

Properties of *Bacillus subtilis* σ^A Factors with Region 1.1 and the Conserved Arg-103 at the N Terminus of Region 1.2 Deleted

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Received 13 October 2003/Accepted 17 December 2003

σ factors in the σ^{70} family can be classified into the primary and alternative σ factors according to their physiological functions and amino acid sequence similarities. The primary σ factors are composed of four conserved regions, with the conserved region 1 being divided into two subregions. Region 1.1, which is absent from the alternative σ factor, is poor in conservation; however, region 1.2 is well conserved. We investigated the importance of these two subregions to the function of *Bacillus subtilis* σ^A , which belongs to a subgroup of the primary σ factor lacking a 254-amino-acid spacer between regions 1 and 2. We found that deletion of not more than 100 amino acid residues from the N terminus of σ^A , which removed part or all region 1.1, did not affect the overall transcription activity of the truncated σ^A -RNA polymerase in vitro, indicating that region 1.1 is not required for the functioning of σ^A in RNA polymerase holoenzyme. This finding is consistent with the complementation data obtained in vivo. However, region 1.1 is able to negatively modulate the promoter DNA-binding activity of the σ^A -RNA polymerase. Further deletion of the conserved Arg-103 at the N terminus of region 1.2 increased the content of stable secondary structures of the truncated σ^A and greatly reduced the transcription activity of the truncated σ^A -RNA polymerase by lowering the efficiency of transcription initiation after core binding of σ^A . More importantly, the conserved Arg-103 was also demonstrated to be critical for the functioning of the full-length σ^A in RNA polymerase.

The RNA polymerase holoenzyme in prokaryotic cells is basically composed of $\alpha_2\beta\beta'$ and σ factor. Designated a core enzyme, $\alpha_2\beta\beta'$ possesses catalytic and regulatory functions. Besides being a transcription factor which confers the specificity of recognition of cognate promoter and initiation of transcription (2, 6, 10, 30, 33, 36), σ factor is involved in open complex formation (16, 24, 28), abortive transcription (13, 29), promoter-proximal pausing (26), and regulation of gene expression (14, 18, 25).

Two families of σ factor (σ^{70} and σ^{54}) have been reported previously (20, 22). In the σ^{70} family, σ factors were classified into the primary and alternative σ factors according to their physiological functions and amino acid sequence similarities (20). The primary σ comprises four conserved regions, each containing two to four subregions (12); however, they are poorly conserved in region 1.1 (20) except for the characteristic acidity. Moreover, some of them are devoid of a 254-amino-acid spacer between regions 1 and 2, the function of which is vague (17). On the basis of the presence or absence of the spacer, the primary σ factor can be further separated into two subgroups, with *Escherichia coli* σ^{70} and *Bacillus subtilis* σ^A referred to as the type members (Fig. 1). However, there is no information about the effect of the above differences on the function of σ factor in the subgroup of σ^A , which is mostly responsible for transcription of the housekeeping genes. Moreover, it remains unclear whether region 1.1 is dispensable for a primary σ in the σ^A subgroup, as has been seen for some

alternative σ factors (11, 20), or if it is essential for transcription initiation at some promoters as reported previously for *E. coli* σ^{70} (1, 32, 35). This study aims to answer whether region 1.1 is essential for the functioning of primary σ factor in the σ^A subgroup and to determine the essential N-terminal boundary amino acid residue for a truncated σ^A to function in RNA polymerase holoenzyme.

We have tried to answer the above questions by constructing and characterizing a collection of the N-terminally truncated σ^A . Here, we present evidences showing that region 1.1, which spans amino acid residues 1 through 99, is not required for the functioning of σ^A either in vitro or in vivo. However, it is able to negatively modulate the promoter DNA-binding activity of σ^A -RNA polymerase. We also show that the conserved Arg-103 at the N terminus of region 1.2 is important to ensure efficient functioning of both the truncated and full-length σ^A in RNA polymerase holoenzyme.

MATERIALS AND METHODS

Construction of truncated *sigA* and *sigA* with Arg-103 being replaced. The truncated *sigA* genes were constructed by PCR using the wild-type (WT) *sigA*-containing plasmid pCD2 as template. The forward primers used for N-terminal truncation are designated *sndx*. They have the sequence 5'-GTCAGAAATCAAGAAGGAGATACAATATG-3', followed by 18 to 21 nucleotides starting from the codon next to the one being deleted, with the EcoRI site underlined, the putative ribosome-binding site italicized, and the initiation codon (ATG) bolded. The reverse primer MI2 has the sequence 5'-CGTACCTGCAGGGATCCGTA TACGCTTACAATAGAAAAT-3', with the BamHI site underlined. Each of the *sigA* DNA fragments was digested with EcoRI and BamHI before being cloned into the compatible sites of the transcriptional fusion vector pT7-5 (31).

Overlapping extension PCR was used to construct *sigA*, with Arg-103 being replaced with Met, Ala, Glu, or Lys. Primers used for the synthesis of the mutant *sigA* are as follows: A, 5'-CTGCAGAGATCTGAATTCGTTGCAAGCTTTG G-3' (the underlined bases represent the EcoRI site); D, 5'-CGTACCTGCAG GGATCCGTATACGCTTACAATAGAAAAT-3' (the underlined bases repre-

