Probing the Importance of Selected Phylum-specific Amino Acids in $\sigma^A$ of Bacteroides fragilis, a Primary $\sigma$ Factor Naturally Devoid of an N-terminal Acidic Region 1.1

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The $\sigma^A$ factor of Bacteroides fragilis is the prototype of a novel subgroup of primary $\sigma$ factors that are essential for growth and ensure the initiation of transcription of the housekeeping genes. This subgroup is confined to the phyla Bacteroidetes and Chlorobi. Its members carry a specific amino acid signature and are notably characterized by a short, basic N-terminal segment instead of the typical acidic region 1.1. Using in vitro mutagenesis, we investigated the importance of this basic segment and of several residues of the signature for the function of $\sigma^A$. We have shown that the conserved residues Phe-61 and Lys-265, located in the core binding and DNA binding subregions 2.1 and 4.2, respectively, are critical for full function of the B. fragilis holoenzyme. With respect to the unusual subregion composition of $\sigma^A$, we have shown that truncation of the basic N-terminal segment, or reversion of its charge, strongly affects the overall transcriptional activity of B. fragilis RNA polymerase in vitro. Our results indicate that the presence of the intact basic segment is required for the formation of RNA polymerase (RNAP)-promoter open complexes, the correct architecture of the transcription bubble, and efficient promoter clearance.

In eubacteria, transcription initiation is brought about by RNA polymerase (RNAP) holoenzyme, which is composed of the five-subunit core ($\alpha_2\beta\beta'\omega$) and one of a variety of transiently associated subunits, the $\sigma$ factors, which are largely responsible for the specificity of promoter recognition and DNA melting (1). On the basis of functional and phylogenetic criteria, the $\sigma$ factors of the major family $\sigma^{70}$ have been assigned to one group of primary and up to four groups of non-essential factors (1–5). In each bacterial species, there is one primary $\sigma$ factor that is essential for growth and closely related to $\sigma^{70}$ of Escherichia coli and that predominantly ensures transcription initiation during the exponential phase of growth, i.e. of the housekeeping genes. In addition, there are variable numbers of the non-essential (including the alternative) $\sigma$ factors that are involved in the transcription mostly of genes activated under various forms of stress or in response to signals from outside of the cytoplasm (5, 6). Forty or fifty odd genes of alternative $\sigma$ factors have been identified, respectively, in the Bacteroides species Bacteroides fragilis and Bacteroides thailandicus (7, 8).

In contrast to the primary $\sigma$ factors studied so far, which interact with promoters comprising the two highly conserved −35 and −10 hexamers (9), the corresponding factor $\sigma^A$ of B. fragilis specifically recognizes the particular vegetative promoters of this species, which comprise the consensus sequences TTTG (−33) and TAnnTTTG (−7) (10, 11). Structurally, $\sigma^A$ of B. fragilis is distinct from most other primary $\sigma$ factors and is the smallest such factor described to date (11). Although containing the conserved regions 1.2 through 4.2 (1, 4), this $\sigma^A$ lacks the strongly acidic region 1.1, which is replaced by a unique short basic segment of ten amino acids. It also displays a typical amino acid signature made up of 35 residues that are identically conserved in the primary $\sigma$ factors of members of the Bacteroidetes and Chlorobi phyla (11). An additional feature of the B. fragilis $\sigma^A$ factor, unusual for the primary $\sigma$ of a Gram-negative bacterium, is the absence of the non-conserved region between regions 1.2 and 2.1.

