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Processivity of T7 RNA Polymerase Requires the C-Terminal Phe^{882}-Ala^{883}-COO$^-$ or "Foot"†

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ABSTRACT: The role of the C-terminal Phe^{882}-Ala^{883} residues of bacteriophage T7 RNA polymerase in specific transcription has been investigated by means of site-directed mutagenesis. A mutant enzyme that lacks the C-terminal Phe^{882}-Ala^{883} residues, denoted the "foot" mutant, has been cloned and overproduced, and the effects of the deletion on promoter recognition, initiation, and elongation have been determined. Gel retardation assays and DNase I footprinting show that the foot mutant specifically recognizes and binds to T7 promoters, although this binding appears to be approximately 30-fold weaker than that of the wild-type enzyme. Transcription assays using oligonucleotide templates that contain the consensus T7 promoter show a dramatic decrease in transcriptional activity for the foot mutant. With templates whose coding region begins CCC..., the mutant synthesizes poly(G) products even in the presence of all four nucleotides. The synthesis of poly(G) products from such templates has previously been observed for the wild-type enzyme when GTP is the sole nucleotide present in the reaction and is thought to occur by a novel mechanism involving slippage of the RNA chain 3′ to 5′ relative to the template [Martin, C. T., Muller, D. K., & Coleman, J. E. (1988) *Biochemistry 27*, 3966-3974]. These data suggest that the loss in transcriptional activity by the foot mutant results from a severe decrease in processivity as well as catalytic efficiency of the enzyme. Removal of the C-terminal Phe and Ala residues from the wild-type enzyme with carboxypeptidase A generates the phenotype of the mutant precisely, proving that all of the properties of the foot mutant derive from the loss of the Phe-Ala-COOH moiety. Protection of the C-terminal residues in the wild-type enzyme from hydrolysis by carboxypeptidase A in the presence of T7 promoter-containing DNA shows that these C-terminal residues may be intimately involved in the DNA–protein interactions, thereby explaining their crucial role in all phases of transcription.

The RNA polymerase from bacteriophage T7 consists of a single polypeptide chain of 883 amino acids that is capable of carrying out all the functions required for the initiation of specific transcription at the phage-specific promoters on the T7 genome. The enzyme recognizes and binds to a phage-specific promoter sequence from −1 to −17 relative to the transcription start site on one face of the DNA helix (Muller et al., 1989). During the early stages of transcription, there is a high probability of dissociation of the ternary enzyme–template–mRNA complex, which leads to abortive cycling (Martin et al., 1988). This dissociation of the ternary complex diminishes dramatically after the incorporation of about 8 bases into the message (Martin et al., 1988; Muller et al., 1988).

Extensive studies on the mechanism of action of T7 RNA polymerase in this laboratory have shown that the protein molecule can be divided into distinct functional domains. The C-terminal (~80K) domain, which can be prepared by partial proteolysis of the enzyme with trypsin, has been shown to contain the promoter recognition and binding function as well as the catalytic center of the enzyme, while the N-terminal

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domain (~20K) is primarily involved in maintaining processivity after the incorporation of ~8 bases (Muller et al., 1988). The topological relationship between the N-terminal 20K domain and the C-terminal 80K promoter-recognizing domain is a critical one. Even a single proteolytic cleavage between Lys^{179} and Lys^{180} significantly reduces the degree of processivity of the T7 RNA polymerase, even though the two domains remain tightly coupled by noncovalent interactions (Muller et al., 1988). Part of the mechanism that prevents the polymerase from dissociating from the ternary complex appears to be an interaction of the emerging mRNA with the N-terminal 20K domain of the polymerase, an interaction that abruptly terminates the abortive cycling or high probability of dissociation of the ternary complex after approximately 8 bases are incorporated (Muller et al., 1988).

In an effort to further delineate the structural features of the enzyme involved in promoter binding, initiation, and processivity, we have deleted the C-terminal Phe^{882}-Ala^{883} sequence. Earlier genetic evidence had suggested that the structure at or near the extreme C-terminus of the enzyme was involved in transcription. The effect of this mutation on the above three aspects of polymerase function are described in this paper.

Materials and Methods

Wild-type T7 RNA polymerase was overproduced in *Escherichia coli* and purified as has been described (King et al., 1986). T7 RNA polymerase concentrations were calculated from an $\epsilon_{280} = 1.4 \times 10^5\ M^{-1}\ cm^{-1}$.

Preparation of a Mutant T7 RNA Polymerase Lacking Phe^{882}-Ala^{883}. The gene for a mutant enzyme minus the C-terminal Phe^{882}-Ala^{883} dipeptide was obtained via a standard cassette replacement method using as the parent plasmid one containing T7 gene 1 modified to carry an *Eco*RI site spanning residues 879–880 ($Asp^{879} \rightarrow Glu^{879}$). The latter was constructed via site-directed mutagenesis by the "gapped duplex" plasmid technique of Morinaga et al. (1984). The $Asp^{879} \rightarrow Glu^{879}$ is a conservative change. While both phage T7 and T3 RNA polymerases have the same C-terminal five amino acids, the related phage SP6 RNA polymerase has a Glu at position 879 instead of Asp. These sequences and that for the mutant are shown below. The mutant gene was reinserted

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     870                 875                 880        883
T7:  Leu-Asn-Leu-Arg-Asp-Ile-Leu-Glu-Ser-Asp  Phe-Ala-Phe-Ala
T3:  Leu-Asn-Leu-Gln-Asp-Ile-Leu-Lys-Ser-Asp  Phe-Ala-Phe-Ala
Sp6: Phe-Asp-Leu-Asn-Glu-Ile-Met-Asp-Ser-Glu  Tyr-Ala-Phe-Ala
"Foot" Mutant of T7 RNA Polymerase
                           -TTA-GAG-TCG-GAA  TTC-GCG-TAG
     Leu-Asn-Leu-Arg-Asp-Ile-Leu-Glu-Ser-Glu  Phe-Ala
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into the plasmid pAR1219 used to produce the wild-type enzyme and overproduced in the same manner (Davanloo et al., 1984). The mutant enzyme, designated the "foot" mutant, was purified as described earlier for wild-type T7 RNA polymerase (King et al., 1986).

