Initiation, Elongation, and Processivity of Carboxyl-Terminal Mutants of T7 RNA Polymerase[†]

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ABSTRACT: Bacteriophage T7 RNA polymerase is a single-subunit enzyme which has a C-terminal amino acid sequence of Phe-Ala-Phe-Ala^{883} (FAFA^{883}). Closely related hydrophobic sequences are present at the C termini of seven other single-subunit RNA polymerases, including the mitochondrial RNA polymerase. Mutations at any of the four C-terminal residues depress initiation rates of T7 RNA polymerase from 50 to 95%, accompanied by large increases in the K_m values for the initiating nucleotide, GTP, as well as the K_m's for promoter DNA. The dramatic drops in initiation rates shown by the mutant enzymes remain after correcting for any alteration in saturation of the enzyme by the initiating nucleotide or the promoter DNA resulting from the changes in K_m. In contrast, the high processivity of the enzyme is not altered by mutations in the last four residues. However, the propensity for the enzyme to add an untemplated nucleotide at the 3′-ends of transcripts is abolished by the A^{880}AFA^{883} mutation. The C-terminal FAFA sequence or foot appears to interact both with the initiating NTP and with the most downstream nucleotides of the promoter, possibly through hydrophobic interactions with the minor groove, in the region where free radical footprinting of the polymerase−promoter DNA complex suggests that the enzyme binds across the minor groove.

The RNA polymerase coded by the genome of bacteriophage T7 is a single polypeptide chain of 883 amino acids (Dunn & Studier, 1983). It recognizes a highly conserved phage-specific promoter sequence whose major recognition and binding elements extend from position −1 to −17 relative to the start of the mRNA at position +1 (Oakley & Coleman, 1977; Martin & Coleman, 1987; Martin *et al.*, 1988; Muller *et al.*, 1988). The polymerase binds to the promoter primarily from one side of the DNA helix as shown by footprinting with the hydroxyl free radical method (Muller *et al.*, 1989). Limited proteolysis of the enzyme has shown that the protein consists of two distinct regions: a C-terminal 80 kDa domain which recognizes the promoter and synthesizes abortive products, but no transcripts longer than eight bases, and an N-terminal 20 kDa domain which binds single-stranded RNA (Muller *et al.*, 1988). Hence, it was concluded that the 80 kDa C-terminal domain, from residue 180 to residue 883 as determined from identifying the newly released N terminus, contains the active site and promoter-binding amino acid residues. However, a single proteolytic cleavage between residues K^{179} and K^{180} significantly reduces the processivity of T7 RNA polymerase on long templates, even though the two domains remain tightly linked by noncovalent interactions and the cleaved enzyme continues to synthesize short transcripts in normal amounts (Muller *et al.*, 1988).

A third structural feature of the polymerase required for rapid initiation is the four C-terminal residues or foot[1] of T7 RNA polymerase (F^{880}A^{881}F^{882}A^{883}-COO$^-$). If the last two, F^{882}-A^{883}, are deleted, the enzyme shows <5% of the activity of the wild-type enzyme and binds 30-fold less tightly to the promoter DNA (Mookhtiar *et al.*, 1991). The crystal structure of the T7 RNA polymerase determined at 3.3 Å resolution demonstrated that there is a C-terminal α-helix extending within a few residues of the carboxyl terminus (Sousa *et al.*, 1993). The location of this helix suggests that the last four residues may lie close to the polymerase active site. The latter has been located on the large C-terminal domain of the molecule by the identification of a short three-stranded *β*-sheet homologous to similar *β*-sheets in the Klenow fragment of *Escherichia coli* DNA polymerase I, reverse transcriptase, and mammalian DNA polymerase *β* (McAllister & Raskin, 1993; Pelletier *et al.*, 1994; Sawaya *et al.*, 1994). The region, referred to as the palm subdomain, contains the DNA binding grooves and contributes D residues believed to bind Mg^{2+} ions required for activity. D^{537} and D^{812} have been assigned this function in T7 RNA polymerase on the basis of sequence homology.

The present paper reports studies of a set of site-specific mutations in the four C-terminal residues of T7 RNA polymerase designed to determine their role in the reaction cycle. Accurate quantitation of individual transcripts from synthetic promoter−templates by phosphorimager analysis as well as kinetic analyses of product formation allowed us to determine the effects of these mutations on formation of the initial diester bond, the elongation steps, the degree of processivity of the enzyme, and the propensity of the enzyme

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[1] Abbreviations: T7 RNAP, T7 RNA polymerase; FAFA^{883}, four C-terminal amino acid residues of T7 RNA polymerase; mutation F880A, A^{880}AFA or AAFA; foot mutants, mutations (or deletions) in any of the four C-terminal residues of T7 RNA polymerase; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; NaDOC, sodium deoxycholate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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to add untemplated nucleotide residues at the 3′-ends of transcripts. The results support a hypothesis in which the $F^{880}A^{881}F^{882}A^{883}$-$COO^-$ fragment participates in formation of a base-binding pocket for the incoming NTP and appears to interact with the promoter DNA as well.

MATERIALS AND METHODS

T7 RNA Polymerase. Wild-type (WT), $FAWA^{883}$, and $FAYA^{883}$ T7 RNAP were prepared from *E. coli* strain BL21 containing plasmids pAR1219, pAR3231, and pAR3232, respectively (gifts of John J. Dunn). All other mutants were generated by a standard cassette mutagenesis method. The cassette vector contained a copy of the T7 gene 1 modified to carry an *Eco*RI site spanning residues 879−880 which results in the D879E mutation (Mookhtiar *et al*., 1991). This change is conservative, since the amino acid sequence of the closely related SP6 RNA polymerase is $E^{879}YAFA^{883}$.

All mutant genes were reinserted into the plasmid pAR1219 used to produce the WT enzyme and the plasmids transformed into *E. coli* strain BL21. Mutant polymerases were overexpressed in the same manner as the WT enzyme (Davanloo *et al.*, 1984; Grodberg & Dunn, 1988). Cells were grown in Super Broth containing 14 g of Bacto-tryptone, 27 g of yeast extract, 10 mL of glycerol, 100 mL of 1 M potassium phosphate at pH 7.3, and 300 mg of ampicillin per liter. Cells were grown at 37 °C until the OD_{600} was 2.0, induced by addition of 1 mM IPTG, and grown for an additional 4 h. Two hours after induction, 5 mL of glycerol was added.

Harvested cells were resuspended in 35−50 mL of lysis buffer containing 50 mM Tris-HCl (pH 8.1), 100 mM NaCl, 2 mM EDTA, and 2 mM DTT. Freshly prepared PMSF (200 μL at 23 mg/mL) was added to the cells just prior to lysis. The cells were sonicated for 2 × 3 min. An additional 100 μL of PMSF was added between runs. The FAAA protein was especially sensitive to proteolysis during sonication. This was overcome by lysozyme treatment of the cells followed by rapid purification of the enzyme which resulted in significantly less proteolysis. Hen egg white lysozyme (15−20 mg), dissolved in 6 mL of lysis buffer, was added to the resuspended cells, and the suspension was mixed slowly at 4 °C for 20 min. Lysis was completed with the addition of NaDOC and incubation at 4 °C for 20 min. A few flakes of DNase I were added to decrease the viscosity, and the solution was mixed for an additional 20 min.

