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T7 RNA polymerase mutants with altered promoter specificities

(cloning vectors/protein–DNA recognition/DNA-binding proteins/hydrogen bonding/phage RNA polymerases)

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ABSTRACT **The amino acid at position 748 in T7 RNA polymerase (RNAP) functions to discriminate base pairs at positions −10 and −11 in the promoter. We have constructed a series of T7 RNAP mutants having all possible amino acid substitutions at this position. Surprisingly, most (13/19) substitutions result in active RNAPs, and many of these exhibit altered promoter specificities. Identification of mutant RNAPs with altered specificities expands the repertoire of highly specific phage RNAPs that are available for use in phage RNAP-based transcription systems and highlights the complexity of sequence-specific DNA recognition.**

T7 RNA polymerase (RNAP) is the best characterized of a family of single-subunit DNA-dependent RNAPs that also includes the RNAPs encoded by bacteriophages T3, K11, and SP6 (1–4). The highly processive bacteriophage enzymes are particularly well suited for studies of RNAP structure and function, as they are able to perform all of the functions that are required for transcription (promoter recognition, initiation, elongation, and termination) in the absence of any auxiliary factors (5).

The phage RNAPs exhibit striking specificity for their promoters, all of which are related to a common 23-base pair consensus sequence (Fig. 1*A*). In the case of T3 vs. T7 promoter specificity, the base pairs at −10 and −11 are the primary determinants of specific promoter recognition, and a T7 promoter variant having the corresponding T3 base pairs substituted at these positions (designated P_{T7} −10C, −11C; the letter denotes the base on the nontemplate strand) is utilized by T3 RNAP but not by T7 RNAP (12). Previous work demonstrated that recognition of these base pairs involves the amino acid residue at position 748 (Asn) and that a T7 RNAP mutant having the corresponding T3 amino acid (Asp) at this position (denoted T7-N748D) preferentially utilizes P_{T7} −10C, −11C over a consensus T7 promoter (P_{T7}) (10, 13, 14). To account for this specificity, it has been proposed that residue N748 makes specific hydrogen bonds with the base pair at −11 (and possibly at −10) in the major groove (10, 11, 14). This model is supported by a preliminary 3.1-Å electron density map of T7 RNAP that places N748 within a putative DNA-binding cleft (15, 16).

To understand further the nature of the interaction(s) between the residue at position 748 and base pairs in the −11 region of the promoter, we constructed a series of T7 RNAPs having each of the 20 amino acids at this position. The activities and promoter preferences of these RNAPs were determined through the use of a collection of T7 promoter variants having all possible single base-pair substitutions at −10, −11, and −12. Most (13/19) amino acid substitutions result in active RNAPs, and many of these exhibit altered promoter specificities. In view of prior observations that altered-specificity mutants are rare among DNA-binding proteins (17), the large number of active RNAP mutants is surprising. A consideration of how the specificities of these mutant RNAPs might arise has contributed to our understanding of promoter recognition by the phage RNAPs and may also be important to a more general understanding of sequence-specific DNA interactions involving other proteins.

MATERIAL AND METHODS

Mutagenesis. pAR1219 (18) was modified to place a silent *Mlu* I restriction site in the RNAP gene at codon 746 and to remove an *Mlu* I site in the plasmid backbone, resulting in pCAR34 (14). RNAP mutants altered at codon 748 were constructed by the PCR using the mismatched primer method (19) and pCAR34 as template. Primer A (5′-CGGAATGGTACCGAAGGA-3′) contains a *Kpn* I restriction site (underlined). Primer B (5′-TCAGACGCGTTTGNN(G/C)CTGATGTTCCTCGGTCAGTTCCGC-3′) contains an *Mlu* I restriction site (underlined) and a degenerate sequence at codon 748 (double underlined). The PCR product AB was digested with *Kpn* I and *Mlu* I and cloned into pCAR34, replacing the interval from the *Kpn* I to *Mlu* I restriction sites in that plasmid. DNA sequencing using the chain termination method (20) was used to confirm the sequence of each of the 20 mutants in the *Kpn* I to *Mlu* I interval. All plasmids were propagated in *Escherichia coli* BL21, which is defective in a surface protease known to cleave the phage RNAP (21).

