Recognition Sites of 3'-OH Group by T7 RNA Polymerase and Its Application to Transcriptional Sequencing*

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When analyzing the elongation mechanisms in T7 RNA polymerase (T7 RNAP) by using site-directed mutagenesis and a protein expression system, we identified the recognition sites of the rNTP 3'-OH group in T7 RNAP. On the basis of three-dimensional crystal structure analysis, we selected and analyzed six candidate sites interacting with the 3'-OH group of rNTP in T7 RNAP. We found that the Phe-644 and Phe-667 sites are responsible for the high selectivity of T7 RNAP for rNTPs. Also, we constructed the protein mutations of these residues, F644Y and F667Y, which display a >200-fold higher affinity than the wild type for 3'-dNTPs. These findings indicate that the phenylalanine residues of 644 and 667 specifically interact with the 3'-OH group. Thus, these mutants, F644Y and F667Y, with incorporation of 3'-dNTP terminators, which is similar to native rNTPs, can offer low backgrounds and equal intensities of the sequencing ladders in our method, called “transcriptional sequencing.”

T7 RNAP1 is a single 99-kDa polypeptide containing in its catalytic domain a highly specific promoter recognition site and a nascent RNA binding domain. T7 RNAP displays a stringent specificity for T7 promoter to initiate transcription. In T7 phage infection and the lytic cycle, T7 RNAP transcribes class II and III of the T7 phage genome (1). Based on three-dimensional structure analysis and mutagenesis of the Klenow fragment, the protein critical regions and structural motifs for polymerase activity have been suggested. Additionally, from sequence homologies of the Klenow fragment, Taq DNA polymerase, and T7 DNA polymerase, structural similarities have also been suggested in motifs A (532–555 aa), B (625–652 aa), and C (805–818 aa) (2). From mutagenesis analysis, the critical sites of primer recognition, metal ion binding, rNTP binding, and polymerase activity have been determined; however these do not necessarily coincide with the homologous motifs. Phenylalanine and tyrosine have been reported to play important roles in discriminating 2'- and 3'-OH groups in Escherichia coli DNA polymerase I (3, 4) and Taq DNA polymerase (5). These findings indicate that the aromatic ring of phenylalanine or tyrosine is needed to discriminate the 2'- and 3'-OH groups. Using mutant characterization and three-dimensional structure analysis (6), the catalytic domain of T7 RNAP was separated into the T7 promoter binding region (674–752 aa), Mg2+ binding sites (Asp-537, Asp-812), and essential regions of polymerase activity (motif A, motif B, motif C). To analyze the mechanism of the polymerase reaction, the interaction sites for the 2'- and 3'-OH group have been extensively examined (3–5).

Recently, Tyr-639 in the active site has been reported to be a discrimination site for 2'-OH groups in T7 RNAP (7). The Y639F mutant retains DNA and RNA polymerase activities but cannot discriminate rNTP from 2'-dNTPs. To further determine the mechanisms of the polymerase reaction, the 3'-OH discrimination site in the elongation step needed to be identified. However, details of the biochemical mechanism to discriminate the 3'-OH group of rNTPs by the T7 RNAP have not been clarified.

Until now, Met-635 on T7 RNAP has been thought to be the interaction site of 3'-OH groups based on homology with the Klenow fragment. This has been the case because the affinity for the rNTP molecule decreases when mutation occurs on Met-635 (8). As mentioned above, Met-635 and Tyr-639 were thought to be the only discrimination sites of 2'- and 3'-OH groups, respectively. They were thought to be a part of the catalytic pocket due to their geometrical alignments.

We found that the Phe-644 and Phe-667 sites that are located downstream of the Y639 site are important sites for interacting with the 3'-OH group of rNTPs in the T7 RNAP. These locations are the opposite of the location previously thought to be only one responsible for discriminating 2'- and 3'-OH groups. These findings suggest that the T7 RNAP may interact in several ways to the 3'-OH group of rNTPs in addition to Met-635. Our findings are in agreement with the reverse compensation theory of 2' - and 3'-OH groups proposed by Tabor and Richardson (5). This discrimination mechanism of the 3'-OH group seems to have been conserved during the process of molecular evolution.

Recently, we developed a novel sequencing method (transcriptional sequencing) using T7 RNAP. This reaction system was improved by the use of F644Y and F667Y mutants to incorporate 3'-dNTPs very efficiently (25).