Purification of T7 RNA Polymerase. Lysed cells were spun down at 13 000 rpm for 30 min. Five milliliters of 2 M ammonium sulfate were added to 40−45 mL of supernatant, followed by dropwise addition of 10% PolyminP to give a final concentration of 0.5%. The solution was stirred at 4 °C for 20 min and spun down at 18 000 rpm for 30 min. The supernatant from the PolyminP step was brought to 55% saturation with ammonium sulfate and stirred at 4 °C for 20 min, and the precipitate was spun down at 8000 rpm for 50 min. The pellet was resuspended in 20 mL of S1 buffer [50 mM HEPES-NaOH (pH 7.0), 50 mM NaCl, 2 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol], dialyzed twice for 1.5 h against 2 L of S1, and loaded onto a 10 mL SP-Sepharose FF column which had been pre-equilibrated with 10 volumes of S1. The column was washed with 9−10 volumes of S1 and the protein eluted with a 400 mL gradient of 50 to 350 mM NaCl. Because of the protease sensitivity of the FAAA mutant, the sample was loaded onto the column in S1 buffer plus 75 mM NaCl and rapidly eluted with a wash of 250 mM NaCl. For the FAAA mutant only, the purest fractions of the polymerase from the SP-Sepharose column were used directly in the transcription assays in order to obtain reliable results as quickly as possible. The other mutants were further purified on a Q-Sepharose FF column as described below.

Scheme 1

The fractions containing T7 RNAP, as monitored by SDS−PAGE, were pooled and dialyzed for 1.5 h twice against 2 L of Q1 buffer [50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 2 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol] and loaded onto a Q-Sepharose FF column which had been pre-equilibrated with 10 volumes of Q1. The column was washed with 9−10 volumes of Q1 and the protein eluted with a 400 mL gradient of 50 to 350 mM NaCl. The average purity of the proteins was 95% as judged by SDS−PAGE. T7 RNA polymerase concentrations were calculated from the OD_{280} using an ϵ of 1.4×10^5 M^{-1} cm^{-1}.

Quantitative RNA Product Profiles and Initiation Rates Measured by Phosphorimaging of Acrylamide Gels. The double-stranded promoter−templates used in the transcription assays are shown in Scheme 1. The single-stranded oligonucleotides were synthesized by the DNA Synthesis Lab, Department of Pathology, Yale University Medical School. The oligonucleotides were annealed by combining equimolar amounts of each strand in the presence of 1 mM NaOAc and heating to 95 °C for 10 min, and the solution was allowed to cool to room temperature slowly (5−6 h). The transcription reaction mix was 30 mM HEPES-NaOH (pH 7.8), 0.05% Tween 20, 100 mM potassium glutamate, 15 mM magnesium acetate, 0.25 mM EDTA, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 0.8 mM GTP and [γ-^{32}P]-GTP, and ATP, UTP, and CTP (0.4 mM each) at 37 °C (Maslak & Martin, 1993, 1994). Formation of poly(G) ladders was carried out by including only 0.8 mM GTP in the reaction mix. The DNA template and NTP substrates were mixed and preincubated at 37 °C for 5 min, and the reaction was started by addition of a 4X enzyme solution and continued for 10 min. An equal volume of formamide/dye solution was added to stop the reaction. The samples were heated to 95 °C for 5−7 min and electrophoresed on a 23% polyacrylamide/8 M urea gel. The gel was then scanned on a Fuji Bas1000 PhosphorImager for quantitation of the products.

The units of the integrated signal stored in the phosphorimager are not readily converted to counts per minute; thus,

freshly diluted [γ-^{32}P]GTP standards of four different concentrations were run on the same gel and scanned into the imager. These standards were used to generate a curve relating the units measured by the phosphorimager to the microcuries associated with each product.

Determination of k_{cat} and K_m with Respect to Promoter DNA for T7 RNA Polymerases. Polymerase reaction rates at each concentration of promoter DNA, NTPs, and polymerase were determined from the rate of [γ-^{32}P]GTP incorporated at the 5′-end of all products in moles per minute per mole of enzyme. The k_{cat}, the K_m for the promoter−template, and the K_m for the initiating nucleotide were determined by the methods previously described (Martin & Coleman, 1987, 1989). Enzyme and promoter DNA concentrations varied from 0.25 to 2.5 μM. The individual rate measurements involve spotting 4 μL of the reaction mix onto 3MM Whatman chromatography paper which had been prespotted with 10 mM EDTA followed by ascending chromatography in 59% ammonium sulfate as described by Martin and Coleman (1987). While in most cases the highest concentrations are not greater than K_m, the curves yielded reasonable fits of the following equation.

$$V = 1/2k_{cat}[D_T + E_T + K_m - [(D_T + E_T + K_m)^2 - 4E_TD_T]^{1/2}] \quad (1)$$

The dependent variable, V, was fit to the two independent variables E_T and D_T, total enzyme and total DNA concentrations, respectively, and the fit parameters, K_m and k_{cat}. Determination of the K_m value for the initiating nucleotide was done by the method of Martin and Coleman (1989). A Lineweaver−Burke plot, $1/V$ vs 1/[GTP], was used to determine the K_m for GTP as the initiating nucleotide.

Circular dichroic spectra were obtained on an AVIV Model 62DS circular dichroism spectrometer. The protein concentration was 0.13 mg/mL (1.3 μM) in 20 mM postassium phosphate and 65 mM NaCl at pH 7.8 and 25 °C. Scan parameters were as follows: 0.2 cm path length, scan from 300 to 197 nm, sampled at 0.5 nm intervals, a band width of 1.5 mm, and a 2.0 s averaging time at each wavelength. Five scans were collected per enzyme sample and averaged. Scan parameters for the detection of thermal transitions were as follows: 0.2 cm path length, the sample temperature raised from 20 to 65 °C in 0.5 °C steps with 2.0 min of equilibration at each temperature, and CD measured at 220 nm with 30 s of averaging time. The protein concentration was 2.5 μM. Spectra were processed to obtain the percent of folded protein and the percent of unfolded protein at each temperature based on the baselines of 100% folded at 20−27 °C and 100% unfolded at 60−65 °C. The melting temperature was taken as the point on the curve where the transition was 50% complete. The degree of cooperativity is defined as the temperature range, ΔT, over which 80% of the transition takes place.

RESULTS

Product Profiles and Kinetic Analysis of Foot Mutants of T7 RNA Polymerase. The function of mutants of a RNA polymerase relative to the WT enzyme can be determined with certainty only if one can compare with reasonable precision the product profiles, initiation rates, elongation rates, as well as processivities and promoter binding affinities of the enzymes. Quantitative product profiles can be determined by phosphorimaging of appropriate ^{32}P-labeled RNA gels. These can be used to measure initiation rates and processivities at specific concentrations of enzyme and promoter−template. The results can be normalized if an independent determination of changes in affinity of the mutant enzymes for the promoter can be made so that corrections for differences in concentrations of the enzyme−promoter complex can be made. In the present work, we did this by determining the K_m value with respect to promoter DNA for each mutant using the method of Martin and Coleman (1987). The K_m value for the promoter DNA was used to calculate the enzyme−promoter concentration present at the concentrations of total enzyme and DNA used (Table 2). In turn, the concentration of complex was used to calculate the initiation rates given in Table 1. The results for five mutants of T7 RNA polymersase in which one of the four C-terminal residues has been altered are summarized in Table 1. In addition, K_m and k_{cat} have been determined for six other foot mutants, including examples in which extra residues have been added to the C terminus of the polymerase (Table 1).

