Bacterial promoters of the extended −10 class contain a single consensus element, and the DNA sequence upstream of this element is not critical for promoter activity. Open promoter complexes can be formed on an extended −10 *Escherichia coli* galP1 promoter at temperatures as low as 6 °C, when complexes on most promoters are closed. Here, we studied the contribution of upstream contacts to promoter complex formation using galP1 and its derivatives lacking the extended −10 motif and/or containing the −35 promoter consensus element. A panel of *E. coli* RNA polymerase holoenzymes containing two, one, or no α-subunit C-terminal domains (αCTD) and either wild-type σ70 subunit or σ70 lacking region 4.2 was assembled and tested for promoter complex formation. At 37 °C, αCTD and σ70 region 4.2 were individually dispensable for promoter complex formation on galP1 derivatives with extended −10 motif. However, no promoter complexes formed when both αCTD and σ70 region 4.2 were absent. Thus, in the context of an extended −10 promoter, αCTD and σ70 region 4.2 interactions with upstream DNA can functionally substitute for each other. In contrast, at low temperature, αCTD and σ70 region 4.2 interactions with upstream DNA were found to be functionally distinct, for σ70 region 4.2 but not αCTD was required for open promoter complex formation on galP1 derivatives with extended −10 motif. We propose a model involving σ70 region 4.2 interaction with the β flap domain that explains these observations.

Most *Escherichia coli* promoters are characterized by the presence of two 6-bp sequence elements centered −10 and 35 nucleotides upstream of the transcription initiation point. These promoters are referred to as −10/−35 class promoters. Interaction of the RNA polymerase (RNAP) σ70 subunit with −10 and −35 promoter elements is responsible for promoter recognition and transcription initiation. σ70 conserved region 4.2 recognizes the −35 promoter element, while σ70 conserved region 2.4 recognizes the −10 promoter element (reviewed by Gross et al., Ref. 1; see also Ref. 2). Multiple alignments of promoter sequences permit the derivation of consensus sequences for the −10 and −35 promoter elements and show that most promoters deviate from the consensus (3). Promoter elements of strong promoters tend to deviate from consensus less than promoter elements of weak promoters. Thus, assuming that promoter elements with consensus sequence are preferred binding sites for σ70 regions 2.4 and 4.2, the strength of regions 2.4 and 4.2 interaction with their respective promoter elements determines the efficiency of promoter complex formation.

For most promoters, RNAP σ70 regions 2.4 and 4.2 interactions with their target promoter elements are sufficient for promoter complex formation. On some promoters, the presence of RNAP α-subunit C-terminal domains (αCTDs) greatly increases transcription initiation beyond the basal level achieved through σ70-promoter element interactions (4). On these promoters, αCTDs make sequence-specific interactions with an A-rich promoter element (the “UP-element”) located upstream of the −35 promoter element (reviewed by Gourse et al., Ref. 5). In the absence of a UP-element, αCTD non-specifically interacts with upstream DNA and the stimulatory effect of these interactions is less significant.

There exists a minor class of promoters that lack recognizable −35 promoter elements and whose −10 elements are extended with an upstream dinucleotide TG. Genetic data show that specific interaction between an additional region of σ70, conserved region 2.5, and the TG motif is required for promoter complex formation on promoters of this class (6). Evidently, this additional contact is strong enough to make promoter complex formation on extended −10 promoters independent of σ70 region 4.2 and −35 promoter element interaction (7).

In order for template-directed RNA synthesis to occur, promoter DNA has to become locally melted (opened). In the catalytically competent open promoter complex, the melting extends from −12 to +3 positions and thus includes the entire −10 promoter element. Promoter opening is temperature-dependent, and promoter complexes formed on −10/−35 type promoters “close” below 15 °C (8). In contrast, promoter complexes on the extended −10 galP1 promoter remain open at temperatures as low as 5 °C (9–12). The reason for this unusual behavior is not completely understood. For example, while it is clear that the extended −10 motif contributes to promoter opening at low temperature (9), it is not sufficient, since open promoter complexes on some extended −10 promoters are sensitive to low temperature (12, 13).

Previous work attempted to compare promoter complexes formed on the −10/−35 class and the extended −10 class promoters (9, 12, 14). However, due to technical constraints, relatively large fragments of promoter DNA were altered. As a result, interpretation of some of the published data is complicated, since promoter complex formation can be affected by...
sequences outside of promoter consensus elements. In this work, we used several derivatives of the galP1 promoter obtained by site-specific mutagenesis and a set of RNAP mutants that lacked a CTD and/or σ70 region 4.2 to study promoter complexes formation in the absence of a CTD-UP element interactions, σ70 region 4.2–35 promoter consensus element interactions and σ70 region 2.5–extended –10 motif interactions. Our results indicate that on the extended –10 galP1 promoter, CTD-DNA interactions and σ70 region 4.2-DNA interactions are functionally equivalent, and at least one of these interactions is necessary for promoter complex formation at physiological temperature of 37 °C. In contrast, galP1 promoter complex formation at low temperature of 6 °C is strictly dependent on the presence of σ70 region 4.2.