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<i>B. subtilis</i>	1	mad-----	-----KQT	HETELTFDQV	K-----EQ	LTESGKKRGV
<i>L. monocytogenes</i>	1	MSDKT-----	---KNTK---	PVAELSVEQV	K-----EQ	LIEEGKKKGI
<i>S. aureus</i>	1	MSDNT-----	---VKIKKQT	IDPTLTLDGV	K-----KA	LIEKGKKEGH
<i>L. interrogans</i>	1	mand-----	---QHTE---	LETEFTLDQV	K-----DQ	LIEQGGKRSS
<i>C. acetobutylicum</i>	1	mknk tevkng	gekKNSK---	KVSKeesake	knekmki vKN	LIDKGGKSGS
<i>H. mobilis</i>	1	-----	-----	-----	-----	-----
<i>E. coli</i>	1	MEQNPQSQLK	L-----	-----	-----	LWTRGKEQGY
<i>S. typhimurium</i>	1	MEQNPQSQLK	L-----	-----	-----	LWTRGKEQGY
<i>B. aphidicola</i>	1	MEHNPKSQLK	L-----	-----	-----	LWTRGKEQGY
<hr/>						
<i>B. subtilis</i>	30	LTYEEIAERM	SSFEIESDQM	DEYYEFLGEG	GVELISENEE	tEDPNIQ---
<i>L. monocytogenes</i>	33	LTYAKIAARL	APFTLDSQM	DEYLEHVGEA	GIEVSDADD	-EDPDET---
<i>S. aureus</i>	36	LSHEEIAEKL	QNFIDSDQM	DDFFDQLMDN	DISLVNEKDS	-SDTDEKlnp
<i>L. interrogans</i>	32	LNYKDIMEKL	SPFDQDPEQM	DEFYEHLDGL	GIEVWENDE	-Evsnlrqge
<i>C. acetobutylicum</i>	48	LTYKEIMDEL	QEVDLGPEQI	EKIYEVLESV	GVDVVGDMHE	-IEVEEEE---
<i>H. mobilis</i>	1	-----	---MTADQV	DDVYEQLGNM	GIDVWSEPAE	-AESMEKAE
<i>E. coli</i>	22	LTYAEVNDHL	PEDIVDSQI	EDI IQMINDM	GIQWEEAPD	-ADDLMLAEN
<i>S. typhimurium</i>	22	LTYAEVNDHL	PEDIVDSQI	EDI IQMINDM	GIQWEEAPD	-ADDLMLAEN
<i>B. aphidicola</i>	22	LTYAEVNDHL	PEEIIDSEI	DDI IQMINDM	GIQWEEAPD	-ADDLILNEI
<hr/>						
Region 1.1 ← → Region 1.2						
<i>B. subtilis</i>	77	-----	---QLAKA	EEE-FDLMDL	SVPPGVKIND	PVRMYLKEIG
<i>L. monocytogenes</i>	79	-----	---ELVKE	ETEsFDLTD	SVPPGVKIND	PVRMYLKEIG
<i>S. aureus</i>	85	s-----	-----	---DL	SAPPGVKIND	PVRMYLKEIG
<i>L. interrogans</i>	81	dhdgndnddf	nfd-----	---DDL	SLPPGIKIND	PVRMYLKEIG
<i>C. acetobutylicum</i>	94	-----	---DL---	---DL	TIPEGIADD	PVRMYLKEIG
<i>H. mobilis</i>	36	PI--IRESEE	eeeeiEV---	---DL	SVPEGVGI	PVRMYLKEIG
<i>E. coli</i>	71	TA--DEDAE	AAAQVLSSVE	SEIGRTT---	-----D	PVRMYMREMG
<i>S. typhimurium</i>	71	TTSTDEDAE	AAAQVLSSVE	SEIGRTT---	-----D	PVRMYMREMG
<i>B. aphidicola</i>	71	NTDDEDAVE	AATQVLSSVE	SELGRTT---	-----D	PVRMYMREMG
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Region 1.2 ← → 254-amino-acid spacer						
<i>B. subtilis</i>	111	RVNLLSAKEE	IAYAQKIEEG	D-----	-----	-----
<i>L. monocytogenes</i>	114	RVDLLTADEE	IALAKRIEAG	D-----	-----	-----
<i>S. aureus</i>	108	RVNLLSAQEE	IELAKRIEQG	D-----	-----	-----
<i>L. interrogans</i>	117	RVPLLSADDE	VELAKRIMNG	D-----	-----	-----
<i>C. acetobutylicum</i>	118	KVPLLSPEEE	IDLAQRKNG	D-----	-----	-----
<i>H. mobilis</i>	73	RVPLLSAEEE	IELAKRMEDG	D-----	-----	-----
<i>E. coli</i>	107	TVELLTREGE	IDIAKRIEDG	INQVQCSVAE	YPEAITYLLE	QYDRVEAEEA
<i>S. typhimurium</i>	109	TVELLTREGE	IDIAKRIEDG	INQVQCSVAE	YPEAITYLLE	QYDRVEAEEA
<i>B. aphidicola</i>	109	TVELLTREGE	IDIAKRIEEG	INQVQSSVSE	YPEAITYLLE	QYDRIk t g q i

FIG. 1. Amino acid sequence alignment of primary σ factors in the σ^A and σ^{70} subgroups. Abbreviations denote the following bacterial species: *B. subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Listeria interrogans*, *Clostridium acetobutylicum*, *Haemophilus mobilis*, *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Buchnera aphidicola*. The primary σ factor can be separated into two subgroups, with those from the top six strains being classified into the σ^A subgroup and those from the bottom three placed in the σ^{70} subgroup. Shown in the figure are region 1.1, region 1.2, and part of the 254-amino-acid spacer, all of which are indicated by arrowheads. Region 1.1 of the *B. subtilis* σ^A spans amino acid residues 1 through 99, while region 1.2 spans amino acid residues 100 through 131. Amino acid residues which are not conserved between the two subgroups of primary σ factor are underlined. The lowercase letters denote residues that are not considered homologous. The conserved arginine residue at position 103 of σ^A is indicated by the asterisk.

sent the BamHI site); Bx (x represents R103M, R103A, R103E, or R103K), 5'-ATACATX₁X₂X₃AACTGGGTCATTGATTTTAACG; and Cx, 5'-GACCCA GTTY₃Y₂Y₁ATGTATTAAAGGAAATCGG-3'. Bx and Cx primers are complementary in the underlined bases. X₁X₂X₃ represents the substituted codon. After restriction enzyme digestion, each of the mutant *sigA* DNA fragments was cloned into the compatible sites of pT7-5 (31).

Construction of plasmids used for complementation assays. The plasmids pCF0, pCF100, and pCF104, designed to express WT σ^A , SND100, and SND104, respectively, were constructed by inserting the corresponding *sigA* DNA fragment into the SalI and HindIII sites of the pHY300PLK plasmid derived from pHY300PLK. The expression of *sigA* on the plasmid was under the control of the P1P2 promoter of the *B. subtilis* *sigA* operon and the ribosome-binding site of *sigA*.

Overproduction and purification of σ^A . Methods used for overproduction and purification of the mutant σ^A were similar to those used for the preparation of WT σ^A (5).

In vitro transcription and abortive transcription assays. Core enzyme containing the His-tagged β' subunit was prepared according to the protocol reported previously (15). The method used for the in vitro transcription assay was modified from a previously reported version (5). For in vitro transcription, 10 μ l

of core enzyme (1 μ g) was mixed with an equal volume of purified σ^A (1.2 μ g) before incubation on ice for 10 min. The molar ratio of core to σ^A was 1:10. Afterward, 20 μ l (0.3 μ g) of pCT20 or pCT24 plasmid harboring the P1P2 promoter of the *B. subtilis* *sigA* operon or the G3b promoter of the *B. subtilis* ϕ 29 phage (7, 19), respectively, was added to the reconstituted holoenzyme. The mixture was then incubated on ice for 10 min before being transferred to a temperature environment of 37°C. Subsequently, 40 μ l of reaction cocktail (40 mM Tris-HCl [pH 7.9]; 10 mM MgCl₂; 160 mM KCl; 0.4 mM dithiothreitol; 0.2 mM each UTP, CTP, GTP, and ATP; 3 μ Ci of [α -³²P]ATP; and 5% glycerol) prewarmed at 37°C was added to start the transcription reaction. The reaction was allowed to proceed for 10 min before adding 160 μ l of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Finally, the RNA products were run on a 6% denaturing polyacrylamide gel, and autoradiography was performed after vacuum drying of the gel.

