Structure−Function Relationship of the Influenza Virus RNA Polymerase: Primer-Binding Site on the PB1 Subunit†

Dmitry M. Kolpashchikov,‡,|| Ayae Honda,§,⊥ and Akira Ishihama*,‡,§

Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan, Division of Molecular Biology, Nippon Institute for Biological Science, Ome, Tokyo 198-0024, Japan, and Nihon University School of Medicine, Itabashi 173−8610, Tokyo, Japan

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ABSTRACT: Influenza virus RNA polymerase is composed of three viral P proteins (PB1, PB2, and PA) and involved in both transcription and replication of the viral RNA genome. The catalytic site for RNA polymerization is located on the PB1 subunit. To identify the primer ATP-binding site, we have employed a highly selective cross-linking technique: three structurally diverse ATP analogues with reactive groups on their phosphate moieties were first cross-linked to the viral RNA polymerase, and the cross-linked nucleotides were then elongated to dinucleotides by adding the second substrate [α-^{32}P]GTP. Only the ATP analogues tethered to the primer-binding site could be elongated to radioactive AG dinucleotides. Using this catalytically competent cross-linking procedure, the PB1 subunit was found to be the only labeled subunit. Limited proteolysis of the labeled PB1 by V8 protease revealed the segment between amino acids 179 and 297 as the only bearer of the radioactive label. Thus, we concluded that this region of PB1 faces the 5′ end of the primer nucleotide. In support of this prediction, the cross-linked dinucleotides were further elongated up to 8−10 nucleotides in length upon addition of all four substrates. This result suggests that the substantial mass of RNA can be accommodated between the binding site for the primer (and nascent RNA) and the catalytic center of RNA polymerization.

Influenza virus RNA polymerase plays an essential role in both transcription and replication of the viral genome [for reviews, see refs *1*, *2*]. The RNA polymerase core enzyme is composed of three viral proteins, PB1, PB2, and PA, which are encoded, in the case of PR8 strain, by the genome RNA segment 2, 1, and 3, respectively (*3*). Transcription is initiated by using capped oligonucleotide primers, which are generated by cleavage of host-cell capped RNA with RNA polymerase-associated endonuclease activity (*4*). In replication, however, the same RNA polymerase core enzyme is involved in both viral RNA (vRNA)-directed[1] synthesis of complementary RNA (cRNA) [first-step reaction of replication] and cRNA-directed synthesis of vRNA [second-step reaction]. ATP serves as a primer for initiation of both vRNA- and cRNA-dependent RNA synthesis (*5*).

The PB1 subunit forms the catalytic center of RNA polymerization (*1*, *2*) and thus can be cross-linked to the nucleoside 5′-triphosphate substrate (*6*, *7*). PB1 contains four conserved sequence motifs, A−D, present in vRNA-dependent RNA polymerases (*8*), and mutation of the conserved residues abolishes the transcription activity (*9*). In fact, extracts from recombinant baculovirus-infected cells expressing PB1 protein show, albeit at low levels, vRNA-directed primer-dependent RNA synthesis activity in vitro (*10*). Moreover, a reporter RNA with vRNA promoter can be transcribed into cRNA in mouse cells expressing PB1 alone (*11*). Recently, Li et al. (*12*) reported that the catalytic center for capped RNA cleavage is also located on the PB1 subunit, even though the RNA cap is recognized by and can be cross-linked to the PB2 subunit (*13*).

The location of the putative nucleotide binding domains of the RNA polymerase has been mapped after cross-linking with 8-N_3-GTP and oxidized GTP (*14*). The amino-terminal proximal binding site (residues 179−358) is located near the motif A of RNA polymerase PB1 subunit (full length, 757 residues), while the carboxy-terminal proximal binding site (residues 458−519) is located in a fragment that contains the RNA-dependent RNA polymerase motif D including the Asp445-Asp446 sequence. One possible explanation for the presence of two GTP-binding sites is that these two fragments form a single GTP-binding site on the tertiary structure. Alternatively, two nucleotide binding sites exist in the PB1 protein, one for the substrate binding and other for the primer (or initiation nucleotide) binding. To localize the primer-binding site of RNA polymerase we have applied, in the

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* To whom correspondence should be addressed. Tel: +81-428-33-1071. Fax: +81-428-33-1072. E-mail: aishiham@nibs.or.jp.

‡ National Institute of Genetics.