Characterization of Mutant RNAPs. Assays of promoter-binding and nonspecific catalytic activities were carried out using cell extracts. Cultures were grown to an absorbance of 0.6 at 600 nm (OD_{600}) in LB broth (22) containing 50 μg of ampicillin per ml and induced with 0.4 mM isopropyl β-D-thiogalactoside for 4 hr. Samples (1.5 ml) were harvested by centrifugation, washed in 1/2 volume of harvest buffer, resuspended in 1/3 volume of lysis buffer, disrupted, and clarified by centrifugation at 15,000 × *g* for 10 min, as described (23). To measure RNAP production, 5-μl portions of the extract were analyzed by electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (24). Nonspecific (promoter-independent) catalytic activity assays were carried out in 10-μl reaction mixtures containing 2 μl of cell extract (≈4 μg of total protein), 5 μg of synthetic poly(dC) template (Pharmacia), and 0.5 mM [α-^{32}P]GTP (0.2 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) (23). Promoter-binding assays (25) were carried out in a reaction volume of 20 μl using 2 μl of cell extract and ≈2 ng of labeled double-stranded oligomer, as described by Gross *et al.* (23).

Transcription reactions to determine promoter specificity were carried out as described by Raskin *et al.* (10) using RNAP that had been purified by chromatography over phosphocellulose (14). The products were resolved by electro-

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Abbreviation: RNAP, RNA polymerase.

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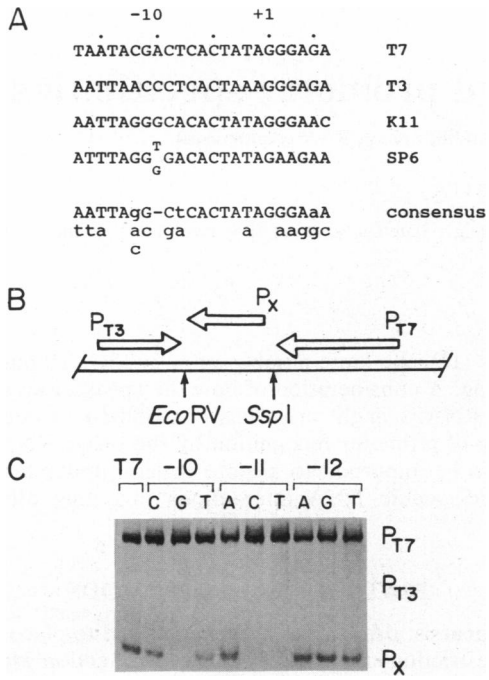

FIG. 1. Promoter structure and construction of template DNA. (*A*) Comparison of consensus promoter sequences for T7, T3, K11, and SP6 RNAPs. The sequences of the nontemplate strand are shown. A consensus sequence shared by all phage RNAPs is indicated at the bottom. The start site of transcription is at +1 (refs. 6–9; J. Rush, personal communication). (*B*) Structure of test templates. Each plasmid contains three promoters: a reference T3 promoter (P_{T3}), a reference T7 promoter (P_{T7}), and a variant T7 promoter (P_X) having one of the four possible base pairs at position −10, −11, or −12 (refs. 10 and 11; see Table 1). Digestion of the plasmid with *Eco*RV and *Ssp* I prior to transcription gives rise to 243-, 297-, and 164-nucleotide runoff products from these promoters, respectively. (*C*) Promoter preference of wild-type T7 RNAP. Plasmid templates, prepared as described above, were transcribed with purified T7 RNAP and the products were resolved by electrophoresis in an 8% polyacrylamide gel. The variant promoter in each plasmid is identified above the lane by noting the base found in the nontemplate strand at the indicated position (T7 signifies use of the consensus promoter sequence as P_X). Transcripts arising from P_{T3}, P_{T7}, and P_X are identified; the different sizes of these transcripts (see above) should be taken into account in interpreting the intensity of the bands.

phoresis and visualized by autoradiography. Each lane of the film was analyzed by densitometry using an LKB densitometer. The intensity of the band arising from P_X was normalized to an internal control (P_{T7} or P_{T3}), and the utilization of each variant promoter was then calculated. Specific activities for each enzyme were determined by measuring the incorporation of substrate into acid-insoluble product (26) in the presence of a template having the optimal promoter for that RNAP.

