Upstream Binding of Idling RNA Polymerase Modulates Transcription Initiation from a Nearby Promoter*

Received for publication, November 24, 2014, and in revised form, February 2, 2015 Published, JBC Papers in Press, February 2, 2015, DOI 10.1074/jbc.M114.628131

Veneta Gerganova‡, Sebastian Maurer‡†, Liubov Stoliar‡§, Aleksandre Japaridze§, Giovanni Dietler¶, William Nasser†, Tamara Kutateladze¶, Andrew Travers‡‡, and Georgi Muskhelishvili‡§

From the ‡School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, D-28759 Bremen, Germany, the §Laboratory of the Physics of Living Matter, EPFL, CH-1015 Lausanne, Switzerland, the ¶UMR5240 CNRS/INSa/UCB, Université de Lyon, F-69003, INSA-Lyon, Villeurbanne, F-69621, France, the †Ivane Beritashvili Centre of Experimental Biomedicine, Gotua str. 14, Tbilisi, Georgia, and the **MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom

Background: The fis promoter upstream region harbors RNA polymerase binding sites of unknown function.

Results: Modifications of the upstream polymerase binding affect fis gene expression in a supercoiling-dependent manner.

Conclusion: Concomitant binding of RNA polymerase at the fis promoter and upstream region acts as a topological device regulating transcription.

Significance: RNA polymerase can act as an architectural factor modulating the activity of transcription initiation complexes.

The bacterial gene regulatory regions often demonstrate distinctly organized arrays of RNA polymerase binding sites of ill-defined function. Previously we observed a module of closely spaced polymerase binding sites upstream of the canonical promoter of the Escherichia coli fis operon. FIS is an abundant nucleoid-associated protein involved in adjusting the chromosomal DNA topology to changing cellular physiology. Here we show that simultaneous binding of the polymerase at the canonical fis promoter and an upstream transcriptionally inactive site stabilizes a RNAP oligomeric complex in vitro. We further show that modulation of the upstream binding of RNA polymerase affects the fis promoter activity both in vivo and in vitro. The effect of the upstream RNA polymerase binding on the fis promoter activity depends on the spatial arrangement of polymerase binding sites and DNA supercoiling. Our data suggest that a specific DNA geometry of the nucleoprotein complex stabilized on concomitant binding of RNA polymerase molecules at the fis promoter and the upstream region acts as a topological device regulating the fis transcription. We propose that transcriptionally inactive RNA polymerase molecules can act as accessory factors regulating the transcription initiation from a nearby promoter.

Regulation of gene transcription by changes of the Escherichia coli RNA polymerase (RNAP)3 holoenzyme composition and its association with transcription factors during the bacterial growth cycle is well understood (1, 2), whereas the role of the arrays of RNAP binding sites in the promoter regions is less appreciated. However, there is ample evidence that the flexibility of genetic regulation can be increased by organizing promoter arrays that are arranged in tandem and/or divergent orientation and often recognized by distinct holoenzymes (3–9). Conceivably, the promoter arrays not only enable the utilization of different RNAP holoenzymes for transcription but also facilitate the integration of multiple physiological signals under the changing growth conditions.

A number of studies in prokaryotes implicated the closely spaced arrays of RNAP binding sites in the regulation of both the strength and the pattern of gene transcription (10–14). Available data suggest that particular arrangements of the RNAP binding sites may have a regulatory function. For example, in the phage λ control region the RNAP molecules binding the closely spaced divergent promoters interfere with each other (15). Such interference was shown to provide an opportunity to regulate divergent genes by means of a single transcription factor binding between the promoters (16). Interference is not unique to divergent promoters, as the promoters arranged in tandem can also show strong transcriptional interference due to the pausing of the upstream initiating polymerase over the downstream promoter (17). Furthermore, in many cases the divergently oriented promoters can cooperate. For example, the diffusion of DNA negative supercoils induced in the wake of the translocating RNAPs (18) can mediate transcriptional coupling of the divergently transcribed operons leading to their coherent expression (19). Overall, during the last decade the role of closely spaced or overlapping promoters in genetic control became increasingly evident both in prokaryotes and eukaryotes (20–24), especially due to their preferential association with regulatory genes (25).

In addition to this regulatory complexity revealed in studies of individual promoter regions, recent genome-wide studies using chromatin immunoprecipitation (ChIP) approaches revealed a significant number of DNA-bound RNAP molecules that could not be associated with ongoing transcription (26–28), as well as numerous unusual promoter-like sites, or promoter “islands,”
of unspecified biological function (29). The emergent view is that the genomic transcriptional landscape is much more complex than previously anticipated (30). Strikingly, over 90% of the identified *E. coli* gene promoters were found surrounded by other promoter-like signals (31). It is possible that these signals serve the purpose of enhancing the local concentration of RNAP in the vicinity of the promoter. However, it is also conceivable that closely spaced RNAP binding sites provide a device for engaging RNAP molecules into higher-order nucleoprotein complexes, analogous to recombination complexes involving multiple recombinase molecules binding adjacent DNA sites and acting both as regulatory and catalytic components of the complex (32–34).

The promoter of the *fis* operon encoding the abundant nucleoid-associated protein FIS belongs to so-called "stringently" controlled promoters (35) characterized by a GC-rich discriminator sequence between the −10 hexamer and the start point of transcription that endows it with exquisite sensitivity to DNA supercoiling (36). This is not surprising given that FIS is a global regulator involved in the homeostatic control of DNA supercoiling (37). The expression pattern of *fis* is remarkable and closely reflected in the variation of FIS protein abundance, which is accumulated in very large amounts (>50,000 copies) on the commitment of cells to growth in rich medium and thereafter sharply drops to about 1% of its maximal concentration (38).