MATERIALS AND METHODS

Plasmids

Plasmid pT7τ (15) was used as a source of wild-type σ70. Plasmid pCYB2τ α-site (15) expressing σ70(α1–565) fused to intein-chitin binding domain (CBD) was used as a source of σ70(1–565) and is described by Severinov and Muir (16). Ampicillin-resistant plasmid pCysHi20 (1–255) expresses N-terminally hexahistidine-tagged σ20 (1–235) according to the protocol described elsewhere (18). Plasmid pET21AsiA (19) expressing C-terminally hexahistidine-tagged AsiA. Plasmid pT719galP1 containing the E. coli galP1 promoter was constructed as follows. The promoter-containing fragment was amplified from the pAA121 plasmid (19) using PCR primers containing engineered EcoRI and HindIII recognition sites, respectively. The amplified fragment was treated with EcoRI and HindIII and cloned into appropriately treated pT719R. Plasmids harboring galP1 promoter derivatives were constructed by site-specific PCR mutagenesis using pT719galP1 as a template and cloned in pT719.

Proteins

σ70 and σ70(1–565)—Wild-type σ70 RNA polymerase subunit was purified from BL21 (DE3) cells harboring the pT7τ plasmid as described (20).

To purify C-terminally truncated σ70(1–565), E. coli XL1-Blue cells were transformed with the pCYB2τ α-site plasmid. Transformants were grown in 1 l of LB with ampicillin (100 μg/ml) at room temperature to an OD600 of 0.6–0.8, and expression was induced by the addition of IPTG to 1 mM. After 6 h, cells were harvested by centrifugation and resuspended in buffer H containing 20 mM Hepes, pH 8.0, 500 mM NaCl, 0.1 mM EDTA. Cells were lysed by sonication; the lysate was clarified by low speed centrifugation and loaded onto a 1-ml chitin column equilibrated in buffer H. The column was washed with 15 ml of buffer H and then quickly flushed with 3 column volumes of freshly prepared buffer H containing 30 mM dithiothreitol. The column outlet was sealed, and the column was left overnight at 4 °C. Pure σ70(1–565) was eluted with 3 column volumes of buffer H without dithiothreitol, dialyzed against buffer H, and stored at –20 °C in the presence of 50% glycerol.

RNAP Core Enzymes—The wild-type RNAP core enzyme and the mutant lacking both αCTDs were prepared by in vitro reconstitution as described (18, 20). The core and holoenzyme fractions were separated on a 1-ml Protein-Pak Q 8 HR column (Waters) attached to a Waters 650 FPLC. RNAP was loaded on the column in TGE buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 5% glycerol) and eluted using a linear 60-ml gradient of NaCl from 0.23 to 0.4 M. Fractions containing RNAP core and holoenzymes were pooled separately, concentrated to 1–2 mg/ml and stored at –20 °C in the presence of 50% glycerol. Heterodimeric RNAP core enzyme containing one wild-type α-subunit and one α-subunit lacking CTD was purified from XL1-Blue strain transformed with pET21AsiA. Transformants were grown in 2 liters of LB with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) at room temperature to OD600 of 0.6–0.8 and expression was induced by the addition of IPTG to 1 mM. After 6 h, cells were harvested by centrifugation and resuspended in buffer C containing 20 mM Tris-HCl, pH 7.9, 5% glycerol, 500 mM NaCl. Cells were lysed by sonication and after a low speed centrifugation, cell extract was loaded onto a 5-ml Mono S HR 5/5 Heparin chromatography column (Amersham Biosciences) loaded with 50 mM NaCl and attached to an FPLC. The column was washed with buffer C containing 20 mM imidazole, and AsiA was eluted with 100 mM imidazole in the buffer. The eluate was diluted 5-fold with buffer D (50 mM Tris-HCl, pH 7.9, 5% glycerol, 50 mM NaCl, 1.65 mM (NH4)2SO4, 1 mM β-mercaptoethanol) and loaded onto a 4 ml phenyl-Tosopearl column equilibrated with the same buffer. The column was washed and eluted with an (NH4)2SO4 gradient from 1.3 to 0 M. Homogeneous AsiAcore protein was eluted from the column at 20–30 mM (NH4)2SO4 concentrated to 1 mg/ml concentration and stored at –20 °C in the presence of 50% glycerol.

In Vitro Transcription

Abortive transcription initiation reactions contained, in 10 μl of transcription buffer (50 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 40 mM KCl), 20 nM RNAP core enzyme and 320 nM σ70 or 640 nM σ70(1–565). Reactions were incubated for 10 min at 37 °C, followed by the addition of 10 nM promoter fragments, 0.1 mM initiating dinucleotide CpA, and 3 μCi (3000 Ci/mmol) of [α-32P]UTP. Reactions proceeded for 10 min at 37 °C and were terminated by the addition of an equal volume of loading buffer containing 9 mM urea. Reaction products were resolved by electrophoresis in denaturing (8 % urea) 20% (19:1) polyacrylamide gel, visualized by autoradiography, and quantified using the Molecular Dynamics PhosphorImager.