EXPERIMENTAL PROCEDURES

Construction and Enzyme Purification of Mutant T7 RNA Polymerases—Mutant polymerase genes were constructed by PCR-mediated site-directed mutagenesis (9). Mutant enzyme expression and large scale purification have been described previously (10, 11).
Fig. 1. Mutation sites of T7 RNA polymerase mutant. The mutated residues and highly conserved motifs among various DNA and RNA polymerases are indicated in this figure. The motifs and mutated sites are located above and below the amino acid sequence, respectively. Motif B, Motif C, Helix Y, Helix Z, and Helix AA are located at 625–652, 805–818, 625–634, 649–658, and 684–699, respectively. The series of T7 RNA polymerase mutants designed in this study consisted of F644Y, F646Y, F667Y, F733Y, F782Y, F882Y, and Y639F.

Transcription Reactions—Nucleotides and radioactive nucleotides were purchased from Amersham Pharmacia Biotech. The transcription assay was done in buffer containing 40 mM Tris-Cl, pH 8.0, 8 mM MgCl2, 5 mM dithiothreitol, 200 μM GMP, and 5 units of T7 RNA polymerase in the presence of rNTP and template DNA at 37 °C for 15 min to 1 h. Before all of the experiments, the quantity of T7 RNAP enzyme was calibrated, and each sample was adjusted with respect to their protein quantities.

Relative activity and processivity were assayed by measuring the amount of the radioactive substrates using DE81 paper binding assay as described elsewhere (7, 12). The amounts of rNTP and template DNA are given in the figure and table legends.

Preference of Each 3'-dNTP Incorporation—Recognition selectivity for each NTP/3'-dNTP was assayed as described (5). The data was analyzed with 8% acrylamide containing 6 M urea and using BAS 2000 analyzing systems (Fuji Photo Film Co., Ltd.).

Transcription Sequencing—The sequencing strategy and method are described elsewhere (25). Sequencing reaction mixtures contained the PCR product of human thyrotropin-β cDNA (13) as sequencing template, the fluorescent 3'-dNTPs (1 μM tetramethyl rhodamine-3'-dUTP, 0.1 μM rhodamine 6G-ATP, 0.1 μM rhodamine 110-GTP, 5 μM X-rhodamine-CTP), 500 μM GTP, 500 μM UTP, 250 μM ATP, 250 μM CTP, 10 units of phosphatase purified from baker yeast and 25 units of T7 RNA polymerases. The reaction was carried out at 37 °C for 1 h. The excess of fluorescent 3'-dNTPs was eliminated by Sephadex G-50 column (Amersham) subjected to ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer Corp.).

RESULTS

Mutation Sites of the Constructed T7 RNAP Mutants—Fig. 1 shows the mutation sites of the constructed T7 RNAP mutants on the primary and three-dimensional structures (6). Bacteriophage T7 RNAP catalyzes the phosphodiester bond formation between the 5'-phosphate group and the 3'-OH group of rNTP, resulting in elongation of the nascent RNA chain. To understand the sites to be mutated, we analyzed the three-dimensional structure, paying attention to the region of helix Y (625–634 aa), loop (635–648 aa) – helix Z (649–658 aa), loop (658–684 aa) in T7 RNAP. It has been reported that the mutants of Lys-631, Tyr-639, Gly-640, Asp-812, and Asp-537 were characterized among the amino acid residues existing on the protein surface (14–17). Among these residues, Asp-812 and Asp-537 were identified as being critical for binding to the metal ion (18). Lys-631, Tyr-639, and Gly-640 were located inside motif B or in its vicinity. The Tyr-639 site is known to be important for rNTP/dNTP discrimination (7). On the basis of the known sites interacting with the 2'- or 3'-OH group, we hypothesized the aromatic residues of polymerases to be 3'-OH group to be candidates for discrimination sites. This concept is supported by reports that the 2'- or 3'-OH discrimination sites are Phe-762 and Phe-766 of E. coli DNA polymerase I (3, 4), Phe-667 of Taq DNA polymerase (5), Phe-155 of Moloney murine leukemia virus reverse transcriptase (19) and Tyr-737 of mycobacterium DNA polymerase I (20). These studies have suggested that the aromatic moiety of phenylalanine and tyrosine residues can discriminate 2'- or 3'-OH groups. We further hypothesized that the para-H group of the aromatic ring of phenylalanine interacts with 2'- and 3'-OH groups of rNTP, and the para-OH group in tyrosine interacts with 2'- and 3'-H groups, with hydrogen bond formation to stabilize these complexes. This stabilization triggers the formation of enzyme-substrate complexes to the catalysis-mediated transition state. With this in mind, we analyzed the aromatic residues in the 625–684 aa region in T7 RNAP and selected Phe-644, Phe-646, and Phe-667 for comparability of each RNA polymerase was measured by the incorporation of [α-32P]UTP using a circular DNA as the template. The reaction mixture was 40 mM Tris-Cl, pH 8.0, 8 mM MgCl2, 5 mM dithiothreitol, 200 μM GMP, and 5 units of T7 RNA polymerase in the presence of 250 μM NTP and 0.2 μCi of [α-32P]UTP (3000 Ci/mmol). In this reaction mixture, 10 μmol of closed circular reeler cDNA clone was used as the template. The final product was subjected to electrophoresis on 8% polyacrylamide gel containing 6% urea and autoradiographed. 1st lane, wild type; 2nd lane, F644Y, 3rd lane, F667Y, 4th lane, Y639F, 5th lane, F667Y, 6th lane, F733Y, 7th lane, F782Y, 8th lane, F644Y/F667Y, 9th lane, F882Y. Finally, we constructed the mutants F733Y, F782Y, and F882Y.