RESULTS

Mutagenesis and Functional Integrity of the RNAP. The gene that encodes T7 RNAP was modified by site-directed mutagenesis to provide a collection of RNAP mutants having each of the 20 amino acids at position 748. All of the RNAPs are soluble, full length, and expressed at normal levels in *E. coli* (Fig. 2*A*).

An important initial consideration was whether the mutant RNAPs retained basic catalytic and/or promoter-binding activity, as it is possible that some alterations might result in extensive changes in protein structure and a generalized loss of enzymatic activity. As shown in Fig. 2*B*, all of the RNAPs exhibit normal levels of nonspecific (promoter-independent) catalytic activity as judged by their ability to synthesize poly(G) from a poly(dC) template. This is consistent with the properties of previously isolated mutants that are affected in this region of the RNAP and with the role of residue 748 in promoter recognition but not catalytic activity (23).

The ability of the mutant RNAP to bind to a consensus T7 promoter was assessed by a gel retardation assay (Fig. 2*C*) and was observed to correlate with the activity of the RNAP in an *in vitro* transcription assay. In general, those RNAPs that were able to initiate transcription at P_{T7} *in vitro* (see below) showed P_{T7}-binding activity, whereas mutant RNAPs that utilized the consensus promoter poorly failed to show binding. We also determined the activity of the mutant RNAP *in vivo* by measuring the expression of a chromosomal chloramphenicol-resistance gene under control of a consensus T7 promoter (23, 28). As before, the activity of the RNAP in the *in vivo* assay correlated with the ability of the RNAP to bind to and initiate transcription from the consensus promoter *in vitro* (data not shown). It thus appears that failure to bind to a consensus promoter is the primary reason for the reduced activity of these mutants (see below).

Specificity of the Mutant RNAPs. To determine the specificities of the mutant RNAPs, we utilized plasmid templates that contained a reference T3 promoter, a reference T7 promoter, and a test promoter (usually a T7 promoter having a nonconsensus base pair at position −10, −11, or −12; see Fig. 1*B* and Table 1). Cleavage of the template with appropriate restriction enzymes prior to transcription results in the synthesis of characteristically sized run-off products from each promoter (Fig. 1*C*).

In the first series of analyses, each enzyme was tested with mixtures of plasmid templates that presented test promoters having all possible single base-pair substitutions at position −10, −11, or −12. The majority of mutants exhibited significant activity in this assay. Active mutants included T7-N748A, C, D, E, G, H, K, Q, R, S, T, W, and Y; mutants with little or no activity included T7-N748V, L, I, P, F, and M (not shown). With the exception of Gly and Ala (which project small side chains) the active mutants possess side chains capable of forming hydrogen bonds, whereas the inactive mutants (except for Pro) have a bulky, nonpolar side chain at this position. It is not known whether the latter mutant RNAPs are inactive because they require more than one base change from the consensus promoter for recognition or because their conformation is sufficiently altered as to disrupt other critical contacts.

The preference of each of the active RNAPs for individual T7 −10, T7 −11, and T7 −12 promoter variants was determined as shown in Fig. 1*C*; the results are summarized in Fig. 3. Each mutant RNAP exhibited a hierarchy of promoter preference that differs from wild-type T7 RNAP. Two general classes of active polymerase mutants were observed—those that retained broad activity on a number of different variant promoters (T7-N748A, C, G, H, K, Q, R, and S, as well as wild-type T7 RNAP) and those that exhibited activity at only one or a few promoters (T7-N748D, E, T, W, and Y).

