Two Aromatic Residues in the PB2 Subunit of Influenza A RNA Polymerase Are Crucial for Cap Binding*

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mRNAs are capped at their 5′-end by a unique cap structure containing N7-methyl guanine. Recognition of the cap structure is of paramount importance in some of the most central processes of gene expression as well as in some viral processes, such as priming of influenza virus transcription. The recent resolution of the structure of three evolutionary unrelated cap binding proteins, the vaccinia viral protein VP39, the eukaryotic translation factor eIF4E, and the nuclear cap-binding protein CBP20 showed that the recognition of the cap structure is achieved by the same general mechanism, i.e. by “sandwiching” of the N7-methyl guanine of the cap structure between two aromatic amino acid residues. The purpose of the present study was to test whether a similar cap recognition mechanism had independently evolved for the RNA polymerase of influenza virus. Combining in vivo and in vitro methods, we characterized two crucial aromatic amino acids, Phe363 and Phe404, in the PB2 subunit of the viral RNA polymerase that are essential for cap binding. The aromaticity of these two residues is conserved in influenza A, B, and C and even in the divergent Thogoto virus PB2 subunits. Thus, our results favor a similar mechanism of cap binding by the influenza RNA polymerase as in the evolutionary unrelated VP39, eIF4E, and CBP20.

Eukaryotic RNA polymerase II transcripts (mRNAs and small nuclear RNAs) are modified at their 5′ extremity by the addition of an N7-methyl guanine cap (m7G)3′ in a 5′-5′ triphosphate linkage. The function of such structures is to protect mRNAs from degradation by 5′-exonucleases (1). Furthermore, cap structures are recognized by specific proteins that are able to distinguish N7-methyl from nonmethylated guanines (2). This is of paramount importance in some of the most central processes for the regulation of gene expression in all eukaryotic organisms including pre-mRNA splicing (3), nuclear export (4), mRNA 3′-end formation (5), and mRNA translation (6). Cap binding is also crucial for some viral functions. Thus, ribose 2′-O-methylation of vaccinia viral mRNAs depends on cap recognition (7). A remarkable example of host dependence is the requirement of a host cap structure for influenza virus transcription (8).

Considerable insight into the mechanism of cap recognition has arisen from a broad set of biochemical and crystallographic studies of three proteins of evolutionary unrelated origin: eIF4E (a eukaryotic cellular factor involved in the initiation of translation), VP39 (a vaccinia virus protein involved in 2′-O-methylation of viral mRNAs), and CBP20 (a eukaryotic nuclear factor involved in mRNA transport in the nucleus) (9–13) (for review, see Ref. 2). In all three cases, the recognition of the cap structure is achieved by the same general mechanism, i.e. by “sandwiching” the N7-methyl guanine between two aromatic amino acid residues (phenylalanine, tyrosine, or tryptophan). This suggests that only one mechanism has evolved to specifically recognize N7-methyl guanine in cap structures. The purpose of this study was to test the hypothesis that a similar cap recognition mechanism had independently evolved for the RNA-dependent RNA polymerase of influenza virus.

Influenza virus RNA polymerase cap recognition is performed by the PB2 subunit of the RNA polymerase (14, 15), yet has specific features distinguishing it from cap recognition by eIF4E, VP39, or CBP20. First, influenza RNA polymerase cap recognition depends on the addition of the viral RNA (vRNA) promoter (16, 17). Without vRNA, no cap recognition occurs. This suggests that a conformational change is required prior to cap binding. Second, the affinity of the binding of the influenza polymerase for cap structures is significantly weaker (at least 100-fold) than that of eIF4E (18, 19), which is similar to that of CBC (the cap binding complex involving CBP20 and CBP80) (20). Thus, it remains possible that alternative cap recognition strategies might have evolved for this unique example of cap recognition by the influenza RNA polymerase. Whether a related “sandwich” recognition or an unrelated mechanism has evolved in the case of the influenza viral RNA polymerase, the cap recognition machinery is of great interest, since if molecular details were known, this would provide a good target for novel specific antivirals to potentially control influenza in the future.