Carboxypeptidase A Digestion of Wild-Type Polymerase. Carboxypeptidase A (40 μM in 96 mM HEPES,[1] pH 7.5/0.4 M NaCl) was treated with 1 mM phenylmethanesulfonyl fluoride for 1 h at room temperature. This sample was diluted 5-fold with water to reduce salt and buffer concentrations. A total of 100 μL of the resulting preparation was added to a mixture containing 200 μL of a solution of T7 RNA polymerase (107 μM in 20 mM potassium phosphate, pH 7.9/0.1 M NaCl/1 mM EDTA/1 mM DTT) and 100 μL of distilled H_2O. This resulted in a polymerase:peptidase ratio of 25:1. The mixture was incubated at 37 °C for 1 h, after which the reaction was quenched by the addition of 2 μL of 1.0 M DTT. Aliquots of the quenched mixture were then analyzed by SDS–PAGE and ^1H NMR. After carboxypeptidase A treatment, there was no change in the gel mobility of the protein and the ^1H NMR of the supernatant showed the signals expected for free Ala and Phe. A time course of digestion of 10 μM polymerase by 0.4 μM carboxypeptidase A was carried out, and the effect on enzyme activity was measured with use of a 22-bp template coding for the message GGACU under conditions described previously (Martin & Coleman, 1987).

Hydrolysis of T7 RNA Polymerase by Carboxypeptidase A in the Presence of T7 DNA. Wild-type T7 RNA polymerase (1.5 pmol in 5 μL of 20 mM potassium phosphate, pH 7.9/100 mM NaCl/1 mM EDTA/1 mM DTT) was incubated with or without T7 DNA (4.4 pmol in 22 μL of TE) at 37 °C for 30 min in a total volume of 45 μL of buffer containing 40 mM Tris, pH 7.8, 30 mM $MgCl_2$, and 10% glycerol. The reaction mix was then cooled to room temperature. Carboxypeptidase A (0.075 pmol in 5 μL of 0.7 mM HEPES, pH 7.5/31 mM NaCl) was added to the reaction mix, and incubation continued at room temperature. Aliquots (10 μL) were removed at 0, 7.5, 15, and 30 min, and the reaction was quenched by the addition of an equal volume of buffer (40 mM Tris, pH 7.8/30 mM $MgCl_2$/10 mM EDTA/10 mM DTT) and 8.8 nM T7 DNA to reaction mixtures that did not already contain DNA. At the end of the time course, all quenched aliquots were incubated at 37 °C for 30 min and the activity measurement was initiated by the addition of 1.25 mM NTPs containing [α-^{32}P]UTP in 5 μL of 40 mM Tris, pH 7.8/30 mM $MgCl_2$. The labeled transcripts were precipitated by 5% trichloroacetic acid and quantitated as previously described (King et al., 1986). Percent activities as a function of time of hydrolysis of carboxypeptidase A were calculated by using the zero time point as 100%. The control reactions contained all ingredients except carboxypeptidase A.

Enzyme Assays. Three types of transcription assays were employed. The first was the standard assay for the incorporation of [α-^{32}P]UTP into RNA with whole T7 DNA as template, followed by precipitation of the transcripts with trichloroacetic acid on a Whatman 3M filter that was washed three times to remove unincorporated nucleotides. The second assay utilized a 22-bp DNA template that contained a specific T7 promoter (positions −17 to −1) and coded for a 5-base message as described in Martin and Coleman (1987). The third assay was performed with a 37-bp template that also contained the T7 promoter and coded for a 20-base message that began with GGG. All assays were carried out at 37 °C as previously described (Martin & Coleman, 1987). Enzyme, substrate, and template concentrations will be described in the figure legends or in Results.

Gel retardation assays were performed as previously described (Muller et al., 1988).

DNase Footprinting of T7 RNA Polymerase on Promoter DNA. Promoter-containing DNA was prepared from the plasmid pUCM22 and the template and non-template strands were end labeled with ^{32}P (Muller et al., 1989). Enzyme (2 μL) dissolved in 20 mM potassium phosphate, pH 7.9/100 mM NaCl/1 mM EDTA/1 mM DTT was mixed with ap-

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; DNase I, deoxyribonuclease I.

Table I: Relative Activities of Wild-Type, Mutant, and Carboxypeptidase A Treated T7 RNA Polymerase

	A. Relative Activity (Percentage of Wild-Type Activity) 37-bp template[a]				
enzyme	[α-^{32}P]UTP, 4 NTPs	[α-^{32}P]-GTP, 4 NTPS	[α-^{32}P]GTP,[b] GTP alone, poly(G) product	[γ-^{32}P]GTP, 4 NTPs	[γ-^{32}P]GTP,[b] GTP alone, poly(G) product
wild type	100	100	100	100	100
mutant	0.2 ± 0.1	3.5 ± 0.5	4.5 ± 0.5	3.0 ± 0.2	4.5 ± 0.5
carboxypeptidase A treated wild type	0.6 ± 0.5	1.3 ± 0.8	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0

	B. Relative Activity (Percentage of Wild-Type Activity)	
	22-bp template	T7 DNA[c]
enzyme	[γ-^{32}P]GTP, 5-base product	[α-^{32}P]UTP, nanomoles of nucleotide incorporated per hour per milligram of enzyme
wild type	100	$3.0 \pm 0.2 \times 10^{-5}$
mutant[d]	5.5 ± 3.5	0[e]
carboxypeptidase A treated wild type	5.7 ± 5.3	0

[a] Assays were carried out as described in the text with use of 2.5 μM template (37 bp producing a 20-base message beginning GGG...; Martin et al., 1988) and 0.5 μM wild type, 5 μM mutant, and 5 μM carboxypeptidase A treated enzymes in the presence of 0.4 mM each ATP, GTP, UTP, and CTP. Activities shown are from at least five determinations and are normalized for enzyme concentration. [b] Assays were carried out with 0.4 mM GTP as the sole nucleotide. [c] These assays were carried out as described in King et al. (1986). [d] The higher variability in the activity of the mutant with the 22-bp template may stem from the short length of the coding region coupled with the weaker promoter binding, which could result in variability in the drop-off rate of the enzyme after initiation. [e] The 0 means that no counts above the level of the blank reaction were detected on the filter.

proximately 40, 120, or 600 fmol of DNA to give a final volume of 20 μL containing 20 mM Tris, pH 8.0, 40 μg/mL BSA, 4% glycerol, 1 mM DTT, 5 mM EDTA, and 2 mM GTP. The mixture was incubated at 37 °C for 30 min. One microliter each of $MgCl_2$ (150 mM) and DNase I (100 μg/mL in 10 mM Tris, pH 7.4/30 mM NaCl) was added successively to the incubation mixture, which was rapidly mixed. The mixture was further incubated at 37 °C for 30 s, and the nuclease cleavage was stopped by the addition of 20 μL of quench solution containing 100 mM EDTA, 4 M ammonium acetate, and 1 mg/mL tRNA and the immersion in a −70 °C bath. The DNA was purified by extraction with phenol/chloroform (1:1) and precipitation with 4 volumes of ethanol, and the pellet was washed with 500 μL of 76% ethanol. The pellet was dried and resuspended in formamide containing bromophenol blue and xylene cyanol, heated to 90 °C for 3 min, and cooled on ice. An aliquot of each reaction mix was counted by liquid scintillation, and volumes containing equal amounts of radioactivity were loaded on a preelectrophoresed 10% polyacrylamide sequencing gel containing 8 M urea. For sequence comparison, the Maxam and Gilbert G + A reaction was carried out for both strands of the radiolabeled promoter-containing DNA.