§ Nippon Institute for Biological Science.

|| Present address: Department of Medicine, Columbia University, 650 W. 168 St., New York, NY 10032.

⊥ Nihon University School of Medicine.

[1] Abbreviations: *o*F-AMP, *o*-formylphenyladenosine-5′-monophosphate; *p*F-ATP, *p*-formylphenyladenosine-5′-triphosphate; ATmP, adenosine 5′-trimetaphosphate; cRNA, complementary RNA; vRNA, viral RNA; RNP, ribonucleoprotein; CBB, Commassie brilliant blue; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

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present study, the method of highly selective labeling of the primer-binding site on RNA polymerase. This method, called "catalytically competent labeling", was originally developed for the study of the functional topography of *Escherichia coli* RNA polymerase (*15*). Briefly, the RNA polymerase bound to a promoter is treated with a chemically reactive analogue of the primer nucleotide. If the nucleotide analogues are recognized as the substrates, they can be cross-linked to the enzyme at the primer (or initiation substrate)-binding site as well as at the substrate-binding site. The cross-linked primer substrates can be elongated to form radio-labeled dinucleotides by adding the second radioactive substrate. Nucleotide analogues cross-linked outside the catalytic center cannot be elongated after addition of labeled substrates and therefore remain invisible during subsequent analysis.

Using the catalytically competent labeling procedure, we have succeeded in radio-labeling the primer (or initiation nucleotide)-binding site on the PB1 subunit and identifying that the radioactive dinucleotides are tethered within the fragment 179−297 of PB1. We then conclude that the N-terminal proximal nucleotide-binding site is the primer-binding site of the influenza virus RNA polymerase.

MATERIALS AND METHODS

Chemicals and Enzymes. *o*-Formylphenyl ester of AMP (*o*F-AMP) and *p*-formylphenyl ester of ATP (*p*F-ATP) were synthesized according to the published procedures (*15*, *16*). ATmP was synthesized as described by Knorre et al. (*17*). Influenza virus ribonucleoprotein core (RNP) was prepared according to the standard procedure (*3*). Expression of PB1 in *E. coli*, its purification from the gel, and V8 digestion were carried out according to ref *14*. V8 protease was a product of Pierce. T7 RNA polymerase was from TAKARA, Japan. All other chemicals used in this study were commercially available products of the highest quality.

Catalytically Competent Labeling of RNP. Reaction mixtures (10 μL) contained the standard components [50 mM HEPES at pH 7.8, 100 mM NaCl, 5 mM magnesium acetate, and 1 mM dithiothreitol (DTT)] and 0.25 mM each of the nucleotide analogues. After addition of 1 μL of influenza virus RNP (5 mg/mL), the mixtures were incubated for 5 min at 30 °C in the case of *o*F-AMP and *p*F-ATP or for 20 min at 25 °C in the case of ATmP. For cross-linking, the reaction mixtures were treated with 1 μL of 0.1 M $NaBH_4$ for 20 min on ice (*18*). When ATmP was used, this treatment was omitted. After addition of 2 μL (10 μCi) of 20 μM [α-^{32}P]GTP, the incubation was continued for 30 min at 30 °C. The samples were subjected to SDS−PAGE on a 9% PAG according to Laemmli (*19*). The dried gel was exposed to an imaging plate. The plates were analyzed with a BAS2000 image analyzer (Fuji, Japan). Demodification of the labeled PB1 subunit at pH 4.9 was carried out according to Godovikova et al. (*20*).

Direct Labeling of RNP. Radioactive dinucleotide with *o*-formylphenyl group at its 5′ end (*o*F-pA^{32}pG) was prepared in an abortive initiation reaction by T7 RNA polymerase. The reaction mixture (100 μL) contained 40 mM Tris-HCl at pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 5 μg/mL template DNA [V84 plasmid (*21*)], 0.1 mg/mL BSA, 0.25 mM *o*F-AMP, and 10 μCi [α-^{32}P]GTP (20 μM). After incubation for 1 h at 37 °C, the dinucleotide was purified by 20% PAGE in the presence of 7 M urea, eluted from the gel, and precipitated with 2% $LiClO_4$/acetone. The total yield was approximately 50%. For cross-linking, RNP (1 μL) was incubated with 1 μCi (0.25 mM) *o*F-pA^{32}pG in 10 μL of the standard transcription mixture for 5 min at 30 °C and then treated with $NaBH_4$ for 20 min on ice. The labeled products were analyzed by SDS 9% PAGE. Dried gels were subjected to autoradiography.