Region 1.1 is considered to be a constant part of the primary $\sigma$ factors (5, 12, 13). This unstructured and largely unconserved region (4, 14, 15) constitutes an autoinhibitory domain that has been reported to prevent free $\sigma^{70}$ of E. coli and free $\sigma^A$ of Thermotoga maritima from binding to DNA unless shortened or otherwise mutationally altered (16–19). This inhibition is thought to result from indirect effects, steric or electrostatic or both, of region 1.1 on region 4.2, which ensures RNAP binding to the −35 promoter element (12, 16). Alternating interaction of the highly negatively charged region 1.1 has been shown to occur with remarkably implicit flexibility with the positively charged double-stranded DNA binding channel in the holoenzyme and with the similarly charged $\beta$ pincer in the RNAP-promoter open complex (20). With respect to function within the holoenzyme, region 1.1 has been suggested to serve as a modulator of promoter binding and to support or inhibit efficient transcription initiation in a promoter-dependent manner.
(15, 21). It has also been found to have a moderating effect on RNAP inhibition by the bacteriophage protein AsIA and on its activation by MotA/AsIA and to substantially increase the stabilization of the interaction between σ70 and the RNAP core in E. coli (12). As for Bacillus subtilis, region 1.1 was concluded not to be required for the function of σ^A either in vitro or in vivo but to be able to negatively modulate the promoter DNA-binding activity of the holoenzyme (22).

In light of the abundant (although not fully conclusive) data on region 1.1 supporting its intrinsic role in the behavior of free σ and on its contribution to the function of previously studied RNAP holoenzymes, it was the aim of the present study to analyze the possible relationship between the unusually short N-terminal region and functional properties of σ^A of B. fragilis. We also tested the importance of some residues of the typical amino acid signature for the function of this factor, the prototype of a novel subgroup of primary σ factors that naturally lack region 1.1.

**EXPERIMENTAL PROCEDURES**

Strains, Plasmids, Standard DNA Manipulations, and Site-directed Mutagenesis — The E. coli strains TG1, ER2566 (New England Biolabs) and XL1 Blue (Stratagene) used in this study for plasmid or protein production were grown under standard conditions with antibiotics added as required. B. fragilis strains 638R (23) and 638R rpoC-His_y (11), grown anaerobically, were used as sources of chromosomal DNA and core RNAP, respectively. Total bacterial DNA was extracted using the Wizard genomic DNA purification kit (Promega). Plasmids were prepared with the QIAprep Spin Miniprep Kit (Qiagen) and introduced into E. coli by electroporation. DNA restriction and modification enzymes were purchased from New England Biolabs. Restriction fragments and PCR amplification products were recovered from agarose gels using the QIAEX II gel extraction kit (Qiagen). PCR products were purified in a Primus 25 thermal cycler (BIO SERV) with Taq, VENT, or Phusion DNA polymerase (New England Biolabs). Plasmids pD2V8 and pD2V29 carrying the B. fragilis promoters P_yg2 and rrsP1, respectively (11), and pD2V30 were used for in vitro transcription assays. Plasmid pD2V30, carrying the rrsP2 promoter of the 16 S rRNA gene of B. fragilis 638R, was constructed by ligating a 128-bp PCR-generated fragment using primers Oli279 (5′-GAATTCGACACAGCATTTAATTTGCAACAG-AGAC-3′; EcoRI site underlined) and Oli280 (5′-GACACCGCGCCAA AGGGTAATCTCAGCAGCATCTACCG-3′; NdeI site underlined). Single point mutations in σ^A were verified by DNA sequencing.

**Holoenzyme Reconstitution** — The σ^A mutant proteins were produced in E. coli ER2566 after intein tagging and purified on chitin beads following the supplier’s instructions, as previously described for σ^A (11). The σ preparations were estimated to be >90% pure after denaturing gel electrophoresis. The proteins were stored at ~80 °C in buffer (Tris-HCl, 10 mM, pH 7.9; MgCl_2, 10 mM; NaCl, 150 mM; EDTA, 0.1 mM; glycerol, 50% (v/v); dithiothreitol, 0.2 mM). B. fragilis core RNAP containing a chromosome-encoded His-tagged β subunit was prepared from strain 638R rpoC-His_y as previously described using nickel affinity, molecular sieve and ion exchange chromatography (11).

Comparative limited proteolysis of wild type and mutant σ^A proteins was essentially carried out as previously described (25) at a σ^A/trypsin weight ratio of 1:1000 in buffer (Tris-HCl, 20 mM, pH 7.9; NaCl, 100 mM).