Results

Structure and Function of a Mutant T7 RNA Polymerase with Phe^{882}-Ala^{883}-COOH Deleted. Of the several mutations of T7 RNA polymerase we have screened, the most unexpected behavior was shown by the mutant that has the two C-terminal amino acids deleted. We have designated this the foot mutant, and in a standard assay using whole T7 DNA as template it displays no activity (Table I). The foot mutant binds in apparently normal fashion to promoter-containing DNA as shown by a gel retardation assay (Figure 1). Like the native protein, the foot mutant forms three retarded bands under the conditions of the gel retardation assay (Figure 1A). These bands have been characterized extensively previously (Muller et al., 1988), and the most rapidly migrating band has been identified as the specific open promoter complex. This is the only band that is not competed for by nonspecific calf thymus DNA. Neither the wild-type nor mutant enzymes form the specific rapidly moving complex with non-promoter-containing DNA (Figure 1A). As shown by the earlier studies, introduction of a single peptide bond cleavage between Lys^{179} and Lys^{180}, a bond that lies on the boundary between the N-terminal domain and the C-terminal promoter-binding domain, abolishes formation of all of the nonspecific complexes (Figure 1B). The singly cleaved enzyme has been termed the 80K–20K species (Muller et al., 1988). The enzyme that carries out this cleavage in many strains of *E. coli* including HMS174 has been identified as the outer membrane endopeptidase known as OmpT (Grodberg & Dunn, 1988). The foot mutant enzyme in its 80K–20K form also forms only the specific fast moving complex (Figure 1B).

DNase Footprinting of the Foot Mutant of T7 RNA Polymerase. Footprinting of the enzyme–promoter complex with DNase I using the foot mutant polymerase shows that its protection pattern of the promoter region is essentially similar to that of the wild-type enzyme (Figure 2). Under these conditions (Materials and Methods), the DNase I footprint for the wild-type enzyme extends from position +13 to −17 on the non-template and +9 to −17 on the template strands. These footprints are generally similar to those previously determined with DNase I (Basu & Maitra, 1986) or (methidium-propyl)-EDTA-Fe (Ikeda & Richardson, 1986, 1987), although a few details are more resolved. For example, there is a clear division of the footprint on the non-template strand into two halves, upstream and downstream of position −8 where the T is less well protected (Figure 2). This division at −8 begins to suggest the two distinct halves of the protection pattern, separated by a half-turn of helix, detected by high-resolution hydroxyl radical footprinting (Muller et al., 1989). The latter allows the conclusion that the polymerase binds the promoter from one side of the double helix. Like previous DNase I footprints, an enhanced cleavage of G at position −9 of the template strand is observed in the complex.

There is in addition a reproducible difference in the general character of the footprint created by the foot mutant consisting of general protection extending upstream of the immediate promoter region to approximately −40 on the template strand, a protection that is not observed for the wild-type enzyme, even when increased concentrations of the wild-type enzyme are used (Figure 3) (see Discussion). While the DNA fragment employed for the footprint on the non-template strand extended only to +21 and therefore did not give a clear picture of possible additional protection downstream by the foot mutant, the DNA fragment used for footprinting the template strand

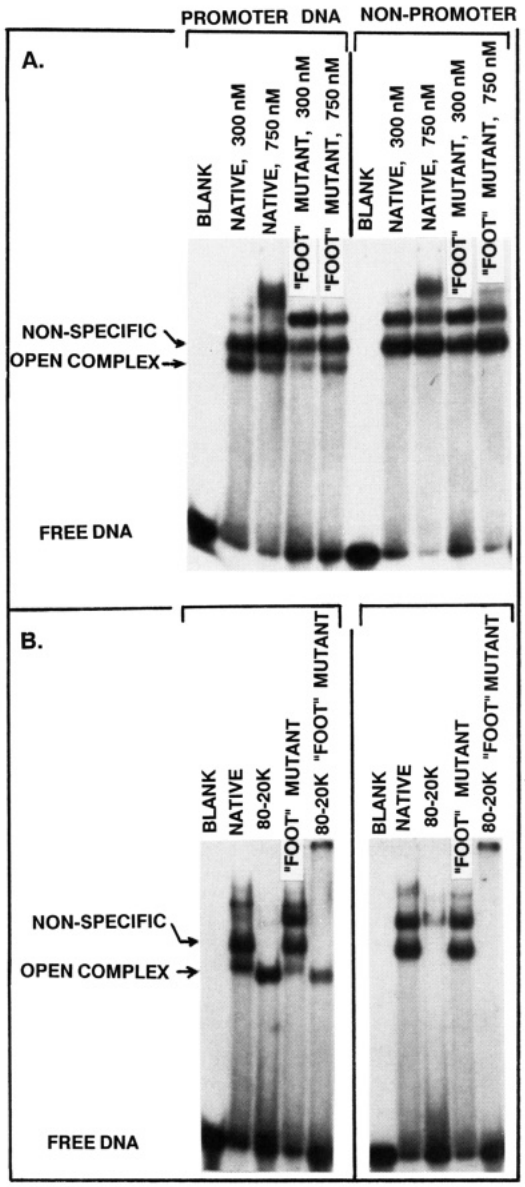

FIGURE 1: Gel retardation assays comparing the complexes formed with 24-bp promoter and non-promoter DNA and (A) native 98K and foot mutant T7 RNA polymerase and (B) their 80K–20K forms. The enzymes were incubated with 100 nM DNA at 37 °C for 10 min in 10 mM potassium phosphate, pH 7.9, containing 1 mM EDTA, 40 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT before loading on a preelectrophoresed 4% polyacrylamide gel and run in 1× TBE. Protein concentrations are indicated above each column.

extended downstream to +193. It does show that protection by the mutant polymerase extends downstream as well to +40. We interpret this as indicating that a nonspecific complex is formed by a second molecule of the mutant enzyme bound adjacent to the true promoter complex. While we have detected several nonspecific complexes formed between the wild-type enzyme and both promoter- and non-promoter-containing DNA by the gel retardation assay (all competed out by calf thymus DNA, see Figure 1), none of these have been reflected in previous footprinting studies by ourselves (Muller et al., 1989) or others (Basu & Maitra, 1986; Ikeda & Richardson, 1986, 1987; Gunderson et al., 1987).