Previously, we identified an array of closely spaced polymerase binding sites organized in tandem and divergent (div) orientation upstream of the canonical *fis* promoter, denoted hereafter as *fisP1* (Fig. 1A). Although potentially all these upstream sites could drive transcription when cloned in episomal reporter constructs, the observed array of RNAP binding sites was proposed to represent a regulatory module (13), consistent with the report that in the native chromosomal context they do not function as *bona fide* promoters (39). In keeping with this notion, mutation of the *div* site strongly binding the RNAP was found to affect the *fisP1*-driven production

![Diagram](https://example.com/diagram.png)

**FIGURE 1.** A, spatial arrangement of RNAP binding sites in the regulatory region of the *fis* operon. The orientation of binding sites is indicated by the order of the RNAP binding −10 and −35 elements. Note that the binding elements of the *fisP2* site and *div* partially overlap. **B** is a stronger binding site and stronger promoter when isolated on an episome compared with *fisP2* (13), therefore we consider in the text only binding of *div*. Only *fisP1* is considered to be engaged in active transcription in vivo. **B**, summary of the modifications in the *fis* promoter region. Substitutions in the −10 and −35 elements of *div* and −10 element of *fisP3* site (italics, dashed box) are indicated in bold; substitutions in the spacer between *fisP1* and *div* are boxed (green dashed box). The position of the 5G/C, 5A/T, and 10A/T insertions are indicated by the red triangle. **C**, sequence of the *fis* promoter region. The *div* −10 and −35 elements are boxed, and *fisP1* −10 and −35 elements are in bold. The IHF binding site is underlined (black dashed line). The CRP binding sites are underlined (blue dashed lines). The FIS binding regions are underlined (red dashed lines). The A/T tracts downstream of *div* are boxed (red boxes).
of β-galactosidase in vivo (40). However, later studies revealed
that the ORF of the first gene of the fis operon, dusB, is essential
for the efficient translation of both the fis mRNA and the substi-
tuted reporter gene message (41–42), casting doubt on the
results obtained with reporter constructs lacking the dusB ORF.
Thus, the regulatory influence of the upstream binding of
RNAP on fis expression remains unclear.

In this study we investigate the influence of the upstream
binding of RNAP on fis expression. We show that RNAP forms
a higher-order complex on simultaneous binding of the
upstream and fisP1 sites in vitro, whereas modifications of the
upstream sequence affect fisP1 transcription both in vivo and in
vitro. This effect depends both on DNA supercoiling and the
spatial arrangement of the upstream and fisP1 binding sites.
Our observations strongly suggest that the upstream binding of
idling RNAP plays a regulatory role in fis transcription.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The E. coli K12 strain CSH50 (F− λ−
ara Δ(lac-pro) rpsl. thi fimE:IS1) was used throughout these
experiments.

Construction of pUCTER—The rrnB terminator was PCR
amplified from pBAD24 (primers 5′-CCCAAGCTTATAAAC-
GAAAGGCTCAGTCG-3′ and 5′-CGCGGATCCGGAAGATCGACGCG-
CCGCTAGGGGATGCATCGCGAAAAAGGCCATCCGTCGAGGATG-3′). The PCR
product was digested by HindIII and BamHI and cloned into a HindIII/BamHI-digested pUC18 back-
bone. The E. coli CSH50 strain carrying a chromosomal fisP-yfp fusion was kindly provided by Dr. Berger and Alissa Respet. The
fisP-dusB-yfp-cat and fisP-yfp-cat fragments were amplified with
Phusion DNA Polymerase (Finnzymes) (primers 5′-GGTGGTGCT-
GCTAACATCCTTG-3′ and 5′-AGGAAACAGCTATGACC-
ATG-3′), and subsequently KpnI digested and cloned into a Smal/
KpnI-digested pUC18 backbone. The resulting plasmid was
subsequently sequenced by Eurofins MWG Operon.

Idling RNAP Binding Modulates Transcription Initiation

AFM Imaging of RNAP Complexes on APTES—AFM
images were collected using a MultiMode SPM with a Nano-
scope III controller (Veeco Instruments, Santa Barbara, CA)
operated in tapping mode in air. The AFM cantilevers used in
air had a spring constant of 5 newton m
−1 (Bruker cantilevers,
TAP150A) with resonance frequencies ranging between 120
and 160 kHz. All the recorded AFM images consist of 512 × 512
pixels with scan frequency

2.5 kHz. AFM images were pro-
duced using Gwyddion* software (version 2.22) (83) and no further
image processing was carried out.

In Vitro Transcription—The in vitro transcription reactions
contained 500 ng of plasmid DNA as a template, 10 μM Tris-
HCl, pH 8.0, 100 mM KCl, 10 μM MgCl₂, 1 mM DTT, 100 μg/ml of
BSA, 8 μM RNA polymerase in a 50-μl volume. Reactions
were preincubated for 10 min at 30 °C. Transcription reactions
were initiated by adding 1/10 volume of 2.5 mM NTP mixture
and incubated for 45 min at 30 °C. The reaction was stopped by
adding 2 volumes of stop solution (1 mM EDTA, 50 mM sodium
acetate, 0.2% SDS). Phenol extraction was followed by precipi-
tation in the presence of 2 μg of glycogen, 0.3 M sodium acetate,
and 2 volumes of ethanol. After precipitation and removal of
residual ethanol, RNA was treated with TURBO DNase (Ambion)
according to the manufacturer’s recommendation.

In Vitro KMnO₄ Reactivity Assay—The reactions were
assembled essentially as for the in vitro transcription assays.
After 30 min of incubation at 30 °C, 0.02 volume of 100 mM
potassium permanganate was added for 30 s. The reactions
were stopped by addition of 0.02 volumes of β-mercaptoetha-
nol. After phenol extraction, the DNA was precipitated as described above and dissolved in water. This sample served as a template for 10 cycles of amplification by *Taq* polymerase with 5'-32P radiolabeled primers 5'-GTAAGTTTTAGACTAT-TTTG-3' and 5'-ATTGCGATGCGATGATTA-3'. The amplification products were analyzed on a 6% sequencing gel.

In Vivo CAA Reactivity Assay—Stationary CSH50 cells harboring the wild type and mutant fis promoter constructs (pWN1 and pWN1-div+ (13)) were diluted into fresh M9 minimal medium and grown for 30 min to an *A*~so~ of 0.1 in 10 ml of medium supplemented with 4 g/liter of casamino acids. CAA was added for 10 min as described (46) to the batch cultures growing at 37 °C under vigorous shaking. The cells were collected by centrifugation and the plasmid DNA was extracted with QIAprep Spin Miniprep Kit. The isolated plasmids (500 ng) were used as a template for 5 cycles of *Taq* polymerase (New England Biolabs) and a 32P-labeled primer 5'-CTGAGCTGATATTGTCCG-3' priming 60 bp downstream of the fis operon transcription initiation site. The samples were separated on a 6% denaturing polyacrylamide gel.