With two exceptions, AAFA^{883} and FAF^{882}, the activities (either directly measured initiation rates or k_{cat}) of these

Table 1: Initiation Rates for Wild-Type and Foot Mutant T7 RNA Polymerases[a]

C-terminal residues	initiation rates[b] (min^{-1})	k_{cat} (min^{-1})	K_m (μM) (template)[c]	K_m (mM) (initiating nucleotide)[d]
FAFA^{883}	44	50 (44−67)	0.02 0.01 = K_s[c]	0.6
FAF^{882}	27	4.0	0.5	3.5
FAYA^{883}	7	0.6	0.7	3.1
FAWA^{883}	11	3−4	0.2−0.7	1.2
FAAA^{883}	0.8	0.3	1.4	15.0[e]
AAFA^{883}	17	−	0.4	
FAFAF^{884}		5.7	1.7	
FAFAFA^{885}		0.3[e]	0.4	
FGF^{882}		0.4	0.2	
FTF^{882}		0.3−0.7	1.1−1.6	
FLF^{882}		1−3	1−3	
FA^{881}		0.2	3.6 0.33 = K_s[c]	

[a] Comparison to k_{cat} and K_m values determined from kinetic analyses. [b] Units for the initiation rates have been converted into moles of [γ-^{32}P]GTP incorporated per minute per mole of enzyme in order to compare to k_{cat}. [c] Both K_m and K_s are with respect to promoter DNA. K_s, the dissociation constant for enzyme−promoter DNA complexes, was determined by titrations of the DNase footprints on promoter DNA as a function of enzyme concentration (Mookhtiar *et al.*, 1991). [d] In the case of the mutant polymerases, reactions were carried out as a function of [GTP] to eliminate major effects of the increased K_m for the initiating nucleotide. FAAA, however, is not compensated (see the caption to Figure 4). These numbers are ±20%. [e] This large number is subject to large errors and is only approximate.

Table 2: Concentrations of Enzyme and Templates Used in Transcription Initiation Assays[a]

enzyme	[E] (μM)	[DNA] (μM)	K_M (μM)
FAFA(WT)	0.50	0.50	0.02
AAFA	0.65	1.25	0.4
FAF	4.50	0.50	0.5
FAYA	3.00	1.25	0.7
FAWA	4.00	0.50	0.7
FAAA	3.50	1.67	1.4

[a] Comparison to K_m for the promoter DNA.

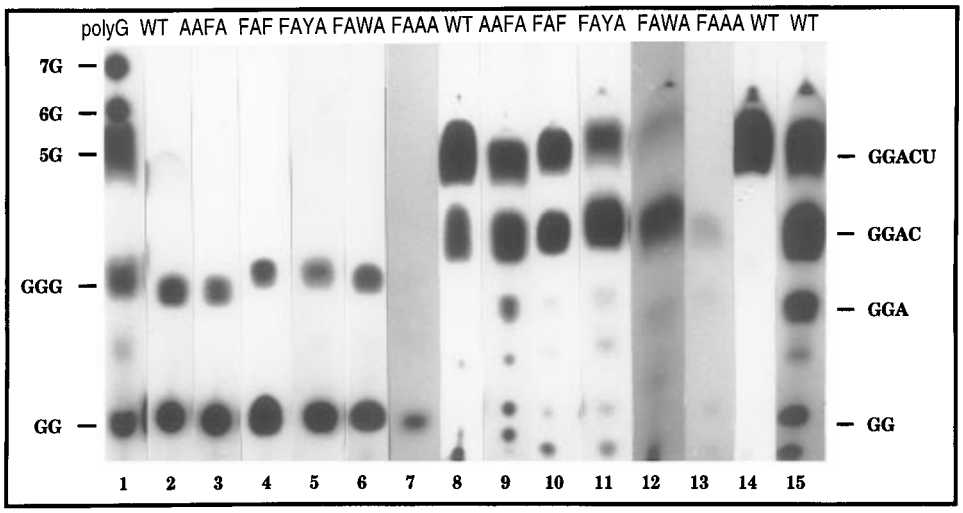


FIGURE 1: Autoradiograph of the gel of transcription products from template 1 produced by WT T7 RNA polymerase and its foot mutants. The complete runoff transcript from template 1 is GGACU as shown in lane 15 ([γ-^{32}P]GTP as the label) and lane 14 ([α-^{32}P]UTP as the label): lane 1, a standard poly(G) ladder of two to seven residues; lanes 2−7, transcription products produced in the presence of GTP alone; and lanes 8−14, transcription products produced in the presence of all four NTPs. The WT or mutant enzymes used are indicated at the top of each lane. In lanes 1−13, [γ-^{32}P]GTP was the label.

eleven foot mutants are <10% of those observed for the WT enzyme. Thus, detailed product profiles or complete kinetic analyses of the mutant enzymes often require reaction mixtures containing enzyme and/or promoter−template DNA concentrations ~10-fold higher than that employed for the WT enzyme. Comparing polymerase assays at widely different polymerase and promoter concentrations poses some practical problems. We and others have observed that the WT enzyme is inhibited at enzyme and promoter concentrations of 10K_m (K. Mookhtiar, unpublished observations). At least part of this inhibition may be related to the fact that at high concentrations T7 RNA polymerase binds to nonpromoter DNA (Mookhtiar *et al.*, 1991).

In the cases of the five mutants for which "initiation" rates were determined both from the phosphorimagining of the products and from k_{cat}, the two numbers are on the same order of magnitude (Table 1). However, with the exception of that of the WT polymerase, the k_{cat} values are somewhat smaller than the initiation rates determined from the normalized phosphorimager data. We believe that quantitation of products by phosphorimaging is a more accurate way of determining absolute rates for these polymerases than the ammonium sulfate filter paper chromatography used in the kinetic analyses, especially for the mutant enzymes where relative amounts and the size profiles of the products vary greatly. In addition, the extrapolated k_{cat} values may be most subject to error because the degree of saturation by the promoter−template is relatively lower for the mutants. Some of the k_{cat} data on the mutants may be distorted through inhibition by the high polymerase concentrations as mentioned above. In fact, DNase footprinting of the original deletion foot mutant showed it to bind nonspecific DNA with greater affinity than the WT enzyme (Mookhtiar *et al.*, 1991).

Quantitation of radioactivity, even by phosphorimaging, does not have a precision greater than ±20% if many replicate assays are performed. A precision of ±20% applies to the rate constants calculated from phosphorimaging data in all the tables in this paper. Note that the k_{cat} for the wild-type enzyme, an average value of 50 min^{-1}, has a range of 44−67 min^{-1} (Table 1). The standard deviation for these data on the WT enzyme is ±26% and can be as high as ±50% for some of the mutants. When kinetic constants were determined on two different promoters or with at least two different preparations of mutant polymerases, the range of kinetic constants observed is given in Table 1.

Initiation of Transcription by Wild-Type and Foot Mutants of T7 RNA Polymerase. Formation of the first phosphodiester bond can be isolated by supplying only GTP, while transcribing from a template which encodes GGACU. T7 RNA polymerase will form a bond between positions +1 and +2 using either GTP or pppGpG as the primer. In either case, the polymerase stalls at position +2 and dissociates after a single nucleotide incorporation. Autoradiographs of the products produced from promoter−template T1 by the WT and five foot mutants of T7 RNA polymerase using the formation of GG plus GGG to measure initiation are shown in Figure 1. As the pppGpG product builds up, it is used as an initiator accounting for the appearance of a pppGpGpG product. Enzyme and promoter concentrations (Table 2) and reaction times in these and subsequent product profiles were adjusted to yield equivalent amounts of total products from the WT and mutant enzymes. This ensures that at least one of the products in each reaction has an intensity in the phosphorimager similar to those of products in the other reactions; thus, low activities are not calculated from low-intensity images. The amount of pppGpGpG found must be multiplied by 2, since each pppGpGpG product represents two independent initiations, the second of which adds no label.