Holoenzymes were reconstituted by incubating typically ~5 pmol of B. fragilis core RNAP with WT or mutant σ^A at a 5-fold molar excess for 20 min at 37 °C. The reconstituted holoenzymes were subjected to limited proteolysis with trypsin and analyzed by 12% SDS-PAGE followed by Coomassie-blue staining. One-dimensional Western blots were carried out using the same conditions, but with 3% SDS and with the same antibodies. 

**In Vitro Mutagenesis of the B. fragilis Primary σ Factor**

The resulting fragments were digested with NdeI/SapI and ligated into the EcoRI/BamHI-digested plasmid pJCD01 (24). All mutations were verified by DNA sequencing.

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zymes were diluted at room temperature in K-Glu buffer (HEPES at 20 mM, pH 8.0, potassium glutamate at 50 mM, magnesium chloride at 10 mM, dithiothreitol at 5 mM, and bovine serum albumin at 500 μg/ml) prior to use for in vitro experiments. Dithiothreitol was omitted from the buffer for permanganate (KMnO₄) reactivity experiments. Determination of σ⁴ Binding to B. fragilis Core RNA Using an Enzyme-linked Immunosorbent Assay—Using a procedure adapted from André et al. (26), 96-well microtitration plates (Maxisorp™, Nunc) were coated overnight at 4°C with increasing amounts (0.1–3 pmol) of purified B. fragilis σ⁴, wild type or mutant, in 100 μl of buffer A (Tris-HCl at 20 mM, pH 7.9, NaCl at 100 mM, and Tween 20 at 0.1% [v/v]). After washing three times with 200 μl of buffer A, the wells were saturated with 250 μl of buffer A containing 1% (w/v) bovine serum albumin (buffer B) for 1 h at room temperature. B. fragilis core RNA (1 pmol) diluted in buffer B (100 μl) was added and the mixture incubated for 1 h at room temperature. The wells were washed three times with 250 μl of buffer A, incubated for 1 h at room temperature with anti-α subunit antibodies (11) diluted 1/500 in buffer B, and washed again with buffer. Core binding was revealed after the addition of peroxydase-labeled anti-rabbit IgG antibodies and H₂O₂/o-phenylenediamine. Absorbency was measured at 490 nm. Runoff Transcription Assays—Plasmids pDV28, pDV29, and pDV30, and linear DNA fragments amplified from each plasmid using primers E7 (5’-TGGCAGATGCCTTCCG-3’) and J7 (5’-GGATTGTGCTCTACTCAGGAG-3’) were used as templates. σ⁴-dependent transcripts (94, 110, and 104 nucleotides, respectively) were expected to end at the rrsBTT1T2 terminators of pCD01. Multiple round transcription assays were performed (in a total volume of 12 μl) with supercoiled or linear template at 10 nm and reconstituted holoenzyme at 50 nm. Each holoenzyme variant was added to DNA to allow open complex formation at 37°C for 30 min. RNA polymerization was started by the addition of a nucleotide solution (200 μM ATP, CTP, and GTP, 20 μM of [α³²P]UTP (4 Ci/mmol) and allowed to proceed for 15 min at 37°C. Single round transcription assays were performed in the presence of heparin (120 μg/ml), with the reaction allowed to proceed for 5 min at 37°C. Reactions were stopped with a mixture of urea (8 M) and xylene cyanol blue (0.5%). After heating to 90°C, the samples were subjected to electrophoresis on a polyacrylamide (8%) gel, followed by autoradiography and imaging with a phosphorimaging device. Abortive Initiation Assays—Abortive initiation was assayed on a linear 287-bp rrsP2 template as for runoff experiments, except that ApG dinucleotide and UTP were added to a final concentration of 0.5 mM and 0.01 mM, respectively. Reactions were allowed to proceed for 30 min and samples submitted to chromatography in WASP buffer (ammonium sulfate at 3.2 M, EDTA, pH 8.0, at 5 mM, isopropanol at 2% [v/v]) on Whatman 3M paper treated with EDTA (100 mM). The trinucleotide