Determination of Open Complex Dissociation Kinetics

Open complex lifetime was measured on linear promoter-containing DNA fragments using an abortive transcription assay. Reactions contained, in 40 μl of buffer (40 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 175 mM NaCl, 2 mM β-mercaptoethanol), 80 nM wild-type and mutant RNAP core enzymes and 80 nM σ70 or 160 nM σ70(1–565). Reactions were incubated for 10 min at 30 °C, followed by the addition of 10 nM promoter fragments and additional incubation for 15 min at 30 °C. A 10-μl reaction aliquot was transferred to tubes containing 0.1 mM dinucleotide CpA, 10 μM UTP, and 3 μCi (3000 Ci/mmol) of [α-32P]UTP. Abortive transcription initiation was allowed to proceed for 10 min and was terminated by the addition of an equal volume of loading buffer containing 9 mM urea. The remainder of promoter complex reaction was supplemented with 100 μg/ml heparin and incubation at 30 °C was continued. 10-μl reaction aliquots were withdrawn immediately after the heparin addition and assayed for abortive transcription as described above. Reaction products were resolved, visualized, and quantified as described above.

DNase I Footprinting

The 147-bp DNA fragments (~96 to +51) harboring the galP1 promoter and its derivatives were amplified by PCR from appropriate plasmids using universal T7 promoter and M13 reverse primers. PCR fragments were digested with HindIII and labeled at nontemplate strand by filling the HindIII sticky end with Klenow enzyme in the presence of [α-32P]dCTP. Promoter complexes were formed in 15 μl of transcription buffer containing 200 nM core enzymes and a 4-fold excess of σ70 or 8-fold excess of σ70(1–565). Reactions were incubated for 10 min at 37 °C, followed, when necessary, by the addition of 1 μM AsiA and further 10 min incubation at the same temperature. Reactions were next supplemented with 10 μM 5′-end-labeled promoter fragment and incubated for 20 min at assay temperature. DNA 1D footprinting reactions were initiated by the addition of 0.1 or 1 unit of DNase I ( Worthington) for 37 and 6 °C reactions, respectively. The reactions proceeded for 30 s at the assay temperature and were terminated by addition of 85 μl stop-mixture (20 mM EDTA, 10 μg of denatured calf thymus DNA, and water) followed by phenol extraction and ethanol precipitation. The DNA samples were dissolved in 1 μl of formamide-loading dye and analyzed using 7% polyacrylamide/8 % urea sequencing gels.

KmO4 Probing

Reactions were set up as described above for DNase I footprinting. Promoter complexes were treated with 1 mM KMnO4 for 15 s at the assay temperature. Reactions were terminated by the addition of β-mercaptoethanol to 300 mM, followed by phenol extraction, ethanol precipitation, and 20 min treatment with 10% piperidine at 95 °C. Reaction products were analyzed using 7% polyacrylamide urea gels.
Fig. 1. Transcription activity of galP1 promoter derivatives in vitro. A, the DNA sequence (top strand) of the galP1 promoter (from −75 to +5) used in this work is shown. The extended −10 consensus sequence, the transcription initiation start point and the sequence corresponding to the −35 promoter box are capitalized. Below the galP1 sequence, site-specific changes introduced in galP1 derivatives used in this work are shown. B, RNAP σ70 holoenzyme was combined with the indicated promoters and abortive transcription initiation assay was performed using Cpa primer and radioactive UTP substrate. The product, CpaPu, was resolved by denaturing PAGE and visualized by autoradiography.

Gel Retardation Assay

The reactions contained, in 20 μl of transcription buffer, 10 nM RNAP wild-type and mutant core enzymes combined with appropriate amounts of σ70 or σ54 (1–565). Reactions were incubated for 15 min at 37 °C, transferred to 6 °C, and incubated for additional 10 min, followed by the addition of 10 μl [32P] end-labeled galP1 promoter fragment. After further 30 min of incubation at 6 °C, reactions were combined with 4 μl of loading buffer (transcription buffer containing 50% glycerol and 0.05% bromphenol blue). When necessary, heparin was added to the final concentration 50 μg/ml, and reactions were immediately loaded on 5% (29:1) polyacrylamide Tris borate/EDTA gel. The gel was run in a cold room and reactions products were revealed by autoradiography.

RESULTS

The galP1 Promoter Derivatives Used in This Work—A galP1 promoter derivative previously constructed and characterized by Burns et al. (12) was used as a starting point of this work. The promoter differs from the natural galP1 promoter by three point mutations. Two substitutions at positions −9 and −8 relative to the start point of transcription create a consensus extended −10 promoter element, TGcTATAAT, instead of the wild-type sequence TGcTATgTgT. The third substitution introduces a T at position −19 and destroys the overlapping galP2 promoter, thus simplifying the analysis of galP1 promoter complexes. We used site-directed mutagenesis to construct “second-generation” derivatives of the optimized galP1 promoter constructed by Burns et al. (Fig. 1A). The first derivative, galP1-35, contains the consensus −35 promoter element, TTGACA, incorporated 18 base pairs upstream of T at position −12, the first base of the −10 consensus element. The second derivative, galP1-TG, was constructed by substituting the TG motif of the extended −10 promoter consensus element for AC. The resulting promoter was named galP1-TG. The final construct, galP1-35-TG, is a derivative of galP1-35 and also has the TG extended −10 motif substituted with AC. Note that our nomenclature differs from that adopted by Kamali-Moghaddam and Geiduschek in the accompanying article (28).