Influenza A virus is a negative strand RNA virus containing eight segments of single-stranded RNA as its genome. The RNA genome is transcribed and replicated in the cell nucleus by the viral RNA-dependent RNA polymerase (for reviews, see Refs. 21–23). The viral polymerase is a heterotrimer composed of three subunits PB1, PB2, and PA. The PB1 subunit plays a central role in both RNA polymerase assembly and RNA polymerization (24–26). The function of the PA subunit remains largely unknown, but mutations in PA have been shown to affect replication (27) and more recently endonuclease activities (28) and elongation (47). The PB2 subunit plays a crucial role in mRNA transcription. PB2 is responsible for recognition of host cap structure.
and binding of the cap structure of host mRNAs (14, 15), which, following endonuclease cleavage 9–17 nucleotides downstream of the cap (29), serve as primers for mRNA synthesis (30, 31). Previous attempts to localize regions of PB2 responsible for cap binding have produced contradictory results. Cap binding was localized to amino acids 533–564 in one cross-linking study (26), but an earlier study also proposed that, in addition, the region 242–252 of PB2 was involved (32). Unfortunately, PB2 shows no significant sequence similarity with the previously crystallized cap-binding proteins (eIF4E, VP39, and CBP20); therefore, amino acid homology searches give no clue as to the regions of PB2 involved in cap recognition. Isolation of PB2 variants of PB2 involved in cap binding, we based our work on the hypothesis that PB2 has evolved a similar cap binding mechanism to eIF4E, VP39, and CBP20. Our approach consisted of a large scale mutagenesis of all aromatic amino acid residues of PB2, that were evolutionary conserved between influenza type A, B, and C and Thogoto virus. This approach was made possible because of an easy method of preparing partially purified recombinant influenza RNA polymerase from human 293T cells (28, 33). We showed that the mutation of just 2 (Phe363 and Phe404) of the 27 evolutionary conserved aromatic residues drastically and specifically inhibit the cap binding activity of PB2. These two residues are present at different positions of PB2 from the positions previously implicated in cap binding using the cross-linking approach (26, 32). Thus, our data suggest that influenza RNA polymerase has evolved a similar general cap binding mechanism to eIF4E, VP39, and CBP20.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids used in this study (pcDNA plasmids encoding the PB1, PB2, PA, PA-His6, or NP proteins; pPOLI plasmids encoding the eight influenza vRNAs; and pPOLI-CAT-RT plasmid) have been described previously (28, 34, 35). Modifications of the PB2 plasmids were prepared by site-directed mutagenesis. PB2 mutant genes have been fully sequenced by standard methods. Details of the mutagenesis strategy and the sequence of mutagenic primers are available on request.

Preparation of Capped RNA for Binding Studies and Endonuclease Assay—A 2′-O-methylated oligoribonucleotide (5′-AmAAUACUAAGC-3′) was chemically synthesized and 5′-diphosphorylated as described (36). For binding studies, the 5′-diphosphorylated oligoribonucleotide was enzymatically capped using [α-32P]GTP (>3000 Ci/mmol) (Amersham Biosciences), as described before (28). For endonuclease assays, the 2′-phospho-capped labeled RNA oligonucleotide was polyadenylated and oligonucleotides with adenosines added at their 3′-end were isolated by oligo(dT)-cellulose chromatography (28). The 5-nucleotide capped capped RNA [α-32P]GpppAmAAU was derived from [α-32P]GpppAmAAUAACAG by RNase A digestion (75 ng/10 μl). RNase A was added immediately after capping, for 30 min at 37 °C, but before purification by 18% PAGE in 7 μl urea.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assay—DNA transfections were performed in 293T cells as described previously (28). Cells were harvested 24 h post-transfection, and cell lysates were used in the CAT assays (28). Quantification was performed visually by comparing activities of serially diluted samples.

Isolation of Partially Purified His-tagged Recombinant Influenza RNA Polymerase (modified from Refs. 28 and 33)—Human kidney 293T cells in suspension in 15-cm dishes (about 1 × 107 cells) were transfected with 20 μg of each of pcDNA-PB1, pcDNA-PB2, and pcDNA-PA-His6 using 90 μl of LipofectAMINE 2000 transfection reagent (Invitrogen) in 36 ml of minimum essential medium containing 10% fetal calf serum and 2 mM l-glutamine. About 48 h post-transfection, cells from one or two dishes were pooled and processed as described previously (28) but with modifications to the lysis buffer (50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 25% glycerol, 0.5% Nonidet P-40, 1 mM β-mercaptoethanol, 1 mM pepstatin (Sigma), 2.5 μg/ml leupeptin (Sigma), and one complete mini-EDTA-free protease inhibitor mixture tablet (Roche Applied Science) per 10 ml). Proteins were eluted from Ni2+–nitrilotriacetic acid-garosor (Qiagen) in 50 μl of elution buffer (50 mM sodium phosphate (pH 8.0), 200 mM NaCl, 10% glycerol, 50 mM imidazole), and 40 μl of glycerol was then added before freezing to −20 °C.