The rates of accumulation of the individual initiation products, GG and GGG, the total initiation rate, [GG] + 2[GGG], as well as the rate of accumulation of the final products (all four NTPs present) using the promoter−template T1 are given in Table 3. While the full-length product on template T1 is GGACU, some premature termination always occurs in the case of a runoff transcript. Thus, the full-length products must include the GGAC product.

If initiation remains rate-limiting for the mutant enzymes, as it does for the WT enzyme, then the rate of accumulation of the full-length product of the transcription should be equal to the initiation rate, i.e. [full-length products]:[initiation products] = 1. The observed ratios are given in Table 3. In

Table 3: Rates of Production of Initiation Products, GG and 2([GGG]), and Final Products, GGAC and GGACU, by T7 RNA Polymeras and Its Foot Mutants on Template 1

	rates for initiation products, [mol of [γ-^{32}P]GTP min^{-1} (mol of enzyme)$^{-1}$]			rates for full-length products [mol of [γ-^{32}P]GTP min^{-1} (mol of enzyme)$^{-1}$]			
enzymes	GG	GGG	total	GGAC	GGACU	total	ratio
FAFA	28	16	44	14	31	45	1.02
AAFA	13	4	17	7	5	12	0.71
FAF	19	8	27	13	12	25	0.93
FAYA	5	2	7	5	2	7	1.00
FAWA	9	2	11	8	2	10	0.91
FAAA	0.7	0.08	0.8	0.4	0	0.4	0.50

the case of the WT enzyme and three of the foot mutants, FAF, FAYA, and FAWA, initiation clearly remains rate-limiting. Two of the mutants, AAFA and FAAA, must have elongation steps that have slowed sufficiently to limit the rate of accumulation of the full transcript (Table 3). If the subsequent bonds of the 5-mer product were made at the same rate as the initial bond, this ratio should be 0.25. Thus, the ratios for FAAA (0.50) and AAFA (0.71) show that, while elongation has slowed, it still remains significantly faster than initiation.

Evaluation of the Initiation and Elongation Rates for Poly-(G) Ladder Formation by the Wild-Type and Foot Mutant T7 RNA Polymerases. If the nucleotide substrate is limited to GTP alone when using a template which codes for GGG at the 5′-end of the RNA, e.g. template T3, T7 RNAP synthesizes a series of poly(G) products, some as long as 20 residues (Figure 2). The mechanism proposed to be responsible is that when the polymerase stalls at position +3, the poly(G) mRNA slips backward relative to the stalled template, thus allowing subsequent "correct" incorporations of GMP residues into the newly emptied +3 position (Martin *et al.*, 1988). This one-dimensional diffusion of the RNA appears to retain an undissociated message only if there are at least two G•C base pairs between RNA and the template at all times, hence the requirement for a template beginning CCC.

Poly(G) ladder formation by the above mechanism begins via a normal initiation process. Thus, if initiation remains 10-fold slower than the rate of one-dimensional diffusion of the poly(G) product along the template, then the rate of accumulation of total 5′-labeled poly(G) products should be the same as the initiation rate. The rates calculated for the production of total poly(G) products from template T3 by the WT and five foot mutant polymerases are given in Table 4 and compared to the initiation rates calculated as described for Table 3. With the exception of the WT enzyme, the rates of poly(G) formation by the foot mutant polymerases are 70−90% of the initiation rate. Note that the real rates of poly(G) formation will be somewhat greater than the numbers given in Table 4, since initiations with pppGpG will occur as this product begins to accumulate. The latter represent repeat initiations for which the γ-^{32}P label is lost. The exact magnitudes of these upward corrections cannot be determined because the average concentration of pppGpG during the reaction is not known.

In contrast to that of the mutant enzymes, total poly(G) product synthesis by the WT enzyme takes place at a rate that is 50% of that of initiation as measured by initial product formation. This suggests that the one-dimensional diffusion required for poly(G) synthesis is much slower than normal forward processive movement of the enzyme, since it slows the rate of total poly(G) product formation by the WT enzyme to about half of that predicted by the initiation rate (Table 4). On the other hand, the mutations in the foot have all slowed initiation sufficiently that the relatively slow one-dimensional diffusion of the poly(G) product is fast enough that it does not decrease the rate of poly(G) product formation much below that predicted by the initiation rate.

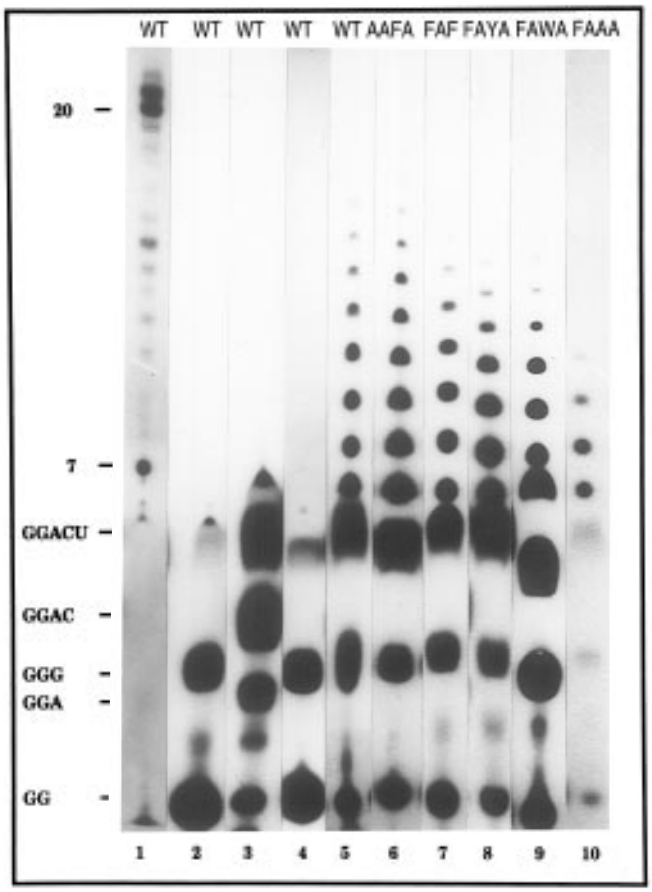


FIGURE 2: Autoradiograph of the gel of poly(G) ladder of products produced from transcription of promoter−template T3 by WT T7 RNA polymerase and its foot mutants in the presence of GTP alone. Lanes 1−5 are controls; lane 1 shows products of normal transcription by the WT enzyme on promoter−template T2 with [α-^{32}P]-UTP as the label. The seven-base RNA is the first product labeled, while the correct runoff transcript is 20 bases in length. Lane 2 shows products of transcription by the WT enzyme on promoter−template 1 with [γ-^{32}P]GTP as the only substrate. Lane 3 shows the same as lane 2 except all four NTPs were present. Lane 4 shows products of transcription by the WT enzyme on promoter−template T2 with [γ-^{32}P]GTP as the only substrate; a 5′-GGA start to the mRNA does not support poly(G) formation. Lanes 5−10 show products of transcription on promoter−template T3 (encodes 5′-GGGA) with [γ-^{32}P]GTP as the only substrate. The WT or mutant enzymes used are indicated at the top of each lane.

Table 4: Comparison of Initiation Rates with Rates of Poly(G) Ladder Synthesis by Wild-Type and Foot Mutants of T7 RNA Polymerase

enzyme	GG + GGG[a]	poly(G) synthesis[a]
FAFA (wild-type)	44	19
AAFA	17	15
FAF	27	21
FAYA	7	4
FAWA	11	7
FAAA	0.8	0.7

[a] Rates are expressed as moles of [γ-^{32}P]GTP incorporatedper minute per mole of enzyme. Thus, all labeled poly(G) products, no matter what the length, are considered equivalent for purposes of calculation of this initiation rate.