The method used for abortive transcription was similar to that used for in vitro transcription. In brief, a 10 \times molar excess of σ^A was reconstituted with 1 μ g of core enzyme. The reconstituted RNA polymerase holoenzyme was then mixed with 0.1 μ g of linear G3b promoter DNA (164 bp in length) that had been synthesized by PCR. The template used for the synthesis of linear G3b promoter was the ϕ 29 phage DNA; the two primers were BC1041 (5'-GAGCTCGGATC

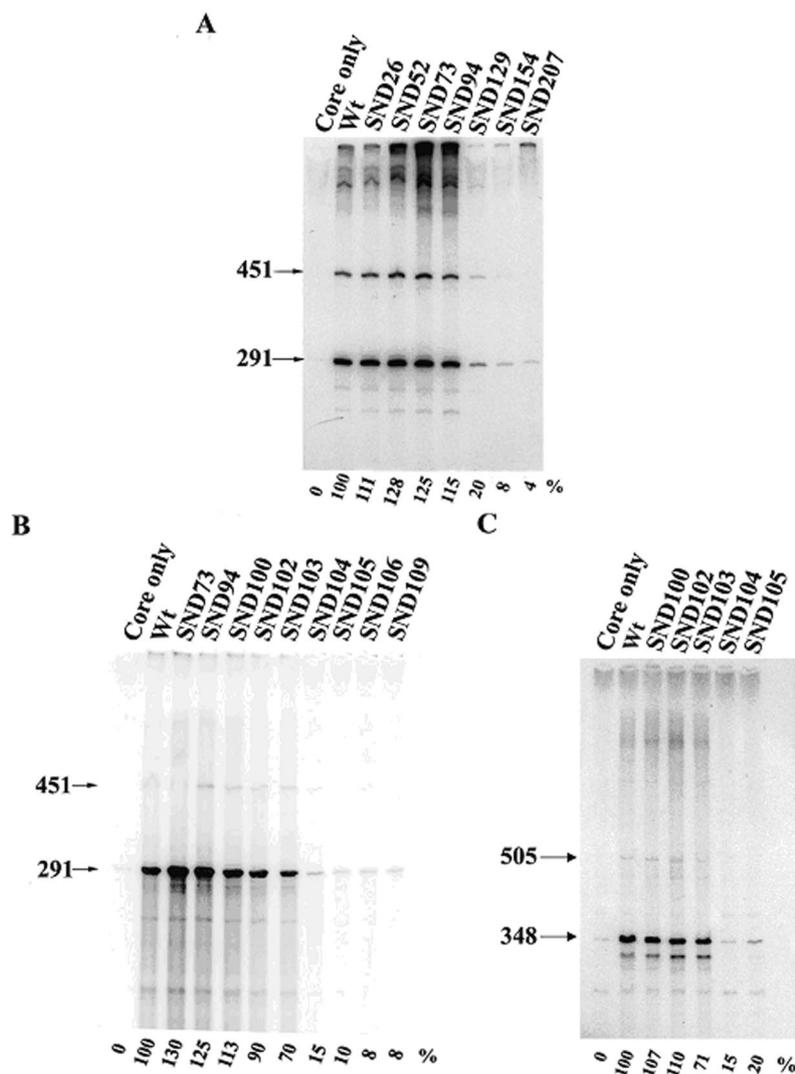


FIG. 2. In vitro transcription assays of the N-terminally truncated σ^A -RNA polymerase holoenzymes. (A and B) In vitro transcription activities of the truncated σ^A -RNA polymerase holoenzymes on the G3b promoter of *B. subtilis* ϕ 29 phage. (C) In vitro transcription activities of the truncated σ^A -RNA polymerase holoenzymes on the PIP2 promoter of the *B. subtilis* *sigA* operon. The method used for the assay is described in Materials and Methods. The truncated σ^A -RNA polymerases assayed are shown at the top of each panel. The arrowheads indicate the mRNA transcripts (451 and 291 bases for the G3b promoter; 505 and 348 bases for the PIP2 promoter) terminated at the TIT2 tandem terminator (19). The value below each lane is the relative transcription activity of the assayed RNA polymerase, with that of the WT σ^A -RNA polymerase referred to as 100%. Each value is the average of at least three individual assays with a standard deviation of less than 10%.

CAGAGAACGTAGACAACCTC-3') and BC1046 (5'-CTGCAGAAGCTTGC CATTCTTCGTCCTCCACT-3'). The transcription reaction was allowed to proceed for 15 min after the addition of reaction cocktail, and the final transcription products were run on an 18% denaturing polyacrylamide gel.

Core-binding activity of σ^A . Equal molar concentrations (5 μ M) of core enzyme and σ^A were mixed and dialyzed against the core-binding buffer (50 mM HEPES [pH 7.9], 0.1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 5% glycerol) at 4°C for 8 h before being subjected to a 5-min incubation at 37°C. The reconstituted RNA polymerase holoenzyme was then injected into a Pharmacia Superdex 200 HR 10/30 gel filtration column to separate core-associated and free σ^A . The fractionated protein samples (1 ml/tube) were then analyzed for the relative contents of β' and σ^A by using Western blot analysis.

Preparation and labeling of G3b promoter DNA for gel retardation assay. The 135-bp linear G3b promoter DNA was synthesized by PCR using the pCoiZA-derived plasmid as template (4). The two primers are BC1041-PstI (5'-GCTGG TCTGCAGAACGTAGACAACAACC-3') and BC1048-XbaI (5'-GCGTCGTC TAGAATTTGTAGACTCTGTATC-3'). To label the G3b promoter, we suspended 2.5 μ g of the DNA fragment recovered by electroelution in 100 μ l of

Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0] and 0.1 mM EDTA). Next, 2 μ l of polynucleotide kinase (10 U/ μ l), 20 μ l of 10 \times kinase buffer (500 mM Tris-HCl [pH 8.2], 100 mM MgCl₂, 1 mM EDTA, 50 mM dithiothreitol, and 1 mM spermidine), 12.5 μ l of [γ -³²P]ATP (10 μ Ci/ μ l), and 65.5 μ l of H₂O were added to the DNA solution. The mixture was incubated at 37°C for 1 h and then extracted twice with phenol-chloroform. The labeled DNA in the upper aqueous phase was precipitated with 0.3 M sodium acetate, washed with 70% ethanol, dried in vacuum conditions, and dissolved in 100 μ l of Tris-EDTA buffer.

Promoter DNA binding of WT and truncated σ^A -RNA polymerases. A gel retardation assay was used to analyze the promoter DNA-binding activities of the WT and truncated σ^A -RNA polymerase holoenzymes. To carry out the experiment, we reconstituted core RNA polymerase with σ^A in binding buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol, 125 mM KCl, and 10% glycerol) on ice for 5 min. Next, the ³²P-labeled G3b promoter was added to the reconstituted holoenzyme. The mixture was incubated on ice for 5 min before being transferred to an environment of 37°C for another 5 min of incubation. Afterward, 3 μ l of loading buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 0.1 mM EDTA, 125 mM KCl, 70% glycerol, and 0.4% bromo-

phenol blue) was added to the binding mixture, and the sample was subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel in $1 \times$ TAE buffer (40 mM Tris-acetate and 2 mM EDTA [pH 8.5]).

Partial proteolysis of N-terminally truncated σ^A . Basically, 15 μ g of truncated σ^A in TEDG buffer (10 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol) supplemented with 0.2 M NaCl was mixed with 15 ng of trypsin or chymotrypsin, incubated at 37°C (final volume, 20 μ l), and quenched at various time points (0, 5, 10, 20, or 30 min) by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The digested protein sample was mixed with 2 \times sample buffer (0.125 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue, and 10% β -mercaptoethanol) and was then run on a sodium dodecyl sulfate–12% polyacrylamide gel. The proteolytic patterns of the truncated σ^A were compared after we stained the gels with Coomassie brilliant blue.