Western Blot Analysis of Recombinant PB2 Proteins—5 μl of cell lysates (from CAT experiments) or 5 μl of purified His-tagged polymerase preparations were analyzed on 8% SDS-PAGE. Proteins were electrophoresed to Hybond-C nitrocellulose membranes (Amersham Biosciences) and probed with a polyclonal anti-PB2 antibody (24). The Western blots performed on His-tagged polymerase preparations allowed us to correct results in subsequent assays for differences in the yield of polymerase antigen obtained with the different PB2 mutants.

Transcription Assays in Vitro—Transcription reactions were performed, essentially as previously described (33), in a total reaction volume of 3 μl containing 1.5 μl of polymerase preparation, 5 mM MgCl2, 2 mM dithiothreitol, 4 units of RNasin (Promega), 1 μg of E. coli tRNA, 1 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 mM GTP, 0.15 μM [α-32P]GTP (>3000 Ci/mmol) (Amersham Biosciences), 4 pmol of 3′-end vRNA promoter (5′-GGCCUGCUUUUGCU-3′) (Dharmanax), 4 pmol of 5′-end vRNA promoter (5′-AGUAGAAAAGGC-3′) (Dharmanax), and 1 mM APg (Sigma). In the globin mRNA–primed transcription assays, the APg was replaced with 0.05 μg of globin mRNA (Invitrogen). In the capped RNA–primed transcription assays, the APg was replaced with a 2′-phospho-labeled 12-nt capped RNA (about 5000 cpm of [α-32P]GpppAmAAUAACAG; see above), and 0.5 mM GTP was used instead of [α-32P]GTP. Transcription reactions were performed at 30 °C for 1 h and analyzed by 18% PAGE in 7 μl urea.

Endonuclease Assays—Endonuclease assays were performed essentially as described (28), in a total reaction volume of 3 μl containing 2 μl of His-tagged polymerase, 32P-capped and polyA-tailed RNA substrate [α-32P]GpppAmAAUAACAGA, (about 5000 cpm; see above), 2 pmol of 5′-end vRNA promoter and 3′-end vRNA promoter (as above), 1 μg of E. coli tRNA, 5 mM MgCl2, 1 mM dithiothreitol, and 40 units of RNasin (Promega). Endonuclease reactions were performed at 30 °C for 30 min and analyzed by 18% PAGE in 7 μl urea.

Cap-binding Assay—A UV-cross-linking assay, modified from Ref. 37, was used to test the cap-binding activity of recombinant His-tagged RNA polymerases. The cross-linking mixture (30 μl) contained 20 μl of partially purified His-tagged polymerase, 1 pmol of 5′-end vRNA promoter, 4 pmol of 3′-end vRNA promoter, 4 μg of Poly(A)-rich tRNA, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 0.1 mM [α-32P]GTP, 0.15 mM MgCl2, 4 mM dithiothreitol, and 10 units of RNase (Promega). Endonuclease reactions were performed at 30 °C for 15 min and transferred into a U-bottom 96-well plate and UV-irradiated on ice for 30 min in a UV Stratalinker (Stratagene) equipped with GST5 bulbs (254 nm). The cross-linked products were analyzed on an 8% SDS-PAGE and detected by autoradiography. To determine which polymerase substrates were crosslinked to the capped RNA, cross-linked complexes were disrupted by 0.1% SDS treatment, and immunoprecipitations were performed with polyclonal anti-PB1, anti-PB2, and anti-PA antibodies, as described previously (37).

Analysis of cRNA, mRNA, and cRNA by Primer Extension Assay and Viral Rescue—Human kidney 293T cells in 35-mm dishes were transfected as for the CAT assays (see above) and harvested 48 h post-transfection, and total RNA was isolated using TRIzol Reagent (Life Technologies, Inc.). Primer extension assays were performed as described (38). Influenza viruses were rescued using the method described in Refs. 34 and 39, recently modified (28).