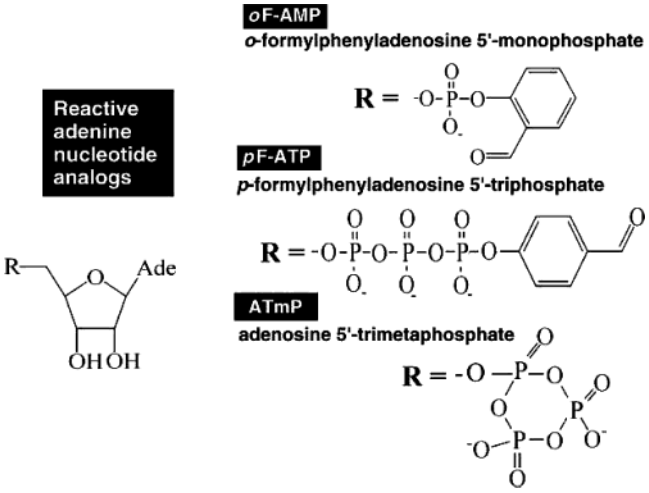

FIGURE 1: Structural formulas of the reactive nucleotide analogues. Three ATP analogues were used for catalytially competent labeling of the primer-binding site of influenza virus RNA polymerase.

V8 Protease Digestion of Labeled PB1 and Analysis of the Cleavage Products. Analysis of the cross-linked peptides was performed essentially according to the published procedure (*14*). Briefly, the gel slice corresponding to the CBB stained and labeled or unlabeled PB1 was cut out and soaked in V8 buffer (125 mM Tris-HCl at pH 6.8, 0.2% SDS, 1 mM EDTA, 10% glycerol, and 0.3% 2-mercaptoethanol) for 3 h. The gel slice containing PB1 was cut into small pieces and loaded into the gel wells together with various amounts of V8 protease in the V8 buffer. Electrophoresis was performed in two steps: 4 h at 30 V in the 5 cm stacking gel, and 4 h at 120 V for peptide separation in 10 cm of the 15% separation gel. After electrophoresis, the gel was CBB stained, dried, and subjected to autoradiography.

Identification the Length of the RNA Product Synthesized by RNA Polymerase Using the Attached Nucleotide as a Primer. ATmP was cross-linked to RNP according to the catalytically competent labeling method in the presence of 2 μL (10 μCi) of 20 μM [α-^{32}P]GTP for 10 min at 30 °C. After addition of 1 μL each of 0.25 mM ATP, CTP, and UTP, the incubation was continued for 30 min at 30 °C. The radioactively labeled PB1 subunit was purified by SDS 9% PAGE and excised from the gel, and the material was eluted from the crushed gel slice in 200 μL of 1% SDS/0.01 M HCl at 37 °C for 2 h. The pooled aqueous phase was shaken with 850 μL of *n*-butanol, dried in a vacuum, and subjected to PAGE on a 20% gel in the presence of 7 M urea.

RESULTS AND DISCUSSION

Priming Activities of the Nucleotide Analogues. The method of catalytically competent labeling of the active site of RNA polymerase uses primer nucleotide analogues