transcripts were quantified using a phosphorimaging device. Electrophoretic Mobility Shift Assays—Linear P₉₄₂, rrsP1, and rrsP2 DNA fragments were amplified by PCR from pDV27, pDV28 and pDV29, respectively, and both strands radiolabeled through 5’-phosphorylation of primers E7 or J7 using phage T₄ polynucleotide kinase (New England Biolabs) and [γ-³²P]ATP (3000 Ci/mmol). The reconstituted holoenzymes (10 to 30 nM) and the DNA fragments (3 nM) were incubated for 30 min at 37°C in K-Glu buffer in a final reaction volume of 10 μl. The reaction mixtures were loaded onto a native polyacrylamide (5.5%) gel after the addition of 2 μl of loading buffer (sucrose at 50%, xylene cyanol at 0.5%, bromphenol blue at 0.5%, and heparin at 100 μg/ml). After electrophoresis, the gels were dried and subjected to autoradiography. DNA was quantified with the phosphorimaging device. Testing of the KMnO₄ Reactivity of DNA—Complexes between the labeled promoter regions (5 nm) and the mutant holoenzymes (50 nm) were allowed to form for 30 min at 37°C in 10 μl of K-Glu buffer. KMnO₄ (3 μl, 18 mM) was added and the reaction stopped after 10 s at 37°C with 3 μl of dithiothreitol (200 mM) and 200 μl of stop buffer (sodium acetate at 0.4 M, EDTA at 2.5 mM, and salmon sperm DNA at 60 μg/ml). The samples were treated with phenol and the DNA precipitated with absolute ethanol and washed with ethanol (70%). The precipitates were resuspended in 100 μl of piperidine (1 M), heated for 30 min at 90°C, evaporated to dryness, and washed with 20 μl of water twice. The samples were resuspended in 10 μl of formamide containing 20 mM EDTA, bromphenol, and xylene cyanol blue. After heating to 90°C, samples (5 μl) were subjected to electrophoresis on a polyacrylamide (8%) gel, followed by autoradiography and imaging with a phosphorimaging device. Molecular Modeling—Modeling using the C-α backbone of the Thermus thermophilus holoenzyme structure (27) (Protein Data Bank code 1W7) was done as previously described (11). RESULTS The Alteration of Several but Not All Amino Acids Constitutively the Typical B. fragilis σ⁴ Signature Affects the Transcriptional Activity of the Holoenzyme—To study the functional relevance of structural features of σ⁴ described previously (11), we constructed 11 mutants with changes in several of the 35 amino acids that make up the Bacteroidetes and Chlorobi σ⁴ signature. Two mutants, ΔNBS and NAS, were altered in the N-terminal segment; a segment of 12 N-terminal amino acids was deleted in ΔNBS and replaced by an acidic segment (R2E, K5E, and K8E) in NAS (Fig. 1A). The nine remaining mutants were point mutants, D19A and D19R in region 2.1, Q116A and Q116R in region 2.4, G133E in region 3.0, H179P in region 3.2, and L256V and K265E in region 4.2; the underlined amino acids are those conserved in the corresponding positions in almost all 35 and 10 box-recognizing primary σ factors (13). The activities of the holoenzymes reconstituted with the individual σ mutants and tested on supercoiled DNA in multiple round runoff transcription using promoters P₉₄₂, rrsP1, and rrsP2 (Fig. 1B) are shown in Fig. 2. The presence of σ⁴ mutants ΔNBS, NAS, and H179P entailed a reduction in transcriptional activity between ~40 and 60%, whereas that of mutants F61L and K265E all but abolished this activity. Mutants D19A, D19R, Q116A, Q116R, G133E, and L256V were at least as active as WTσ⁴, and there were no important promoter-dependent differences in the relative transcriptional activities except for rare ~20–50% increases with P₉₄₂ or rrsP1 (Fig. 2B).
In Vitro Mutagenesis of the B. fragilis Primary ω Factor

Very similar results were obtained with all mutants in single round runoff transcription (data not shown).