RESULTS

Selection of Influenza PB2 Mutants—The conservation of the cap binding structural motif (2 aromatic residues) in the structures available (VP39, eIF4E, and CBP20) suggested that this motif might have evolved in the PB2 subunit of the viral polymerase from different types of influenza virus. An alignment of PB2 subunits of influenza A, B, and C viral types and also of the less related PB2 of Thogoto virus was performed (Fig. 1). All amino acids whose aromatic property was conserved between the four strains were selected for mutational analysis (11 mutants at positions 34, 49, 55, 98, 99, 130, 135, 217, 240, 404, and 572). This selection was further extended to aromatic residues that were not strictly aligned (five mutants at positions 115, 168, 363, 446, and 694) and also to aromatic residues whose aromatic property was not conserved in the divergent
To test the function of the PB2 mutants, we initially used an in vivo transcription/replication assay that is based on in vivo reconstitution of PB2 into viral ribonucleoprotein complexes and the expression of a CAT reporter gene. Mutant PB2 proteins were co-expressed in 293T cells with influenza PB1, PA, and NP proteins (the minimal set of influenza viral proteins required for transcription and replication of vRNA) and a vRNA-like CAT RNA, encoding a negative-sense CAT reporter gene (see "Experimental Procedures"). Cell extracts were tested for CAT activity 24 h post-transfection (Table I).

Eight positions (Tyr34, Phe168, Phe325, Phe363, Phe404, Phe446, Tyr531, and Tyr550) were initially selected for further studies, since nonconservative mutants at these positions lead to <1% of CAT activity, whereas conservative mutants retained full CAT activity.

At 11 other positions, alanine mutants had 1% CAT activity, whereas conservative mutants showed either partial activity (10%) (F119W, F217W, W240F, Y572W, and F656W), or minimal activity (1%) (F130W, F135W, W537F, Y592W, F621W, and F633W). These positions, nine of which involved the introduction of a tryptophan residue, remained potential candidates and were also selected for further analysis (see below and Fig. 2C). Additionally, all alanine mutants were tested in a primer extension analysis as a further check that
positions involved in cap binding had not been excluded (see below and Fig. 5B).

Eight other positions were no longer considered as candidates, since their alanine mutants showed partial (W49A, W98A, W99A, Y115A, and Y658A) or full activity (Y55A, W552A, and Y694A), reflecting a partial or full cap binding activity.

ApG-primed Transcriptional Activity in Vitro—The eight initially selected pairs of mutants (at positions Tyr34, Phe168, Phe225, Phe363, Phe404, Phe446, Tyr531, and Tyr550) were tested in ApG-primed transcription using the His-tagged polymerase preparations, partially purified by nickel affinity chromatography (see “Experimental Procedures”). Previous work showed that transcription by influenza RNA polymerase could be primed in vitro by the addition of ApG, thus bypassing the requirement for cap binding (for reviews, see Refs. 21–23). Therefore, mutants active in ApG-primed transcription would remain candidates for cap binding, whereas mutants defective in ApG-primed transcription could be discarded, because they must have affected functions other than cap-dependent transcription. Four mutant pairs, corresponding to amino acids Phe603, Phe404, Phe446, and Tyr531 (Fig. 2, A and B), remained candidates, since they retained a high level of ApG-primed transcription activity, whether the mutation was conservative or not. On the other hand, four alanine mutants, corresponding to positions Tyr34, Phe168, Phe225, and Tyr550, were discarded under this criterion, since the alanine mutations led to weak ApG-primed transcription (Fig. 2, A and B).

At the additional 11 positions (Phe119, Phe130, Phe135, Phe177, Trp182, Trp203, Trp217, Tyr372, Tyr485, Phe633, Phe635, and Phe656), we selected only alanine mutants to check whether these mutants were negative in ApG-primed transcription, because this would exclude these positions as candidates. None of them remained candidates, since they all showed weak (<20%) ApG-primed transcription activity (Fig. 2C). It was unnecessary to test the conservative mutants at these 11 positions, because the results, whether positive or negative, would not be diagnostic. However, a further analysis was performed later using primer extension as a final check (see below and Fig. 5B).