The ability of promoter fragments containing galP1 and its derivatives to serve as templates for the synthesis of CpaPu from the Cpa primer and the UTP substrate by E. coli RNAP σ70 holoenzyme was investigated (Fig. 1B). DNA fragments containing galP1, galP1-35 as well as galP1-35-TG supported high and comparable levels of CpaPu synthesis. In contrast, little transcription was detected when RNAP was combined with the galP1-TG fragment (less than 5% compared with other promoters used here). These result indicate the following. (i) galP1 has no functional −35 promoter consensus element. (ii) The −10 consensus promoter element alone, in the absence of the TG motif, is insufficient for transcription initiation, and (iii) the consensus −35 element sequence introduced in galP1-35-TG promoter is functional.

Promoter Complex Formation by RNAP Mutants Lacking αCTD and/or σ70 Region 4.2 on galP1 Derivatives—We wished to determine the contribution of RNAP domains capable of interaction with upstream promoter sequences, the αCTD and the σ70 subunit region 4.2, to promoter complex formation on galP1 and its derivatives. To this end, 5 mutant RNAP holoenzymes that lack one or both αCTDs and/or σ70 region 4.2 and wild-type σ70 RNAP holoenzyme were prepared. RNAP holoenzymes were reconstituted in vitro by combining RNAP core containing two copies of the wild-type α subunit, RNAP core that has one α and another copy truncated at amino acid 235, and RNAP core that contains two copies of truncated α with either wild-type σ70, or with a σ70 mutant that is truncated at amino acid position 565, σ70Δ1–565, and thus lacks region 4.2 (16, 22). Native PAGE analysis showed that reconstituted RNAP holoenzymes contained less than 5% RNAP core (data not shown). The ability of mutant holoenzymes and wild-type RNAP control to form promoter complexes at 37 °C with DNA fragments containing galP1 and its active derivatives was investigated by DNase I footprinting. The footprinting results obtained with DNA fragments that contained functional promoters galP1, galP1-35, and galP1-35-TG are presented in Fig. 2, A–C, respectively. On all three promoters, the wild-type
Promoter Complex Formation on galP1 Promoter and Its Derivatives

Promoter complexes formed at 37 °C using the indicated RNAP holoenzymes on DNA fragments containing indicated functional galP1 promoter derivatives. Promoter fragments were 32P-end labeled at the top (non-template strand). Promoter complexes were footprinted with DNase I, reaction products were separated by electrophoresis in a 7% sequencing gel and revealed by autoradiography.

**Fig. 2.** Promoter complex formation on galP1 promoter derivatives using mutant RNAP holoenzymes lacking αCTD and/or α70 region 4.2. Promoter complexes were formed at 37 °C using the indicated RNAP holoenzymes on DNA fragments containing indicated functional galP1 promoter derivatives. Promoter fragments were 32P-end labeled at the top (non-template strand). Promoter complexes were footprinted with DNase I, reaction products were separated by electrophoresis in a 7% sequencing gel and revealed by autoradiography.

hologenzyme protected DNA from +20 to −23 (Fig. 2, A–C, lane 2). DNase I hypersensitive sites centered at positions −25, −26, −44, −57, and −67 were also observed in complexes formed on all three promoters with wild-type RNAP. The DNA between the hypersensitive sites was protected (Fig. 2, A–C, lane 2). This periodic pattern of protection and hypersensitivity has been attributed to wrapping of upstream DNA around RNAP (Attey et al., Ref. 11). The hypersensitive site at position −25 was very prominent in the galP1-35 and galP1-35-TG complexes, and was less pronounced in galP1 complexes. DNase I hypersensitivity at about −25 is a common feature of promoter complexes formed by bacterial RNAP (3); it may result from DNA bending that arises when RNAP establishes simultaneous contacts with the −35 and −10 promoter consensus elements (2). If this interpretation is correct, weaker hypersensitivity in the galP1 complexes is expected, since α70 region 4.2 does not make specific contacts with DNA on galP1.

In the galP1-35, and galP1-35-TG complexes, but not in galP1 complexes, position −5 was exposed to DNase I attack (compare, for example Fig. 2, A–C, lane 2).

On all three promoters, complexes formed by α70 holoenzymes lacking one of the αCTDs were very similar to the corresponding wild-type RNAP complexes, though protection of a DNase I-sensitive band at −52 was decreased somewhat (Fig. 2, A–C, compare lanes 2 and 3). In complexes formed by α70 holoenzyme lacking both αCTDs, protection at −52 and −48 was further decreased (Fig. 2, A–C, compare lane 4 with lanes 2 and 3), suggesting that in the wild-type RNAP complexes, protection of AT-rich sequence between positions −52 and −48 is due to αCTD binding, in agreement with earlier results (11).