RNA Extraction and Quantitative Real Time PCR—RNA was extracted from batch cultures of cells grown in parallel to those used for the CAA reactivity assay. RNA was extracted with the RNAEasy® kit. The amount of transcript produced from fisP1 was measured by quantitative real-time PCR (qRT-PCR) with *Taq* polymerase (New England Biolabs) and a 32P-labeled primer 5'-CTGAGCTGATATTGTCCG-3', amplifying the first 100 bp of the fisP1 transcript. The *yfp* transcript was measured from the strains carrying chromosomal insertions of the loop modifications with primers 5'-GAGTCCTGACCTCACGATATTG-3' and 5'-ACGCTGGAAAAAGGCTGCTG-3', amplifying the first 100 bp of the fisP1 transcript. The *yfp* transcript was measured from the strains carrying chromosomal insertions of the loop modifications with primers 5'-GAGTCCTGACCTCACGATATTG-3' and 5'-ACGCTGGAAAAAGGCTGCTG-3'. qRT-PCR was carried out using QuantiTeco SYBR Green one-step Q-PCR (Qiagen GmbH, Hilden, Germany), and a Mx3000PTM Real Time cycler (Stratagene, La Jolla, CA). The PCR program consisted of the following cycles: 1 × 50 °C for 10 min; 1 × 95 °C for 15 min; and 40 × 95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s. The ΔΔ*C* method was used for the quantification of the results (84),

$$ R = \frac{\text{WT efficiency}}{\text{Target efficiency}} \quad (\text{Eq. 1}) $$

where WT is the reference sample. In the normalized expression level graphs, the ln of the derived R values were plotted. Thus a value of +1.0 and -1.0 indicate a 2-fold increase and decrease of expression, respectively.

Treatment with Topoisomerase I—The topoisomerase I treatment was performed under following conditions: 500 ng of DNA, 1× New England Biolabs 4 buffer, 1× BSA, and 0.5 μg of *Escherichia coli* topoisomerase I (kind gift of Monika Glynkowska). The reaction was performed at 37 °C for 30 min and stopped by heat inactivation (65 °C for 20 min).

Nicking Reaction—Nicking was performed with NtBspQI endonuclease (New England Biolabs). The reaction mixture contained 1 μg of DNA, 1 unit of enzyme, 1× New England Biolabs 3 buffer. The mixture was incubated at 50 °C for 1 h and heat inactivated at 80 °C for 20 min.

High-resolution Agarose Gel Electrophoresis—The plasmid topoisomers were separated on 1% horizontal agarose gels run for 21 h at 45 V and 4 °C in 1× TBE buffer in the presence of 0.5 μg/ml of chloroquine. The DNA bands were visualized under UV light after staining with ethidium bromide.

Real Time Promoter Activity Measurements—All reporter plasmids were freshly transformed before each measurement. Bacterial strains carrying constructs inserted in the chromosomal loci were directly inoculated from glycerol stocks. The cells were grown on chloramphenicol LB agar plates for 18 h, after which several colonies were picked and grown in LB liquid medium overnight cultures for a maximum of 16 h. The measurements of the mutated pUC-fis4 were performed according to the following protocol. The cultures were diluted 1:1000 in a 96-well microtiter plate NUNC in LB medium and measured on time-resolved fluorometer Victor X5 technologies (PerkinElmer Life Sciences). Several colonies (4 to 5) were picked and analyzed per experiment. Fluorescence measurements were corrected with the background fluorescence of plasmid, carrying the fis6 construct (see Fig. 5). OD measurements were corrected with the respective medium blank. The cultures were diluted 1:200 in a 96-well microtiter plate NUNC in LB medium and measured on Tecan Infinite 200 Pro Plate Reader. Fluorescence measurements were corrected with the background fluorescence of the used parent CSH50 strain. OD measurements were corrected with the respective medium blank.

Absolute activity was defined as the product of the corrected fluorescence over the corrected *A*~so~. The number of active hours is a value defined as the number of hours from time point 0 until the time point where the promoter activity loses oscillation. This represents the total number of hours when the main absolute activity peak is formed. The end point of the peak is the beginning of a plateau in the absolute activity representations. The measurements forming a plateau are selected as 12 or more consecutive time points whose respective values could be rounded to the same number, thus representing a "loss" in activity, because there are no fold-differences between the measurements. Data analysis was performed with Microsoft Excel. Graphs were generated via Origin Pro Lab 8.6. Error bars represent a S.E.

RESULTS

AFM Analyses of the RNAP-fis Promoter Interactions Reveal a Higher Order RNAP Complex—We asked whether the specific arrangement of the RNAP binding sites in the fis promoter region supports formation of higher-order RNAP complexes. For this purpose we imaged the RNAP nucleoprotein complexes by AFM using a 816-bp DNA fragment comprising the fis promoter sequence from -108 to +106 with respect to fisP1 initiation start site at +1 flanked by vector sequences. On this fragment the upstream *div* site is located near the fragment center and fisP1 is closer to one end. Analysis of 434 RNAP-fisP1 complexes by AFM revealed a distinct variety of structures (Fig. 2A–E). The measured diameter of a single RNAP molecule bound to DNA was 43 ± 5 nm, however, only 42% of all the complexes fitted these dimensions. Furthermore, ~13% of the complexes were about twice the diameter of a single RNAP molecule (Fig. 2E). To distinguish these complexes we generated three values designated A, B, and C for each measured
complex, where $A$ and $U$ represent the contour lengths of bound and unbound DNA, respectively (Fig. 2F). From these values the binding position (arm ratio $B/C$ or $C/B$), the occupied space ($U-B$), and the DNA contour length shortening ($U-A$) were calculated.

Considering a distance of about 90 bp between the $-10$ hexamers of fisP1 and div we assume a separating borderline in the middle of this 90-bp sequence to distinguish fisP1 and div binding at a theoretical arm ratio of 1.9 (Fig. 2G). All complexes with higher arm ratios were assumed to be RNAP-fisP1 complexes (56% of total), whereas those with lower ratios (44% of total) were considered the RNAP-div complexes. We calculated the theoretical arm ratios for RNAP binding at fisP1 and div as 2.4 and 1.4, respectively. Our measurements indeed showed a ratio of 1.4 for RNAP-div complexes, but a higher ratio (3.1) was obtained for the RNAP-fisP1 complexes (Fig. 2H). This might be explained by differential wrapping of the DNA due to the bending anisotropy of the fis promoter region (43). Nevertheless, the average shortening of the contour length was around 35 nm in both cases, similar to that observed with the λPR promoter (44). However, when we separately analyzed the complexes having twice (or more) the diameter of a single RNAP molecule we found an arm ratio of 1.9 and shortening of contour length by 65.4 nm (Fig. 2H). In general we observe a high variance of complexes formed on the fis promoter fragments (Fig. 2I) and a higher variance of contour length shortening and arm length distributions in comparison to the control λPR DNA complexes (Fig. 2J).