The AAFA mutant is one of the most active foot mutants (Table 1), but unlike those of the WT enzyme, the initiation rate (GG + 2[GGG]) and the rate of synthesis of total poly-(G) products are approximately the same for the AAFA mutant. Thus, the intrinsic initiation rate for AAFA must have fallen at least 50%. The kinetic data are not as complete for AAFA, and while the K_m for the promoter DNA is significantly larger than that for the WT enzyme, the range is 0.1−0.4 μM. A K_m of 0.4 μM is an upper limit; otherwise, the rate of poly(G) synthesis would exceed that of the WT enzyme when calculated on the basis of the concentration of the enzyme−promoter−template complex.

K_m Values for the Initiating Nucleotide for Foot Mutant T7 RNA Polymerases. Since mutations of the four C-terminal residues could change the afffinity of the enzyme for the initiating nucleotide as well as that for the promoter DNA, the K_m values for the initiating nucleotide for five representative foot mutants were determined. In the study of Martin and Coleman (1989), the K_m measured for GTP with the WT enzyme was 0.6 mM. For the foot mutants FAF, FAYA, and FAWA, K_m for the initiating GTP increases, but no more than 5-fold (Table 1). The FAAA mutant is a dramatic exception with the K_m for the initiating nucleotide increasing by ~15-fold. The deviations in the data for the measurement of this large K_m are greater than for the other mutant enzymes.

Processivity of the Wild-Type and Foot Mutants of T7 RNA Polymerase. In order to profile the products of "normal" forward processive transcription on a template by the foot mutants of T7 RNA polymerase, we chose template T2 which codes a 20-base runoff transcript. This template begins with CCA and thus avoids complications from synthesis of poly-(G) products by the mutant enzymes, many of which form detectable amounts of poly(G) products even in the presence of all four NTPs when transcribing on a template beginning with CCC. When transcripts terminate because the enzyme falls off the end of the template, WT T7 RNA polymerase invariably produces significant amounts of transcripts which are one nucleotide shorter as well as one nucleotide longer than the expected length (Figures 3 and 4). The transcripts one residue shorter are due to premature dissociation of the polymerase as it approaches the end of the template. The transcripts one residue longer than expected are due to the addition of an untemplated nucleotide, most frequently AMP or CMP as determined by sequencing the products (Milligan *et al.*, 1987). Excluding FAAA, which makes no long products, most foot mutants, like the WT, make full-length products of 19−21 bases (Figures 3 and 4). The full-length transcripts made by the AAFA mutant are altered in the fact

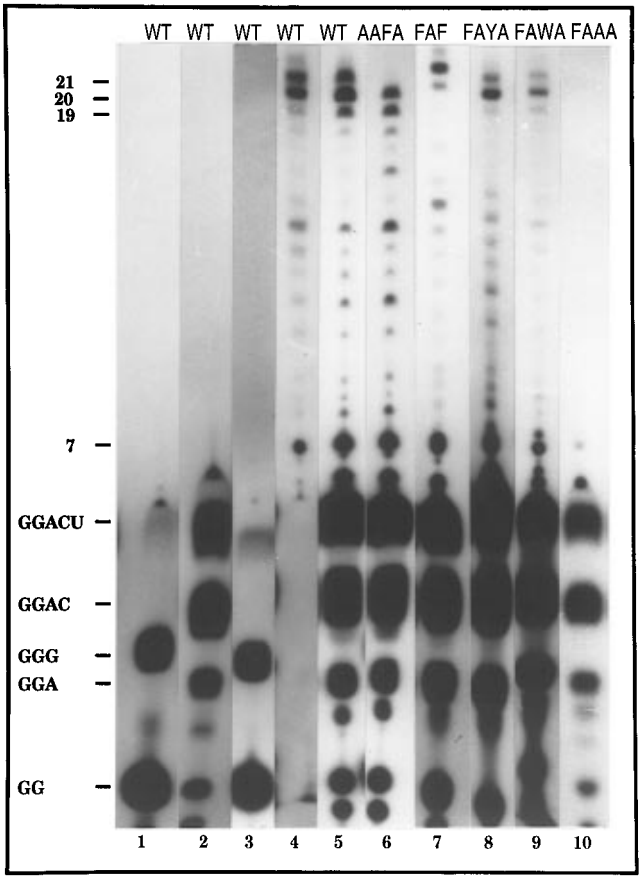


FIGURE 3: Autoradiograph of the gel of the products of transcription of promoter−template T2 by T7 RNA polymerase and its foot mutants. Promoter−template T2 encodes a message start of 5′-GGA and a 20-base runoff transcript. With the exception of lane 4, all products are labeled with [γ-^{32}P]GTP. Lanes 1−4 are controls: lane 1, products of the WT enzyme on promoter−template T1 with GTP as the only substrate; lane 2, the same as lane 1 but with all four NTPs present; lane 3, products of the WT enzyme on template 2 with GTP as the only substrate; and lane 4, same as lane 3 but with all four NTPs present and [α-^{32}P]UTP as the label (the seven-base RNA is the first product labeled). Lanes 5−10 show products of transcription on promoter−template T2 in the presence of all four NTPs. The WT or mutant enzymes used are indicated at the top of each lane.

that the enzyme makes no 21-base transcript. The "abortive" phase is also similar to that of the WT enzyme for four of the foot mutants; i.e. the percentage of transcripts showing premature termination decreases abruptly after seven bases are incorporated. In contrast, FAAA does not escape from the abortive phase and synthesizes no detectable products longer than seven or eight bases (Figure 4).

A processivity for each step of the complete transcription can be calculated by determining the fraction of the RNA product of a specific length, n, that is extended to the $n + 1$ length (Table 5). For the WT enzyme, the first three bonds are formed with a relatively high processivity, 0.74−0.93. There is then a dramatic drop to processivities between 0.27 and 0.56, associated with a large number of abortive transcripts. After incorporation of the 7th nucleotide, the processivity suddenly increases to >0.90 and remains at 0.95−1.00 through the incorporation of the 20th nucleotide. This pattern of processivity is maintained by all the foot mutants tested except FAAA (Table 5).

The entries in Table 5 for template position 20 are those for the addition of an untemplated NMP by T7 RNA