Transcription Primed by a 12-Nucleotide Capped RNA or by Globin mRNA in Vitro—Transcription primed by a 12-nt capped RNA depends on the capacity of the polymerase to bind capped RNA. Therefore, we tested the four remaining candidate pairs of PB2 mutants in transcription primed with a capped RNA (Fig. 3A). Conservative as well as alanine mutants corresponding to positions Phe146 and Tyr331 were as active in transcription primed with a 12-nt capped RNA as with ApG (Fig. 2A), suggesting that these amino acids were not participating in the specific cap-binding process. The mutants corresponding to the two other amino acids at positions 363 and 404 remained candidates, since the alanine mutants showed a decreased capacity to transcribe RNAs primed by a 12-nt capped RNA but still retained high ApG-primed transcription activity, whereas the conservative mutants, F363Y and F404Y, retained both activities (Fig. 3D). Thus, Phe363 and Phe404 are the two remaining candidates.

We then performed globin mRNA-primed transcription on mutants at these two remaining positions. Both mutants F363A and F404A were unable to mediate globin mRNA-primed transcription (Fig. 3B). Mutants at position Phe146 were also tested as positive controls. This confirmed the importance of positions 363 and 404.

Endonuclease Activity in Vitro—An endonuclease assay (see “Experimental Procedures”) was used to confirm these results. The endonuclease capacity of the polymerase depends on its capacity to bind a capped RNA. The results expected from these assays would be the same as in transcription primed by 12-nt capped RNA and globin mRNA, since for these three assays the cap-binding capacity was a common limiting step. Fig. 3, C and D, confirmed that mutants at positions 363 and 404 gave the expected results in the endonuclease assays (i.e., F363A and F404A have significantly reduced endonuclease activity, whereas F363Y and F404Y show nearly wild-type activity).

Cross-linking Experiments in Vitro—As a final confirmation of the specific importance of the two candidate amino acids,
Phe³⁶³ and Phe⁴⁰⁴, in cap-binding, the ability of the two pairs of mutants to bind a capped RNA in a competitive UV-cross-linking assay was tested. Two bands were observed at about 90 kDa on SDS-PAGE upon cross-linking the³²P-labeled 12-nt long capped RNA to the wild-type polymerase preparation (Fig. 4A, lane 1). Immunoprecipitation of the cross-linked RNA polymerase confirmed that the band with lower mobility corresponded to PB1, and the band with higher mobility corresponded to PB2 (Fig. 4A, lanes 2 and 3). As expected, no signal was obtained after immunoprecipitation with an anti-PA antibody (26). To avoid background binding of the capped RNA by both PB1 and PB2, a cross-linking experiment was set up with a³²P-labeled 5-nt capped RNA, m ⁷GpppAmAAU (see “Experimental Procedures”). Immunoprecipitation of the cross-linked RNA polymerase subunits revealed that the short capped RNA was bound only to PB2 (Fig. 4A, lane 7). This short capped RNA was used in competitive cross-linking experiments with an unlabeled cap dinucleotide analogue, m7GpppG. As shown in Fig. 4B, the addition of 0.05 and 0.5 mM concentrations of competitor m7GpppG to the cross-linking mediated by wild-type polymerase led to a 60 and 80% decrease of the cross-linked PB2 signal, respectively (Fig. 4G). On the other hand, both mutants F363A and F404A mediated very low, nonspecific binding (<5% of wild type), which was not competed out with m⁷GpppG (Fig. 4, C and E). As expected, if Phe³⁶³ and Phe⁴⁰⁴ were the correct candidates, the two conservative mutants, F363Y and F404Y, led to efficient binding that was competed specifically by m⁷GpppG (Fig. 4, D and F).