FIGURE 2. AFM images of RNAP-fisP DNA complexes (260 x 260 nm, Z-scale 3 nm) and statistical data obtained for RNAP-fisP DNA complexes. A–E, upper panels show the 70° angled views, lower panels show the top views. Note that two distinct positions (one more central, another near the DNA fragment end) are occupied by RNAP in A and B, whereas both positions appear occupied in the complex shown in C–E, the RNAP dimer complexes. F, statistical analysis of complexes. The parameters used for statistical analysis are indicated by colored dotted lines. B and C, arm lengths RNAP-fisP DNA complex, $A$ = contour length of fragment with bound RNAP, and $U$ = contour length of free DNA fragment. G, schematic of the fisP DNA fragment used for AFM. The assumed borderline between fisP1 and div binding sites (B/C arm ratio $r = 1.9$) and the location of corresponding $-10$ elements are indicated. H, summary of measurements of RNAP-fisP complexes. I, comparison of the distributions of DNA contour lengths in RNAP-fisP and the RNAP-λPR complexes (the λPR data taken for comparison (53)). J, distribution of the contour and arm lengths in the RNAP-fisP DNA and RNAP-λPR DNA complexes on linear fragments.

Idling RNAP Binding Modulates Transcription Initiation

MARCH 27, 2015 • VOLUME 290 • NUMBER 13

JOURNAL OF BIOLOGICAL CHEMISTRY 8099

ASBMB
Idling RNAP Binding Modulates Transcription Initiation

FIGURE 3. RNAP binds the div site in vitro and in vivo. A, in vitro KMnO₄ reactivity assay using the wild type and div⁺ promoter constructs. Lanes 1 and 3, KMnO₄ reactivity of the RNAP complexes formed at the wild type and div⁺ plasmid constructs, respectively. Lanes 2 and 4 show the reactions with free DNA. B, in vivo CAA reactivity assay. The primer extension reactions using RNAs isolated from cells after CAA treatment are shown with corresponding sequencing reactions. Lanes 1 and 3, CAA reactivity of the wild type and div⁺ mutant fis promoter constructs, respectively. Lanes 2 and 4 show the reactions with RNA isolated from untreated cells. The −10 and −35 elements of the div site are indicated. Red boxes indicate the positions of the div⁺ mutation and the original base. C, the overlay of scans of lane 1 (black) and lane 3 (red). Note the stronger untwisting in the −10 element of div carrying the div⁺ mutation.

however, because RNAP tightly binds and readily untwists the div −10 region on linear fragments when no fisP2 untwisting is evident (40), we assume that RNAP predominantly binds at the div site. Taken together, these data are in keeping with previous findings showing that RNAP can simultaneously bind at the fisP1 and div sites in vitro (40) and demonstrate the formation of a higher-order complex on simultaneous binding of RNAP at fisP1 and the upstream transcriptionally inactive site.

RNAP binds the div site in vivo—RNAP binds the div site with higher affinity than the fisP1 site in vitro (38), but whether this site is bound by RNAP in vivo is unclear. Previously we found that binding of RNAP strongly increases the potassium permanganate reactivity of the div −10 region in vitro indicative of DNA untwisting (40). To facilitate the detection of such an untwisting effect in vivo we introduced an “up” mutation in the div −10 element (hereafter designated div⁺) changing it to the consensus sequence (5′-GATAAT-3′ to 5′-TATAAT-3′).

As expected, the div⁺ mutation increased the potassium permanganate reactivity of the DNA around the −10 element of div on binding of RNAP to plasmid constructs in vitro (Fig. 3A). No noticeable opening of the div site by RNAP in vivo instead of permanganate we used chloracetaldehyde (CAA) for modifying the bases in single-stranded DNA regions (46). After transformation of the wild type and div⁺ constructs in E. coli, the exponentially growing cells were treated with CAA for 10 min (see “Experimental Procedures”). The isolated plasmids were subjected to asymmetric PCR using a radiolabeled primer hybridizing 60 bp downstream of the div site. Visualization of the CAA reactivity signatures of the wild type and div⁺ promoter constructs on sequencing gels demonstrated that the div⁺ mutation strongly enhanced the reactivity of the div −10 element also in vivo (Fig. 3, B and C). We infer that the div site is capable of binding RNAP in the exponentially growing cells actively expressing fis.

Mutations of Upstream RNAP Binding Sites Modulate the fisP1-driven Reporter Gene Expression in Vivo—To test whether the upstream binding of RNAP has any physiological relevance, we investigated the effect of mutations in RNAP recognition elements of upstream sites on fis expression. Because recent studies showed that dusB is essential for the efficient translation of both the fis mRNA and the substituted reporter gene message (41, 42), we first generated the fis promoter constructs by substituting the dusB ORF (fisP-yfp) once and the fis ORF (fisP-dusB-yfp) once by the ORF of the yellow fluorescent protein (YFP) and examined the production of YFP from the promoter constructs inserted both in the chromosomal locus and also on the plasmids. We observed that lack of the dusB ORF on the fisP-yfp construct essentially abolished YFP production, whereas in the presence of dusB ORF the YFP expression showed a characteristic transient fis expression pattern (Fig. 4, A and B). We inferred that our fisP-dusB-yfp construct faithfully reproduces the fis expression pattern and can be safely used for further studies. In addition, we observed a direct correlation between the levels of YFP expression from plasmids and the duration of the lag period. A decrease in YFP expression leads to the shortening of the lag phase due to a lower YFP protein load, and vice versa, a higher YFP load results in a delay of growth (Figs. 4 and 5).
To reveal the impact of the upstream RNAP binding on fis expression, we introduced mutations in the /H11002/10 and /H11002/35 elements of the div site (Fig. 1B) and tested them in vivo. The fluorescence produced from the wild type and mutant promoters was monitored during the entire growth cycle after the transformation of reporter constructs into E. coli K12 CSH50 cells. Clear effects were observed with “down” mutations in the /H11002/10 (5'-GATAAT-3' to 5'-GATCAT-3') and /H11002/35 (5'-TTG-CAT-3' to 5'-CTGCAT-3') RNAP recognition elements of the div site, and also in the /H11002/10 element of the putative fisP3 binding site (5'-TAATAT-3' to 5'-GAATAT-3') located upstream of div. This latter mutation was shown to increase the untwisting of the div -10 element by RNAP in vitro (13). The down mutations including those altering the div -10, div -35 recognition elements, and the fisP3 -10 element significantly reduced YFP production, whereas div+ exerted no significant effect (Fig. 4, C and D). We infer that modulation of the upstream RNAP binding is relayed, either directly or indirectly, to the fisP1 promoter.