To rule out the possibility that the altered transcriptional activities of ΔNBS, NAS, F61L, H179P, and K265E were connected with substantial disturbance of the global folding of the σ^ω mutants or of their binding to the RNAP core, we carried out limited proteolysis with trypsin and assayed σ-core binding in an enzyme-linked immunosorbent assay test. No gross differences between the digestion patterns of WT σ^ω and the mutants and notably of the most affected, F61L and K265E, were observed, with the exception of NAS (supplemental Fig. S1). Digestion of this mutant yielded a fragment with an apparent molecular weight of ~22 kDa, with the N-terminal sequence MEQLE as determined by Edman degradation (the analysis was carried out at the Plate-forme Analyse et Microsequençage des Protéines, Pasteur Institute). The particular migration of this fragment is suspected to result from decreased SDS binding due to its high negative charge. The size of its wild type equivalent of ~20 kDa (supplemental Fig. S1) is compatible with proteolysis at the σ^ω 4 loop of the linker domain (28), and its variant migration behavior is therefore not considered to reflect an alteration of the mutant protein structure. There were also no notable alterations in the amounts of core RNAP that were bound by the σ^ω mutants (supplemental Fig. S2). These observations then suggested that the residues, the replacement of which resulted in reduced transcriptional activity, should be involved in steps at or after holoenzyme binding to DNA.

To study open complex formation in greater detail, runoff transcription was carried out with linear templates. Although the absolute amounts of transcript were reduced ~2-fold (data not shown), the relative amounts with respect to the wild type holoenzyme were hardly altered (compare Figs. 2 and 4; see below).

Heparin-resistant Complex Formation Is Not Correlated with Transcriptional Activity of ΔNBS-containing Holoenzyme—
The binding of holoenzyme reconstituted with σ^ω mutants to radiolabeled DNA fragments containing promoters P_942, rrsP1, or rrsP2 was tested for in an electrophoretic mobility shift assay after treatment of the preformed complexes with heparin. The fractions of bound DNA (Fig. 3A) were measured and normalized with respect to the DNA bound by the WT holoenzyme (Fig. 3B). Promoter binding of the holoenzyme was only marginally affected when H179P, which afforded a close to 40% reduction in transcriptional activity, was present. The reduction in transcriptional activity in the presence of mutants NAS and F61L was accompanied by a substantial reduction in promoter binding, although there was no perfect correlation between the two activities. On the other hand, in the presence of mutant K265E, there was no promoter binding and no transcriptional activity. Mutant ΔNBS displayed an unexpected behavior, in that promoter binding in its presence was increased by factors between 2 and 3.5 (depending on the individual promoter tested; see Fig. 3B), whereas the transcriptional activity was reduced by almost one-half (Fig. 2). This suggested the possibility that the formation of unusually heparin-resistant complexes resulted in impaired initiation or elongation or both.

Holoenzyme with ΔNBS, F61L, or K265E Synthesizes Disproportionately High Amounts of Abortive Transcripts—Abortive transcription was assayed with linear template containing rrsP2, the only promoter (of the three used in this study) that supported transcription initiation with adinucleotide, i.e. ApG. Trinucleotide (ApGpU) synthesis, as related to overall transcriptional activity, is shown in Fig. 4. Although runoff and abortive transcriptional activities were reduced in
In Vitro Mutagenesis of the B. fragilis Primary σ Factor

A

1 2 3 4 5 6 7

B

FIGURE 3. DNA binding activities of wild type- and mutant σA-containing holoenzyme of B. fragilis. A, binding activities of holoenzyme (30 nM) in the presence of heparin were compared using an electrophoretic mobility shift assay with radiolabeled linear DNA. DNA-holoenzyme complexes were separated on native polyacrylamide (5.5%) gels. The enzymes contained no σ (lanes 1); wild type σA (lanes 2); ΔNBS (lanes 3); NAS (lanes 4); F61L (lanes 5); H179P (lanes 6); and K265E (lanes 7). The promoters are indicated below the gels. The amounts of free and bound DNA were determined using a phosphorimaging device, and the ratio was calculated for each enzyme and normalized with respect to the wild type enzyme.

