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Promoter specificity determinants of T7 RNA polymerase

(DNA binding protein/processivity/transcription/base pairing)

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ABSTRACT **The high specificity of T7 RNA polymerase (RNAP) for its promoter sequence is mediated, in part, by a specificity loop (residues 742–773) that projects into the DNA binding cleft (1). Previous work demonstrated a role for the amino acid residue at position 748 (N748) in this loop in discrimination of the base pairs (bp) at positions −10 and −11 (2). A comparison of the sequences of other phage RNAPs and their promoters suggested additional contacts that might be important in promoter recognition. We have found that changing the amino acid residue at position 758 in T7 RNAP results in an enzyme with altered specificity for the bp at position −8. The identification of two amino acid:base pair contacts (i.e., N748 with the bp at −10 and −11, and Q758 with the bp at −8) provides information concerning the disposition of the specificity loop relative to the upstream region of the promoter. The results suggest that substantial rearrangements of the loop (and/or the DNA) are likely to be required to allow these amino acids to interact with their cognate base pairs during promoter recognition.**

The single subunit DNA-dependent RNA polymerases (RNAPs) that are encoded by bacteriophage T7 and its relatives (e.g., T3, SP6, K11) are highly specific for their individual promoter sequences (for review, see ref. 3). Although each promoter consensus sequence is related to a common sequence that extends from −17 to +6, significant differences in the interval from −8 to −11 suggest that this region may be critical to the discrimination of the promoter by its respective RNAP (Fig. 1). Indeed, in earlier work the bp at −10 and −11 were found to be the primary determinants of specificity for T7 versus T3 promoters, and the bp at −8 and −9 were found to be the primary determinants of specificity for SP6 versus T7 promoters (4, 5).

Promoters for the phage RNAP seem to consist of two functional domains: a binding domain that extends from −17 to −6 and an initiation domain that extends from −5 to +6 (6, 7). In general, variations in the binding domain affect the affinity of the RNAP for the promoter but have little effect on initiation (k_{cat}), whereas variations in the initiation region affect k_{cat} but have little effect on binding (6, 7). A variety of experimental results indicate that the binding region is recognized as a double strand duplex upstream from −6 and that the initiation region is melted open very rapidly upon (or simultaneously with) polymerase binding (7–11). During the early stages of transcription, T7 RNAP engages in repeated cycles of abortive initiation in which short RNA products are synthesized and released before the polymerase clears the promoter and forms a stable elongation complex (12–14). Footprinting studies with methidiumpropyl EDTA-Fe(II) have shown that the polymerase protects the promoter as far upstream as −21 during this process and that these contacts are maintained until the polymerase isomerizes into a processive elongation complex (15).

The topology of T7 RNAP:promoter contacts in the binding region has been characterized in some detail (see Fig. 1). Hydroxyl radical footprinting and chemical modification interference studies reveal contacts located predominately on one face of the double-stranded DNA helix, centered on the major groove in the vicinity of the bp at −9 (16, 17). A consideration of the hierarchy of permissible base substitutions (18–20) and studies involving incorporation of base analogs at defined positions (7, 21, 22) have identified functional groups in the major groove that are important to promoter binding. Contacts between the RNAP and the promoter are made on the nontemplate (NT) strand at −11 and −10 but cross to the template (T) strand side at −9 and track along this side of the major groove until −6/−5, where the transition to a melted form of the DNA in the initiation region is expected to begin (7).