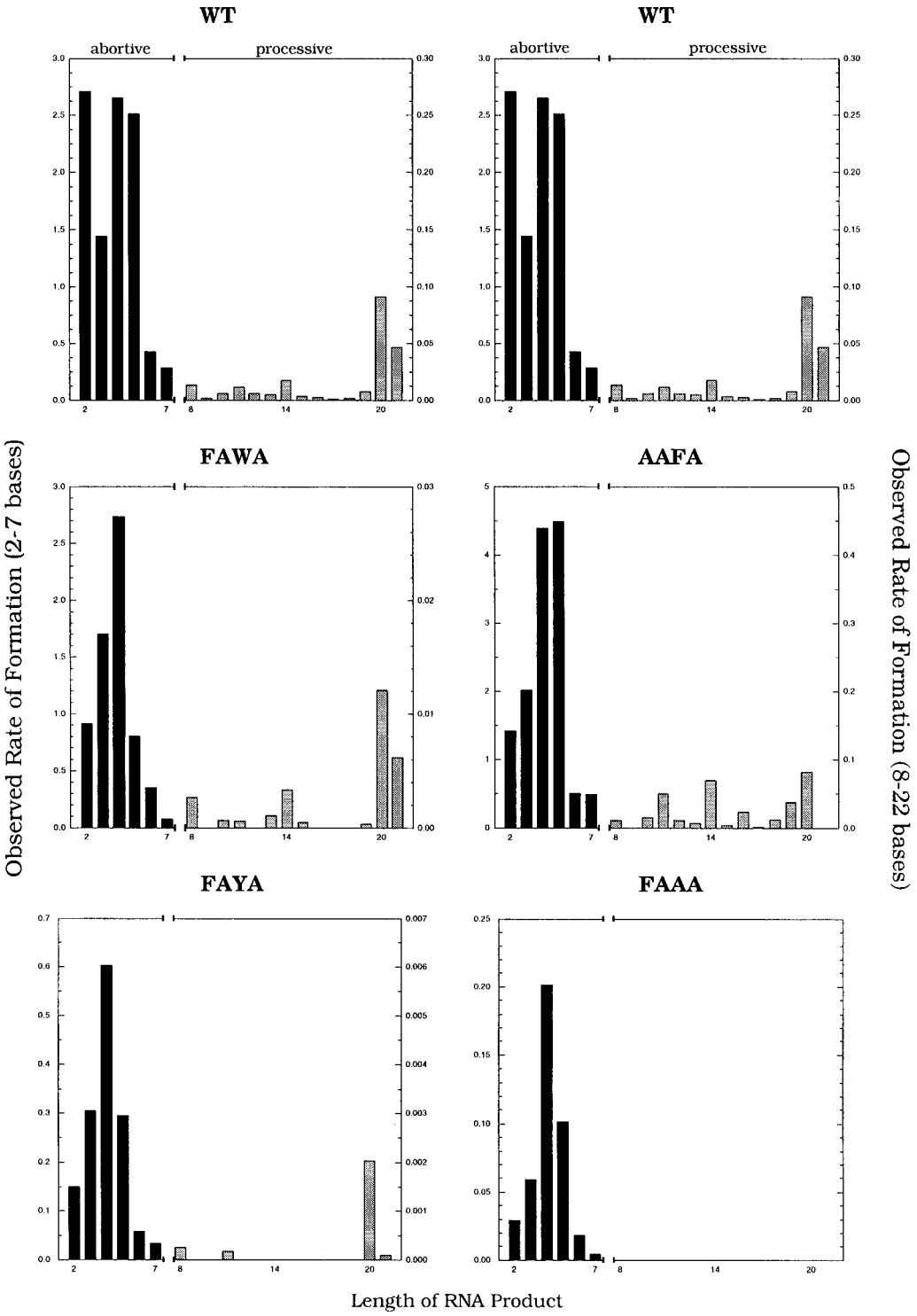


FIGURE 4: Bar graph of the amounts of the transcription products as a function of length for each transcription reaction in Figure 3. Labels at the top of panels indicate the use of the WT or mutant enzyme. The ordinates at the left of the panels apply to products two to seven bases in length (the abortive phase), and the ordinates at the right of the panels apply to all longer products. The units are the rate of formation of each mRNA in moles per minute per mole of enzyme. The ordinate size has been expanded or contracted for each panel to normalize the maximum bar height in each. While the mutant FAAA is not saturated with respect to the initiating GTP, the effect on the amounts of abortive and the longer mRNAs should be the same.

polymerase which occurs ~30% of the time for the WT enzyme. The most striking finding among the foot mutants is that the AAFA enzyme does not incorporate an untemplated nucleotide, while the other foot mutants which make full-length transcripts (all with F^{880} in place) add an untemplated 3′-residue (Figure 4, Table 5). The absence of this activity associated with the AAFA enzyme is more fully documented by the detailed autoradiograph comparing the full-length transcripts of the WT enzyme to those of the AAFA mutant (Figure 5).

Processivity during the Formation of the Ladder of Poly-(G) Products. Normal forward processivity of the T7 RNA polymerase, after incorporation of the 7th NMP, is very high (Table 5). On the other hand, for poly(G) ladder formation, the enzyme remains stalled at position +3 of the template, while the homopolymer message peels off the stalled

Table 5: Processivity of Wild-Type and Foot Mutant T7 RNA Polymerases at Each Step in the Synthesis of a 20-Base mRNA[a]

template position	NTP	FAFA	AAFA	FAYA	FAWA	FAAA
2	G	0.74	0.90	0.90	0.86	0.93
3	A	0.81	0.84	0.77	0.70	0.85
4	A	0.56	0.57	0.39	0.32	0.38
5	A	0.27	0.23	0.24	0.36	0.18
6	G	0.54	0.62	0.38	0.23	0.18
7	U	0.43	0.40	0.07	0.26	0.00
8	C	0.94	0.97	0.90	0.90	−
9	U	0.99	1.00	1.00	1.00	−
10	G	0.97	0.95	1.00	0.97	−
11	U	0.94	0.84	0.92	0.98	−
12	A	0.97	0.96	1.00	1.00	−
13	C	0.97	0.97	1.00	0.95	−
14	C	0.90	0.70	1.00	0.85	−
15	A	0.98	0.97	1.00	0.97	−
16	G	0.98	0.85	1.00	1.00	−
17	A	1.00	0.99	1.00	1.00	−
18	C	0.99	0.90	1.00	1.00	−
19	G	0.95	0.68	1.00	0.98	−
20	U	0.34	0.00	0.04	0.34	−

[a] The processivity is defined as the fraction of total mRNA products of the indicated length which are extended by the addition of the next nucleotide.

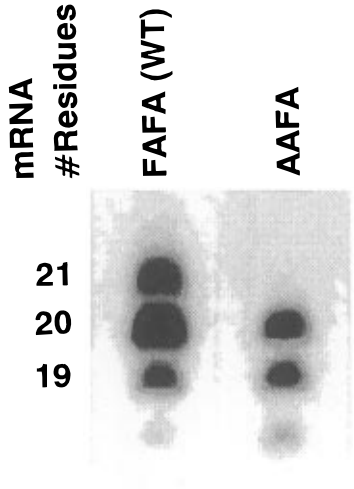


FIGURE 5: Autoradiograph of the gel of the full-length runoff transcripts produced by WT T7 RNA polymerase and the A^{880}-AFA^{883} mutant transcribing from promoter−template T2. The label was [γ-^{32}P]GTP.

template. One might predict a constant rate for this mechanism once the first two bonds have been formed. This model is supported by the observation of an exponential decrease in the amounts of the poly(G) products as they become longer (Figure 2). This is made clear by plotting the quantity of each poly(G) product in log mole percent vs the length of the product according to McClure and Chow (1980) which shows that the processivity describing the postulated one-dimensional diffusion process is constant as predicted (Figure 6). This processivity is also much lower, 0.37−0.62, than the normal forward processivity of the enzyme (Table 5). In contrast to the normal high processivity of the enzyme, that associated with the one-dimensional diffusion and the exclusive incorporation of GMP is significantly changed by these foot mutations. $FAFA^{883}$, $AAFA^{883}$, and FAF^{882} show processivities from 0.52 to 0.62, while all mutants with substitutions of the side chain at 882 decrease this processivity substantially.