The relative specific activity of each mutant RNAP at its preferred promoter (as compared to the activity of wild-type T7 RNAP at P_{T7}) ranged from 0.36 to 0.04 (Fig. 3). It should be noted that the stringent requirements of T7-N748D, E, T, W, and Y for a particular base pair at either position −10 or −11 may obscure the detection of secondary preferences at other positions and may result in an underestimate of the activities of these enzymes. For example, T7-N748D requires a C·G base pair at −11, and in the absence of this base pair no activity is detected from any of the −10 variants (Fig. 3). However, if a C·G base pair is presented at −10 in combi-

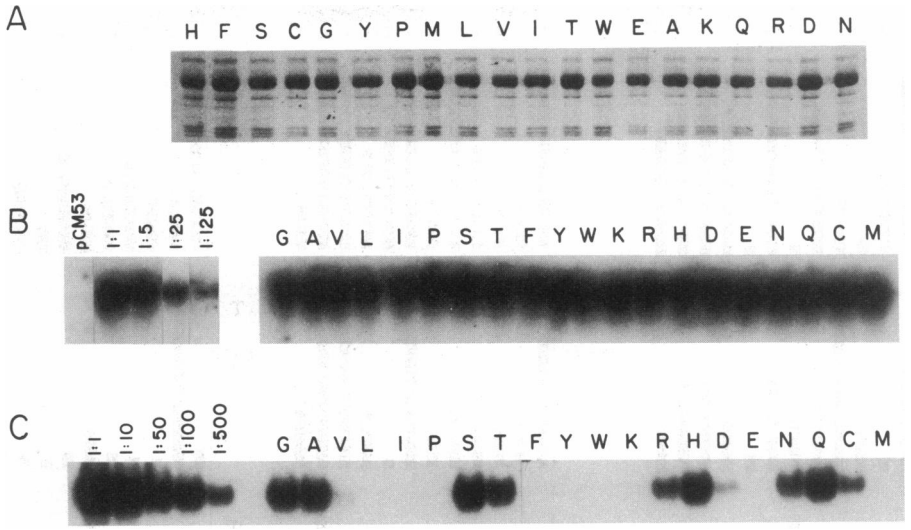

FIG. 2. Functional and structural integrity of mutant RNAP. Cultures were induced with isopropyl β-D-thiogalactoside and cell extracts were prepared as described by Gross *et al.* (23). (*A*) Integrity and solubility of RNAP. Samples (5 μl) of the extract were resolved by electrophoresis in a 10% polyacrylamide gel and stained with Coomassie blue (24). The major band corresponds to T7 RNAP. (*B*) Nonspecific (promoter-independent) catalytic activity. The products of reactions containing 1 μl of cell extract, poly(dC) as template, and [α-^{32}P]GTP as substrate were analyzed by electrophoresis in a 20% polyacrylamide gel. The autoradiogram reveals the synthesis of poly(G) products >150 nucleotides that migrate near the origin of the gel (23). Dilution of extracts that contain wild-type T7 RNAP in a null extract lacking RNAP (pCM53; ref. 27) indicates that this assay can detect nonspecific catalytic activity over a 125-fold range of polymerase concentrations. (*C*) Promoter-binding activity. Cell extracts were incubated with a 24-bp ^{32}P-labeled oligonucleotide that contains a T7 promoter, and the protein/DNA mixtures were resolved by electrophoresis under conditions in which specific binding of the oligomer results in retardation of its mobility (23). Dilution of the cell extract as described above demonstrates that this assay can detect promoter binding over a 500-fold range of polymerase concentrations.

nation with a C·G base pair at −11, the activity of this enzyme is increased ≈2-fold (10, 14).

DISCUSSION

The key finding of this work is that substitution of different amino acids at position 748 generates T7 RNAPs with altered promoter specificities. Some of these RNAP mutants have promoter preferences not previously observed for any known phage RNAP. Others, such as T7-N748K and R, have specificities for the base pairs at −10, −11, and −12 that are similar to those of previously characterized RNAPs—e.g., the K11 and SP6 RNAPs, which have Lys and Arg at the homologous positions, respectively (3, 4). This suggests that the K11 and SP6 RNAPs may generate specificity for these base pairs in a manner similar to that of T3 and T7 RNAP (10, 14). The broad spectrum of specificities exhibited by the mutant RNAPs has contributed to our understanding of the mechanisms of promoter recognition (see below) and may enrich our understanding of sequence-specific protein–DNA interactions in general. Furthermore, by expanding the repertoire of highly specific RNAPs that are available for the synthesis of RNA probes, or for use in phage RNAP-based expression systems, these findings may be of practical significance.