To reveal the impact of the upstream RNAP binding on fis expression, we introduced mutations in the -10 and -35 elements of the div site (Fig. 1B) and tested them in vivo. The fluorescence produced from the wild type and mutant promoters was monitored during the entire growth cycle after the transformation of reporter constructs into E. coli K12 CSH50 cells. Clear effects were observed with “down” mutations in the -10 (5'-GATAAT-3' to 5'-GATCAT-3') and -35 (5'-TTG-CAT-3' to 5'-CTGCAT-3') RNAP recognition elements of the div site, and also in the -10 element of the putative fisP3 binding site (5'-TAATAT-3' to 5'-GAATAT-3') located upstream of div. This latter mutation was shown to increase the untwisting of the div -10 element by RNAP in vitro (13). The down mutations including those altering the div -10, div -35 recognition elements, and the fisP3 -10 element significantly reduced YFP production, whereas div+ exerted no significant effect (Fig. 4, C and D). We infer that modulation of the upstream RNAP binding is relayed, either directly or indirectly, to the fisP1 promoter.

**FIGURE 4.** In vivo analysis of the promoter activity of the native fisP1 region and mutations introduced upstream of the canonical fisP1. A, absolute promoter activity (fluorescence/OD580) of constructs with and without the dusB ORF in the native chromosomal locus of fis over time. B, absolute promoter activity (fluorescence/OD580) of constructs with and without the dusB ORF, expressed from a plasmid backbone over time. C and D, div+ is an up mutation in the -10 region of div, -99T/C is a down mutation in the -10 region of div, -74A/G is a down mutation in the -35 region of div, and -126T/G is a down mutation introduced in the -10 region of fisP3. C, absolute promoter activity (fluorescence/OD580) of the mutated constructs over time. Note that all down mutants show a steep decline in promoter activity. Growth curves are depicted as dashed line, plotted on the right y axis. D, scatter plot of the number of active hours for each indicated sample. The value is calculated as the time (in hours) from the beginning of promoter activation until a detectable lack of oscillation (a plateau) of expression is reached. The faster loss of activity results in a smaller value of the number of active hours, as seen for all down point mutations.

Idling RNAP Binding Modulates Transcription Initiation

MARCH 27, 2015 • VOLUME 290 • NUMBER 13

JOURNAL OF BIOLOGICAL CHEMISTRY

8101
separating the −35 RNAP hexamers of the fisP1 and div (Fig. 1, B and C). Because the inserted A/T stretches could be utilized by RNAP as fortuitous A + T-rich UP elements (48), we also inserted a stretch of five guanines (5G/Cs) in the same position. Notably, both the 5A/T and 5G/C insertions would alter the helical phasing between the bound polymerase molecules, whereas 10A/T would not. In addition, we substituted Gs for all Ts, and Cs for all As within a 19-bp region (positions −46 to −66 with respect to the start point of fisP1 transcription) between the fisP1 and div sites generating the “GC-rich spacer” construct, in which the thermodynamic stability of the DNA in the linker region between the divergent RNAP binding sites would be increased without altering their helical register (Fig. 1C).

All these constructs were tested for their influence on the fisP1 activity both in vivo and in vitro. Interestingly, we observed that both the 5A/T and 5G/C insertions significantly impaired fisP1 activity, insertion of a 10A/T had no noticeable effect, whereas the substitution of bases without altering the linker length (GC-rich spacer) only slightly decreased transcription (Fig. 5, A–C). The finding that insertion of half, but not a full helical turn of the DNA exerted a negative effect on transcription suggested that spatial orientation of the upstream and the fisP1 RNAP binding sites is pertinent to the fisP1 transcriptional activity.

Insertions in the div-fisP1 Linker DNA Region Affect the Promoter Untwisting by RNAP—To reveal the functional relevance of the observed constraints imposed by local DNA geometry on
the RNAP binding and transcriptional activity, we next investigated the effects of the insertions on promoter opening. The plasmid constructs were incubated with increasing RNAP concentrations and the promoter opening was monitored by potassium permanganate reactivity assay. Previously it was shown that the div site is readily untwisted at low RNAP concentrations, whereas higher RNAP concentrations are required for the untwisting of the stringent fisP1 promoter (13, 40). As expected, with all the used promoter constructs we observed a RNAP concentration-dependent untwisting of the /H11002 element of div, being most pronounced for the 10A/T insertion mutant (Fig. 8, A and C). The 10A/T construct also demonstrated a significantly increased untwisting of the fisP1/H11002 element without much dependence on the RNAP concentration in the used range (25–75 nM). Higher RNAP concentrations were necessary to observe the opening of the wild type fisP1, whereas even at these high concentrations the promoter opening in both the 5A/T and 5G/C insertion constructs was strongly impaired (Fig. 8, B and C). Thus, whereas none of the 5-bp insertions impaired the div –10 hexamer untwisting, they both impaired the opening of the fisP1 promoter. We infer that the spatial organization of the div and fisP1 sites and thus, the local geometry of the DNA constrained by the RNAP nucleoprotein complex, is critical for the fisP1 promoter opening.