Structural analysis of σ^A protein. The circular dichroism (CD) spectra of the truncated σ^A were measured by using a Jasco 710 CD spectrophotometer. Each of the purified σ^A samples was dialyzed against TEDG buffer supplemented with 0.2 M NaCl and adjusted to a final concentration of 0.2 mg/ml before CD analysis. The spectral data were analyzed by using Jasco software.

RESULTS

Effect of N-terminally truncated σ^A on transcription activity of σ^A -RNA polymerase. The *B. subtilis* housekeeping σ^A factor belongs to a subgroup of primary σ factors which are devoid of a 254-amino-acid spacer between regions 1 and 2 (Fig. 1). In order to assess whether region 1.1 of this σ factor is dispensable for its function as a known alternative σ factor which lacks region 1.1, we first constructed a series of N-terminally truncated σ^A factors. To avoid the possible effect of extra amino acid residues fused at the N terminus of the truncated σ^A on its function, the pT7-5 vector (31), which is suitable for transcriptional fusion, was adopted so as to overproduce the truncated σ^A . In this way, the most N-terminal amino acid residue of the truncated σ^A would be methionine or the amino acid residue directly C terminal to that designed for deletion, depending on whether N-terminal processing of the truncated σ^A occurred. After purification of the overproduced σ^A , each of them was analyzed for in vitro activity on the G3b promoter after reconstitution with core RNA polymerase. As shown in Fig. 2A, SND26, SND52, SND73, and SND94, with part of region 1.1 deleted, increased the transcription activity of the truncated σ^A ; however, further deletion of region 1.2 or region 2 (such as SND129, SND154, and SND207) drastically reduced the activity, indicating that elimination of amino acid residues from the N terminus to a certain position within amino acid residues 94 to 129 of σ^A is detrimental to σ^A function.

To precisely map the position, more N-terminally truncated σ^A variants (SND100 to SND109) were created, overproduced, N-terminally sequenced (Table 1), and analyzed for the activities on both the G3b promoter of *B. subtilis* ϕ 29 phage (Fig. 2B) and the P1P2 promoter of the *B. subtilis* *sigA* operon (Fig. 2C). Our data showed that the truncated σ^A -RNA polymerases can be divided into three activity groups (Fig. 2B). SND73, SND94, SND100, and SND102, from which most or all of region 1.1 was deleted, belonged to the high-activity group and were at least as active as the WT. Furthermore, SND103, with the conserved Arg-103 (Fig. 1) replaced with methionine (Table 1), had a moderate activity which was about 70% of that of the WT. SND104 to SND109, with Arg-103 removed, were in the low-activity group. Less than 15% of WT activity was retained by RNA polymerases containing these truncated σ^A variants. Similar results were obtained for the truncated σ^A -

TABLE 1. N-terminal amino acid sequences^a of truncated σ^A factors

σ^A derivative	Amino acid residue at the N terminus of region 1.2 ^b at position no.:								
	100	101	102	103	104	105	106	107	
WT	D	P	V	R	M	Y	L	K	
SND100	– ^c	P	V	R	M	Y	L	K	
SND102		–	M	R	M	Y	L	K	
SND103			–	M	M	Y	L	K	
SND104				–	M	Y	L	K	
SND105					–	M	L	K	

^a The N-terminal sequence of each truncated σ^A factor was determined by using an amino acid sequencer (ABI model 494) at National Chung-Hsing University.

^b Region 1.2 spans residues 100 through 131 of the *B. subtilis* σ^A factor.

^c –, the specific amino acid residue and those N terminal to it have been deleted.

RNA polymerases on the P1P2 promoter (Fig. 2C). Since the nucleotide sequences of the G3b and P1P2 promoters are different, especially at the –35 region (7, 19), it was assumed that the transcription defect caused by deletion of Arg-103 in SND104 is promoter independent. Moreover, our results clearly manifested that the conserved Arg-103 is an essential N-terminal boundary amino acid residue for the truncated σ^A ; the loss of this arginine, rather than its preceding amino acid sequence, is detrimental to σ^A function.

With region 1.1 deleted, σ^A is functional in vivo. The dispensability of region 1.1 for σ^A to function in vitro suggested that the truncated σ^A with region 1.1 deleted might be also functional in vivo. To test this idea, we used the plasmids pCF0, pCF100, and pCF104, which were able to express WT σ^A , SND100, and SND104, respectively, in *B. subtilis* (data not shown), to complement the growth defect of *B. subtilis* DB3200, in which the WT *sigA* is under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *spac* promoter (19). As expected, *B. subtilis* DB3200 was low in growth yield in minimal medium lacking IPTG (Fig. 3). Introduction of pCF0 or pCF100 increased the growth yield of *B. subtilis* DB3200 to about the same level as that observed in the presence of IPTG. However, pCF104 only partially restored the growth of *B. subtilis* DB3200. The difference in capability of pCF100 and pCF104 to complement the growth defect of *B. subtilis* DB3200 in vivo correlated well with the difference in transcription activity of the corresponding truncated σ^A -RNA polymerases in vitro (Fig. 2). These results suggest that the truncated σ^A with region 1.1 deleted is functional in vivo and that further deletion of the conserved Arg-103 in region 1.2 is somehow detrimental to the functioning of the truncated σ^A in vivo.

Importance of conserved Arg-103 at the N terminus of region 1.2 to functional properties of N-terminally truncated σ^A . The drastic reduction in transcription activity of SND104 σ^A -RNA polymerase suggests that SND104 must be defective at a certain step of transcription initiation. To clarify this point, we first analyzed the core-binding efficiency of the truncated σ^A . In the analyses, equal numbers of moles of core enzyme and truncated σ^A were mixed and then subjected to molecular sieving, during which σ^A can be separated into the free and core-associated states. As shown in Fig. 4, about 20% of the

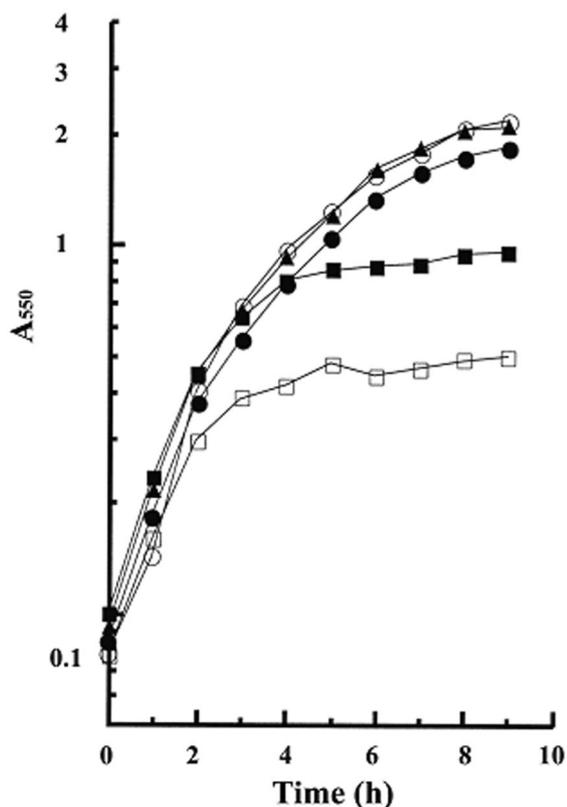


FIG. 3. Complementation of the growth defect of *B. subtilis* DB3200 by the N-terminally truncated σ^A in vivo. The plasmids pCF0, pCF100, and pCF104 (see Materials and Methods) were introduced separately into *B. subtilis* DB3200. The growth curve of each strain of *B. subtilis* in minimal medium was then measured. \circ and \square indicate the growth of *B. subtilis* DB3200 in the presence and absence of IPTG, respectively. \bullet , \blacktriangle , and \blacksquare represent the growth of *B. subtilis* DB3200/pCF0, DB3200/pCF100, and DB3200/pCF104, respectively, in the absence of IPTG. A_{550} , absorbance at 550 nm.