We have carried out a complete titration of the promoter-containing DNA with the foot mutant and the wild-type enzyme, measuring the development of the footprint as a function of enzyme concentration from 0.2 to 7 μM. A titration by Gunderson et al. (1987) for the wild-type enzyme over a concentration range of 0–0.08 μM was used previously to determine a binding constant of 1×10^8 M^{-1}. Thus, a titration from 0.2 to 7 μM enzyme would be expected to yield 90–100% protection, as is observed (Figure 3). In contrast, the same concentration range for the foot mutant is in the middle of the range for the development of the footprint. This titration indicates an association constant for the mutant enzyme–promoter complex of $\sim 3 \times 10^6$ M; i.e., the foot mutant has about 30-fold less affinity for the T7 promoter sequence than the wild-type enzyme. At 7 μM wild-type enzyme, there is very little nonspecific DNA binding downstream of the promoter (Figure 3C). On the other hand, 7 μM mutant enzyme shows almost complete protection both upstream and downstream of the promoter by nonspecific binding of the enzyme (Figure 3B,C). This difference is more apparent at a 5-fold lower DNA concentration (Figure 3A). Furthermore, this nonspecific binding appears to have a much steeper enzyme concentration dependence than the promoter protection by the wild-type enzyme (compare the relative changes in lanes 4 and 5 to those in lanes 9 and 10 of Figure 3C).

Transcriptional Activity of the Foot Mutant T7 RNA Polymerase. In the standard assay employing whole T7 DNA, the foot mutant is completely inactive (Table I). Total inactivation of the mutant refers to the failure to detect any counts above background in the usual T7 polymerase assay that utilizes the precipitation of relatively long [α-^{32}P]-labeled RNAs on filter paper (see Table I). We have previously pointed out that such filter assays do not detect the short products made by the enzyme under many conditions (Muller et al., 1988). Even the relatively mild decrease in the degree of processivity occurring in the case of the 80K–20K enzyme reduces the percentage of transcripts retained on the filter paper to less than 5% of that shown by the wild-type enzyme (Muller et al., 1988), even though the average mRNA synthesized by the 80K–20K enzyme is $\sim$1200 nucleotides in length. A true assessment of the catalytic potential of "inactive" mutants, therefore requires gel assays that can isolate short products as well as determine their time rate of appearance.

The transcripts produced by the wild-type and the foot mutant enzyme in the more sensitive assays developed by Martin and Coleman (1987) and Martin et al. (1988) are shown in Figure 4. The first uses a 22-bp template producing a 5-base message, GGACU, which the wild-type enzyme does very efficiently (Figure 4, lane 1), at a rate of 50 min^{-1}, which corresponds to the initiation rate (Martin & Coleman, 1987). Since the label employed was [γ-^{32}P]GTP, all abortive products are observed. The foot mutant also makes similar products (Figure 4, lane 7) but requires higher enzyme concentrations, 0.5 μM for wild type vs 5 μM for mutant. On this as well as other templates, the foot mutant produces significantly more 2–4-base products relative to the 5-mer than the wild-type enzyme (Figure 4).

The second template is a 37-bp template beginning GGG, which is expected to form a 20-base mRNA by falling off the end. Complete sequences for these promoter–templates are given in Martin et al. (1988). As discussed earlier this "fall off" is often ±1 base (Milligan et al., 1987; Martin et al., 1988) (see Discussion). The frequency of dissociation (or abortive product formation) before the mRNA is $\sim$8 bases long is evident for the native enzyme (Figure 4, lanes 2, 3, and 4). While the foot mutant makes none of the normal full-length products for the 37-bp template, it does make products (Figure 4, lane 8), some of which appear to correspond to the ladder of poly(G) products made by the native enzyme when provided with GTP alone and a template beginning CCC (Figure 4,

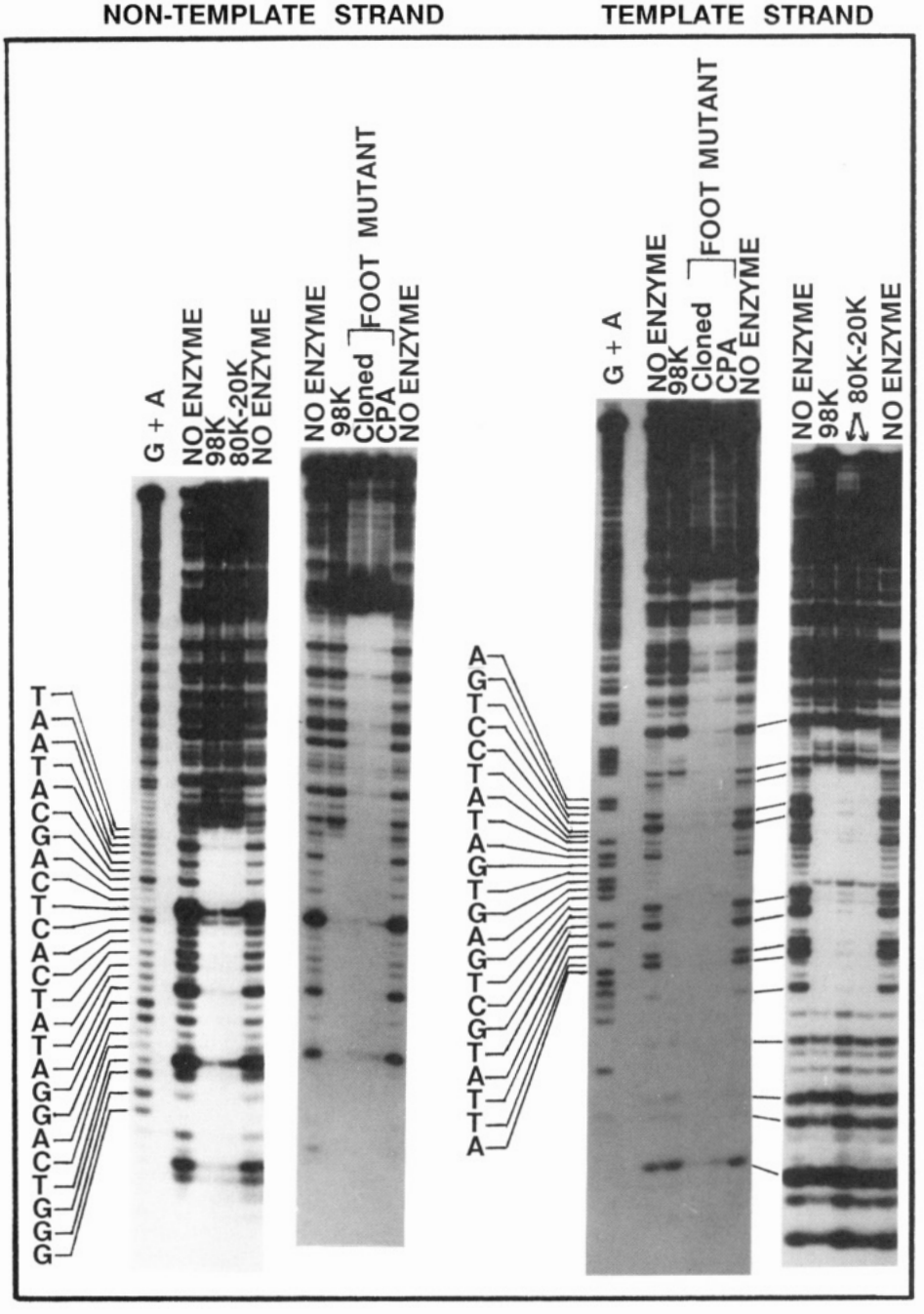

FIGURE 2: DNAse I footprints of 2 nM T7 promoter-containing DNA by wild-type and foot mutant T7 RNA polymerases. The two outside panels show the footprints obtained for wild-type 98K (0.2 μM) and wild-type 80K–20K (0.2 and 0.5 μM) enzymes and the non-template (left) and the template strands (right). The internal panels compare the footprints obtained for the wild-type 98K enzyme (0.2 μM) with those for the cloned foot mutant (0.75 μM) and carboxypeptidase A treated wild-type 98K (2 μM) enzymes. Maxam and Gilbert G + A sequencing reactions were used for band assignments.