FIGURE 4. Comparative abortive and runoff transcriptional activities. Activities were determined using linear rrsP2-containing DNA. Abortive transcription was assayed in the presence of the dinucleotide ApG, UTP, and radioactive UTP. Abortive and runoff transcripts from three independent experiments were quantified and normalized with respect to those obtained with the wild type σA-containing holoenzyme. In the abortive transcription assay, 35 mmol of ApGpU/mol of UTP added were synthesized by the wild type holoenzyme in 15 min. In the runoff assay, the enzyme produced 7 mmol of rrsP2 transcripts/mol of UTP added in 15 min. Eo designates B. fragilis core RNAP with no σ factor.

the other hand, abortive transcription with ΔNBS-containing holoenzyme was increased by almost 50% with respect to the WT enzyme and as such in apparent agreement with the increased promoter binding (Fig. 3). This would point to a decreased ability of the ΔNBS-containing holoenzyme to proceed from initiation to elongation. By contrast, the failure of the F61L- and K265E-containing enzymes to support productive elongation would likely be due to highly unstable open complexes.

The Basic N-terminal Segment of σA Plays a Role in Open Complex Formation—In light of the differences in the capacity of the holoenzyme variants to form heparin-resistant complexes, the nature of the transcription bubble on the linear rrsP2 template was investigated by analyzing the thymine sensitivity to KMnO4. With WT holoenzyme, three reactive thymines were observed on the coding strand and two on the alternatively radiolabeled template strand. The thymines at positions −9 on the template strand and −8 and −4 on the non-template strand were the most reactive, whereas those at position +2 on the coding strand and −1 on the template strand were the least sensitive (Fig. 5). The strongly reduced thymine reactivity in the presence of F61L and K265E could be expected considering the likelihood that the corresponding holoenzymes form only highly unstable open complexes. The reactivity in the presence of NAS was similarly low, whereas it was only marginally decreased in the presence of H179P, which supported the formation of somewhat more stable open complexes than NAS. The presence of ΔNBS resulted in a quite different sensitivity pattern. Although the reactivity was reduced overall (by ~40%), the sensitivity of the individual thymines was altered noticeably in that it was increased at positions +2 on the coding and −1 on the template strand relative to what was observed with the WT enzyme. This observation would be compatible with a shift in the contacts between the DNA-holoenzyme variant and the promoter region during or after open complex formation when the basic N-terminal segment is absent from σA.

DISCUSSION

Taking σA of B. fragilis as a prototype, we have probed the functional importance of a set of amino acids specifically conserved in the novel subgroup of primary σ factors found in the phyla Bacteroidetes and Chlorobi. We have analyzed the properties of 11 σA mutants in vitro, as no mutant for the temperature-sensitive production of σA has yet been isolated in B. fragilis. This σA being unable to recognize typical −10 or −35
In Vitro Mutagenesis of the B. fragilis Primary α Factor

In vivo or in vitro (11), we have used three B. fragilis promoters, two ribosomal RNA promoters, rsP1 and rsP2, and P942, which drives the expression of carbapenem and nitroimidazole resistance in this species (Fig. 1B). The discrete differences among the sequences of these three promoters did not substantially affect any of the σ^A functions tested here. Seven B. fragilis σ^A-specific amino acid residues (11) located across the four conserved σ regions (4) were replaced by the residues found at the corresponding positions in most primary σ factors, including those of E. coli, B. subtilis, Thermus aquaticus and T. thermophilus.