No footprint was observed when α70(1–565) holoenzymes were combined with galP1-35-TG (Fig. 2C, lanes 5–7). This result is expected, since the interaction between α70 region 4.2 and the −35 promoter consensus element is essential for promoter complex formation on the −10′−35 class promoters. In contrast, footprints were readily observed when α70(1–565) holoenzymes reconstituted from wild-type core enzyme or from core enzyme lacking one αCTD were combined with galP1 and galP1-35, also as expected (Fig. 2, A and B, lanes 5 and 6). These footprints are distinct from the corresponding α70 holoenzyme footprints in that position −37, which is fully protected in the presence of α70 RNAP complexes, is hypersensitive in the presence of α70(1–565) RNAP, presumably because specific interactions between α70 region 4.2 and the −35 promoter element (galP1-35) or nonspecific interactions between α70 region 4.2 and promoter DNA (galP1) are lacking. In complexes formed by α70(1–565) RNAP holoenzymes containing both αCTDs, protection at around −40′−50 is present (Fig. 2, A and B, lane 5), suggesting that αCTD is able to interact with its binding site independently of α70 region 4.2. Hypersensitivity at −67 and at −57 is absent in α70(1–565) complexes. The reasons for this are unclear, since α70 region 4.2 is only expected to interact with promoter DNA −35 base pairs upstream of the transcription start point (1).

Surprisingly, RNAP holoenzyme lacking both αCTDs and α70 region 4.2 produced no footprints on extended −10 galP1 or galP1-35 promoters (Fig. 2, A and B, lane 7). Since the removal of these RNAP domains individually had no effect on promoter complex formation, we conclude that on galP1 and galP1-35, αCTD, and α70 region 4.2 interactions with upstream promoter DNA can substitute for each other. However, the removal of both of these interactions prevents promoter complex formation even in the context of a consensus extended −10 promoter.

Stability of Promoter Complexes—The dissociation kinetics of promoter complexes formed on galP1 and its two active derivatives, galP1-35 and galP1-35-TG, was investigated. Promoter complexes were formed, challenged with DNA competitor heparin, and the amount of complexes that survived heparin chal-
Heparin-induced dissociation of RNAP-promoter complexes formed on galP1 promoter derivatives. A, promoter complexes were formed on indicated promoter fragments using wild-type σ70 RNAP holoenzyme. Complexes were challenged with heparin (+hep). At the indicated times after heparin addition, reaction aliquots were withdrawn and combined with substrate mixture containing CpA and [α-32P]UTP. Reactions were allowed to proceed for 10 min, reaction product, CpApU, was separated by denaturing PAGE and quantified. Each experiment was repeated at least three times. Mean values are presented. On the right, an autoradiograph of a representative gel is shown. B, experiment was performed as in A using mutant RNAP holoenzymes.

The results are consistent with the idea that interactions between σ70 region 2.5 and the TG motif contribute to promoter complex stability. The results also suggest that specific interactions between σ70 region 4.2 and the −35 promoter
consensus element increase promoter complexes ability to withstand heparin challenge (see, however, below).

We next measured the dissociation kinetics of promoter complexes formed by RNAP mutants (Fig. 3B). As can be seen, $\sigma^{70}$ holoenzymes on galP1, galP1-35 and galP1-35-TG dissociated with the same kinetics irrespective of the presence of oCTD(s). Similarly, the dissociation kinetics of promoter complexes formed by $\sigma^{565}$ holoenzymes on galP1 and galP1-35 did not depend on the presence of cCTD. Thus, cCTD interactions with upstream galP1 promoter DNA do not contribute to promoter complex ability to withstand heparin challenge, in agreement with data obtained on other promoters (23).

Curiously, comparison of $\sigma^{70}$(1–565) and $\sigma^{70}$ RNAP complexes on galP1 (or galP1-35) revealed no differences in stability. Furthermore, $\sigma^{70}$(1–565) RNAP-galP1-35 complexes were more stable than $\sigma^{70}$(1–565) RNAP-galP1 complexes. It therefore follows that increased stability of galP1-35 complexes compared with galP1 complexes is not caused by $\sigma^{70}$ region 4.2 interactions with the −35 promoter consensus element. We conclude that $\sigma^{70}$ region 4.2 interactions with galP1 promoter DNA do not contribute to promoter complex stability as measured by heparin challenge assay. Increased stability of galP1-35 complexes compared with galP1 complexes must be due to intrinsic properties of galP1-35 DNA and/or due to galP1-35 DNA interactions with RNAP domains other than cCTD or $\sigma^{70}$ region 4.2.

**Promoter Complex Formation at Low Temperature**—Promoter complexes formed on galP1 at temperatures as low as 6 °C are open, as judged by KMnO_4 probing (9). This is an unusual behavior since promoter complexes formed on most promoters are insensitive to KMnO_4 at this temperature, and are therefore closed. KMnO_4 probing of promoter complexes formed by the wild-type RNAP at 6 °C revealed the presence of KMnO_4-sensitive bands in galP1-35, but not in galP1-35-TG complexes (Fig. 4A, compare lanes 2 and 6). Complexes formed on galP1 were also open at 6 °C (data not shown). We conclude, in agreement with earlier data (12), that the TG motif is essential for low temperature opening.