Modifications of the Linker DNA between the div and fisP1 Sites Modulate the Binding of Plasmid Constructs by RNAP—To understand the impact of the insertions on the RNAP nucleoprotein complex formation at the fis promoter we carried out AFM imaging experiments quantifying the RNAP binding to the plasmid constructs carrying the 5G/C, 5A/T, and 10A/T insertions, as well as the GC-rich spacer construct. To facilitate the visualization of the complexes, the plasmids were relaxed by Topo II before the complex formation and imaged on the APTES surface (see “Experimental Procedures”), which due to strong hydrophobic interactions traps the complexes more rapidly and thus better conserves their solution configuration (49, 50). At the used RNAP concentrations about 60–75% of plasmids were bound by a single RNAP molecule, whereas of the remaining 25–40% one-half were bound by two RNAP molecules and another half by two RNAP molecules in close vicinity, consistent with the putative oligomer. For the distinct constructs these distributions were not significantly different, whereas under the same conditions no significant RNAP binding was observed with plasmids lacking the fis promoter sequences (Fig. 9 and data not shown). The measurements of the arm ratios of complexes formed on linear fragments imaged on APTES at the same RNAP concentrations (Fig. 10) supported the notion that in complexes formed on plasmid constructs the RNAP was bound either at div, or fisP1, or both div and fisP1 sites. We found that both the 5A/T and 5G/C plasmid constructs were significantly impaired in RNAP binding, whereas the 10A/T and GC-rich spacer constructs showed no difference to the wild type (Fig. 11). This significant difference in binding of the constructs differing only by modifications in the fis regulatory region indicates that we indeed observe the variation in occupation of the div and fisP1 sites and not of any plasmid-borne promoters. We thus infer that alteration of the spatial organization of RNAP binding sites by insertion of half a helical turn of the DNA between div and fisP1 interferes with RNAP binding.
Idling RNAP Binding Modulates Transcription Initiation

DISCUSSION

In this study we describe the role of the upstream binding of RNAP in regulating the promoter of the fis operon encoding the pleiotropic regulator FIS (51, 52). By AFM imaging of complexes formed on linear promoter fragments we revealed a higher-order assembly consistent with a RNAP oligomer, presumably a dimer, as judged by the measurements of the contour length shortening, binding the divergently oriented fisP1 and div sites. The centers of the −10 RNAP binding elements of these divergent sites are separated by approximately nine helical turns, suggesting a particular local geometry of the complex with closely approaching RNAP molecules binding nearly on the same face of the DNA helix. We find that RNAP binding engages about 90 bp of DNA in this complex (Fig. 2), in good agreement with the wrapping of about 180 bp of DNA in this complex (Fig. 2), in good agreement with the wrapping of about 90 bp observed for binding of individual RNAP molecules at the div site (58, 59), whereas a more recent observation suggested formation of transcriptionally active dimers by interactions between the RNAP core enzymes stacking “side by side” (60). It is noteworthy that the early electron microscopy observations of a V-shaped structure of the RNAP dimer, as well as the structure of a dimer unit observed in recent crystallographic studies (61, 62) would be consistent with the possibility of physical interactions between the RNAP molecules binding the divergent fisP1 and div sites.

Relevance of the Upstream RNAP Binding and the Spatial Arrangement of the Upstream and fisP1 Sites to fis Transcriptional Control—Our data suggest that the upstream binding of RNAP has a role in the control of fis expression. First, we found that up-mutation in the div site (div+) increased the untwisting of the div − 10 element in vivo (Fig. 3), indicating that this site can be occupied by polymerase during the exponential growth when the expression of fis is maximal (35, 38). Second, point mutations affecting the RNAP binding consensus sequences of the div and fisP3 sites modulated the fisP1 transcription, albeit to a different extent (Fig. 4, C and D). Importantly, the RNAP recognition elements of the div site partially overlap those of the fisP2 site, organized in tandem with fisP1 (see Fig. 1A). However, because div is both a stronger binding site readily

4 J. T. Finch and A. Travers, unpublished observations.
forming open complexes in vitro (Fig. 3) and acts as a substantially stronger promoter than fisP2 when isolated from native chromosomal context on plasmid constructs in vivo (13), we assume that these mutations primarily affect div binding. We cannot rule out that these mutations affect the binding of transcriptional regulators of fis expression the binding sites of which overlap the div and fisP3 RNAP binding elements including FIS itself, the global repressor H-NS (63), and the pleiotropic regulators IHF and CRP (Fig. 1C). This complex question merits separate study and is out of the scope of this paper. The effects of disruptions in the helical phasing of FIS binding sites on transcription have been previously studied (64). It was shown that the assembly of a specific nucleoprotein complex, comprising a regulatory protein, RNAP, and DNA, requires three helically phased FIS binding sites in the promoter upstream region. In the case of the fis promoter region, however, the multitude of regulators and the entanglement of their binding sequences with those of the RNAP required the use of insertion mutations, which did not affect any of the known regulator binding sites. We observed a coherent response of the fisP1 promoter to the insertion mutations both on plasmids and in the chromosomal context in vivo and also in vitro (Figs. 5 and 6) with only the insertions of half a helical turn (5A/T and 5G/C) but not the full turn (10A/T) of DNA decreasing the fisP1 activity.

In E. coli the A + T-rich UP elements binding the C-terminal domain of the RNAP α subunit are normally located between positions −40 and −60 from the transcription start point (48). At fisP1 a sequence between positions −52 and −39 (−52ATTGGTCAAAAGTTT−39) could serve as a presumptive proximal UP element-like subsite, but this is unlikely because the substitutions of Gs for Ts and Cs for As in this sequence in

---

**FIGURE 8.** Potassium permanganate reactivity of the fisP1 and div complexes formed on plasmid constructs in vitro. A and B, Phosphorimager scan of a representative gel showing the permanganate reactivity of the −10 elements of the div site (A) and fisP1 (B) detected for the wild type (WT), 5A/T, 10A/T, and 5G/C insertion constructs at different RNAP concentrations indicated in units (1 unit = 16 nM RNAP). C, statistics derived from four independent experiments. The band intensity for each sample at each RNAP concentration was normalized to that of wild type (WT div or WT fisP1) set to 100%. In the presented quantification an average of the detected individual mutant to wild type signal ratios over all the RNAP concentrations is shown. All data analysis was done using the AIDA software and Microsoft Excel.
the GC-rich spacer construct (\(-^{52}CGGGGGGAATTTT^{39}\)) did not impair fisP1 activity in a purified \textit{in vitro} system (Fig. 5D). In principle, the 5A/T and 10A/T insertions, which are located upstream of this sequence (at position \(-53\); Fig. 1B), could create a fortuitous UP element. However, such a fortuitous UP element would be expected to exert a strong activating effect on fisP1 as observed with other stringent promoters (65), whereas for the 10A/T insertion we observed either no effect, or only moderate activation, respectively, \textit{in vivo} and \textit{in vitro}, and for the 5A/T insertion construct we observed an inhibition. In addition, not only the 5A/T, but also the 5G/C insertion elicited inhibitory effects both \textit{in vivo} and \textit{in vitro}. Because the primary contribution to the C-terminal domain of the RNAP subunit binding is mediated via the interactions with the DNA minor groove (66), and because the 5A/T and 5G/C sequences would have a substantially different (narrow and wide, respectively) minor groove geometry (67), our data suggest that it is not the specific sequence, but rather its extent, that is relevant.