Four residues of σ^A (Asp-19, Gln-116, Gly-133 and Leu-256) could be changed without substantially affecting transcription, and the corresponding mutants were not studied further. The amino acid at the position corresponding to Asp-19 in region 1.2 plays a crucial role in σ^A of B. subtilis (Arg-103) but not in σ^O of E. coli (Arg-99) (22, 29). The position of Gln-116 in region 2.4 is occupied in σ^O by Arg-441, which is part of the arginine triad (Arg-436, -441, -451) required for closed complex formation (30) and likely makes promoter contact upstream of the −10 element. Interestingly, the Q116R mutant, (but not Q116A) in B. fragilis had increased transcriptional activity on P942, a promoter with a G immediately upstream of the −7 element, which might interact with Arg-116. Residue Gly-133 of σ^A in region 3.0 corresponds to Glu-458 in σ^O, which is involved in the recognition of the TG motif at the extended −10 element (31). The G133E substitution in σ^A had no adverse effect on transcription. It is conceivable that the Bacteroides promoters, which possess the equivalent of a −10 extended region at their downstream end (Fig. 1B) have a lesser requirement for the classical −10 upstream extended region. Finally, Leu-256, which can be altered into valine without effect (Fig. 2) corresponds to Val-407 in the turn of the HTH motif of the T. aquaticus σ^A, a residue not in direct contact with the −35 element (28).

There was one point mutation in σ^A, H179P, which affected the transcriptional activity of the corresponding holoenzyme moderately. In σ^O, Pro-504 at the corresponding position is located in the N-terminal part of region 3.2, a flexible linker that is completely embedded within the holoenzyme. This region blocks the path for RNA exit and must be displaced by the initiating RNA. A P504L mutant had a decreased core affinity, and the holoenzyme formed unstable open complexes and showed reduced ability to produce abortive, as compared with full-length, transcripts (32–34). No substantial change in the ratio of productive to abortive transcripts was seen with the B. fragilis H179P RNAP (Fig. 4). However, the amount of stable heparin-resistant complexes was somewhat reduced, as was the amount of productive transcripts (Figs. 2 and 3).

Two σ^A mutations, F61L and K265E, affected the runoff transcriptional RNA polymerase activity drastically. The mutants were less deficient in short abortive product synthesis primed with the dinucleotide ApG at rsP2. The first amino acid, Phe-61, is located in region 2.1 known to be involved in core binding but on the opposite face of the residues that make close contacts with the core. In the molecular model of B. fragilis σ^A bound to promoter DNA (11), Phe-61 is at 3.6 Å from Ile-103 (supplemental Fig. S3A), indicative of a van der Waals interaction. This interaction seems particular to the Bacteroidetes and Chlorobi primary factors, because the equivalent residues in T. aquaticus σ^A (Leu-209 and Ser-251) and in E. coli σ^O (Leu-386 and Ser-428) are >5 Å apart. The substitution F61L in B. fragilis σ^A would weaken this interaction and lead to a non-optimal closed and open complex formation. Hence, we conclude that the F61L mutant, although not noticeably deficient in core binding, leads to a holoenzyme disturbed in promoter DNA binding and melting. The second mutation, K265E, is located in region 4.2, which binds the −35 promoter element (28). The dramatic decrease in productive transcription suggests the involvement of the lysine in binding the −33 element of B. fragilis promoters. The change from a basic to an acidic residue would abolish any possible electrostatic Lys-265-DNA interaction (supplemental Fig. S3B). Consequently, the stability of the holoenzyme binding to the −33 region might be decreased, leading to unstable complexes. The K265E mutant-containing holoenzyme did not form heparin-resistant complexes, and no reactive T bases were visible in the −7 region. Strikingly, the primed synthesis of abortive products ApGpU was decreased only 2-fold, compared with the wild type holoenzyme (Fig. 4), suggesting that DNA opening is stabilized by the template-bound initiator dinucleotide, not an unprecedented case (28, 35). How-
ever, in our case, the increase in abortive synthesis was not accompanied by substantial full-length transcription. We therefore are tempted to ascribe the odd synthesis of abortive products observed in the particular case of the F61L and K265E mutant-containing holoenzymes to a branch pathway leading mainly to unstable moribund, instead of productive, complexes (36).