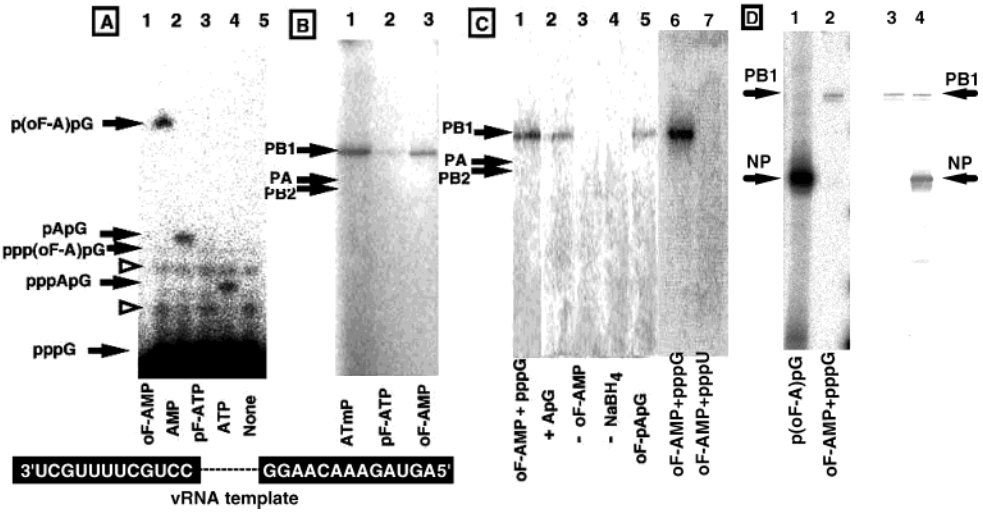

FIGURE 2: Substrate properties of ATP analogues and their use for the labeling of the primer-binding site of influenza virus RNA polymerase. (A) Transcription initiation assay was carried out for 1 h at 30 °C in the standard reaction mixture (10 μL), which contained 10 μCi [α-^{32}P]GTP, 0.25 mM each of the primer nucleotides, *o*F-AMP (lane 1), AMP (lane 2), *p*F-ATP (lane 3), ATP (lane 4), or no primer (lane 5), and 1 μg of viral RNP. The 3′- and 5′-terminal sequences conserved among all eight vRNA segments are shown at the bottom. The reaction products were separated by PAGE on a 20% gel in the presence of 7 M urea. The gel was exposed to an imaging plate, and the plate was analyzed with a BAS 2000 image analyzer (Fuji, Japan). Dinucleotides synthesized in abortive transcription initiation by T7 RNA polymerase were used as mobility makers (note that the migration behaviors of the dinucleotides containing modified bases are different from those consisting of regular nucleotides). The migration positions of substrates and products are indicated by arrows, while triangles show the trace amounts of radioactive contaminants in [α-^{32}P]GTP used. (B) RNP was subjected to catalytically competent cross-linking of initiation dinucleotides using ATmP (lane 1), *p*F-ATP (lane 2), or *o*F-AMP (lane 3) as primers. The cross-linked RNP was further incubated with [α-^{32}P]GTP for the formation of initiation dinucleotides prior to gel electrophoresis. (C) RNP was subjected to the catalytically competent cross-linking by *o*F-AMP as described in the Materials and Methods. Lanes 1 and 6, the complete reaction using *o*F-AMP and [α-^{32}P]GTP; lane 2, the presence of 0.25 mM ApG; lane 3, *o*F-AMP was omitted; lane 4, $NaBH_4$ was omitted; lane 5, the cross-linking of RNP immediately after formation of *o*F-pA^{32}pG; and lane 7, reaction carried out using [α-^{32}P]UTP in place of [α-^{32}P]GTP. (D) Cross-linking of RNP with externally added *o*FpA^{32}pG, which was synthesized in the abortive initiation reaction by T7 RNA polymerase. Migration positions of PB1, PB2, PA, and NP are identified by immuno-blotting of the gel against anti-PB1, anti-PB2, anti-PA, and anti-NP antibodies. The immuno-blot patterns against anti-PB1 alone and a mixture of anti-PB1 plus anti-NP antibodies are shown in lanes 3 and 4, respectively. For quantitative comparison, the time of gel exposure was set the same from A to D.

bearing reactive groups at their phosphate moiety (*15*, *16*). In our study, we used *o*-formylphenyladenosine-5′-monophosphate (*o*F-AMP), *p*-formylphenyladenosine-5′-triphosphate (*p*F-ATP), and adenosine 5′-trimetaphosphate (ATmP) (for the structures see Figure 1). The formylphenyl groups of the first two analogues allow Schiff base formation with the ϵ-amino group of Lys, which is stabilized by a mild reduction with sodium borohydride. On the other hand, ATmP is a highly reactive phosphorylating agent, which can form stable covalent bonds with His and Lys residues, at neutral pH, although the bonds are hydrolyzed under acidic conditions (*16*). This set of the three ATP analogues with reactive groups at α, β, or γ phosphates allows a comprehensive study of the interaction between the triphosphate moieties of the priming nucleotides and the surroundings of the primer-binding site on the vRNA polymerase.