Table 1. Sequences of variant T7 promoters

Promoter	Sequence	Plasmid
	−15 −10 −5 +1 +5	
P_{T7}	taatacgactcactatagggaga	pRKD258
P_{T7} −10C	taatacg**C**ctcactatagggaga	pRKD256
P_{T7} −10G	taatacg**G**ctcactatagggaga	pGD15
P_{T7} −10T	taatacg**T**ctcactatagggaga	pGD16
P_{T7} −11A	taatac**A**actcactatagggaga	pGD17
P_{T7} −11C	taatac**C**actcactatagggaga	pRKD247
P_{T7} −11T	taatac**T**actcactatagggaga	pGD19
P_{T7} −12A	taata**A**gactcactatagggaga	pRKD243
P_{T7} −12G	taata**G**gactcactatagggaga	pGD22
P_{T7} −12T	taata**T**gactcactatagggaga	pGD23

The sequence of the nontemplate strand is shown. Positions in the promoter are numbered relative to the start site for transcription (+1). Uppercase characters (in boldface) indicate changes from the consensus promoter sequence (P_{T7}).

Recognition of the Base Pair at −11. Biochemical and genetic studies indicate that the side chain of the Asn residue at 748 generates specificity for the G·C base pair at −11 through direct interaction with the nontemplate guanine (10, 14). Replacing N748 with glycine, which lacks a functional side chain (a "loss of contact" substitution), is expected to reduce or eliminate discrimination of this base (17, 29, 30). As anticipated, T7-N748G, unlike T7 RNAP or any of the active mutants, is able to utilize a P_{T7} −11T promoter about as well as a consensus T7 promoter despite the presentation of a methyl group by thymine in the major groove. Although T7-N748G exhibits reduced discrimination against P_{T7} −11C, the mutant enzyme retains a significant bias against this promoter. The latter observation suggests that additional features of the RNAP may contribute to recognition of the base pair at −11.

T7-N748H exhibits enhanced specificity for the consensus promoter (i.e., nonconsensus base pairs are generally less well tolerated by T7-N748H than by the wild-type enzyme). The wild-type specificity of T7-N748H is consistent with the similar side-chain lengths of His and Asn and with the ability of the δ nitrogen on the His side chain to mimic the hydrogen-bonding capabilities of Asn (30). The specificity of T7-N748E is qualitatively similar to T7-N748D, and it is likely that both of these mutants make the similar major groove contacts at −10 and −11.

The ability of T7-N748Q to utilize P_{T7} −11C is somewhat unexpected, given the chemical similarity of the Gln side chain to that of the wild-type Asn. The increased length of the Gln side chain may alter the orientation of the carbamide group in the major groove or allow increased flexibility in the positioning of hydrogen bonds.

Recognition of the Base Pair at −10. Previous experiments suggested that the nontemplate base at position −10 may also be contacted by N748 (10, 14). Consistent with this, replace-