The crystal structure of T7 resembles a cupped right hand, with fingers, palm, and thumb domains that form a putative DNA binding cleft features that seem to be common with other polymerases studied to date (1, 23). In previous work, we identified a specific residue in T7 RNAP (N748) that is responsible for discrimination of the bp at −10 and −11 (2, 24). Substitution of this amino acid with the corresponding residue found in T3 RNAP resulted in an enzyme (T7-N748D) that preferred T3 bp at −10 and −11, and the complementary modification in T3 RNAP (T3-D749N) resulted in a similar switch in specificity for that enzyme. In the crystal structure of T7 RNAP, N748 lies on an extended loop (residues 742–773) that projects out from one wall of the binding cleft (the fingers domain) and extends within 4 Å of the opposite wall (which is composed of residues in the N-terminal domain). This information, together with the identification of mutations that affect the active site (which must interact with the promoter near +1), allowed the orientation of the RNAP with respect to the direction of transcription to be determined (see Fig. 2 and ref. 1). In this work, we sought to identify other residues in the specificity loop that might be involved in base-specific contacts with the promoter.

MATERIALS AND METHODS

Generation and Purification of Mutant RNAPs. Mutant RNAPs were generated by oligonucleotide-directed site-specific mutagenesis as previously described (25); DNA sequences of the relevant plasmids are available upon request. All enzymes were in a histidine-tagged background and were

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Abbreviation: RNAP, RNA polymerase.

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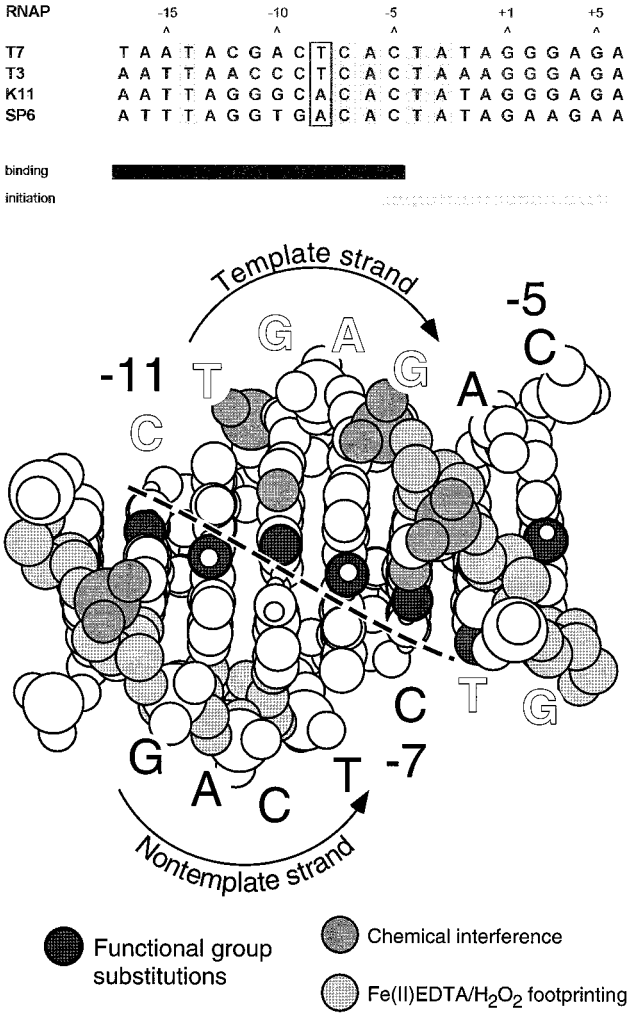

FIG. 1. Promoter structure. (*Upper*) Alignment of the consensus promoter sequences for T7, T3. K11, and SP6 RNAPs. The sequence of the nontemplate strand is presented; the transcription start site is at +1 (for review, see ref. 3). Positions at which bp are conserved in all phage promoters are shaded; the bases at −8 are enclosed in a box. The solid bar below the sequences denotes the binding region, which is recognized in a double-stranded form; the stippled bar denotes the initiation region, which is thought to be melted open from about −5 to +3 during RNAP binding and initiation (6, 7). (*Lower*) Summary of promoter recognition contacts (modified from ref. 7; drawing courtesy of Dr. Craig T. Martin). The promoter region from −13 to −5 is modeled as B-form DNA. Sugars protected by bound RNAP in hydroxyl radical footprinting experiments are indicated in light gray (16); guanine N7 and phosphate groups identified by chemical modification interference studies are in medium gray (17); base functional groups identified via incorporation of base analogs are in dark gray (7, 21, 22). A dashed line separates the interface between bases in the template and nontemplate strands.

purified as described in He *et al.* (25). The presence of the amino-terminal histidine tag has no effect upon promoter binding or polymerization kinetics.