FIG. 2. Processivity of T7 RNA polymerase mutants. The assay method used in this study was described previously (12). The processivity of each RNA polymerase was measured by the incorporation of [α-32P]UTP using a circular DNA as the template. The reaction mixture was 40 mM Tris-Cl, pH 8.0, 8 mM MgCl2, 5 mM dithiothreitol, 200 μM GMP, and 5 units of T7 RNA polymerase in the presence of 250 μM NTP and 0.2 μCi of [α-32P]UTP (3000 Ci/mmol). In this reaction mixture, 10 μmol of closed circular reeler cDNA clone was used as the template. The final product was subjected to electrophoresis on 8% polyacrylamide gel containing 6% urea and autoradiographed. 1st lane, wild type; 2nd lane, F644Y, 3rd lane, F667Y, 4th lane, Y639F, 5th lane, F667Y, 6th lane, F733Y, 7th lane, F782Y, 8th lane, F644Y/F667Y, 9th lane, F882Y.

By sequencing several clones obtained by PCR, we found that our sequence was different form that present in the GenBank data base. Additionally, the sequence of T7 genome matched our data. For example, proline 665 was substituted by leucine in the region estimated for the rNTP binding. Since at site 665 there may be leucine in the sequencing at the T7 genome, we considered T7 RNAP with leucine at the 665 site to be the wild-type T7 RNAP. We also constructed an add-back mutant substituting with proline at Leu-665 (L665P) for comparison
with respect to processivity and 2'- or 3'-OH discrimination efficiencies. No differences were noted between the L665P and wild type (data not shown).

**Mutational Effects on the Biological Activity of the Wild Type**—We analyzed the effects of processivity, relative activity, and 3'-dATP incorporation efficiencies in each mutant polymerase (Fig. 2, Table I, and Table II). Table I shows the correlation between the disruptive effects of the relative activity by the mutations. Among the constructed mutants, F882Y showed marked reduction in relative activity to the wild type. The Phe-882 site has been reported to be the contact site with the PvuII as the template. ND indicates that no activity was detected. Total reaction aliquots were spotted onto DE81 paper, and after washing, the retained radioactivity of DE81 paper was counted.

**Specific Recognition of the 3'-OH Group by Phe-644 and Phe-667**—As shown in Table II and Fig. 3, the Phe-644 and Phe-667 residues specifically recognizing the 3'-OH group were determined by analyzing the 3'-dATP incorporation rate in polymerases. Table II shows the 3'-dATP incorporation efficiencies to the elongation chain with plasmid DNA pBS linearized with PvuII as the template. The different properties for recognizing the 3'-dATP substrate among the mutants were determined by analyzing the inhibitory effects by 3'-dATP on the extension of the elongation chains. This assay shows that the mutations of F644Y and F667Y affect the ability of the enzyme to recognize the structural differences between rNTPs OH and the 3'-dATP H groups. Thus, 3'-dATP is a good substrate for F644Y and F667Y mutants. In comparison, the substitution of tyrosine at 646 reduced the incorporation rate of 3'-dATP. With the F644Y and F667Y mutants, the incorporation rates of 3'-dATP differed and the specificity for recognizing the 3'-OH group was better for the F644Y than for the F667Y mutant. This shows that the mechanism for recognizing the 3'-OH group differs between the two residues.