First, we examined the ability of these adenine nucleotide analogues to be used as primer substrates for initiation of RNA synthesis by influenza virus RNA polymerase. RNP was incubated with ATP (the natural initiation substrate to be incorporated at the primer site in de novo initiation) and [α-^{32}P]GTP (the second substrate that should be incorporated at the substrate site), and radioactive pppA^{32}pG was formed (lane 4 of Figure 2A). The de novo initiation of RNA synthesis by the RNP-associated RNA polymerase was detectable, albeit at low levels (*21*, *22*). When ATP was replaced by AMP, pA^{32}pG was formed (lane 2 of Figure 2A), indicating that the binding site of the initiation nucleotide recognizes the base moiety of incoming nucleotides. The addition of *o*F-AMP, in place of ATP or AMP, gave a radioactive band of *o*F-pA^{32}pG (lane 1 of Figure 2A). The level of *o*F-AMP incorporation was as high as those of the natural substrate ATP. In contrast, the level of *p*F-ATP incorporation was very low (lane 3 of Figure 2A; note that dinucleotide ppp(*p*F−A)pG overlaps with an as yet unidentified contaminant in [α-^{32}P]GTP preparation), suggesting that the bulky *p*-formylphenyl group attached at γ phosphate of *p*F-ATP interferes with its recognition by the RNA polymerase. We then concluded that *o*F-AMP is as active as AMP and ATP in priming de novo initiation of RNA synthesis by the influenza virus RNA polymerase. The total yield of the synthesis of dinucleotides in the presence of AMP, *o*F-AMP, or ATP was estimated to be about 1.5% of [α-^{32}P]GTP added. The priming activity of ATmP was not studied because this nucleotide derivative has high reactivity toward water and is rapidly degraded under the conditions employed.

Specific Labeling of the PB1 Subunit with Priming Nucleotides. To increase the level and specificity of cross-linking, we employed the catalytically competent labeling method, in which a priming nucleotide analogue was first cross-linked to proteins and then the second substrate [α-^{32}P]-GTP was added to allow the formation of initiation dinucleotides. The cross-linking of radioactive dinucleotides containing the reactive nucleotide analogues was always detected at the PB1 subunit (Figure 2B), as expected from the functional map of influenza virus RNA polymerase (*1*, *2*).

The most intensive labeling of PB1 was with use of ATmP (lane 1 of Figure 2B), whereas the labeling efficiency was 5