lanes 5 and 6). While the ladder made by the foot mutant shows heterogeneity (Figure 4, lane 8), the foot mutant enzyme is capable of making a homogeneous poly(G) ladder from the 37-bp template when given GTP alone (Figure 4, lane 9). When [α-^{32}P]UTP is used as the label, the foot mutant synthesizes small amounts of products 7–20 bases long that appear to be normal but shortened transcripts (Figure 4, lane 13). They are first labeled at the 7-mer level, as expected, since the first A in the template strand is at position 7. For comparison, the assays of this same preparation employing [γ-^{32}P]GTP as the labeled substrate in the presence of all four NTPs or with GTP alone are shown in lanes 15 and 16.

When measured by the chromatography assay described in Martin and Coleman (1987), the mutant enzyme makes the 5-mer, GGACU (which in the case of the wild-type enzyme is a measure of initiation, 50 min^{-1}), at a rate of only $\sim$0.8 mol of GGACU/min per mole of enzyme. When making the poly(G) ladder on the 37-bp template, the mutant synthesizes the poly(G) products at a rate corresponding to the incorporation of $\sim$0.68 mol of [α-^{32}P]GMP/min per mole of enzyme, under conditions where the wild-type enzyme incorporates $\sim$17 mol of [α-^{32}P]GMP/min per mole of enzyme. These numbers, however, are specific activities only under specified conditions and are not directly comparable to the kinetically determined k_{cat} values we have presented previously for the wild-type enzyme on these templates (Martin & Coleman, 1987; Martin et al., 1988). Therefore, in order to give a picture of activity for the mutant relative to the wild type, we have

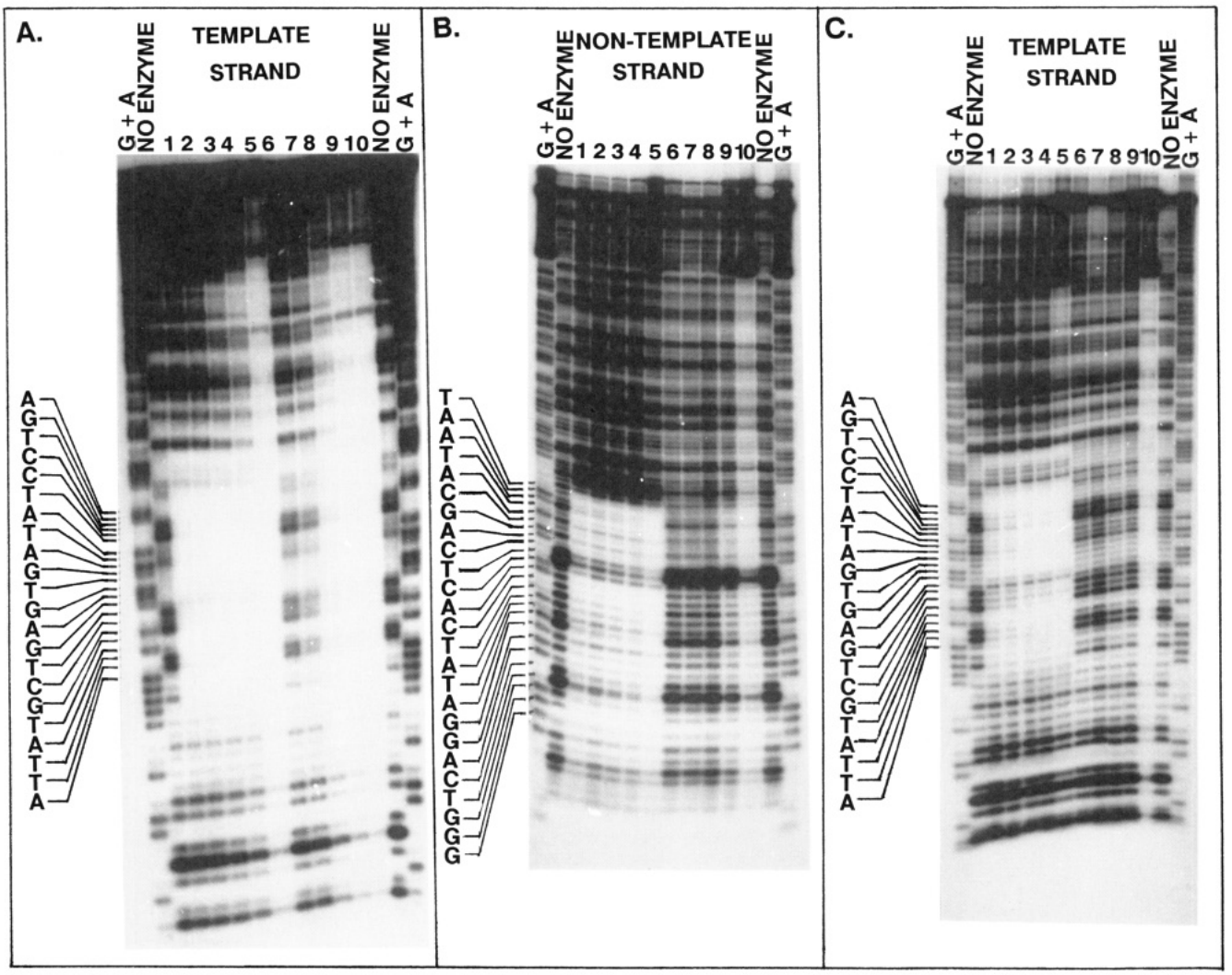

FIGURE 3: DNAse I footprints of wild-type and foot mutant T7 RNA polymerase on (A) 6 nM template, (B) 30 nM non-template, and (C) 30 nM template strands of promoter-containing DNA as a function of enzyme concentration. Lanes 1–5 are for 0.2, 0.5, 1.5, 3.0, and 7.0 μM wild-type enzyme, and lanes 6–10 are for 0.2, 0.5, 1.5, 3.0, and 7.0 μM foot mutant. Lanes with no added enzyme are indicated. Maxam and Gilbert G + A sequencing reactions were used for band assignments.

expressed the activities of the mutant on the several templates and with the various NTP mixtures as percentages of the wild-type enzyme assayed under conditions as similar as possible. The precision of the assays with the large amounts of mutant enzyme required is less than with the wild-type enzyme. The mutant under all conditions has an average of less than 5% the activity of the wild-type enzyme (Table I).