![Fig. 3. Chain termination study for the preference of the incorporation of 3'-dNTPs and rNTPs in F644Y and wild-type T7 RNA polymerase.](image)

**Table I**

| Mutation site | Relative activities of RNAP activity comparing with wild type |
|---------------|-------------------------------------------------------------|
| F882Y         | ND                                                          |
| F733Y         | 0.65                                                        |
| F782Y         | 0.31                                                        |
| F646Y         | 0.59                                                        |
| F644Y         | 0.63                                                        |
| F667Y         | 0.56                                                        |
| F644Y/F667Y   | 0.45                                                        |
| Y639F         | 0.12                                                        |
| Wild type     | 1.00                                                        |

**Table II**

| Mutation site | Relative reduction of RNAP activity by 3'-dATP |
|---------------|-----------------------------------------------|
| F644Y         | 5.130                                         |
| F667Y         | 4.711                                         |
| F644Y/F667Y   | 5.580                                         |
| F733Y         | 1.173                                         |
| F782Y         | 1.075                                         |
| F646Y         | 0.459                                         |
| Y639F         | 0.930                                         |
| Wild-type     | 1.000                                         |

**Fig. 3** shows the quantitative comparison of inhibitory effects to the transcription of mutant and wild-type polymerases when the 3'-dATP was added to the transcriptional reaction using the closed circular reeler cDNA clones as the template (22). The incorporation rates of wild-type polymerase for 3'-dATP as the substrate were clearly lower than those of F644Y. As shown in Table II, this mutant increases the 3'-dATP incorporation rates. Whereas transcripts produced by the wild-type enzyme extended almost to the terminal end of the template, most transcripts produced by F644Y polymerase terminated at approximately 100 base pairs, and the full-length transcript yield produced by F644Y decreased to a few percent of all transcripts produced (Fig. 3). Similar results were found with F667Y (data not shown). These results indicate that Phe-644 and Phe-667 are important sites for 3'-OH group discrimination in T7 RNAP. In a similar assay system using E. coli DNA polymerase I, Taq DNA polymerase, and T7 DNA polymerase, effects similar to those in Fig. 3 were reported by Tabor and Richardson (5). In our examination, F644Y and F667Y did not incorporate
2'-dNTP. This indicates that Phe-644 and Phe-667 might not be involved in the 2'-OH recognition (data not shown).

Recently, Tyr-639 residue has been reported to be a discrimination site for 2'-OH groups in T7 RNAP (7). In this work, we also examined whether or not Y639F is a discrimination site for 3'-dATP and NTPs. As a consequence, the Y639F mutant showed no differences in the incorporation rate between 3'-dATP and NTP (Table II), as reported recently by Huang et al. (23). This mutant also showed no changes in its ability to discriminate and incorporate 3'-dCTP, 3'-dGTP, 3'-dUTP substrates (data not shown).

**Recognition Selectivity of F644Y and F667Y for 3'-dNTP and rNTP**—Table III shows the differences of 3'-dNTP recognition rates among the wild type and the mutants. We determined the inhibitory effects on transcription by adding 3'-dATP at various concentrations and by using the closed circular reeler cDNA clone as the template. We found that the incorporation efficiencies of 3'-dNTP by F644Y and F667Y were affected by the structural differences in their base moieties. However, activities of 3'-dNTP incorporation for F644Y and F667Y were >100-fold greater than the wild-type polymerases.

The Tyr-639 mutant was similar to its wild-type T7 RNAP with respect to 3'-dNTP discrimination (data not shown). The scale of the effect of 3'-dNTP incorporation on F644Y and F667Y activity was 3'-dUTPs > 3'-dCTPs > 3'-dATP > 3'-dGTP. The F644Y mutant led to more efficient incorporation than the F667Y mutant with respect to the discrimination rate of 3'-dNTP versus rNTP. The relative affinity among 3'-dNTP and rNTP in the mutants was >100-fold greater than in comparison to the wild type. Similar findings were reported for E. coli DNA polymerase I (5). In consideration of all these findings, we suggest that Phe-644 and Phe-667 are the key residues in 3'-OH discrimination.

**Effect of F644Y and F667Y Mutants on Transcriptional Sequencing**—We recently developed a novel sequencing method (transcriptional sequencing) to resolve the problems encountered in existing sequencing methods using DNA polymerase (25). Transcriptional sequencing offers the advantages of a high throughput, time savings, and automation for genome projects and clinical diagnosis. In transcriptional sequencing, the extension reaction is carried out with T7 RNAP, in contrast to existing methods that use T7 DNA polymerase or *Taq* DNA polymerase (24). The method being reported on (25) is based on the chain termination reaction, performed by using 3'-dNTPs as the terminator instead of deoxy-NTPs in DNA polymerase-based sequencing. In the DNA chain termination reaction method, native *Taq* DNA polymerase had the problem of imbalance of incorporation efficiencies for deoxy-NTPs on the elongating chain, leading to a loss of uniformity in the sequencing ladder, and consequently, obtained signals that were difficult to analyze. Recently, these problems were overcome by thermo sequenase, which produces a better uniformity of incorporation (24). Transcriptional sequencing using F644Y and F667Y overcame problems in a similar fashion to those reported by Tabor and Richardson (5). This improvement of the sequencing pattern can be seen from Fig. 4, which presents the sequencing analysis of the human thyrotropin-β cDNA (13) PCR product by transcriptional sequencing with F644Y in comparison with its wild type. The use of F644Y mutant allowed us to obtain uniform and long sequence signals. These signals were similar to those obtained with the F667Y mutant (data not shown). In Fig. 4, the sequence signals indicated as arrowheads are high background signals, which did not change. Only the intensity of the correct signals increased for the F644Y mutant, thus yielding results of higher accuracy and confidence levels when the F644Y and F667Y mutants were used (25).