Transcription Assays. Test plasmids having a mutant T7 promoter and a reference T7 promoter (ref. 18 and D. Parrotta, personal communication) were digested with *Eco*RV and *Ssp*I, treated with proteinase K, extracted with phenol and chloroform, and precipitated with ethanol (26). Transcription reactions were carried out in a volume of 10 μl in 30 mM Hepes, pH 7.8, 100 mM potassium glutamate, 15 M $Mg(OAc)_2$, 0.25 mM EDTA, 1 mM DTT, 0.05% Tween-20 (27) containing 0.5 mM ATP, CTP, GTP, and UTP (Pharmacia Ultrapure), 2 μCi of [α-^{32}P]ATP (specific activity 800 Ci/mmol; New England Nuclear), 10 ng of RNA polymerase, and 0.3 μg of each plasmid template (for mixed template reactions) or 0.5 μg of a single plasmid (for reactions having an individual template). Reactions were incubated at 37°C for 10 min, and the products were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea as previously described (25).

RESULTS

To identify potential contacts between amino acids in the specificity loop and bp in the upstream region of the promoter, we compared the DNA sequences of the T7, T3, SP6, and K11 promoters (Fig. 1) and the amino acid sequences of these RNAPs in the region comprising the specificity loop (Fig. 2). We noted that the SP6 and K11 promoters differ from the T7 and T3 promoters at position −8, where they both have an A in the nontemplate strand (−8A) as compared with a T in the T7 and T3 promoters; we also noted that the SP6 and K11 RNAPs share common amino acid residues (KM) at positions 758 and 759, whereas the residues QP occupy the corresponding positions in the T7 and T3 RNAPs. The location of residue Q758 in the specificity loop is such that it seemed a reasonable candidate for participating in a contact with the bp at −8 (Fig. 2).

To test this prediction, we engineered a variety of substitutions in this region of T7 RNAP (Table 1). We then determined the effects of these changes on RNAP specificity by transcription of