Generation of the Foot Mutant by Digestion of T7 RNA Polymerase with Carboxypeptidase A. In early studies of T7 RNA polymerase we identified the C-terminal amino acids of the protein as Phe-Ala by carboxypeptidase A digestion, information that was used to identify the termination codon for gene 1 (Osterman & Coleman, 1981). This work established that the carboxy terminus of the enzyme was reasonably exposed to solvent, at least in the enzyme uncomplexed with promoter. Therefore, the close association between the carboxy-terminal Phe-Ala and the active center implied by the dramatic loss of activity shown by the foot mutant appeared at first surprising. Removal of the C-terminal amino acids by carboxypeptidase A, however, does inactivate the enzyme in a rapid time course that correlates with the removal of only Phe and Ala from the C-terminus.

A typical time course for a carboxypeptidase A inactivation is shown in Figure 5, in which the template was the 22-bp fragment producing the GGACU message. The standard conditions we adopted for producing the modified enzyme are given in Materials and Methods. Since the C-terminal sequence of T7 RNA polymerase is Phe-Ala-Phe-Ala-COOH, the proteolysis product is undoubtedly heterogeneous in terms of removal of one or both Phe and Ala residues, but it behaves almost exactly as the foot mutant produced by site-directed mutagenesis. The comparable activities of the carboxypeptidase A treated enzyme are included in Table I, and radioautographs of the 5-mer produced from the 22-mer template and the poly(G) ladders produced from the 37-mer template by the carboxypeptidase A treated enzyme are included in Figure 4 (lanes 10–12). The poly(G) ladders and the activity profiles produced by the carboxypeptidase A treated enzyme are identical with those shown by the foot mutant. The altered footprint on the T7 promoter DNA shown by the foot mutant is also reproduced by the carboxypeptidase A treated polymerase (Figure 2). The relative activities of the wild-type, the cloned foot mutant, and the carboxypeptidase A treated foot mutant mimic are quantitatively compared in Table I for synthesis of both normal transcripts and the poly(G) ladder.

The Presence of T7 DNA Protects T7 RNA Polymerase from Inactivation by Carboxypeptidase A. If the conditions of carboxypeptidase A digestion are moderated from that producing the most rapid inactivation of the polymerase, it

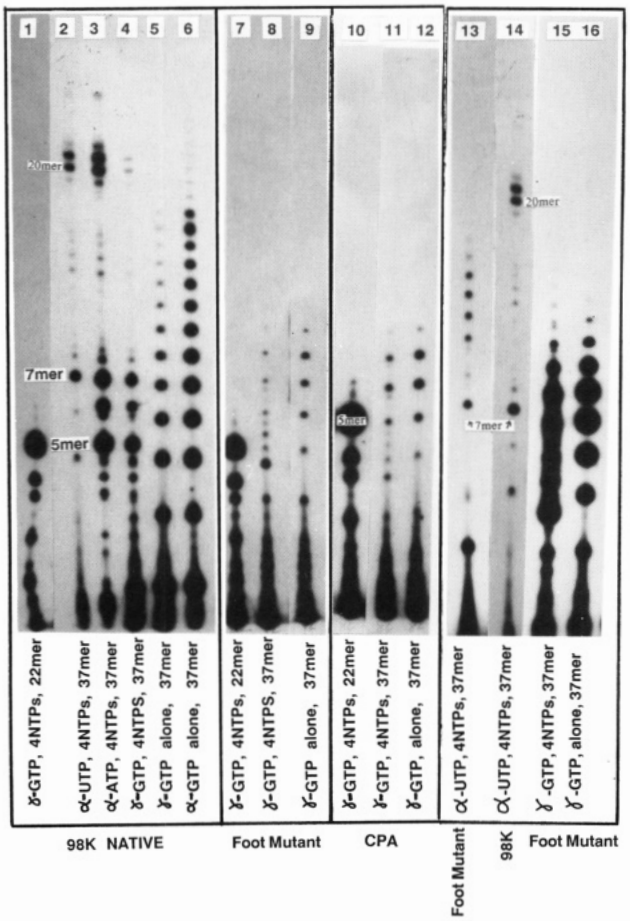

FIGURE 4: Gel analysis of the transcribed messages produced from 22-bp (lanes 1, 7, and 10) and 37-bp (all other lanes) templates that code for a 5-base and a 20-base product, respectively. Transcripts produced by the wild-type enzyme, 98K (0.5 μM), are shown in lanes 1–6 and 14. Transcripts from the cloned foot mutant (6.25 μM) are shown in lanes 7–9, 13, 15, and 16. Transcripts from the carboxypeptidase A treated wild-type enzyme (6.25 μM) are shown in lanes 10–12. Transcription was carried out for 10 min at 37 °C with 2.5 μM template, 0.4 mM each of the four NTPs (lanes 1–4, 7, 8, 10, 11, and 13–15), or 0.4 mM GTP alone (lanes 5, 6, 9, 12, and 16). The [^{32}P]-labeled NTP used is shown under each lane. The reaction was quenched by the addition of an equal volume of formamide, heating at 95 °C for 5 min, and chilling prior to loading on a 20% sequencing gel.

is possible to significantly protect T7 RNA polymerase from inactivation by the prior addition of whole T7 DNA (Figure 5). Each T7 DNA molecule carries 19 promoters. If enough DNA is added to titrate all of the polymerase molecules, a concentration of carboxypeptidase A that would normally cause 40% inactivation in 30 min results in only ~20% inactivation in the same time period (Figure 5).