WT σ^A was found to associate with core enzyme, as assessed by its coelution with the β' subunit of RNA polymerase; however, much higher levels of core binding were observed for SND103 (77%) and SND104 (65%) (Fig. 4). These results indicate that the drastic reduction in the activity of SND104 σ^A -RNA polymerase (Fig. 2) is not attributed to the core binding of SND104. The reduction should be due to a certain defect arising after core binding. Thus, the activities of promoter DNA binding of WT, SND100, SND102, SND103, and SND104 σ^A -RNA polymerases were further analyzed. To diminish the effect of possible differences in core binding of the truncated σ^A on promoter DNA binding of the corresponding σ^A -RNA polymerases, we used RNA polymerase holoenzymes reconstituted from a $10\times$ molar excess of σ^A for gel retardation assays. As shown in Fig. 5, a slight increase in the promoter DNA-binding activity was observed for both SND100 and SND102 σ^A -RNA polymerases (Fig. 5A). However, it was difficult to assess the promoter DNA-binding activities of SND103 and SND104 σ^A -RNA polymerases since the retarded RNA polymerase-DNA complexes started to disappear when the concentration of RNA polymerase was above 10 nM, especially for SND104 σ^A -RNA polymerase (Fig. 5B, upper panel). This phenomenon might be attributed to aggregation of the binary complexes, which was eliminated as heparin was incorporated into the binding buffer. With the improvement, a clearly lower promoter DNA-binding activity was observed for SND104 σ^A -RNA polymerase than that of the WT or SND103 σ^A -RNA polymerase (Fig. 5B), indicating that removal of Arg-103 from the truncated σ^A will affect promoter DNA binding of the truncated σ^A -RNA polymerase and/or the stability of the binary complexes thus formed.

Interestingly, two extra bands with electrophoretic mobilities faster than those of the binary complexes but slower than those of the free promoter DNA were observed in binding mixture containing SND103 or SND104 σ^A -RNA polymerase (Fig. 5B,

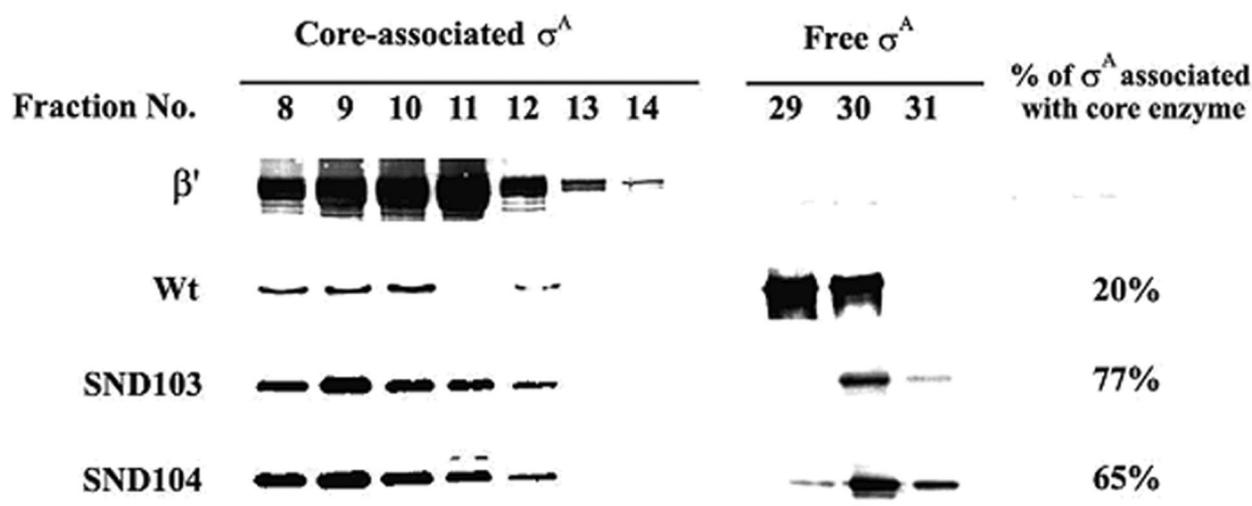


FIG. 4. The core-binding efficiency of the WT and truncated σ^A . The method used for measuring the core-binding efficiency of σ^A is described in Materials and Methods. Sample collection was started after elution of the molecular sieving column with buffer equal to the void volume. The fractionated samples (1 ml/tube) were analyzed for the contents of β' and truncated σ^A by Western blotting. The core-associated σ^A was assessed by its coelution with β' . The percentage of core-associated σ^A was calculated by dividing the band density of core-associated σ^A by the total band densities of core-associated and free σ^A .