The modifications in mutants ΔNBS and NAS of σ^70 had more comprehensible effects and demonstrated the requirement of the intact, phylum-specific N-terminal basic segment for full σ^70 function. Noticeably, and in contrast to the region 1.1 of the primary σ factor in Thermosynechococcus elongatus, which contains both an acidic region (as do almost all of these factors) and a basic patch located immediately adjacent to region 1.2 (37), the presence of the N-terminal basic sequence in σ^70 of B. fragilis does not enable the free factor to bind to DNA nor does its absence promote DNA binding (data not shown), as has been shown for the N-terminally truncated equivalents of the primary σ factors of E. coli, B. subtilis, and T. maritima (16–18, 22). On the other hand, truncation of the basic N terminus (ΔNBS) or reversion of its charge (NAS) leads to the formation of holoenzyme with only about half of its transcriptional activity (Fig. 2), again at variance with what has been observed with σ^70 of T. elongatus and with σ^30 of E. coli at certain promoters when truncated at their N-terminal limits of region 1.2 (21, 37).

Permanganate reactivity showed that a significant amount of the complexes formed with the ΔNBS RNAP were open, although they were less abundant than seen with the wild type enzyme. The high reactivity of the thymines in close vicinity to the transcription start site in the presence of the ΔNBS mutant (Fig. 5) suggested a different architecture of the transcription bubble due to the absence of the basic segment. This would mean that the presence of the N-terminal basic patch in the wild type impedes the accessibility of thymine (T) −1 and T + 2 to potassium permanganate. There is recent evidence with E. coli and T. aquaticus RNAPs (38, 39) that σ^70 region 1.2 in the open complex makes additional contacts with the non-template strand in the proximity of the transcription start site. A specific sequence, GGGGA, located immediately downstream of the −10 hexamer was identified as the major determinant for binding to region 1.2 of the σ factor of T. aquaticus (38). It is highly plausible that the conserved downstream TG motif in the −7 box plays a similar role in B. fragilis, and we speculate that region 1.2 is displaced in the absence of the NBS, allowing permanganate access to T −1 and T + 2. Alternatively, the N-terminal basic patch could make direct contacts with DNA close to the transcription start. The greater resistance to heparin of the ΔNBS complex could be due to the fact that the corresponding holoenzyme with less positive charges binds less heparin. A chase experiment with the open complex formed with radiolabeled rrsP2 and an excess of cold promoter DNA (data not shown) indicated that the complex with the ΔNBS holoenzyme is more stable than the complex with the wild type holoenzyme. A similar observation was reported for a region 1.1 deletion mutant of σ^70 (Rutherford and Gourse, as cited in Haugen et al. (39)). Considering also that the ΔNBS mutant is deficient in promoter escape, synthesizing almost twice as many abortive transcripts as the wild type (Fig. 4), our results show that the integrity of the basic N-terminal sequence of B. fragilis σ^70 is required for efficient promoter escape and productive transcript synthesis.

Taken together, the present analysis of the major σ factor of B. fragilis shows the essential character of the two signature amino acids Phe-61 and Lys-265 for full RNAP function, with their deduced contribution of subregion 2.1 to open complex formation and of subregion 4.2 to DNA binding, respectively. Less expected is the apparent insensitivity to mutational alteration of four (Asp-19, Gln-116, Gly-133, and Leu-256) of the seven individual signature amino acids probed. The salient structural feature of the B. fragilis σ^70, i.e. the presence of a short basic instead of a rather long, highly acidic N-terminal segment is shown to have a functional impact on the formation of RNAP-promoter open complexes, the correct architecture of the transcription bubble, and efficient promoter clearance.

The presence of the poorly conserved N-terminal region 1.1 has been considered a constant attribute of the prokaryotic primary σ factors (4, 5, 12–15). This region is, however, not strictly ubiquitous and when it is present, its functions do not appear to be unique (22, 37). A primary factor devoid of region 1.1 was initially observed in Chlorobium tepidum (40). It is now clear that this is not an isolated case and that the corresponding σ factors of at least 25 species (supplemental Table S1), all confined to the phyla Bacteroidetes and Chlorobi and containing a common specific amino acid signature, lack this region.

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In Vitro Mutagenesis of the B. fragilis Primary α Factor

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