active polymerase complex in vivo from individual components. In this system, the component to be tested is presented as plasmid DNA under the control of the T7 promoter and is introduced into cells by transfection. Thus it is possible to analyze many mutations rather quickly without preparing the recombinant vaccinia virus each time. The data presented in this paper show that the assay is consistently reproducible and that the CAT activity after transfection compares favorably with that obtained by using all recombinant vaccinia viruses.

Using this system, we have analyzed the functional motifs of PB1. Initially we have chosen PB1 for a number of reasons. First, PB1 appears to form the core catalytic component of the polymerase complex involved in chain elongation (8, 23). Second, and more important, comparative sequence analysis demonstrated the presence of four conserved motifs (the polymerase module) only in PB1 and not in PB2 or PA of influenza virus (35). Since no data demonstrating the structure-function relationship of PB1 were available, our first goal was to determine whether the conserved motifs were important in PB1 function and define their relationship to other known viral polymerases. Although conserved motifs have been observed among many RNA polymerases, including the reverse transcriptase of retroviruses and RNA-dependent RNA polymerases of double-stranded and positive-strand RNA viruses (naked or enveloped) and of negative-strand RNA viruses (segmented or nonsegmented), experimental data demonstrating the involvement of these motifs in polymerase activity are available for relatively few virus families. Furthermore, no consensus sequence except the four invariant amino acids (one in each motif) has been found (35).

Data presented here show that the invariant amino acids in motifs I, II, III, and IV are critical in PB1 function. For motif I, a similar conclusion was drawn for the human immunodeficiency virus reverse transcriptase from studies involving a D→E mutation (25) and by linker insertion, GDAYF→GD GIPYF (16). For motif II, some mutation, PQGW→GQGW (7), and linker insertion, PQGW→PPEFRW (16), in this region were lethal whereas other alterations, PQGW→ PNGW, could be tolerated (7). Similarly, inactivation of L-A virus (double-stranded RNA virus in yeasts) by mutation of G as well as other adjacent residues within this region also underscores the importance of motif II in polymerase activity (39).

Sequence comparison, structure analysis, and experimental data suggest that motif III forms the core of the transcriptase/replicase activity (3, 35). It has the invariant DD (or DN) sequence flanked by hydrophobic residues on either side and possesses a strand-loop-strand (the beta hairpin) structure with YGDD contained in the exposed loop (35). This motif is presumed to be involved in one or more functions of the active site such as metal binding, template binding, phosphate binding, or other catalytic functions (3, 20). The active sites of DNA polymerases of many human DNA viruses (YGDTD) also have a similar secondary structure and sequence and possess similar functions (3, 20).

Motif III of influenza virus PB1 including the flanking sequences of DD obeys the rules proposed for this region and possesses a similar secondary structure and flanking hydrophobic sequence (3, 35). However, this core also contains the signature sequence of individual RNA virus polymerases e.g., YMDD (retroviruses), YGDD (positive-strand RNA viruses), XGDD (double-stranded RNA viruses), QGDN (unsegmented negative-strand RNA viruses), and SSDD (influenza viruses). We therefore made a number of mutations in this core site to examine the sequence requirement of this motif. Overall our data support the conclusion obtained from studies with other viral polymerases. In particular, both DDs appear to be absolutely critical, because alteration of either D residue makes the protein inactive. A similar conclusion was obtained from mutation of DD in human immunodeficiency virus reverse transcriptase (7, 25, 36) and yeast L-A virus RNA polymerase (39). However, the second D is not invariant, since QGDN is found in the L protein of the unsegmented negative-strand RNA viruses (34, 35) and the protein remains moderately active after the mutation QGDN→QGDD (42). Mutation of the flanking position (SDD→GDD) abrogates polymerase activity, indicating that GDD, which is common in most viral polymerases, is not compatible with influenza virus

PB1. It should be noted that mutations of GDD→A/SDD and MDD→V/S/ADD could be partially tolerated in poliovirus (19) and retrovirus (47), respectively. Mutation of other upstream adjacent sequences shows that some alterations could be tolerated whereas others could not. For example, S-443→A did not affect PB1 activity but S→T reduced it considerably. A similar effect was observed when Y of YGDD was mutated in poliovirus (18). Downstream of DD, conservative changes (L-449→P or V-451→A) did not affect the PB1 activity greatly. A similar conclusion was reached after mutation of the L protein of vesicular stomatitis virus in this region (42). However, caution should be exercised in drawing conclusions from this kind of mutational analysis since changes must be conservative and multiple changes of the same residues should be examined. Alanine scanning has been proposed to be useful in making neutral changes without significantly affecting the structure (39). Such studies are under way to define the boundaries of the motifs. Furthermore, motifs I, II, III, and IV may work cooperatively, and the intermotif distances may also be critical for the correct folding and three-dimensional structure (35).

In conclusion, studies reported here have demonstrated that four conserved motifs, present in viral polymerases, are also important in influenza virus PB1 activity and that, of the signature sequence SSDD in motif III, SDD is invariant for influenza virus polymerase. Future studies will investigate the boundaries of each motif and the specificity of the sequence among influenza type A, B, and C, viruses.

ACKNOWLEDGMENTS

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