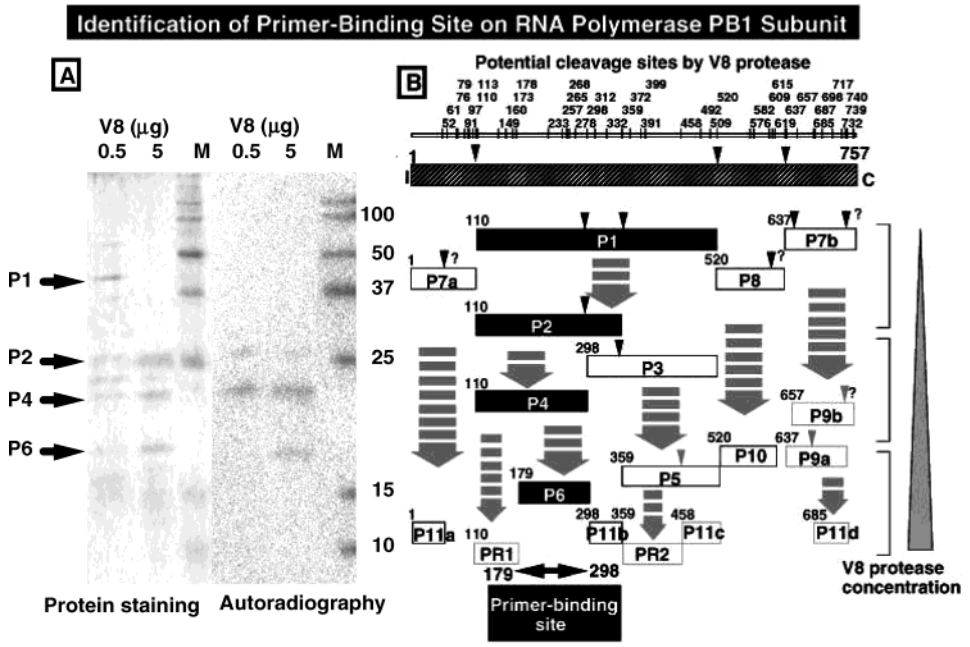

FIGURE 3: Proteolytic cleavage mapping of initiation dinucleotide cross-linked PB1 with V8 protease. (A) RNP was subjected to catalytically competent cross-linking with *o*F-AMP and [α-^{32}P]GTP as in Figure 2, and the radio-labeled PB1 was isolated by SDS 9% PAGE. The gel slice containing labeled PB1 was mixed with unlabeled PB1 that was purified from PB1-expressing *E. coli*. The indicated amount of V8 protease was added into each well together with a sample-loading buffer. Note that V8 protease (28 kDa) migrates in the gel together with P2. The mixtures were subjected onto SDS 15% PAGE. Lane M represents the molecular size markers. After electrophoresis, the gel was stained with CBB (lanes 1 and 2) and subjected to autoradiography (lanes 3 and 4). Each gel band was analyzed by microsequencing. Position of the peptides and molecular weight markers are indicated on the left and right, respectively. The intensive CBB staining in the P2 region in the 5-μg V8 lane gel is attributable to the V8 protease that was added. (B) Cleavage mapping of PB1. PB1 contains a total of 42 potential cleavage sites by V8. Cleavage mapping was performed by adding increasing amounts of V8 and microsequencing each cleaved fragment as described by Asano and Ishihama (*14*).

and 1.5 times lower with *p*F-ATP and *o*F-AMP, respectively (lanes 2 and 3 of Figure 2B). The low yield of PB1 labeling with *p*F-ATP is in good agreement with its poor activity as the priming substrate (see lane 3 of Figure 2A). No labeling of PB1 was observed when the reactive analogue *o*F-AMP (lane 3 of Figure 2C) or the sodium borohydride treatment was omitted (lane 4 of Figure 2C). The addition of 0.25 mM ApG, which is routinely used as an artificial primer for RNA synthesis in vitro, at the first stage of the catalytic competent labeling reaction, reduced the labeling efficiency by two times (lane 2 of Figure 2C), suggesting that ApG and *o*F-AMP compete with each other for binding to the primer nucleotide-binding site of PB1. When [α-^{32}P]GTP was replaced by [α-^{32}P]UTP, no radioactivity was detected at the PB1 band (lane 7 of Figure 2C), indicating that the complementary to template vRNA (see the template sequence at the bottom of Figure 2A) of the incoming nucleotide at the substrate site is required for dinucleotide formation with the priming nucleotide, which is cross-linked at the primer site, in good agreement with our previous observations (*23*). When the cross-linking was performed immediately after initiation of transcription after simultaneous addition of both *o*F-AMP and [α-^{32}P]GTP, the initiation dinucleotide was cross-linked only to the PB1 protein, albeit at a reduced rate, compared with the prior cross-linking of *o*F-AMP (lane 5 of Figure 2C). This indicates that the initiation dinucleotide synthesized in vitro remains associated with the catalytic center of RNA polymerase at least for the reaction time (30 min at 30 °C) employed. When the preformed dinucleotide *o*F-pA^{32}pG, which was synthesized with T7 RNA polymerase, was used at a high concentration for affinity-labeling of RNP, the majority was cross-linked to the abundant basic protein NP (Figure 2D).

When taken together, we concluded that the catalytically competent labeling procedure allows the selective labeling of the catalytic subunit PB1 of influenza virus RNA polymerase. Even using the catalytically competent labeling procedure, the overall yield of cross-linking of reactive dinucleotide (*o*F-pApG) was, however, still at a low level because the activity of de novo initiation by RNP-associated RNA polymerase is low. The similar controls with *p*F-ATP gave us essentially the same results (data not shown).

Identification of the primer nucleotide-binding site on PB1 to identify the primer-binding site on PB1, we performed by V8 cleavage mapping of the PB1 protein, which was labeled by the catalytically competent procedure using each of three reactive ATP analogues. The cross-linked PB1 was isolated by SDS−PAGE (see Figure 2), mixed with unlabeled PB1 that was purified from *E. coli* expressing PB1 (14), and subjected to limited proteolysis by V8. The data for *o*F-ATP cross-linking are presented in Figure 3A.