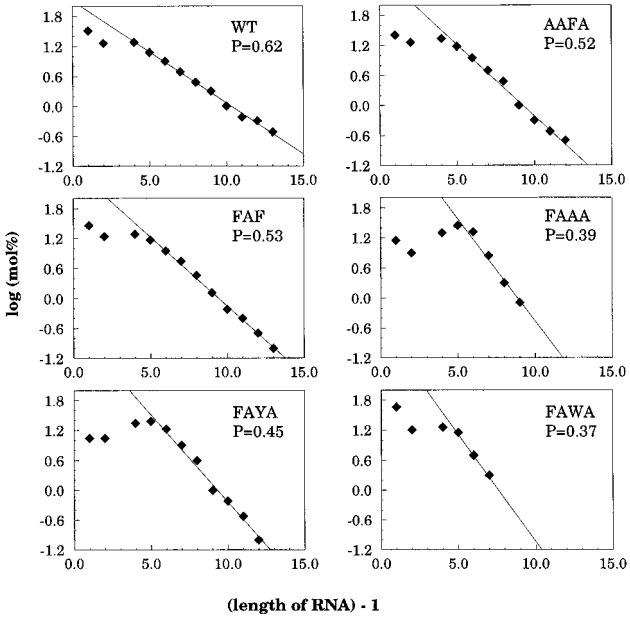


FIGURE 6: Processivity graphs applying to poly(G) ladder formation by T7 RNA polymerase and its foot mutants on promoter−template T3 plotted according to McClure and Chow (1980). The log mole percent of the total products remaining after each step (the ordinate) are plotted vs the length of the RNA minus one residue ($n - 1$). The line in each panel was fit to the poly(G) lengths of six bases or greater ($n - 1 \geq 5$). The slope of this line is equal to $-\log P$, where P is the processivity.

There is one unexplained observation; the GGGG product is missing from all poly(G) ladders, which is perhaps associated in some way with the switch from the movement of the template to diffusion of the mRNA (Figure 2). While migration of most four-base transcripts is relatively normal in this buffer system (Figures 1−3), the possibility that tetra-(G) migrates extremely anomalously has not been ruled out.

Evidence for the Absence of Major Structural Changes in the Foot Mutants of T7 RNA Polymerase. As in all studies of mutant enzymes, unless a crystal structure of each mutant protein is obtained, one cannot rule out the possibility that significant conformational changes have occurred in the protein at some distance from the changed or missing side chain. In order to rule out gross conformational changes consequent to mutations of the four C-terminal residues of T7 RNA polymerase, the circular dichroism in the near-ultraviolet was determined for the purified foot mutants. The near-ultraviolet CD spectra for the WT and the mutants FAAA, FAYA, FAWA, and AAFA are superimposable; thus, these mutations do not cause major changes in the secondary structure of the protein.

Despite a CD spectrum like that of the native protein, the FAAA mutant showed a uniquely high susceptibility to proteolytic degradation during and after sonication of the *E. coli* cells which suggested to us that structural changes not detected by the CD were present in this mutant. To determine the comparative stability of the mutant and WT enzyme, we carried out temperature-induced unfolding of the two proteins following the transition by circular dichroism. The melting curve of the WT protein is highly cooperative, the unfolding transition occurring over a range of 6 °C with a T_m of 48.5 °C. The unfolding transition of the FAAA mutant is less cooperative, occurring over a range of 12 °C with a T_m of 45.1 °C (Figure 7).

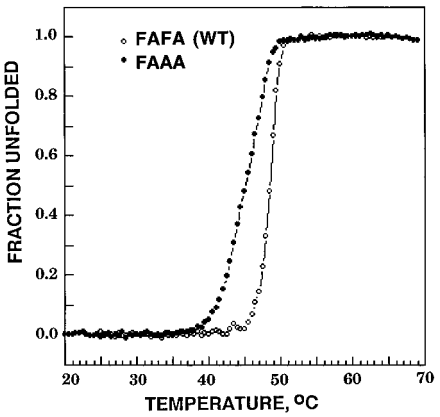
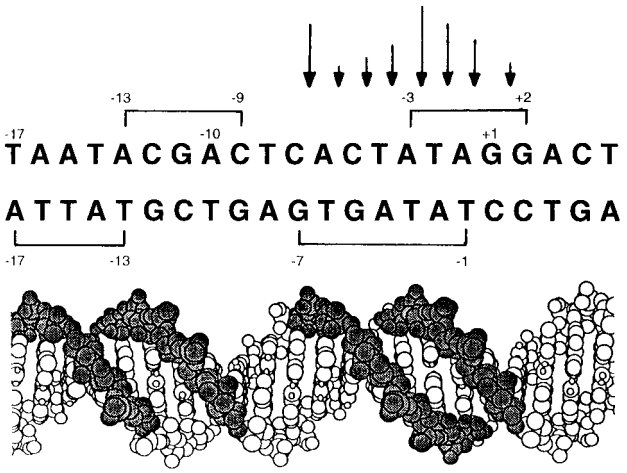


FIGURE 7: Thermal unfolding of WT T7 RNA polymerase and the FAAA mutant. The ellipticity of the proteins at 220 nm is plotted vs temperature.

Scheme 2



DISCUSSION

Substitution or deletion of any of the side chains of the four C-terminal amino acid residues of T7 RNA polymerase causes a dramatic decrease in the rate of initiation of transcription (Table 1). Initiation is the rate-limiting step(s) in the synthesis of mRNAs by the WT T7 enzyme (Martin & Coleman, 1987). The crystal structure of T7 RNA polymerase at 3.3 Å resolution shows that the position of a C-terminal helix, which ends approximately at residue 878, appears to place the $F^{880}AFA^{883}$ C terminus near the active center of the enzyme (Sousa *et al*., 1993). A small three-stranded β-sheet which is homologous to those occurring in the "palm" subdomains of the active centers of Klenow fragment, reverse transcriptase from HIV, and eukaryotic DNA polymerase β identifies the approximate location of the active center (McAllister & Raskin, 1993; Sousa *et al.*, 1993; Swaya *et al.*, 1994). This β-sheet contributes aspartyl side chains which coordinate Mg^{2+} and are D^{537} and D^{812} in T7 RNA polymerase (Bonner *et al.*, 1992; Osumi-Davis *et al.*, 1992). While model building allows placement of the F^{880} and F^{882} side chains above the β-sheet at the active center without steric clashes, no electron density has been published for the immediate C-terminal residues (see the discussion of flexibility below).

Functions of the four C-terminal residues based on the mutation data are postulated to be (1) participation in binding of the initiating nucleotide and (2) interactions with the promoter DNA near the downstream end of the TATA sequence through hydrophobic interactions within the minor groove. Large increases in K_m for the initiating NTP due to mutations of the foot (Table 1) and the finding that the AAFA mutant does not add an untemplated nucleotide at the end of a runoff transcript support the first conclusion (Figure 5). A base-binding pocket on the enzyme surface would appear to be a requirement for adding an untemplated nucleotide residue, and the presence of the F^{880} side chain is essential for this function. In the absence of base pairing with the template, it is not surprising that the processivity of this step is dramatically lowered (Table 5).

The second conclusion is supported by the fact that all changes made within the last four residues of T7 RNA polymerase cause the K_m with respect to promoter DNA to rise by at least 10-fold (Table 1). The accompanying dramatic changes in the initiation rate are not due to the resulting changes in the concentration of the enzyme−promoter complex, since the latter have been factored into the calculation of the initiation rates of the mutants (Table 1). Furthermore, the kinetically determined k_{cat} values are maximum velocities. In the case of the original deletion foot mutant, $FAFA^{883} \rightarrow FA^{881}$, the loss of affinity for promoter DNA was confirmed directly by a DNase footprint on promoter DNA as a function of enzyme concentration for both the WT and FA^{881} mutant under identical conditions (Table 1). The K_d value for the enzyme−promoter complex rose from 0.01 μM for the WT enzyzme to 0.3 μM for the mutant (Mookhtiar *et al*., 1991). An additional finding was that the affinity of the FA^{881} mutant for nonspecific DNA actually rose compared to that of the WT enzyme, as if this terminal FA^{883} is involved in the recognition of a specific DNA sequence within the promoter.