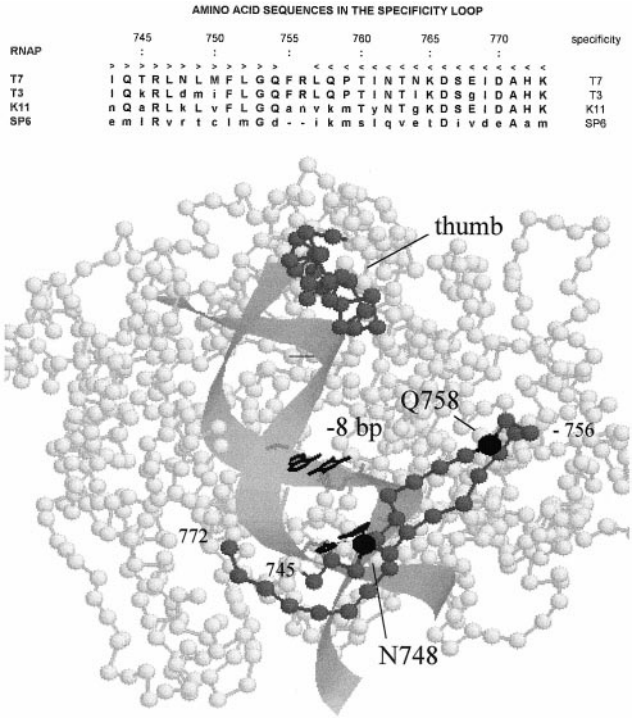

FIG. 2. RNA polymerase structure. (*Upper*) The amino acid sequences of the T7, T3, K11, and SP6 RNAPs in the region of the specificity loop (T7 residues 742–773) are aligned (31–34); lowercase letters indicate positions where the sequences differ from that of T7, and dashes indicate a gap that has been inserted into the SP6 sequence to optimize the alignment. The arrowheads along the top indicate travel along the loop from the "fingers" wall of the DNA binding cleft to the tip (residue 756) and return. (*Lower*) The alpha carbon backbone of T7 RNAP is represented as connected spheres (coordinates are from the Brookhaven Protein Databank). Residues 745–772 in the specificity loop (1) and residues 369–390 in the "thumb" motif (35) are shaded in gray. The positions of residues N748 and Q758, which are involved in recognition of the bp at −11 and −8, are indicated in black. Computer modeling of B-form DNA in the binding cleft was carried out as proposed by Patel *et al.* (28), placing the specificity loop on top of the DNA and residue N748 near the bp at −11. The base pairs at −8 and −11 are depicted in wire frame.

plasmid templates that carry a variant T7 promoter having an alternate bp at a particular position. Representative results obtained with the mutant T7 RNAP Q758K are shown in Fig. 3. In the first analysis (a mixed promoter assay; *B*) the wild-type or mutant enzyme was presented with a mixture of plasmid templates that each carried a promoter having one of the three nonconsensus bp at a particular position, as well as a reference promoter (see Fig. 3*A*). In this assay, the wild-type enzyme utilized the reference promoter efficiently and showed characteristic specificity for promoters having bp substitutions from −12 to −6. Thus, whereas the wild-type enzyme will tolerate some substitutions at −12 and −6 (recognized by the presence of an RNA product that results from initiation at the test promoter, P_X), it shows little tolerance for promoters having substitutions from −11 to −7 (little or no production of RNA from P_X) (18–20). In contrast, Q758K did not utilize the reference promoter at all and showed activity only with the set of templates having altered bp at −8. When the activity of Q758K was tested with each of the −8 promoter variants individually (*C*), it was observed that this enzyme utilized only a T7 promoter having a C/G bp at −8 (i.e., P_{T7} −8C; promoter variants are identified by indicating the base in the nontemplate strand at the relevant position).

A number of other amino acid substitutions at position 758 were subsequently found to result in enzymes with altered specificities for the bp at −8 (see Table 1 and Fig. 4). Although most nonpolar substitutions (e.g., Q758A, Q758V) and mutations that resulted in multiple substitutions gave rise to RNAPs with little promoter-dependent activity, these enzymes retained nonspecific catalytic activity, as evidenced by their ability to transcribe poly(dC) as a template (data not shown), demonstrating the functional integrity of the active site in these enzymes. Furthermore, mutant RNAPs with altered specificities (i.e., Q758K and Q758R) exhibited decreased affinity for a synthetic promoter having the consensus sequence in a gel retardation assay and an increased affinity for P_{T7} −8C (M.R., unpublished observations). These results indicate that the effects of these changes are mediated through promoter binding and not as a result of alterations in catalytic activity.

DISCUSSION

The effects of amino acid substitutions at position 758 clearly demonstrate a role for this residue in the recognition of the bp at −8. What might the nature of this recognition be? Characterization of synthetic promoters in which base analogs have been incorporated has demonstrated a critical role for the 6-amino group of the adenine in the template strand as an important contact at −8, apparently requiring a hydrogen bond acceptor in the polymerase (7). An attractive hypothesis is that the carboxamide oxygen of Q758 in wild-type T7 RNAP supplies this function. In the SP6 and K11 promoters, the template strand adenine found in the T7 promoter at −8 is replaced with a thymine, which presents a 4-carbonyl group in approximately the same position. Hydrogen bonding with this group would require a hydrogen bond donor, such as the ε-amino group of the lysine found in SP6 and K11 RNAPs at the position homologous to Q758 in T7 RNAP. However, a T7 RNAP mutant with a Q758K substitution (which we expect to behave like the SP6 and K11 RNAPs with regard to its specificity for the bp at −8) showed preference not for P_{T7} −8T but for P_{T7} −8C. The only hydrogen bond acceptors on the major groove surface of P_{T7} −8C are the N7 and O6 of the guanine in the template strand. The N7 also occurs in the