Discussion

The single-subunit T7 RNA polymerase carries out all the functions necessary for the transcription of the T7 phage genome from the phage-specific promoters located in the 3′ 86% of the genome. The enzyme has one of the most rapid elongation rates known for a RNA polymerase, 200–300 s^{-1} (Martin & Coleman, 1987). The enzyme shows a high degree of processivity, capable of synthesizing a significant number of transcripts 15×10^3 nucleotides in length from whole T7 DNA in vitro (Golomb & Chamberlin, 1974; Niles et al., 1974; Oakley et al., 1975). When incorporating the first 8–10 nucleotides, however, the degree of processivity is not so high and the enzyme releases a significant number of short products due to a greater probability of dissociation of the ternary

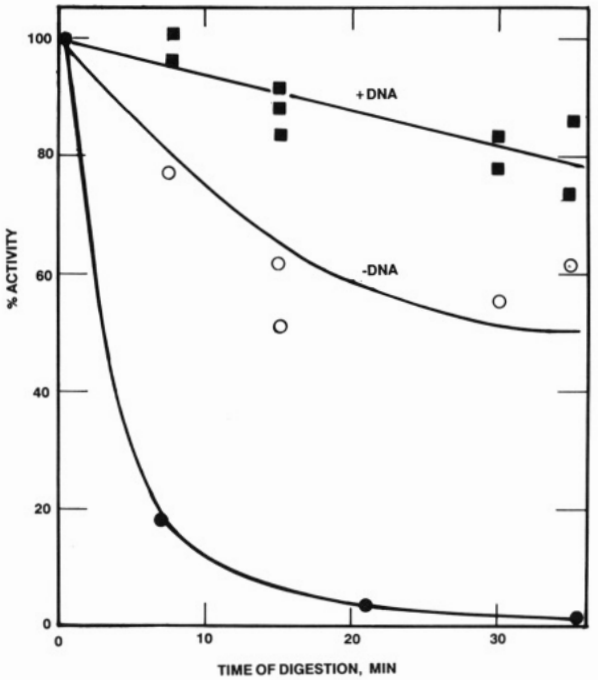

FIGURE 5: Protection of the Phe^{882}-Ala^{883} foot of T7 RNA polymerase from hydrolysis by carboxypeptidase A by whole T7 DNA. Reactions were carried as described under Materials and Methods in the presence (■) and absence (○) of T7 DNA; the reaction was quenched, and the samples were assayed with use of T7 DNA as template. The inactivation of T7 RNA polymerase under conditions used to produce the foot mutant (●) is shown also. The activity of the enzyme was assayed by use of the 22-bp template synthesizing the 5-mer GGACU.

complex (Martin et al., 1988). High processivity is established after the mRNA is 8–10 nucleotides in length. From the product profiles and from the probing of the melted region of the dsDNA template with a single-stranded endonuclease, the enzyme appears to move downstream on the template at each elongation event (Strothkamp et al., 1980; Martin et al., 1988).

Kinetic analyses of T7 RNA polymerase reactions with synthetic promoters have shown that progress of the reaction can be described by the following three phases: (1) the rapid highly specific binding of the enzyme to its promoter sequence to form an open promoter complex; (2) the initiation of the catalytic event by formation of the first phosphodiester bond in a relatively slow step; and (3) the establishment of the highly processive elongation reaction as the polymerase moves along the template. Reaction rates of T7 RNA polymerase as functions of template or substrate concentrations require analysis by integrated forms of the Michaelis–Menten equation, since enzyme and template concentrations rarely approach saturating conditions (Martin & Coleman, 1987). Such analyses have shown that the polymerization can be formulated as

$$\text{enzyme} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{enzyme–DNA} + \text{NTP} \xrightarrow{k_{cat}} \text{enzyme} + \text{DNA} + \text{message}$$

where $K_m = (k_{-1} + k_{cat})/k_1$ and k_{cat} is the initiation rate if product accumulation rates are measured for a 5-base message with one [α^{32}P] label or with [γ-^{32}P]-labeled messages (Martin & Coleman, 1987). Initiation rates, k_{cat}, are ~50 min^{-1}, while K_m values for promoters with the T7 concensus sequence are $\sim 1 \times 10^{-7}$ M (Martin & Coleman, 1987). The fact that the reaction fits such kinetics in no way limits the number of intermediates leading to the formation of the open promoter complex or the number of individual steps collected in k_{cat}. Much evidence suggests that the isomerization from the closed

to the open promoter complex is a relatively slow step in the case of *E. coli* RNA polymerase (von Hippel et al., 1984). While the rate of this isomerization in response to accessory proteins may be an important factor in the control of the rate of transcription in *E. coli*, this transition appears to be a rapid preequilibrium step in the case of T7 RNA polymerase with the equilibrium greatly in favor of the open promoter complex.

Previous work has shown that the T7 enzyme consists of at least two distinct domains, an N-terminal 20K domain (residues 1–179) and a C-terminal 80K domain (residues 180–883). The 80K domain, produced by removal of the 20K domain with trypsin, has been shown to contain the promoter recognition and binding region as well as the catalytic center of the enzyme (Muller et al., 1988). Thus, the proteolyzed 80K is fully capable of initiating transcription from specific promoters. The 80K domain alone, however, is not fully processive. It is unable to synthesize transcripts longer than ~8 bases and therefore cannot escape the process defined as abortive cycling (Martin et al., 1988; Muller et al., 1988). The 20K domain apparently confers a high degree of processivity on the reaction by forming a complex with the emerging mRNA chain after ~7 diesters have formed, thus decreasing the probability of dissociation of the ternary complex (Muller et al., 1988). A precise spatial relationship between the 20K and 80K domains is required for maximum processivity; even a single peptide bond cleavage at the bond between Lys^{179} and Lys^{180} causes significant premature termination. Although not as dramatic as the removal of the 20K domain, this single cleavage causes the average mRNA length produced in vitro from whole T7 DNA as template to fall from 3000–5000 bases to ~1200 bases (Muller et al., 1988). A Gaussian distribution of transcripts, ~600 to ~1500 bases in length, is produced by the 80K–20K enzyme.

Since the initial work showing the division of T7 RNA polymerase into two large domains, we have attempted to further separate and define the specific structural features on the enzyme involved in the various steps of the transcription mechanism by constructing a number of site mutants of the polymerase. The most striking result of site-specific mutation of T7 RNA polymerase thus far is the total inactivation of the enzyme produced by removal of the C-terminal Phe^{882}-Ala^{883}-COO^- (Table I). By increasing the concentration of the mutant enzyme, the profile of products made by the mutant enzyme can be easily established (Figure 4).

Since the C-terminal Phe^{880}-Ala^{881}-Phe^{882}-Ala^{883}-COO^- residues are freely available to removal by carboxypeptidase A, a modification that produces the same phenotype as the mutant (Figures 2 and 4), such a dramatic involvement in the mechanism by the two terminal residues located in an exposed and flexible C-terminal region was unexpected. The observation that these residues are protected from carboxypeptidase A digestion by binding of promoter-containing DNA suggests that the C-terminus undergoes a conformational change upon promoter binding and that the C-terminal Phe-Ala-Phe-Ala-COO^- may become intimately involved with the promoter DNA (Figure 5).

While this result suggests a general structural basis for how a flexible C-terminus becomes involved in the transcriptional mechanism, even a preliminary assessment of the precise function of the Phe-Ala-COO^- required application of all the probes that have been devised for examining each separate part of the multistep transcription reaction. The results have led to the conclusion that the C-terminal Phe-Ala foot appears to be affecting all the stages of the polymerase reaction from promoter binding to processivity. Processivity is affected at both the early stages of transcription (indicated by enhanced formation of 2-mer, 3-mer, and 4-mer products) and the later elongation stage (indicated by the failure to produce normal transcripts of 20 bases or more coupled with the appearance of a significant number of transcripts in the 12–14-base range) (Figure 4).

