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E. coli SSB Activates N4 Virion RNA Polymerase Promoters by Stabilizing a DNA Hairpin Required for Promoter Recognition

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Summary

Bacteriophage N4 virion RNA polymerase transcription of double-stranded promoter-containing DNAs requires supercoiled template and E. coli single-stranded DNA–binding protein (EcoSSB); other single-stranded DNA–binding proteins cannot substitute. The DNA determinants of virion RNA polymerase binding at the promoter comprise a small template–strand hairpin. The requirement for EcoSSB is surprising, since single-stranded DNA–binding proteins destabilize hairpin structures. DNA footprinting of EcoSSB on wild-type and mutant promoters indicates that EcoSSB stabilizes the template–strand hairpin owing to the hairpin–loop sequences. Other single-stranded DNA–binding proteins destabilize the promoter hairpin, explaining the specificity of EcoSSB activation. We conclude that EcoSSB activates transcription by providing the appropriate DNA structure for polymerase binding. The existence of small hairpins stable to single-stranded protein binding suggests a novel mechanism that provides structural determinants for specific recognition in single-stranded DNA transactions by an otherwise nonspecific DNA-binding protein.

Introduction

Coliphage N4 early transcription is carried out by a virion-encapsulated, phage-coded RNA polymerase, which is injected into the host along with the viral genome (Falco et al., 1977). This enzyme is unable to transcribe any double-stranded DNA, including duplex genomic N4 DNA (Falco et al., 1980). However, denatured or single-stranded promoter-containing templates are transcribed accurately and efficiently (Haynes and Rothman-Denes, 1985), indicating that the N4 virion RNA polymerase is a sequence-specific single-stranded DNA–binding protein (Glucksmann et al., 1992).

N4 virion RNA polymerase promoters share sequence homology from position −18 to +1, which includes a set of short inverted repeats centered at −12 (Haynes and Rothman-Denes, 1985; see Figure 2A). Analysis of a large series of mutant promoters revealed that DNA secondary structure, specifically a 3 base loop, 5–7 bp stem hairpin on the template strand, is required for virion RNA polymerase promoter recognition. However, double-stranded rather than single-stranded DNA is likely to serve as the in vivo template for N4 virion RNA polymerase. Two host factors are required for N4 early transcription: Escherichia coli DNA gyrase (Falco et al., 1978) and E. coli single-stranded DNA–binding protein (EcoSSB) (Markiewicz et al., 1992). In vitro, supercoiled promoter-containing templates do not support virion RNA polymerase activity unless EcoSSB is present (Markiewicz et al., 1992). Activation is specific to EcoSSB; other single-stranded DNA–binding proteins cannot substitute (Markiewicz et al., 1992). Based on these results, we proposed a model for the utilization of promoters by N4 virion RNA polymerase. First, a hairpin forms at the promoter region, which has been rendered single stranded owing to the negative superhelicity of the template. Subsequently, EcoSSB binds to this region to yield an "activated promoter," in which virion RNA polymerase recognizes the hairpin structure and a subset of the conserved bases present at −18 to +1 on the template strand (Glucksmann et al., 1992). The requirement for EcoSSB is puzzling, since single-stranded DNA–binding proteins function in replication and recombination, in part, by binding to single-stranded DNA and destabilizing secondary structures.

To elucidate the basis for the role of EcoSSB in transcription activation of N4 early promoters, we have studied the interaction of virion RNA polymerase and EcoSSB with single-stranded N4 early promoter–containing templates. We show that EcoSSB, although not required, activates N4 virion RNA polymerase transcription on single-stranded DNAs containing early promoters. We have performed DNA footprinting experiments in the presence of EcoSSB or virion RNA polymerase (or both) using wild-type and mutant promoters. The results obtained indicate that EcoSSB, unlike other single-stranded DNA–binding protein, is unable to destabilize the template–strand hairpin required for N4 virion RNA polymerase promoter recognition. In addition, these results explain the specificity of EcoSSB activation: EcoSSB is a transcriptional activator because it provides the correct template structure for N4 virion RNA polymerase binding.

Results

EcoSSB Activates N4 Virion RNA Polymerase Transcription on Single-Stranded, Promoter-Containing Templates

We have previously shown that N4 virion RNA polymerase promoters cloned in a plasmid are utilized by the virion RNA polymerase only if the plasmid is supercoiled and EcoSSB is present (Markiewicz et al., 1992) or if the template is single stranded (Haynes and Rothman-Denes, 1985; Glucksmann et al., 1992). Thus, a linear, double-stranded DNA template bearing promoter P1 is not transcribed by N4 virion RNA polymerase in the

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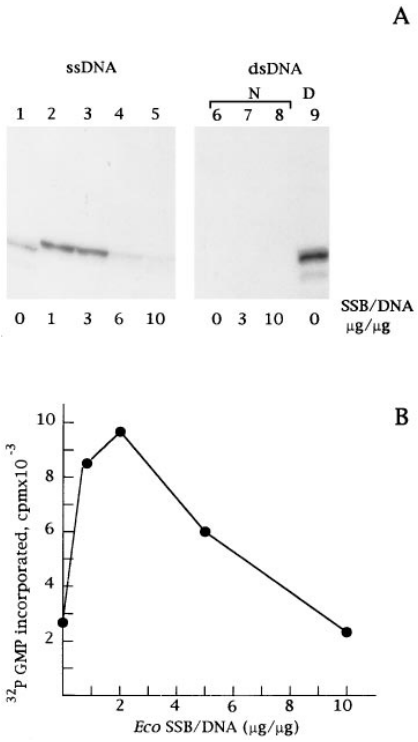

Figure 1. EcoSSB-Induced Stimulation of Virion RNA Polymerase Transcription on PM103

(A) Run-off transcription assays on viral BamHI-restricted PM103 DNA (single-stranded DNA [ssDNA]), BamHI-restricted PM103 replicative form native DNA (double-stranded DNA [dsDNA], N), or heat-denatured double-stranded DNA (dsDNA, D).