While the changes in K_m for both the initiating nucleotide and the promoter DNA accompanying mutations of the last four residues of the polymerase are striking, there must be changes that specifically affect the catalytic steps to explain the low initiation rates (Table 1). Thus, the conformation of the foot must have important roles in determining the placement of the single-stranded template and the nucleotides base paired at positions +1 and +2 relative to any catalytic groups of the enzyme, e.g. enzyme-bound Mg^{2+}. The template sequence in this region is 3′-TGATATCC-5′ in which CC are positions +1 and +2. Probing of the T7 RNA polymerase−promoter complex with a single-stranded endonuclease has shown that the nontemplate strand, 5′-CACTATAGG-3′, is exposed to single-stranded endonuclease digestion for a length of eight nucleotides (sugar phosphates from nucleotides −7 to +2) in the region of the TATA box (Scheme 2). The diester bonds of the nontemplate strand cleaved by the endonuclease are indicated by the arrows above the nontemplate strand in Scheme 2. The length of the arrrows indicates the sensitivity of each bond to cleavage. In contrast, the complementary region of the template strand, 3′-GTGATATCC-5′, is fully protected from endonuclease cleavage (Strothkamp *et al.*, 1980; Osterman & Coleman, 1981). This finding suggests that the double strand is melted in this region.

Despite the dramatic effect of most of the mutations in the four C-terminal residues of T7 RNA polymerase on the steps that constitute initiation, they have little effect on the processivity pattern of the polymerase (Figure 4, Table 5). With the exception of the FAAA, all the foot mutants can

establish a very high processivity, 0.95−1.00, after the incorporation of the first seven nucleotides. This suggests that the interactions between the emerging mRNA and the polymerase which determine this high degree of processivity are not located in the immediate vicinity of the polymerase active center. This conclusion is compatible with the suggestions that the high processivity associated with mRNA longer than seven bases is due either to interactions of the emerging mRNA with the thumb subdomain of the enzyme as has been suggested recently (Bonner *et al.*, 1994) or to an interaction of the mRNA with the N-terminal 20 kDa domain of the polymerase, $M^{1}-K^{179}$ (Muller *et al.*, 1988). Even a single cleavage of the $K^{179}-K^{180}$ peptide bond significantly decreases this high processivity (Muller *et al.*, 1988).

Since the TATA box and the mRNA start site are not separated in the T7 promoter, it is not surprising that the FAFA structure could participate in binding of the promoter DNA as well as in binding of the initiating nucleotide. Further postulates concerning the function of the FAFA foot rest on some general properties of the foot mutants. Both size and conformation of the foot are severely restricted. Addition of a F^{884} residue drops k_{cat} by 10-fold, while addition of an extra two residues, $F^{884}A^{885}$, drops k_{cat} by 150-fold (Table 1). Deletion of the C-terminal A^{883} creates a reasonably active enzyme that makes full-length products, a relatively normal poly(G) ladder, and retains a relatively high processivity within the abortive phase (Figures 2 and 4, Table 1). However, deletion of $F^{882}A^{883}$ together disables the protein as dramatically as does the FAAA mutation (Table 1). While much of the mutation and deletion data suggest that the F^{880} and F^{882} residues are the most important in maintaining the activity of the polymerase, the intervening residue, A^{881}, has special properties as well. Substitutions of G, T, or L in the relatively active FAF mutant drops k_{cat} another 10-fold (Table 1). These data suggest that the size and hydrophobicity of the foot are important, and the restriction to a CH_3 side chain at position 881 implies a highly specific packing of this structure into the ternary polymerase−promoter−NTP complex. There are seven single-subunit phage RNA polymerases and the single-subunit mitochondrial RNA polymerase which contain FXFX or YXFX as the C-terminal four residues, where X is a hydrophobic or aromatic residue (Butler & Chamberlin, 1982; Kotani *et al.*, 1987; Masters *et al.*, 1987; Oeser & Tudzynski, 1989; Dietz *et al.*, 1990; Lerbs-Mache, 1993).

Footprinting with hydroxyl free radicals generated by the Fe-EDTA method reveals that the sugar−phosphate backbone of the promoter DNA is protected by T7 RNA polymerase from radical-induced cleavage in two staggered patterns separated by 11 base pairs or about one turn of the DNA helix (shown in Scheme 2 by brackets) (Muller *et al.*, 1989). The staggered pattern of the footprints, one beginning at +2 and the second at −9 on the nontemplate strand, is indicative of ligand−DNA interactions taking place across the minor groove of the DNA as seen in the projection of the hydroxyl radical footprint on B-form DNA (Scheme 2). The most downstream of the two regions straddles the TATAG sequence that appears to be close to the target for the interaction of the $FAFA^{883}$ structure; the G is position +1.

Several crystal and NMR-determined structures of DNA−protein complexes have been published which illustrate that proteins can bind to DNA via minor groove intercalation using Leu, Phe, or Trp side chains. The most notable of these is the TATA binding protein or TBP (Kim, Y., *et al.*, 1993; Kim, J. L., *et al.*, 1993), but similar interactions between the minor groove and the protein are found for SRY, the protein encoded by the human testis-determining gene (Werner *et al.*, 1995a), the human oncogene product, ETAS1 (Werner *et al.*, 1995b), and the purine repressor protein, PurR, from *E. coli (*Schumacher *et al.*, 1994). Thus, a set of hydrophobic residues like the FAFA at the C terminus of T7 RNA polymerase may be involved in hydrophobic or even partially intercalative interactions with the promoter via the minor groove.

The hydroxyl radical footprint of the polymerase on the DNA in this region extends from the G at −7 on the template strand to the G at +2 on the nontemplate strand, thus protecting both sides of the minor groove (Scheme 2). Interactions of FAFA with the downstream end of this sequence, 5′-CACTATAGG-3′, could be involved in the binding of both the initiating NTP and the promoter DNA, accounting for the large decrease in affinity of the enzyme for the promoter caused by mutations in the FAFA sequence (Table 1). It is noteworthy that the hydrophobic side chains constituting the "glove-fit" protein−DNA interaction of TBP tolerate substitution of polar or larger side chains very poorly (Reddy & Han, 1991; Yamamoto *et al.*, 1992; Poon *et al.*, 1993), a characteristic of the FAFA sequence as well (Table 1).

The FAFA structure may be part of a sequence or base recognition device, but the complete set of interactions between T7 RNA polymerase and the promoter in the region of the 5′-ACTATAGG-3′ sequence must be more extensive, since the DNA helix is melted over these eight base pairs centered on the TATA sequence as the endonuclease probe shows (Osterman & Coleman, 1981). Hydrophobic interactions including partial intercalation of the $F^{880}AFA^{883}$ structure in the minor groove beginning at the 3′-end of this sequence could be associated with the melting of this region of the promoter.

The hydrophobicity and stringent requirements on the size and nature of the side chains which are functional at residues 880−883 of the T7 polymerase, as well as the apparent direct effect of changes in the $FAFA^{883}$-COO^{-} structure on promoter binding, might suggest that this C-terminal foot must be present in a fixed structure at the active center. However, there is significant evidence that this is not the case in the free enzyme. Treatment of the free enzyme with carboxypeptidase A removes A^{883} and F^{882} and results in a loss of activity to the level shown by the authentic deletion mutant, FA^{881}-COO^{-} (Mookhtiar *et al.*, 1991). While the addition of promoter-containing T7 DNA protects against carboxypeptidase A digestion, the protection is only partial. Some type of interaction between the hydrophobic C terminus and the protein surface seems likely on the basis of the decreased stability of the FAAA mutant (Figure 7); however, the foot may be relatively flexible and may become fixed only in the ternary enzyme−promoter−NTP complex. If the interaction of FAFA-COO^{-} is initially with the downstream region of the promoter as postulated, the foot is likely to undergo cycles of conformational change as the template moves through the active center.

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