To determine whether RNAP contacts with upstream DNA contribute to open complex formation at low temperature, we performed KMnO_4 probing of galP1 complexes formed by different $\sigma^{70}$ and $\sigma^{70}$(1–565) RNAP holoenzymes at 37 and 6 °C. Since RNAP holoenzymes containing one cCTD behaved indistinguishably from the corresponding holoenzymes containing two cCTDs (data not shown), we only present data obtained with holoenzymes that have or lack both cCTDs. As expected from DNase I footprinting results, all RNAP holoenzymes, with the exception of the mutant lacking both cCTDs and $\sigma^{70}$ region 4.2, formed open complexes at 37 °C (Fig. 4B, compare lanes 2, 3, and 6 with lane 7). $\sigma^{70}$ holoenzymes formed open promoter complexes at low temperature irrespective of the presence of cCTD (Fig. 4B, compare lanes 4 and 5). $\sigma^{70}$(1–565) holoenzyme did not form open complexes at low temperature irrespective of the presence of cCTD (Fig. 4B, lanes 8 and 9). Thus, at low temperature, upstream DNA binding by $\sigma^{70}$ region 4.2 but not by cCTD is necessary for open complex formation on galP1.

The inability of RNAP holoenzyme lacking $\sigma^{70}$ region 4.2 to form open complexes at 6 °C can be due to its inability to perform localized DNA melting, which can become rate-limiting at lower temperatures. Alternatively, the lack of open complex formation at lower temperatures can be caused by the inability of the mutant enzyme to bind to promoter DNA. To determine which of these possibilities is realized, we first footprinted galP1 complexes formed at 6 °C with DNase I (Fig. 4C). The results indicate that only $\sigma^{70}$ holoenzymes protected DNA from DNase I digestion at low temperature. The overall pattern

**Fig. 4.** Promoter complex formation and promoter opening at low temperature. A, promoter complexes were formed at indicated temperatures using the indicated $\beta^{32}$P-endlabeled (top-strand) promoter-containing fragments and wild-type $\sigma^{70}$ RNAP holoenzyme. Complexes were probed with KMnO_4, reaction products were separated on a sequencing gel and revealed by autoradiography. B and C, the indicated RNAP holoenzymes were used to form complexes with $\beta^{32}$P-endlabeled (top strand) galP1 promoter containing fragment at 6 or 37 °C (B) or 6 °C (C), probed with KMnO_4 (B) or footprinted with DNase I (C), and reaction products were analyzed as above. D, promoter complexes were formed at 6 °C using the indicated RNAP holoenzymes with $\beta^{32}$P-endlabeled galP1 promoter containing fragment. Reaction products were separated, with or without heparin challenge, on a native 5% polyacrylamide gel. The gel was run at 6 °C. Reaction products were revealed by autoradiography.
of protection was very similar to the 37 °C complexes protection pattern (compare Fig. 4C, lanes 2 and 3, with Fig. 2A, lanes 2 and 4). In contrast, the pattern of DNase I digestion of promoter DNA in the presence of α70(1–565) RNAP holoenzymes was similar to the pattern obtained with naked DNA.

The results presented in Fig. 4C suggest that the low temperature melting defect exhibited by α70(1–565) RNAP holoenzymes may simply be a consequence of their inability to bind to promoter DNA at these conditions. An alternative explanation would be that closed promoter complexes formed by enzymes that lack α70 region 4.2 are so short-lived that they are not detected during the 15-s DNase I footprinting reaction. We therefore attempted to follow closed complex formation using gel retardation assay, hoping that the caging effect during gel electrophoresis may stabilize complexes that were not detected by footprinting (Fig. 4D). RNAP holoenzymes were combined with radioactively labeled galP1 promoter fragment at 6 °C, complexes were allowed to form, and reactions were separated by electrophoresis in a native polyacrylamide gel kept at 6 °C. To monitor open complex formation, reactions were treated with heparin prior to loading on the gel. Reactions that were subjected to electrophoresis without heparin treatment were expected to reveal the presence of both closed and open promoter complexes, as well as complexes formed due to end-binding and other non-promoter complexes. To decrease the amounts of non-promoter complexes, reactions were set up in the presence of excess promoter DNA over RNAP holoenzymes. Electrophoretic separation of reaction products containing α70 holoenzymes revealed, in the absence of heparin challenge, free promoter DNA and two shifted bands (Fig. 4D, lanes 2 and 3). Both shifted bands disappeared upon addition of heparin and a single band with intermediate mobility appeared. This heparin-resistant band must correspond to open promoter complexes detected by KMnO4 probing and DNase I footprinting (Fig. 4, A–C). Bands seen in the absence of heparin must also correspond to open complexes, because addition of heparin did not result in significant increase in free DNA present in reactions (Fig. 4D, compare lanes 2 and 3 to lanes 3 and 5, correspondingly). Apparently, the addition of heparin changes the complex mobility, either because heparin interacts with the complex, or because some RNAP-DNA contacts are broken in the presence of heparin.