We suggest that the insertion mutations modulate fisP1 transcription by altering the spatial organization of the RNAP upstream and fisP1 binding sites. This notion is supported by several lines of evidence. First, we observed that insertions of one-half but not a full helical turn of DNA impair fisP1 transcription. Second, the substitution of bases within a 19-bp sequence in the linker DNA between the divergent fisP1 and \textit{div} sites without altering their helical arrangement (GC-rich spacer construct) does not affect fisP1 transcription \textit{in vitro} and is less detrimental than the 5A/T and 5G/C insertions (Figs. 5 and 6). Furthermore, this dependence of promoter activity on helical phasing is lost if the plasmid DNA is relaxed by topoisomerase action or by DNA nicking. This latter observation is especially relevant, because fisP1 is a highly supercoiling-dependent promoter (13, 68), whereas supercoiling affects the preferred helical repeat and so the local geometry of the DNA (67). In addition, we observe that on plasmid constructs the 5A/T and 5G/C insertion mutants are significantly impaired in RNAP binding (Fig. 11). Finally, we observe that the 5-bp insertion mutants are also impaired in the open complex formation at fisP1, despite the untwisting of the \textit{div} \(-10\) element DNA being fairly similar to wild type (Fig. 8). Taken together these observations strongly suggest that the alteration of local geometry of the DNA in the \textit{div}-fisP1 linker region destabilizes the binding of the RNAP at
**Idling RNAP Binding Modulates Transcription Initiation**

**REFERENCES**

1. Ishihama, A. (2000) Functional modulation of *Escherichia coli* RNA polymerase. *Annu. Rev. Microbiol.* **54**, 499–518

2. Ishihama, A. (2012) Prokaryotic genome regulation: a revolutionary paradigm. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **88**, 485–508

3. Tse-Dinh, Y. C., and Beran, R. K. (1988) Multiple promoters for transcription of the *Escherichia coli* DNA topoisomerase I gene and their regulation by DNA supercoiling. *J. Mol. Biol.* **202**, 735–742

4. Lesley, S. A., Jovanovich, S. B., Tse-Dinh, Y. C., and Burgess, R. R. (1990) Identification of a heat shock protein in the topA gene of *Escherichia coli*. *J. Bacteriol.* **172**, 6871–6874

5. Goodrich, J. A., and McClure, W. R. (1991) Competing promoters in prokaryotic transcription. *Trends Biochem. Sci.* **16**, 394–397

6. Liebig, B., and Wagner, R. (1995) Effects of different growth conditions on the *in vivo* activity of the tandem *Escherichia coli* ribosomal RNA promoters P1 and P2. *Mol. Gen. Genet.* **249**, 328–335

7. Qi, H., Menzel, R., and Tse-Dinh, Y. C. (1997) Regulation of *Escherichia coli* topA gene transcription: involvement of a σS-dependent promoter. *J. Mol. Biol.* **267**, 481–489

8. Govantes, F., Orjalo, A. V., and Gunsalus, R. P. (2000) Interplay between three global regulatory proteins mediates oxygen regulation of the *Escherichia coli* cytochrome d oxidase (cydAB) operon. *Mol. Microbiol.* **38**, 1061–1073

9. Cameron, A. D., Kröger, C., Quinn, H. J., Scally, I. K., Daly, A. J., Kary, S. C., and Dorman, C. J. (2013) Transmission of an oxygen availability signal to the *Salmonella enterica* sensicaovar *Typhimurium* fs promoter. *PLoS One* **8**, e94382

10. Le Grice, S. F., and Sonenshein, A. L. (1982) Interaction of *Bacillus subtilis* RNA polymerase with a chromosomal promoter. *J. Mol. Biol.* **162**, 551–564

11. Hanamura, A., and Aiba, H. (1991) Molecular mechanism of negative autoregulation of *Escherichia coli* crp gene. *Nucleic Acids Res.* **19**, 4413–4419

12. González-Gil, G., Kahmann, R., and Muskhelishvili, G. (1998) Regulation of *cpr* transcription by oscillation between distinct nucleoprotein complexes. *EMBO J.* **17**, 2877–2885

13. Nasser, W., Rochman, M., and Muskhelishvili, G. (2002) Transcriptional regulation of *fs* operon involves a module of multiple coupled promoters. *EMBO J.* **21**, 715–724

14. Li, M., Wang, J., Geng, Y., Li, Y., Wang, Q., Liang, Q., and Qi, Q. (2012) A strategy of gene overexpression based on tandem repetitive promoters in *Escherichia coli*. *Microb. Cell Fact.* **11**, 19

15. Strainie, M. G. Jr., Sullivan, J. J., Collado-Vides, J., and deHaseth, P. L. (2000) Promoter interference in a bacteriophage lambda control region: effects of a range of interpromoter distances. *J. Bacteriol.* **182**, 216–220

16. El-Robh, M. S., and Busby, S. J. (2002) The *Escherichia coli* cAMP receptor protein bound at a single target can activate transcription initiation at divergent promoters: a systematic study that exploits new promoter probe plasmids. *Biochem. J.* **368**, 835–843

17. Palmer, A. C., Ahlgren-Berg, A., Egan, J. B., Dodd, I. B., and Shearwin, K. E. (2009) Potent transcriptional interference by pausing of RNA polymerases over a downstream promoter. *Mol. Cell* **34**, 545–555