Synthesis of mRNA, cRNA, and vRNA in Vivo by Polymerases with Mutant PB2 Subunits—A recently developed primer extension assay (see “Experimental Procedures”) allowed the transcription/replication of mutants of the two remaining PB2 candidates to be assessed in vitro as further confirmation of the in vitro results (Fig. 5A). The alanine mutants, F363A and F404A, showed mRNA levels less than 5% of wild type, while retaining significant replication activity (Fig. 5). The conservative mutations, F363Y and F404Y, retained near wild-type mRNA levels. Tryptophan mutations at positions 363 and 404 were also assayed to check whether other aromatic amino acids could replace F363 and F404. The mutant F363W showed near wild-type levels, but the mutant F404W showed very low mRNA levels (Fig. 5A). Thus, the introduction of a tryptophan residue can also lead to a loss of cap binding activity, possibly because tryptophan is bulkier than either phenylalanine or tyrosine, and may be sterically hindered from forming a functional aromatic sandwich. Because of this result, we had to consider the possibility that we had omitted potentially interesting candidate mutants at positions in which an alanine mutation led to <1% of CAT activity and where a conservative mutation also significantly impaired CAT activity (Table I). Therefore, we tested all 25 other alanine mutants (Table I) in primer extension (Fig. 5B).

Eight mutants (W49A, Y55A, W98A, Y115A, W552A, F656A, Y658A, and F694A) showed nearly wild-type levels of mRNA, cRNA, and vRNA. Two mutants (Y34A and W99A) showed low levels of cRNA but still significant mRNA levels. Since all of these alanine mutants led to significant mRNA levels, it can be assumed that these 10 aromatic amino acids are not directly involved in cap binding.

Two other alanine mutants (F446A and Y531A) showed re-
Importance of the Aromatic Ring of Phe\textsuperscript{363} and Phe\textsuperscript{404}—Here we have shown that both Phe\textsuperscript{363} and Phe\textsuperscript{404} are important for cap binding. Further evidence for aromaticity can, in theory, be obtained by studying leucine mutants, since leucine is considered to be partially isosteric with phenylalanine. We therefore constructed F363L and F404L (see “Experimental Procedures”). These two mutants generate &lt;1% CAT activity (Table I). Further, when rescued into recombinant virus, F363L and F404L produced only pinhead-sized plaques on MDBK cells. Thus, the aromatic ring of Phe\textsuperscript{363} and Phe\textsuperscript{404} is important for the function of these amino acids and cannot be replaced by leucine.

**DISCUSSION**

We tested, in this paper, the hypothesis that cap recognition by the PB2 subunit of the influenza RNA polymerase involved an aromatic sandwich. This idea has gained in strength, since a similar sandwiching cap binding motif has recently been characterized from crystallographic studies of three evolutionary unrelated proteins, eIF4E, VP39, and CBP20 (9–13). Nevertheless, influenza virus RNA polymerase cap recognition mechanism has specific features that differ from the previously characterized recognition by eIF4E, VP39, and CBP20. Thus, it remains possible that influenza RNA polymerase had developed an alternative cap recognition strategy. This question was addressed here by a large scale mutagenesis study of the evolutionary conserved aromatic amino acids of the PB2 subunit of the RNA polymerase, since PB2 has been shown in many studies to be responsible for the specific recognition of host mRNA cap structures (for reviews, see Refs. 21–23). The selected aromatic amino acids were mutated into a conservative and a nonconservative (alanine) mutation. This was based on the assumption that only alanine mutants of aromatic residues directly involved in the aromatic sandwich would affect cap binding, whereas the corresponding conservative mutants would retain all or part of their cap binding activity.

Two crucial assays allowed us to identify two amino acids involved in capped RNA recognition: the ApG-primed and capped RNA-primed transcription assays. The specific involvement of these two residues in cap structure recognition was confirmed in vitro by competitive binding experiments with a
cap dinucleotide analogue (Fig. 4, B–F). Furthermore, these results were strengthened by an in vivo analysis of the ability of these mutants to transcribe and replicate a vRNA-like CAT reporter RNA (Fig. 5). We also confirmed by primer extension, performed on all of the 27 selected mutants, that no conserved PB2 aromatic residues other than Phe363 and Phe404 were specifically involved in mRNA synthesis. The fact that we could rescue influenza virus with the mutations F363Y, F404Y, and the double mutation F363Y/F404Y in the PB2 subunit, whereas their respective alanine mutants F363A and F404A either could not be rescued or produced only pinhead-sized plaques, was consistent with our hypothesis that these residues are directly involved in cap binding.