As expected from KMnO4 probing and DNase I footprinting data, little or no heparin-resistant complexes was observed when α70(1–565) RNAP holoenzymes were used for promoter complex formation (Fig. 4D, lanes 9 and 10). A different situation was observed in the absence of heparin. The α70(1–565) RNAP holoenzyme that lacked both αCTDs was unable to form promoter DNA (Fig. 4D, lane 8), while the enzyme that had wild-type α produced two bands with the same mobility as those observed in α70 holoenzyme lanes (Fig. 4D, lane 7). We take these results as evidence that α70(1–565) holoenzyme reconstituted with wild-type RNAP core fails to form an open promoter complex at low temperature because it fails to isomerize to open promoter complex. One the other hand, the double mutant holoenzyme that lacks α70 region 4.2 and αCTDs fails to bind promoter DNA.

**Effect of T4 AsiA on Promoter Complex Formation**—Bacteriophage T4 anti-σ AsiA binds to two sites in α70, in regions 4.1 and 4.2. AsiA binding to α70 region 4.2 prevents the recognition of the −10/−35 class promoters by interfering with α70 region 4.2 interaction with the −35 promoter element (19, 24). AsiA does not prevent promoter complex formation on extended −10 promoters because region 4.2 interactions with upstream DNA are not critical on these promoters (19, 24). Interaction of AsiA with region 4.1 (in the context of α70(1–565) holoenzyme) stimulates transcription from bacteriophage T4 extended −10 class middle promoters through an undefined mechanism (22). We studied promoter complexes formed by various RNAP holoenzymes in the presence of AsiA. Promoter complexes were formed in the presence of 5-fold excess of AsiA over α70 present in the reaction. The amounts were sufficient to completely block promoter complex formation on galP1-35-TG by wild-type RNAP holoenzyme (data not shown). The DNase I footprinting experiment presented in Fig. 5A was conducted on the galP1-35 promoter at 37 °C. An identical result was obtained on galP1 (data not shown). As can be seen, promoter complexes formed by α70 RNAP holoenzymes in the presence of AsiA differed from complexes formed in its absence primarily by increased accessibility of DNA positions −34 and −35 to DNase I attack (compare lanes 2 and 3, and lanes 4 and 5). In addition, AsiA abolished DNase I hypersensitivity at −67 and −57, consistent with the results in Fig. 2 that show that hypersensitivity of these sites requires full-length α70. The addition of AsiA to α70 RNAP lacking both αCTDs diminished, but did not completely prevent promoter complex formation (Fig. 5A, compare lanes 6 and 7). Since no promoter complexes were formed when both αCTDs and α70 region 4.2 were missing (Figs. 2A and 4B), the result implies that α70 region 4.2 is able to interact with DNA even in the presence of bound AsiA. Alternatively, AsiA itself may interact with DNA (25).

Promoter complexes formed by αCTD-containing α70(1–565) RNAP holoenzymes in the presence of AsiA differed from complexes formed in its absence primarily by decreased accessibility of DNA position −37 and increased accessibility of DNA positions −34 and −35 (Fig. 5B). As a result, αCTD-containing α70(1–565) holoenzymes produced very similar footprints in the presence of AsiA. Addition of AsiA did not allow the α70(1–565) holoenzyme that lacked both αCTDs to form a promoter complex (Fig. 5B, compare lanes 6 and 7).

Our last experiment investigated the effect of AsiA on low temperature promoter opening by α70(1–565) holoenzyme. As shown above (Fig. 5B), the binding defect of RNAP that lacked α70 region 4.2 and αCTDs could not be overcome by the addition of AsiA at 37 °C. Likewise, AsiA had no effect on promoter binding by this mutant enzyme at 65 °C (data not shown). Since RNAP containing the wild-type α but lacking α70 region 4.2 appeared to bind promoter DNA normally at 6 °C, we asked if AsiA could stimulate open complex formation by this enzyme. The addition of AsiA stimulated promoter opening (Fig. 5C, compare lanes 4 and 5) and thus allowed to partially overcome the defect caused by the absence of α70 region 4.2.

**DISCUSSION**

Several conclusions about the galP1 promoter complex formation by E. coli RNAP α70 holoenzymes can be drawn from the experiments presented herewith. First, at physiological temperature, the αCTD upstream promoter DNA interactions and α70 region 4.2 upstream promoter DNA interactions are functionally interchangeable on galP1 derivatives containing extended −10 motif. At least one of these interactions is necessary for efficient open promoter complex formation. Mechanistically, each of these interactions may serve to recruit RNAP holoenzyme to promoter and allow α70 region 2.5 to engage the extended −10 element. The presence of these upstream interactions has no effect of the dissociation kinetics of preformed promoter complex as measured by heparin challenge experiments.