18. Liu, L. F., and Wang, J. C. (1987) Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7024–7027

**Acknowledgment**—We thank Prof. Jürgen Fritz for providing the AFM facility.
19. Opel, M. L., and Hatfield, G. W. (2001) DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the ilvYC operon of Escherichia coli is proportional to promoter strengths and transcript lengths. Mol. Microbiol. 39, 191–198
20. Yamada, M., Kabir, M. S., and Tsunodomi, R. (2003) Divergent promoter organization may be a preferred structure for gene control in Escherichia coli. J. Mol. Microbiol. Biotechnol. 6, 206–210
21. Bae, J. Y., Laplaza, J., and Jeffries, T. W. (2008) Effects of gene orientation and use of multiple promoters on the expression of XYL1 and XYL2 in Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 145, 69–78
22. Nakagawa, H., Tategu, M., Yamauchi, R., Sasaki, K., Sekimachi, S., and Yoshida, K. (2008) Transcriptional regulation of an evolutionary conserved intergenic region of CDT2-IN75. PLoS One 3, e1484
23. Seila, A. C., Core, L. J., Lis, J. T., and Sharp, P. A. (2009) Divergent transcrip- tion: a new feature of active promoters. Cell Cycle 8, 2557–2564
24. Reppas, N. B., Wade, J. T., Church, G. M., and Struhl, K. (2006) The expression of promoters of transcriptional regulators. EMBO J. 25, 384–387
25. Mooney, R. A., Davis, S. E., Peters, J. M., Rowland, J. L., Ansari, A. Z., and Struhl, K. (2009) RNA polymerase: evidence for anion-linked conformational transitions in the transcription elongation complex. J. Mol. Biol. 392, 1616–1635
26. Grainger, D. C., Hurd, D., Harrison, M., Holdstock, J., and Busby, S. J. (2005) Studies of the distriution of Escherichia coli cAMP-receptor protein and RNA polymerase along the E. coli chromosome. Proc. Natl. Acad. Sci. U.S.A. 102, 17693–17698
27. Lee, H. M., Ohm, S. J., Koh, C. H., and Lee, S. H. (2006) The expression of promoters of transcriptional regulators. Mol. Microbiol. 59, 258–266
28. Shavkunov, K. S., Masulis, I. S., Tutukina, M. N., Deev, A. A., and Ozoline, O. N. (2009) Gains and unexpected lessons from genomes-scale promoter mapping. Nucleic Acids Res. 37, 4919–4931
29. Rice, P. A., Mow, K. W., Montaño, S. P., Boocock, M. R., Rowland, S. J., and Stark, W. M. (2010) Orchestration of epigenetic silencing by histone modifications in different regions of large bacterial genomes. PLoS Genetics 6, e1001000
30. Savchuk, L. P., and Biblishvili, R. Sh. (1979) Influence of ionic strength on RNA-polymerase structure. Mol. Biol. (Mosk) 13, 509–518
31. Donnellon, S. R., and Bull, D. M. (2004) Homeostatic regulation of supercoiling sensitivity coordinates transcription of the bacterial genome. EMBO Rep. 5, 710–717
32. Nasser, W., Schneider, R., Travers, A., and Muskhelishvili, G. (2001) CRP modulates fis transcription by alternate formation of activating and repressing nucleoprotein complexes. J. Biol. Chem. 276, 17878–17886
33. Opel, M. L., and Hatfield, G. W. (2001) DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the ilvYC operon of Escherichia coli is proportional to promoter strengths and transcript lengths. Mol. Microbiol. 39, 191–198
34. Yamada, M., Kabir, M. S., and Tsunodomi, R. (2003) Divergent promoter organization may be a preferred structure for gene control in Escherichia coli. J. Mol. Microbiol. Biotechnol. 6, 206–210
35. Bae, J. Y., Laplaza, J., and Jeffries, T. W. (2008) Effects of gene orientation and use of multiple promoters on the expression of XYL1 and XYL2 in Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 145, 69–78
36. Nakagawa, H., Tategu, M., Yamauchi, R., Sasaki, K., Sekimachi, S., and Yoshida, K. (2008) Transcriptional regulation of an evolutionary conserved intergenic region of CDT2-IN75. PLoS One 3, e1484
37. Seila, A. C., Core, L. J., Lis, J. T., and Sharp, P. A. (2009) Divergent transcrip- tion: a new feature of active promoters. Cell Cycle 8, 2557–2564
38. Reppas, N. B., Wade, J. T., Church, G. M., and Struhl, K. (2006) The expression of promoters of transcriptional regulators. EMBO J. 25, 384–387
39. Mooney, R. A., Davis, S. E., Peters, J. M., Rowland, J. L., Ansari, A. Z., and Landick R. (2009) Regulator trafficking on bacterial transcription units in vivo. Mol. Cell 33, 97–108
40. Shavkunov, K. S., Masulis, I. S., Tutukina, M. N., Deev, A. A., and Ozoline, O. N. (2009) Gains and unexpected lessons from genomes-scale promoter mapping. Nucleic Acids Res. 37, 4919–4931
41. Rice, P. A., Mow, K. W., Montaño, S. P., Boocock, M. R., Rowland, S. J., and Stark, W. M. (2010) Orchestration of epigenetic silencing by histone modifications in different regions of large bacterial genomes. PLoS Genetics 6, e1001000
42. Savchuk, L. P., and Biblishvili, R. Sh. (1979) Influence of ionic strength on RNA-polymerase structure. Mol. Biol. (Mosk) 13, 509–518
43. Donnellon, S. R., and Bull, D. M. (2004) Homeostatic regulation of supercoiling sensitivity coordinates transcription of the bacterial genome. EMBO Rep. 5, 710–717
44. Nasser, W., Schneider, R., Travers, A., and Muskhelishvili, G. (2001) CRP modulates fis transcription by alternate formation of activating and repressing nucleoprotein complexes. J. Biol. Chem. 276, 17878–17886
45. Opel, M. L., and Hatfield, G. W. (2001) DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the ilvYC operon of Escherichia coli is proportional to promoter strengths and transcript lengths. Mol. Microbiol. 39, 191–198
46. Yamada, M., Kabir, M. S., and Tsunodomi, R. (2003) Divergent promoter organization may be a preferred structure for gene control in Escherichia coli. J. Mol. Microbiol. Biotechnol. 6, 206–210
47. Bae, J. Y., Laplaza, J., and Jeffries, T. W. (2008) Effects of gene orientation and use of multiple promoters on the expression of XYL1 and XYL2 in Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 145, 69–78
48. Nakagawa, H., Tategu, M., Yamauchi, R., Sasaki, K., Sekimachi, S., and Yoshida, K. (2008) Transcriptional regulation of an evolutionary conserved intergenic region of CDT2-IN75. PLoS One 3, e1484