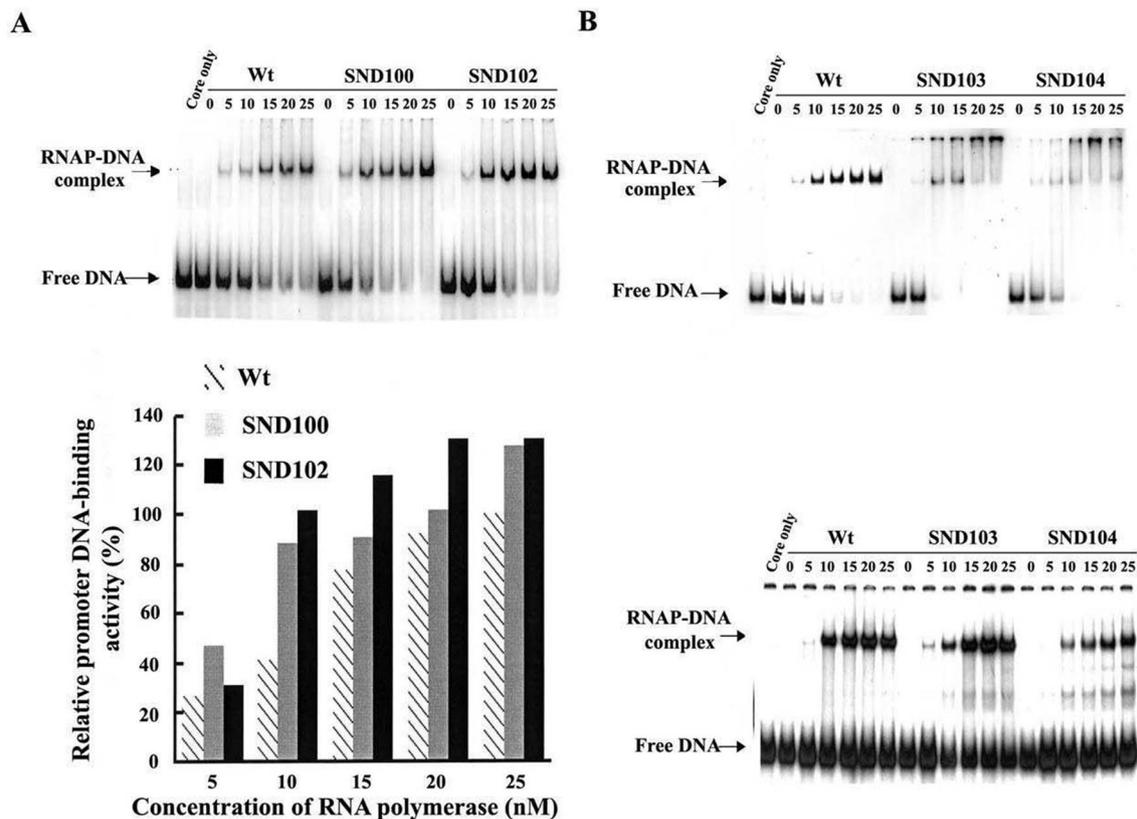


FIG. 5. The promoter DNA-binding activities of the WT and N-terminally truncated σ^A -RNA polymerase holoenzymes. (A) Promoter DNA-binding activity of the WT, SND100, or SND102 σ^A -RNA polymerase in the absence of heparin. In the assay, a 1 nM concentration of 32 P-labeled G3b promoter DNA was mixed with various concentrations (0, 5, 10, 15, 20, and 25 nM) of the reconstituted RNA polymerase holoenzyme. The molar ratio of σ^A to core was 10:1 for enzyme reconstitution. The gel retardation assay used for measuring the promoter DNA-binding activity is described in Materials and Methods. The relative promoter-DNA binding activity shown in the bottom panel was calculated by dividing the band density of the binary complexes formed at the indicated concentration of the truncated σ^A -RNA polymerase by that formed by a 25 nM concentration of the WT σ^A -RNA polymerase. Each value is the average of three individual tests. (B) Promoter DNA-binding activities of the WT, SND103, and SND104 σ^A -RNA polymerase holoenzymes in the absence (top panel) or presence (bottom panel) of heparin. The method used for the assay was the same as that used for panel A, except that a 0.1 nM (0.6 μ g/ml) concentration of heparin was incorporated in the binding buffer when needed. The two bands indicated by asterisks are putative complexes formed by the G3b promoter and free SND103 or SND104. RNAP, RNA polymerase.

bottom panel). These two bands seem to be the complexes formed by the G3b promoter and free SND103 or SND104, since they were also detectable in the absence of core enzyme (data not shown).

Besides promoter DNA binding, other transcription properties such as open complex formation and abortive transcription were also analyzed for the truncated σ^A -RNA polymerases. The efficiency of open complex formation was estimated by single-cycle transcription, while the activity of abortive transcription was estimated by measuring the radioactivity of abortive transcripts. Parallel to the decrease in promoter DNA binding (Fig. 5B, bottom panel), a significant reduction in the activity of single-cycle transcription (Fig. 6A) and abortive transcription (Fig. 6B) was detected for SND104 σ^A -RNA polymerase. The efficiency of open complex formation of SND104 σ^A -RNA polymerase ranged from about 25 to 60% of that of the WT depending on the time of incubation prior to heparin addition (Fig. 6A, bottom panel). Moreover, the activity of abortive transcription of SND104 σ^A -RNA polymerase, as assessed by measuring the total radioactivity of abortive

transcripts, was only 20% of that of the WT (Fig. 6B). The above results indicate that the RNA polymerase containing SND104 is defective in transcription initiation after core binding.

Importance of conserved Arg-103 at the N terminus of region 1.2 to structure of N-terminally truncated σ^A . To understand the reasons for the drastic change in transcription properties of SND104 σ^A -RNA polymerase, we analyzed the overall conformation of the truncated σ^A by using limited proteolysis. No significant difference in trypsin or chymotrypsin digestion pattern was observed among SND100, SND102, SND103, and SND104 (Fig. 7A), suggesting that the gross structures of the truncated σ^A were similar despite the differences at their N termini (Table 1). Since the data obtained from limited proteolysis could not rule out the possibility of a minor but detrimental structural change in SND104, CD was adopted for further analysis. As shown in Fig. 7B, the CD spectra of the truncated σ^A were separated into three groups according to their superimposability. SND100 and SND102, with high transcription activity, had very similar CD spectra.