Table 1. Promoter specificities of mutant RNAPs

RNAP*	Plasmid†	Relative promoter utilization‡				Relative specific activity§
		−8T	−8A	−8C	−8G	
Wild type	pBH117	1	0.01	0.01	0.01	1
Q758C	pMR74	0.15	0.43	0.06	1	0.71
Q758S	pMR57	0.15	1	0.01	0.86	0.53
Q758R	pMR64	0.03	0.07	1	0.04	0.31
Q758E	pMR75	1	0.01	0.01	0.01	0.23
Q758N	pMR65	0.07	0.08	0.01	1	0.16
Q758K	pMR50	0.03	0.04	1	0.01	0.15
Q758G	pMR55	0.33	0.73	0.16	1	0.11
Q758A	pMR72	0.26	0.97	0.33	1	0.08
Q758V	pMR62	0.66	0.67	0.29	1	0.04
Q758H	pMR71	0.30	1	0.47	0.47	0.03
Q758L	pMR66	1	0.49	0.31	0.96	0.03
Q758I	pMR56	0.53	0.19	0.23	1	0.02
Q758Y	pMR76	0.71	1	0.66	0.85	0.01
Q758T	pMR70	0.99	1	0.01	0.96	0.01
Q758D	pMR73	ND	ND	ND	ND	<0.01
Q758W	pMR63	ND	ND	ND	ND	<0.01
Q758F	pMR54	ND	ND	ND	ND	<0.01
Q758P	pMR67	ND	ND	ND	ND	<0.01
subs(755-761)	pBH212	ND	ND	ND	ND	<0.01
subs(755-757)	pMR49	ND	ND	ND	ND	<0.01
subs(758-759)	pBH213	ND	ND	ND	ND	<0.01
subs(743-777)	pMR106	ND	ND	ND	ND	<0.01

*Individual amino acid substitutions at position 758 are identified. In the RNAPs encoded by pBH212, pMR49, pBH213, and pMR106, the amino acid residues in T7 RNAP in the range indicated have been replaced with the corresponding residues from K11 RNAP (see Fig. 1).

†The plasmid that encodes the indicated RNAP.

‡The preference of the RNAP for a promoter having the indicated bp at position −8 was determined as shown in Fig. 3*C*. The radioactivity in each electrophoretic species was quantified by exposing the gel to a PhosphorImager screen (Molecular Dynamics) taking into account the base composition and sizes of the individual transcripts. The amount of RNA produced from the test promoter relative to that of the reference promoter was determined [(P_{T7}X)/(P_{T7}ref)], and the data in each set were normalized to the strongest promoter in that series (24). For mutant enzymes that had very low activity at any promoter (<0.01 that of the wild-type enzyme), these values were not calculated (ND).

§The activity of each mutant enzyme at its strongest promoter relative to the activity of the same amount of WT enzyme at a consensus promoter.