**DISCUSSION**

We have shown here that the Phe-644 and Phe-667 sites on T7 RNAP are important for 3'-OH group discrimination. In the protein structure, Phe-644 is located in the region of the loop (635–647 aa) between helix Y (624–634 aa) and helix Z (648–658 aa) in the catalytic domain. The loop between helix Y and helix Z contains important residues discriminating the rNTP molecule because Tyr-639 is located in this loop. The Phe-667 site is located in the region of the loop (659–664 aa) between helix Z (648–658 aa) and helix AA685–700 aa. Three-dimensional analysis suggests that these loops are positioned in the neighborhood of the rNTP molecule. Thus, Phe-644 and Phe-667 in the two loops seem to closely interact to the 3'-OH group of the elongating rNTP molecule during the 3'-dNTP discrimination phase of the reaction.

Experimental results using *in vitro* mutagenesis have shown that Met-635 (8) and Tyr-639 (7) interact with 3'-OH and 2'-OH groups, respectively. The geometries of Met-635 and Tyr-639 are very important to interact with 3'-OH and 2'-OH groups. However, our data indicated that the geometrical alignment of T7 RNAP and the rNTP molecule also lies downstream of Tyr-639. This geometrical alignment to Phe-644 and Phe-667 is the opposite of that between Met-635 and Tyr-639. These findings suggest that in T7 RNAP, there are at least two ways to access the 3'-OH group. This data agrees with the reverse compensation theory of the OH and H groups (5) on the polymerase-substrate complex, because the phenylalanine on T7 RNAP was found to be a very good target for discriminating the
3'-OH group. Thus, this mechanism, which makes use of the aromatic moiety, is conserved in the process of molecular evolution of polymerases.

Mutation of Phe-644 and Phe-667 to tyrosine increased the 3'-dNTP incorporation, but F644Y showed better incorporation than F667Y and Phe-644, which is located in the neighborhood of the Tyr-639, which was reported to be the 2'-OH discrimination site (7). This suggests that the Phe-644 directly interacts with the 3'-OH group, whereas the Phe-667 has indirect discrimination activity, such as that via metal ions. In addition, mutation of both sites to tyrosine (F644Y/F667Y) increased the incorporation rate of 3'-dATP better than that for F644Y or F667Y mutants. As shown in Tables II and III, these results suggest that Phe-644 and Phe-667 residues access the 3'-OH group from different directions.

Until now, Met-635 on T7 RNAP was considered to be an important site for interacting with the 3'-OH group because of its sequence homology to E. coli DNA polymerases I and the fact that mutation of Met-635 to alanine on T7 RNAP causes a large increase in $K_m$ values for rNTP (8). However, these results could not be confirmed directly for 3'-dOH discrimination; only the mutant of this site, Met-635, has low affinity to rNTP, and the direct evidence has not yet been shown that Met-635 recognizes 3'-OH group directly.

We found that Phe-644 and Phe-667 mutated to tyrosine, F644Y and F667Y, in order to incorporate 3'-dNTPs on the order of 100–270-fold greater in comparison to the wild type. Similar results were obtained when the 3'-OH discrimination residue in DNAP I (Phe-762) was mutated (5).

In this report, we have shown that two sites in T7 RNAP (Phe-644, Phe-667) are key residues for 3'-OH discrimination. This suggests that other DNA polymerases, RNA polymerases, and reverse transcriptases may have direct or indirect sites for 3'-OH discrimination and such residues have not yet been identified. Our observations suggest that further work on elongation analysis and 2' and 3'-OH discrimination mechanisms is required.

By using the F644Y and F667Y mutants, highly accurate and confident signals were obtained because of the lower discrimination between rNTPs and 3'-dNTPs. As reported (25), transcriptional sequencing has advantages for the genome field.

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