As a final check on the importance of Phe363 and Phe404 in cap binding, we have mutated them into leucine, since some nucleotide-binding proteins (e.g. DNA ligases and RNA guanylyltransferases) (41, 42) have a hydrophobic leucine residue in their binding pocket. Thus, leucine could, conceivably, substitute for phenylalanine in an aromatic sandwich of PB2. However, we found that these mutants could not restore, even partially, the activity of the polymerase, showing the importance of the aromatic ring for the function of Phe363 and Phe404. It should also be noted that both Phe363 and Phe404 are absolutely conserved in the PB2 sequence of influenza A, B, and C, whereas Thogoto virus PB2 has tyrosine at these two positions (Fig. 1). Thus, it is likely that a similar cap binding mechanism has independently evolved in eIF4E, VP39, CBP20, and the PB2 subunit of influenza virus.

A detailed analysis of the behavior of mutants of these two aromatic residues showed that they did not affect cap binding in an identical manner. F363A does not affect cap binding, as assayed by endonuclease activity and transcription primed by capped primers, to the same extent as F404A in vitro (Fig. 3). On the other hand, the conservative mutation, F404W, diminished the cap binding activity extensively, as seen in primer extension (Fig. 5A). F404Y showed an intermediate pattern between wild type and F404A, although F363W and F363Y retained almost wild-type activity (Fig. 5A). Such asymmetrical behavior of the two aromatic residues implied in capped RNA binding has recently been observed in studies of another cap binding enzyme, VP39 (11, 40). Indeed, the alanine mutation of the first aromatic residue of VP39 (Tyr22) did not abolish cap binding (11), whereas the tryptophan mutation of the second aromatic residue (Phe180) led to a much greater loss of enzymatic activity (40). Such asymmetry could be linked to the lower affinity of VP39 for m7GTP compared with that of eIF4E and CBC (19, 20, 40). This more labile interaction between VP39 and a capped substrate may be essential to an inherent turnover requirement, VP39 being an enzyme (11, 40). In influenza virus, the turnover requirement for the polymerization activity might also be reflected in the low affinity of influenza polymerase for cap structures (when compared with that of eIF4E and CBP20) (18–20). Such low affinity is consistent with our data showing that two phenylalanine residues are crucial for cap binding because previous studies of VP39 and eIF4E showed that tryptophan was usually preferred to tyrosine (which was preferred to phenylalanine) for efficient cap binding (40, 43, 44). The presence of tyrosine residues at positions equivalent to 363 and 404 in Thogoto virus PB2, we speculate, might lead to higher affinity of the polymerase for cap structure. More efficient binding of the polymerase to cap structures might be required in the case of Thogoto virus to compensate...
for the fact that endonuclease cleavage occurs much closer to the cap structure in Thogoto virus than in influenza viruses (45).

The two aromatic residues characterized in this study, Phe363 and Phe404, are localized centrally in the PB2 subunit. This contradicts previous data in which two regions located closer to the N terminus (residues 242–252) (32) or to the C terminus (residues 538–577 or 533–564) (26, 32) were reported to be involved in cap binding, based on cross-linking results. In our studies, none of the alanine mutants of either a conserved amino acid (at position 572) or semiconserved aromatic amino acids (at positions 537, 550, and 552) within these previously proposed regions were specifically defective in cap binding. Thus, we speculate that these previously characterized regions may be involved in cap binding in an indirect way, possibly by binding to structures adjacent to the m'G of the cap structure, e.g., to the 2'-O-methyl group of the residue adjacent to the m'G or to the triphosphate linkage of the cap structure or to phosphate backbone residues of the RNA primer. Taking the mutagenesis reported here together with the previous cross-linking results, we suggest that the cap binding region is localized to a central region of PB2, with residues Phe363 and Phe404 forming the sandwich motif.

In summary, our results favor the direct involvement of Phe363 and Phe404 in binding the m'G of the cap structure in an aromatic sandwich. It is also known that another acidic residue, Asp364, is immediately adjacent to Glu362, which is needed to neutralize the positive charge of m7G of the cap structure (33). It is also known that another acidic residue, Asp364, is immediately adjacent to Glu362, which is needed to neutralize the positive charge of m7G of the cap structure. It is also known that another acidic residue, Asp364, is immediately adjacent to Glu362, which is needed to neutralize the positive charge of m7G of the cap structure. It is also known that another acidic residue, Asp364, is immediately adjacent to Glu362, which is needed to neutralize the positive charge of m7G of the cap structure. It is also known that another acidic residue, Asp364, is immediately adjacent to Glu362, which is needed to neutralize the positive charge of m7G of the cap structure.