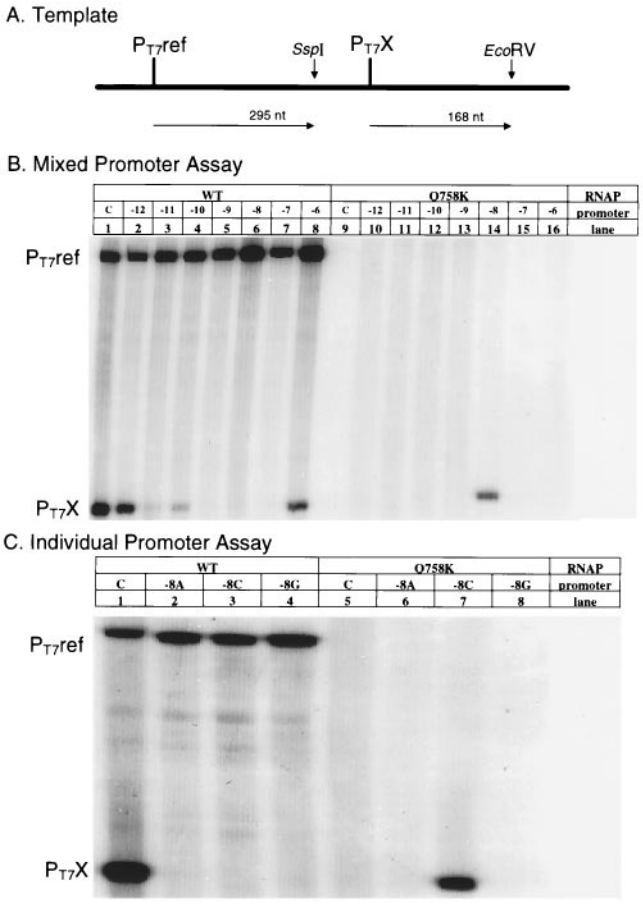

FIG. 3. Altered promoter specificity of T7–Q758K. (*A*) Plasmid templates that carry a reference T7 promoter (P_{T7}ref) and a test promoter (P_{T7}X) were digested with *Ssp*I and *Eco*RV; transcription from each promoter is expected to give rise to runoff products of the lengths indicated (18). (*B*) Mixtures of three plasmid templates each having a promoter with one of the alternate bp at the test position (P_{T7}X), in addition to the reference promoter (P_{T7}ref), were transcribed by wild-type T7 RNAP or Q758K, as noted. The identification of the test promoter set is given by position; thus, for example, the reaction shown in lane 2 utilized a mixture of templates having test promoters with nonconsensus bp at −12. The reactions shown in lanes 1 and 9 utilized a control template (C) having a consensus T7 promoter at the test promoter location. The products were resolved by electrophoresis and visualized by exposure to a PhosphorImager screen. (*C*) Each reaction contained one plasmid template having a single bp substitution in P_{T7}X at position −8, as indicated. The control template (lanes 1 and 5) has a consensus promoter (P_{T7} −8T) at the test promoter location.

template strand adenine of the consensus promoter. Because T7-Q758K is unable to recognize the consensus promoter, it is likely that the substituted lysine is involved in the formation of a hydrogen bond with guanine O6. The chemical nature of the protein–promoter interaction is therefore altered to that of the corresponding residues of the SP6 and K11 RNAPs, but the spatial position of the interaction remains characteristic of wild-type T7 RNAP. The promoter preference of Q758R, which similarly substitutes a hydrogen bond donor for the native carboxamide, is virtually identical to Q758K (Table 1). It seems likely, then, that the correct spatial positioning of the residue at 758 involves interaction with other amino acid residues in the polymerase and that the specificity of the contact depends on the geometry as well as the chemical nature of the amino acid. In addition to single aa substitutions at position 758, we have also generated T7 RNAP mutants in which multiple residues in the specificity loop were exchanged