Removal of the foot does not appear to alter the ability of the enzyme to specifically recognize the T7 promoter. Even such details of the DNase I footprint as the hyperreactive G at position −9 of the template strand and the less-well-protected T at −8 of the non-template strand are preserved (Figure 2). On the other hand, the footprints as a function of increasing concentrations of the mutant enzyme show that overall binding affinity for the promoter has decreased about 30-fold (Figure 3). Previous work, however, suggests that such a decrease in K_s or K_m alone is unlikely to be responsible for a dramatic decrease in k_{cat}, since alterations in promoter sequence can have the effect of decreasing apparent binding affinity of the enzyme for the promoter by 30-fold without affecting k_{cat}; i.e., normal activity can be restored by increasing enzyme and/or template concentration (Martin & Coleman, 1987). The decrease in binding affinity of the mutant does suggest that the C-terminal foot is closely associated with the promoter DNA at the active site. The observation that the removal of the foot leads to a significant increase in nonspecific binding to DNA (Figure 3C), despite the decrease in promoter-specific binding, suggests a discriminatory role for these residues. These results suggest that the wild-type enzyme possesses some topological feature that allows the enzyme to discriminate against the binding of non-promoter DNA. This topological feature, associated with the C-terminal Phe-Ala, must increase the affinity of the enzyme for the promoter DNA (probably as the open promoter complex), while at the same time decreasing the affinity for nonspecific DNA (probably in the closed form). Such topological discrimination would appear highly desirable for an RNA polymerase.

The proof that the foot mutant still retains the catalytic ability to form phosphodiester bonds was provided by the discovery that it is capable of synthesis of a poly(G) ladder (Figure 4). We have previously described the formation of a series of poly(G) nucleotides from 3 to 16 bases in length by the native enzyme when the template begins CCC and the enzyme is supplied only with GTP as the substrate (Martin et al., 1988). This was postulated to occur by a mechanism in which the RNA slips relative to the template with the enzyme stalled at position +3 such that only two Gs remain base-paired at positions +1 and +2 of the template, allowing an additional G to be attached at the 3′ end, now over the catalytic center. In the case of the foot mutant, however, the poly(G) ladder also forms when all four NTPs are present, again only if the template begins CCC (Figure 4). However, when all four NTPs are used there is heterogeneity of the G ladder. This heterogeneity could easily be explained, however, by the simultaneous synthesis of some fraction of correct transcripts. In fact, using [α-^{32}P]UTP as the label shows that transcripts in addition to the poly(G) ladder are being made (Figure 4). The fact that the more normal products are made in much smaller amounts that the poly(G) products and that the majority of them are between 7 and 14 bases long suggests that one of the defects in the foot mutant is a severe drop in the degree of processivity. Synthesis of a poly(G) ladder, on the other hand, would not be expected to be altered as much by a decrease in processivity alone; thus, synthesis of the poly(G) ladder becomes a relatively significant process even in the presence of all four NTPs. In fact, under conditions

where a relatively inefficient, poorly processive normal synthesis is competing with poly(G) ladder formation by a slippage mechanism, it is reasonable to predict the formation of heterogeneous products by a combination of poly(G) synthesis and the addition of other NMP residues at the end of the poly(G) by normal processive synthesis. Such products of varying sequence would be expected to migrate slightly differently than other transcripts and poly(G) oligomers of the same length as is observed when all four NTPs are available (Figure 4, lane 8). Thus, the C-terminal Phe-Ala-COO^- foot is a second structural feature of the enzyme, in addition to the N-terminal 20K domain, required for a high degree of processivity of the T7 RNA polymerase.

By the formation of whatever transcript one chooses to measure the catalytic activity of the foot mutant, 5-mer, poly(G) ladder, or UMP-containing transcripts, there is a decrease in activity to 3–5% of that of the native enzyme (Table I), suggesting that in addition to a radical effect on processivity there is also a direct effect of the missing C-terminal residues on the catalysis of new diester bond formation. While formation of the 5-mer measures the overall initiation rate, rather than elongation, for the wild-type enzyme (Martin & Coleman, 1987), if elongation became slow enough this step could control 5-mer production. On the other hand, synthesis of the poly(G) ladder by the slippage mechanism should measure largely the continuous repeat of diester formation at positions +2–+3 of the template. The foot mutant, however, shows a dramatic fall in the rate of poly(G) as well as 5-mer formation (Table I), suggesting a fundamental defect in diester formation no matter what further sequential process is occurring. We have observed, however, that poly(G) ladder formation by the foot mutant is often more efficient than the synthesis of normal transcripts and thus may dominate the products, e.g., compare lanes 13, 15, and 16 in Figure 4. The exact ratio of poly(G/U)-labeled transcripts is quite variable however (see lanes 7–12 in Figure 4 and Table I). The decrease in the intrinsic catalytic efficiency of the enzyme could result from a subtle difference in the binding of the mutant to the DNA to form a transcriptionally active complex. We cannot rule out the possibility that the foot is responsible for the precise placement of catalytic residues for maximal activity in the open promoter complex. Thus, the differences in the binding of the mutant enzyme to the promoter as compared to the wild type, detected by footprinting (Figure 3), could also result in the observed decrease in catalytic efficiency.

The directional processivity of an RNA polymerase must be coupled in some fashion to the scission of the α–β phosphate bond of the NTP, the release of the pyrophosphate moiety, or the binding of the next NTP (von Hippel et al., 1984). In the case of the T7 RNA polymerase, this coupling must result in a conformational change in the protein that moves the template relative to the enzyme surface by one nucleotide frame. It is possible that the C-terminal foot participates in this coupling. It is certainly flexible as assayed by carboxypeptidase A susceptibility, and it also appears to be closely associated with or immobilized by the promoter DNA (Figures 3 and 5), both features one might predict for a structural feature of the protein involved in such a conformational coupling. Since the foot mutant enzyme is bound less tightly to the promoter DNA than the wild-type enzyme, it is highly likely that it is also less tightly bound to the melted template DNA. On thermodynamic grounds alone, the decrease in binding energy could lower the degree of processivity. These findings suggest that there are a number of mutational alterations of the C-terminal foot region of T7 RNA polymerase that should shed additional light on the function of the C-terminus. Deletion or insertion of amino acid residues somewhat before the Phe-Ala-Phe-Ala-COO^- sequence may reveal whether the length of the arm carrying the foot is critical. Change of the amino acids in the foot, especially the Phe residues, will reveal how critical the side chains are to function. Do the Phe side chains interact with the bases positioned at the catalytic center of T7 RNA polymerase?

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