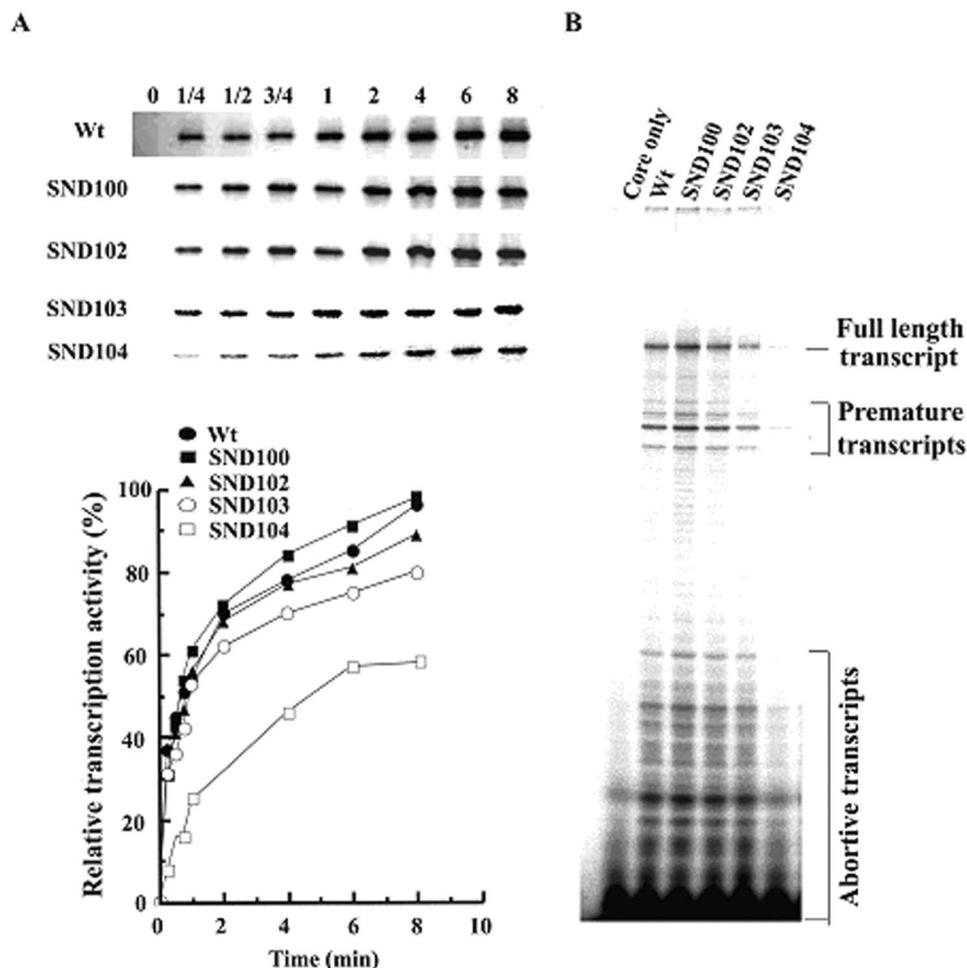


FIG. 6. Single-cycle transcription and abortive transcription assays of the N-terminally truncated σ^A -RNA polymerases. (A) Single-cycle transcription activities of the truncated σ^A -RNA polymerases. The method used for the single-cycle transcription assay was similar to that used for in vitro transcription. Briefly, 2 μg of σ^A -RNA polymerase holoenzyme was mixed with 0.8 μg of pCT24 plasmid, which harbors the G3b promoter. The reaction mixture (40 μl) was then incubated at 37°C for 0, 1/4, 1/2, 3/4, 1, 2, 4, or 8 min before 0.3 μg of heparin (0.1 $\mu\text{g}/\mu\text{l}$) was added. The molar ratio of heparin to G3b promoter was 200:1. Subsequently, 37 μl of reaction cocktail was added, and the reaction was allowed to proceed for 5 min at 37°C. The top panel shows the band densities of in vitro transcript (291 bases) generated by each of the σ^A -RNA polymerase holoenzyme. The bottom panel shows the relative transcription activities of the tested RNA polymerases, with that of SND100 σ^A -RNA polymerase obtained at 8 min referred to as 100%. Each value is the average of three individual tests, and the deviation is less than 10%. (B) Abortive transcription activities of the truncated σ^A -RNA polymerases. The method used for abortive transcription is the same as that described in Materials and Methods. The full-length transcript is 61 bases in length. The RNA polymerase tested is as indicated at the top of each lane.

SND104 and SND105, with low transcription activity, were also superimposable in CD spectra. SND103, with moderate transcription activity, had an absorption curve falling just between those for the two above-mentioned groups. The relatively higher negative absorptions of SND103, SND104, and SND105 (also SND106 and SND109 [data not shown]) in CD spectra demonstrate that the substitution (SND103) or deletion (SND104) of Arg-103 has increased the content of stable secondary structures of the truncated σ^A with moderate and low activities.

Arg-103 is critical for full-length σ^A to function. To rule out the possibility that the effect of Arg-103 deletion on transcription of SND104 σ^A -RNA polymerase is due to the creation of an unusual N terminus of the truncated σ^A —and to see whether Arg-103 is also important for the functioning of full-

length σ^A in an RNA polymerase holoenzyme—we constructed, overproduced, and compared the transcription activities of a set of full-length σ^A variants in which Arg-103 was replaced with Lys (K), Met (M), Ala (A), or Glu (E). Here, R103E was designed to enable analysis of the importance of the positive charge of the arginine side chain, and R103A allowed analysis of both the positive charge and size of the side chain. As shown in Fig. 8, the transcription activity of R103E or R103A σ^A -RNA polymerase on the G3b promoter was about 15 or 27%, respectively, of that of the WT, indicating that both the positive charge and bulky side chain of Arg-103 are important for σ^A to function in a holoenzyme. This notion was supported by the facts that the R103K σ^A -RNA polymerase, the Arg-103 of which was replaced with a positively charged and comparably sized lysine, was 10% more active than the

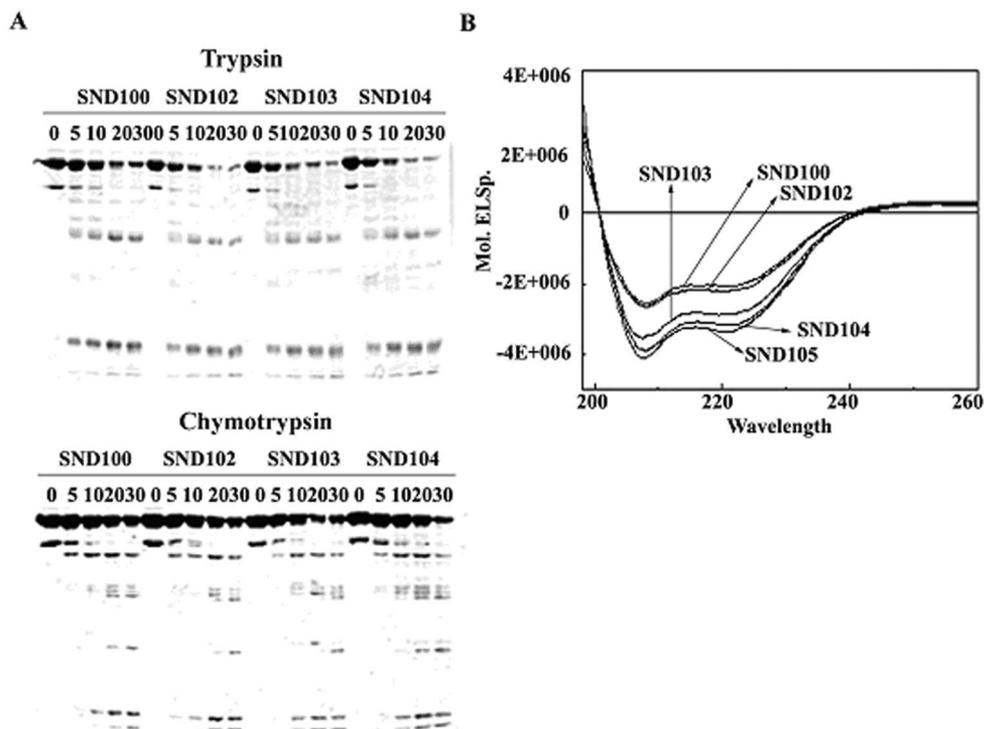


FIG. 7. The structural properties of the N-terminally truncated σ^A . (A) Partial proteolysis analyses of the N-terminally truncated σ^A . The truncated σ^A subunits were partially digested with trypsin (top panel) or chymotrypsin (bottom panel) (see Materials and Methods). The time points (in minutes) to stop the proteolytic reaction are shown on top of each panel. (B) CD spectra of the N-terminally truncated σ^A . Each of the N-terminally truncated σ^A (0.2 mg/ml) subunits was scanned with UV light from 190 to 260 nm. The absorption curves for the truncated σ^A are as indicated.

WT and that the R103M σ^A -RNA polymerase, of which the positive charge but not the size of the amino acid side chain was removed, retained about 89% of the activity of the WT (Fig. 8). Taken together, these results demonstrate that the conserved Arg-103 at the N terminus of region 1.2 is also important for the full-length σ^A to function in an RNA polymerase holoenzyme.

DISCUSSION

We have herein characterized a collection of the *B. subtilis* N-terminally truncated σ^A factors. Our results demonstrate that region 1.1 is not required for σ^A to function either in vitro or (probably) in vivo. Furthermore, the conserved Arg-103 at the N terminus of region 1.2 is critical for the N-terminally truncated σ^A to function in vitro. Deletion of this arginine residue will increase the content of stable secondary structures of the truncated σ^A and greatly reduce the transcription activity of the truncated σ^A -RNA polymerase by lowering the efficiency of transcription initiation after σ^A and core association. Moreover, the conserved Arg-103 is also important for the functioning of full-length σ^A in an RNA polymerase holoenzyme.