αCTD upstream promoter DNA interactions and α70 region 4.2–35 promoter consensus element interactions are functionally distinct on a galP1 promoter derivative lacking the extended −10 TG motif: α70 region 4.2–35 promoter consensus
element interaction is required for open complex formation on this promoter, while αCTD upstream promoter DNA interaction is dispensable. It is possible that this difference is simply a consequence of different strengths of αCTD and σ70 region 2.4 interactions with their targets. Alternatively, σ70 region 2.4–35 promoter element interaction but not αCTD upstream promoter DNA interaction may allow σ70 region 2.4 to productively engage the −10 promoter element and initiate promoter melting in the absence of specific interactions between region 2.5 and the TG motif. The differential ability of αCTD and σ70 region 4.2 DNA interactions to allow to engage the −10 promoter element could be due to the fact that αCTDs are attached to RNAP through unstructured and highly flexible tethers, while σ70 region 4.2 is connected to RNAP through an extensive protein-protein interaction with the β flap domain (2, 26, 27). The β flap has only limited mobility and may therefore allow “signal transduction” from the −35 promoter element to the −10 promoter element.

Analysis of promoter complex formation at low temperature of 6°C demonstrates that even on an extended −10 promoter σ70 region 4.2 and αCTD play different roles in promoter complex formation. Our results show that even though the extended −10 TG motif is necessary for low temperature galP1 open complex formation, it is not sufficient, and σ70 region 4.2 (and presumably its interaction with upstream DNA) is also required. Both sequence-specific and non-specific σ70 region 4.2 interactions are sufficient for low temperature open complex formation in the context of extended −10 promoter since low temperature complexes form on galP1-35 and galP1. The latter promoter lacks a functional −35 promoter element as evidenced by the inactivity of the galP1-TG promoter (Fig. 1). In contrast to strict requirement for σ70 region 4.2 for promoter complex formation at low temperature, αCTDs are not required, in agreement with accompanying work of Kamali-Moghaddam and Geiduschek (28). In this respect, low temperature complex formation on extended −10 galP1 derivatives is similar to the situation observed on a −10/−35 derivative at 37°C.

Since αCTD does not substitute for σ70 region 4.2 at 6°C, σ70 region 4.2 somehow contributes to low temperature DNA opening. The gel retardation results appear to support change for this, since an enzyme reconstituted from wild-type RNAP core and σ70 lacking region 4.2 forms heparin-sensitive closed complexes but fails to isomerize to heparin-resistant complexes. Since RNAP holoenzyme reconstituted from wild-type core and σ70 lacking region 4.2 readily forms open complexes at physiological temperature, there must exist a cold-sensitive step in promoter complex formation that is overcome in the presence of σ70 region 4.2. The movement of the β subunit flap domain could be a potential candidate for such a step. We propose that in the context of σ70(1–565) holoenzyme, the β flap can adopt several (at least two) conformations relative to the rest of RNAP core molecule. One of these conformations is not productive and does not allow promoter complex formation on extended −10 promoters. This conformation is infrequent at 37°C, but becomes predominant at low temperature. In the context of the σ70 RNAP holoenzyme, the β flap interaction with σ70 region 4.2 interaction stabilizes an alternative active conformation of the β flap, at both the low and the high temperatures. According to this view, AsiA may stimulate low temperature melting by σ70(1–565) holoenzyme not because AsiA interacts with promoter DNA but because AsiA helps stabilize the active conformation of the β flap at low temperature. Indeed, specific binding of AsiA to the β flap has recently been reported (29).
Acknowledgments—We thank Dhruti Savalia for constructing some of the galP1 derivatives used in this work, Melanie Barker for advice on promoter complex stability assays, and E. P. Geiduschek, T. Heyduk, A. Hochschild, S. Nechaev, and R. Saecker for careful reading and constructive criticism of the manuscript.

REFERENCES
1. Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. (1998) *Spring Harb. Symp. Quant. Biol.* 63, 141–155
2. Murakami, O. N., and Tsyganov, M. A. (1995) *Nucleic Acids Res.* 23, 4533–4541
3. Attey, A., Belyaeva, T., Savery, N., Hoggett, J., Fujita, N., Ishihama, A., Severinov, K., and Gourse, R. L. (1993) *Science* 262, 1407–1413
4. Ross, W., Gosink, K. K., Salomon, J., Igarashi, K., Zou, K., Ishihama, A., Severinov, K., and Gaal, T. (2000) *Mol. Microbiol.* 37, 687–695
5. Belyaeva, T., Griffiths, L., Minchin, S., Cole, J., and Busby, S. (1993) *Biochem. J.* 296, 851–857
6. Burns, H. D., and Minchin, S. D. (1994) *Nucleic Acids Res.* 22, 3840–3845
7. Vassylyev, D. G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M. N., Borukhov, S., and Yokoyama, S. (2002) *Nature* 417, 712–719
On the Role of the *Escherichia coli* RNA Polymerase σ^70^ Region 4.2 and α-Subunit C-terminal Domains in Promoter Complex Formation on the Extended –10 galP1 Promoter

Leonid Minakhin and Konstantin Severinov

*J. Biol. Chem.* 2003, 278:29710-29718.  
doi: 10.1074/jbc.M304906200 originally published online June 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304906200

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/278/32/29710.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 29 references, 14 of which can be accessed free at

[http://www.jbc.org/content/278/32/29710.full.html#ref-list-1](http://www.jbc.org/content/278/32/29710.full.html#ref-list-1)