The PB1 protein contains a total of 42 potential cleavage sites by V8, of which the initial cleavage takes place at three positions, 110, 520, and 637, yielding the limited cleavage products, P7a, P1, P8, P7b, in this order from the N terminus (Figure 3B) (*14*). The radioactivity was detected for P1, P2, P4, and P6 (Figure 3B) after confirmation by N-terminal microsequencing. The smaller fragments, P2, P4 and P6, are all derived from the largest fragment P1, and the smallest labeled peptide P6 is a part of P1, P2, and P4. After the protein staining intensity and the radioactivity for each labeled peptide were measured, we concluded that P6

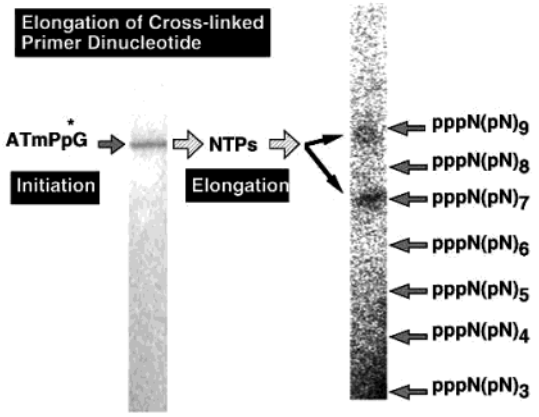

FIGURE 4: Elongation of the cross-linked primer nucleotide on PB1. RNP was subjected to catalytically competent cross-linking of the initiation dinucleotide using ATmP. An aliquot was analyzed by SDS−PAGE followed by autoradiography. The reaction was continued by adding all four substrates for another 30 min at 30 °C. RNA-cross-linked PB1 was isolated by SDS−PAGE. Cross-linked RNA products were released from PB1 by treatment with pH 2.0 and analyzed by PAGE in the presence of 7 M urea. The size markers were prepared in the abortive initiation reaction with T7 RNA polymerase in the presence of [α-^{32}P]GTP and the different combinations of the incomplete set of elongating NTPs.

between M179 and E298 is the only peptide bearing the initiation dinucleotide.

Essentially the same results were obtained in experiments that used *p*F -ATP and ATmP as the primer nucleotide analogues (data not shown). ATmP is cross-linked to either Lys or His residues. The P6 fragment contains 1 His and 13 Lys residues. To identify the species of the amino acid residue within the P6 fragment that was involved in primer binding, we examined the stability of affinity-labeled ATmP-containing dinucleotides under acidic conditions. The PB1-tethered radioactivity was stable at pH 4.9 at room temperature, whereas raising the temperature to 50 °C led to the complete release of the radioactivity after incubation for 5 h. This kinetic profile is the same as the hydrolysis pattern of the phosphoamide bond of ATP γ-methylamide (*15*), suggesting that ATmP was cross-linked to one of the Lys residue rather than the His residues within the P6 fragment. Taking into account that *o*-formylphenyl groups are cross-linked exclusively to Lys residue, we concluded that all three reagents are cross-linked to a segment between Lys197 and Lys297 within the P6 fragment (from M179 to E298). This segment is positively charged and contains a total of 13 Lys residues.

Elongation of the Cross-Linked Initiation Dinucleotides. The dynamic movement of the transcription complex along template DNA has been studied in detail using *E. coli* RNA polymerase. During the elongation of nascent RNA chains, the domain containing the catalytic site migrates in advance of the enzyme mainframe, but the movement of the two parts is not always synchronized, reflecting conformational plasticity of the transcription machine (*24*). In the early stage of transcription, the RNA polymerase stays on the promoter until the catalytic domain reaches to the position of about +9, producing the nascent transcript of about 9 nucleotides in length (*25*). This finding suggests that a substantial mass of RNA can be accommodated within the active center domain.

We then examined the elongation of the cross-linked dinucleotides within the influenza virus RNA polymerase. For this purpose, we used ATmP as a reactive initiation nucleotide because the cross-link can be cleaved under acidic conditions. After the elongation reaction by adding all four substrates, the radioactive PB1 subunit was separated by SDS−PAGE and the isolated PB1 was treated at pH 2.0 to cleave the cross-linking. The released radioactive RNA was then analyzed by PAGE in the presence of 7 M urea. As shown in Figure 4, the cross-linked dinucleotide was found to be extended up to 8−10 nucleotides in size. A low level of the 9-nucleotide-long product is due to the efficient elongation at this point. This result further confirms our prediction that the nucleotide-binding site determined by the catalytically competent cross-linking method represents the primer-binding site on the influenza virus RNA polymerase. The result also suggests that nascent RNA chains can be accommodated within the catalytic center domain including the primer-binding site between Lys179 and Lys297, which faces the 5′-phosphate terminus of RNA.

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