Three functions have been ascribed to region 1.1 of *E. coli* σ^{70} , which include autoinhibition of promoter binding by free σ^{70} (8, 9), modulation of formation of stable polymerase-promoter complexes (32), and enhancement of efficiency of transcription initiation (35). Similar to that reported for *E. coli* σ^{70} ,

autoinhibition of promoter binding by region 1.1 was observed for free σ^A (Fig. 5B, bottom panel). However, region 1.1 of the *B. subtilis* σ^A is not essential for transcription initiation of σ^A -RNA polymerase in vitro, including promoter DNA binding (SND100 in Fig. 5A), open complex formation (SND100 in Fig. 6A), and abortive transcription (SND100 in Fig. 6B). The fact that region 1.1 is dispensable for the functioning of σ^A in vitro is consistent with the finding that pCF100 is able to fully complement the growth defect of the WT σ^A -deficient mutant, *B. subtilis* DB3200 (Fig. 3), and the temperature-sensitive *sigA* mutant, *B. subtilis* DB1001, in vivo (data not shown). The dispensability of region 1.1 for σ^A to function is similar to that happening to the alternative σ , which is devoid of region 1.1 and a long spacer (20), and also to that recently reported for the *E. coli* σ^S , which has region 1.1 but no spacer (11).

Regions 1.1 of the primary σ factor are poorly conserved in amino acid sequence, albeit with a preservation of the characteristic acidity. Moreover, this region is assumed to form a disordered structure since it is undetectable in the electron density maps of the cocrystal of *Taq* RNA polymerase and promoter DNA (23). Actually, region 1.1 of the *B. subtilis* σ^A is also largely disordered, as predicted by a protein disorder predictor (data not shown). The dispensability of this region for *B. subtilis* σ^A to function (Fig. 2, 3, 5, and 6) implies that it can be independent and separated from other domains of σ^A . Support for this idea came from in situ proteolysis of the purified *Taq* σ^A in crystal trials, in which region 1.1 was shown to separate completely from other fragments consisting of the

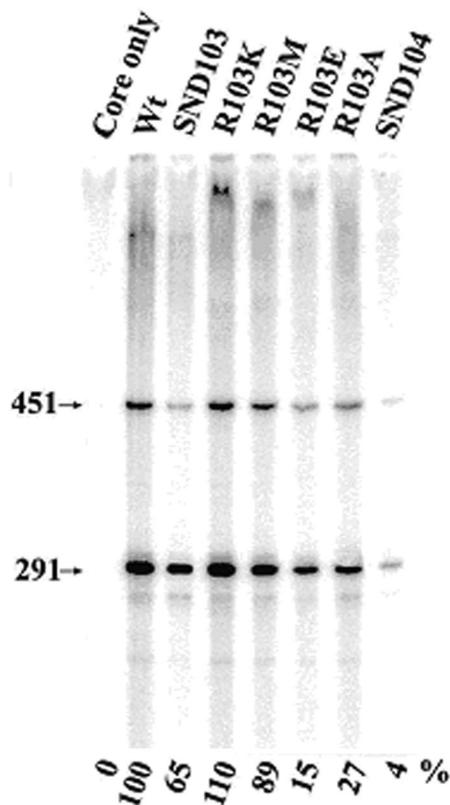


FIG. 8. In vitro transcription activity of mutant σ^A -RNA polymerase holoenzyme, with Arg-103 of σ^A replaced with Lys (K), Met (M), Ala (A), or Glu (E). The method used for in vitro transcription is the same as that described for the truncated σ^A . The mutant σ^A -RNA polymerases assayed are shown at the top. The arrowheads indicate the two mRNA transcripts (291 and 451 bases long) generated from the G3b promoter. The value below each lane indicates the relative transcription activity of the assayed RNA polymerase, with that of the WT σ^A -RNA polymerase referred to as 100%. The standard deviation for three individual tests was less than 10%.

conserved regions 1.2 to 2.4, 3.0 to 3.1, and 4.1 to 4.2 (3); further support also comes from our previous study, in which Arg-103 was shown to be more accessible to trypsin digestion (34). It has been proposed that the acidic region 1.1 of *Taq* σ^A localizes directly inside the main channel and interacts with the basic surface of the channel wall of the RNA polymerase core enzyme (23). If this is true, there would be extra positive charges on the channel surface of the N-terminally truncated σ^A -RNA polymerase, which would lead in turn to an enhanced interaction of the truncated σ^A -RNA polymerase with the negatively charged promoter DNA. Our observation of an enhanced promoter DNA-binding activity of SND100 or SND102 σ^A -RNA polymerase comparing with that of the WT (Fig. 5A) strongly supports this notion. Thus, region 1.1 of σ^A is able to modulate the promoter DNA-binding activity of σ^A -RNA polymerase.

The disappearance of binary complexes at relatively high concentrations of SND103 or SND104 σ^A -RNA polymerase is interesting (Fig. 5B). It is unlikely that this event is attributed to the loss of region 1.1, as no such phenomenon was observed for SND100 or SND102 σ^A -RNA polymerase. In other words, this event does not result simply from the exposure of extra

positive charges on the channel wall of SND103 or SND104 σ^A -RNA polymerase as mentioned previously. It is most probably triggered by a combination of the extra positive charges on the channel wall of SND103 or SND104 σ^A -RNA polymerase and the conformation of each of the two truncated σ^A factors in RNA polymerase holoenzyme.

Our results demonstrate that the conserved Arg-103 in region 1.2 is important for both the N-terminally truncated (Fig. 2) and full-length (Fig. 8) σ^A forms to function in an RNA polymerase holoenzyme. The high conservation of this arginine in known primary σ factors (Fig. 1) suggests that it may play similar roles for all of the primary σ factors. The relatively high efficiency of core binding of SND104 (Fig. 4) but low promoter binding of SND104 σ^A -RNA polymerase (Fig. 5B, bottom panel) implies that a certain transcription defect(s) arises in the SND104 σ^A -RNA polymerase after core and σ^A association. Most likely, the SND104 σ^A -RNA polymerase does not assume a conformation suitable for recognition and binding of the promoter DNA. Since SND103 σ^A -RNA polymerase remains efficient in promoter DNA binding (Fig. 5B), we propose that the conserved Arg-103, rather than its preceding amino acid residues, is critical for σ^A to undergo a proper conformational change in the core enzyme. Moreover, the differential effect of substitution of Arg-103 for Lys, Met, Glu, or Ala in σ^A on the transcription activity of σ^A -RNA polymerase (Fig. 8) suggests that both the positive charge and bulky side chain of the conserved Arg-103 are important for ensuring a proper conformational change of the RNA polymerase holoenzyme.

Although many structural and functional aspects of σ factors have been investigated, some important issues, including the binding of free σ factor to the promoter, the conformational change of σ factor upon core binding, and the DNA melting by σ factor, remain to be solved. The mutant σ^A obtained in the present study may enable us to study the conformational change of σ^A during core binding. The direct binding of SND103 or SND104 to the G3b promoter in the presence of heparin (Fig. 5B) implies that the binding strength may be strong enough for future footprinting study of σ^A . Moreover, the heparin resistance of the binary complexes containing the G3b promoter and free SND103 or SND104 (Fig. 5B) implies that the truncated σ^A alone may be able to melt the double-stranded DNA (21, 27). Detailed analyses of the above properties are now being undertaken.

ACKNOWLEDGMENT

This research was supported by the National Science Council, Taiwan, Republic of China (NSC 90-2311-B-005-008).

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