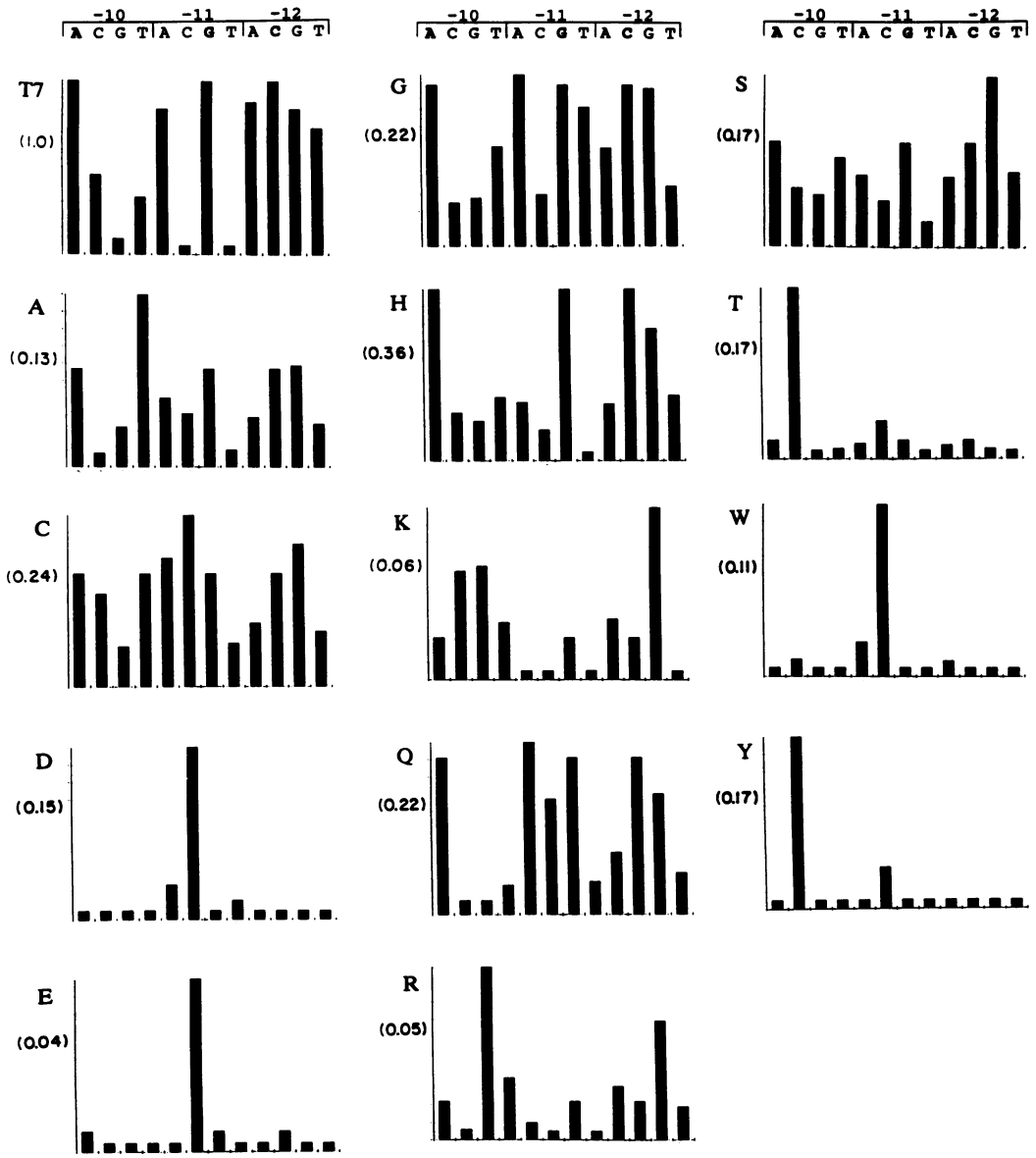

FIG. 3. Specificity and activity of wild-type and mutant RNAPs. Plasmid templates were transcribed with purified RNAP and the products were resolved by electrophoresis as described in the legend to Fig. 1. The letter to the left of each panel indicates the amino acid found at position 748 in the mutant RNAP; T7 signifies the wild-type enzyme. The base found in P_X on the nontemplate strand is indicated above each lane; boldface letters denote the consensus base at that position. Each lane of the autoradiogram was analyzed by densitometry and the intensity of the band arising from P_X was normalized to an internal control in that lane (P_{T7} for most mutants, P_{T3} for T7-N748D and W). The height of the bar represents the strength of each promoter relative to the strongest promoter in the series. The relative specific activity of each RNAP at its best promoter (as compared to the activity of wild-type T7 RNAP at P_{T7}) is given in parentheses.

ment of N748 with A, K, R, T, and Y results in RNAPs with altered specificities for this base pair. However, replacing N748 with C, G, H, Q, or S does not dramatically alter discrimination of the base pair at position −10, indicating that for these mutants (and possibly for the wild-type enzyme) recognition of this base pair may involve additional contacts. The observation that T7-N748G does not exhibit altered specificity for the base pair at −10 is important in this regard.