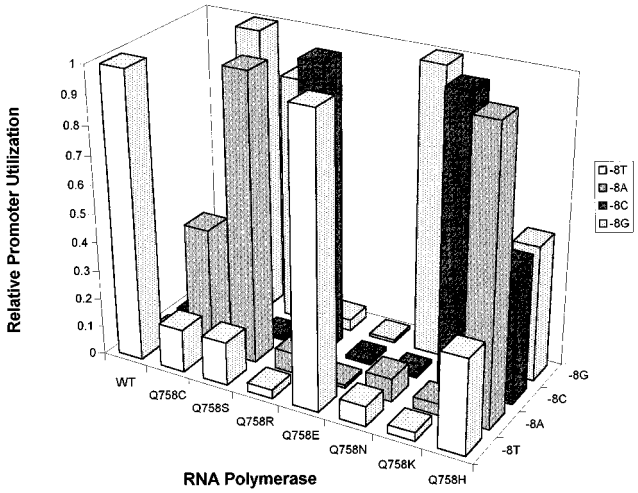

FIG. 4. Specificities of mutant RNAPs for the bp at −8. The relative preferences of each mutant RNAP for a promoter having the base indicated in the nontemplate strand are presented (data are from Table 1).

with the corresponding residues from K11 RNAP, including one mutant in which the entire loop (residues 743–777) was replaced (Table 1). These mutant RNAPs retained nonspecific catalytic activity but were inactive in promoter-dependent transcription assays, suggesting that the specificity loop may not function independently of other structural elements in the RNAP. A more detailed understanding of the interactions between the amino acid residue at position 758 and the bp at −8 will require the characterization of the interaction of RNAP mutants having altered specificities with chemically modified promoters [see for example, Li *et al.* (7)].

Although Q758 is involved in discrimination of the bp at −8, it is possible that it may also be involved in discrimination of adjacent bp, for example by interaction of the amido group of Q758 with the 6-carbonyl group of guanine at −7 or −9. Such a situation has been observed with N748, which is primarily involved in discrimination of the bp at −11 but is also thought to contribute to specificity at −10 (2, 22). However, we have found that Q758E (which continues to utilize the consensus bp at −8) does not exhibit an altered preference for the bp at −7 or −9 (Table 2), making this possibility less likely. Furthermore, none of the mutant RNAPs tested were able to utilize promoters having bp substitutions at other positions from −12 to −6 to a significant level (data not shown).

A co-crystal structure of T7 RNAP docked with its promoter has not yet been obtained. However, two views as to how the template DNA might be modeled into the binding cleft have been proposed, both of which align the DNA along the axis of the cleft. Whereas Sousa *et al.* (1) placed the DNA on top of the specificity loop, Patel *et al.* (28) noted that there is sufficient room under the loop to accommodate a B-form helical structure. The distinction is important for a number of reasons, one of which is that if the specificity loop were on top of the promoter, it would partially encircle the DNA in the binding cleft and could contribute to stabilization of the elongation complex once the upstream promoter contacts have been released (28, 29). Although hydroxyl radical footprinting studies suggest that the RNAP interacts predominately with one side of the helix, favoring a "DNA on top" model (16), other studies suggest that the RNAP may make contacts on both sides of the helix in the binding region (30). The finding that residues N748 and Q758 are responsible for discriminating the bp at −11 and −8 does not allow us to resolve this issue, as it is not possible to align these amino residues with their cognate base pairs in either scenario. For example, a line drawn

Table 2. Relative promoter utilization by wild-type T7 RNAP and Q758E

	RNAP	
Promoter	Wild-type	Q758E
−9C	1	1
−9A	0.01	0.01
−9G	<0.01	0.01
−9T	<0.01	<0.01
−8T	1	1
−8A	0.01	0.01
−8C	0.01	0.01
−8G	0.01	0.01
−7C	1	1
−7A	<0.01	<0.01
−7G	0.01	<0.01
−7T	0.01	0.01

from N748 to Q758 is almost perpendicular to the axis of DNA in the putative binding cleft and, more importantly, to the [−11]G:O6–[−8]A:N6 axis (see Fig. 2 and ref. 28). Furthermore, although the distance from the centers of the bp at −11 and −8 is about 10.5 Å, the distance from amino acid residues 748 to 758 (α-carbon to α-carbon) is 20.5 Å. Thus, a substantial rearrangement of the loop and/or the DNA must occur during promoter recognition to allow these amino acids to interact with the base pairs at −8 and −11.

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