Recognition of the Base Pair at −12. Although the base pair at position −12 is highly conserved among all T7 promoters, substitutions of alternate base pairs at this position result in only mild reductions in utilization by T7 RNAP (refs. 10–12, 14; see Fig. 3). In our earlier work, there was no evidence to support an interaction between residue N748 and the base pair at −12 (10, 14). Compared to wild-type T7 RNAP, most mutants with broad promoter specificity exhibit a preference for C·G and G·C base pairs (as opposed to A·T and T·A base pairs) at −12. This observation may reflect the unmasking of a preexisting preference for G·C and C·G base pairs at this position as a result of reduced specificity for the base pairs at −10 and/or −11. The bias against A·T and T·A base pairs at −12 may be noteworthy, as this pattern of discrimination is often associated with minor groove contacts (11, 31).

General Considerations. It is possible for the center of an aromatic ring to function as a hydrogen bond acceptor (32). Such an interaction (which is estimated to be energetically half as strong as a typical hydrogen bond) could be an important component of the specificity of T7-N748W if the cytosine N4 atom of −11C were to interact with either aromatic ring of tryptophan. Compared to its activity at P_{T7} −11C, T7-N748W exhibits enhanced activity at P_{T7} −10C, −11C (14). This effect might result from an interaction of each aromatic ring of tryptophan with one of the cytosine N4 atoms at −10C and −11C. The specificity exhibited by

T7-N748Y might also involve an aromatic hydrogen bond to the cytosine N4 atom of −10C (or −11C). Alternatively (or in addition), the tyrosyl hydroxyl group might contribute to the unusual specificity of T7-N748Y through conventional hydrogen bond interactions (see below). Aromatic hydrogen bonds have been observed in protein–protein and protein–drug interactions (33–35) but have not yet been demonstrated as the basis for sequence-specific DNA–protein interactions. It has recently been proposed that aromatic hydrogen bonds may contribute to the specificity of several catabolite gene activator protein mutants (R. Ebright, unpublished material). T7-N748W may provide an additional model for studies of aromatic hydrogen bond-mediated DNA recognition.

It is important to note that the base-pair preferences of the mutant RNAP were determined in the context of a T7 promoter (i.e., using T7 promoter variants having only a single base-pair change from the consensus sequence). Although T7-N748C, D, E, G, Q, T, W, and Y will all utilize T3 base pairs at either −10 or −11, T7-N748C, G, Q, and T do not utilize a consensus T3 promoter, whereas T7-N748D, E, W, and Y do (14). Thus, despite their tolerance for T3 base pairs at −10 and −11, the former enzymes must retain some other features of T7 RNAP that are important for discrimination against a T3 promoter. It is possible that long-range conformation differences in the protein–DNA interface contribute to promoter specificity or that there are alterations in protein and/or DNA conformation that occur subsequent to initial binding. These conformation changes may be important in the later steps of the transcription process, including catalysis, and may contribute to the apparent specificity of the mutant RNAP. The ability of the RNAP to participate in these interactions may depend not only upon the appropriate hydrogen bond capabilities of residue 748 but also upon the interaction of this amino acid residue with the rest of the protein.

As shown in Fig. 3, T7-N748T and Y exhibit nearly identical specificities for variant T7 promoters. Although this might suggest a role for the hydroxyl group present in both of these amino acids in promoter recognition, this group is located at different positions relative to the α-carbon backbone in Thr and Tyr. The similar specificities of these RNAPs for the base pairs at −10 and −11 would thus have to arise by formation of hydrogen bonds to different determinants in these base pairs or by a different orientation of Thr and Tyr in the protein. These potential subtleties in recognition are reflected in the observation that T7-N748T will utilize a T7 promoter but not a T3 promoter, whereas T7-N748Y will use both promoters weakly (14). Similarly, T7-N748T (but not T7-N748Y) can bind to a consensus T7 promoter *in vitro* (Fig. 2). Consistent with the discussion above, these differences may result from subtle changes in polymerase structure caused by the presence of negatively charged or aromatic amino acid residues (i.e., D, E, W, or Y) at position 748.

In conclusion, it is likely that recognition of the −11 region of the promoter by T7 RNAP involves multiple mechanisms of protein–DNA recognition, among which direct readout of the sequence by hydrogen bond interactions is the most important. Other mechanisms such as indirect readout and induced fit (36–45) may also contribute to the recognition process. The RNAP mutants described in this work provide a rich resource with which to characterize these interactions.

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