(B) Quantitation of the EcoSSB stimulation of run-off transcription on viral BamHI-restricted PM103 DNA. Conditions are described in Experimental Procedures.

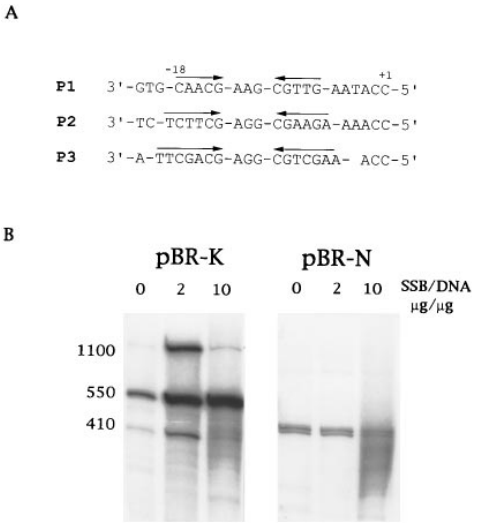

Figure 2. Effect of EcoSSB on Activation of Different Virion RNA Polymerase Promoters

(A) The sequence of the N4 virion RNA polymerase promoters (Haynes and Rothman-Denes, 1985).

(B) pBR-K contains promoters P1 and P2, followed by terminators t1 and t2. Promoter P1 yields two RNAs 410 and 1100 nt in length. Promoter P2 yields a 550 nt transcript. pBR-N contains promoter P3. pBR-K and pBR-N were restricted with BamHI and heat denatured. Conditions are described in Experimental Procedures.

presence or absence of EcoSSB (Figure 1A, lanes 6–8). EcoSSB is not required for transcription on single-stranded (Figure 1A, lane 1) or denatured, double-stranded (lane 9) templates. However, addition of EcoSSB within a critical concentration range produced a 4-fold increase in transcriptional activity when single-stranded template was used (Figure 1A, lanes 2 and 3, and Figure 1B). Addition of EcoSSB-specific antibody abolished transcription (data not shown). EcoSSB saturates single-stranded DNAs at a protein to DNA ratio of 10:1 (w/w) (Chrysogelos and Griffith, 1982). Therefore, EcoSSB activation of P1 transcription on single-stranded template occurs at subsaturating protein to DNA ratios. At high EcoSSB to DNA ratios, transcription is severely inhibited (Figure 1A, lanes 4 and 5).

The extent of activation is promoter dependent; stronger promoters are less activated by EcoSSB than weaker promoters (P1> P2> P3). Addition of EcoSSB has little effect on transcription initiation from promoter P3 (present in pBR-N), which is the strongest promoter (Figure 2B, right). Transcription of pBR-K, which contains both promoters P1 and P2, shows that P1 is more sensitive to activation than P2 (Figure 2B, left). Transcription from P1 yields two RNAs (of 410 and 1100 nt), which terminate at terminators t1 and t2, respectively, while transcription from P2 yields only one transcript (550 nt) terminating at t2 (Markiewicz et al., 1992). Inhibition of transcription occurs at high EcoSSB to DNA ratios. Transcription from promoter P1 is most sensitive to inhibition at high EcoSSB to DNA ratios (Figure 2B). Transcription from stronger promoters is less sensitive to inhibition than from weaker promoters.

To determine whether activation was specific to EcoSSB, other single-stranded DNA–binding proteins were tested. The T4 gene 32 protein (T4 gp32) (Kowalczykowski et al., 1981) and its T4 gp32* derivative (Lonberg et al., 1981), which has 47 amino acids deleted from the carboxyl terminus, the T7 single-stranded DNA–binding protein (T7 gp2.5) (Kim et al., 1992), the F episome single-stranded DNA–binding protein (SSF) (Chase et al., 1983), and the N4 single-stranded DNA–binding protein (N4SSB) (Lindberg et al., 1989) do not stimulate transcription (data not shown). Instead, these proteins inhibited transcription, even at subsaturating protein to DNA ratios. Therefore, activation of the single-stranded template is EcoSSB specific.

EcoSSB Binding Alters the DNA Conformation, and Virion RNA Polymerase Protects the Promoter Region

To define the role of EcoSSB in transcription activation of N4 early promoters, we performed DNA footprinting experiments in the presence of EcoSSB or virion RNA polymerase (or both) on single-stranded DNA templates. The results of DNase I cleavage of a 5′ end–labeled, single-stranded BamHI fragment (136 nt in length) containing promoter P1 placed 34 nt from the 3′ end are shown in Figure 3A. In the absence of EcoSSB and RNA polymerase, a weak DNase I cleavage signal appears

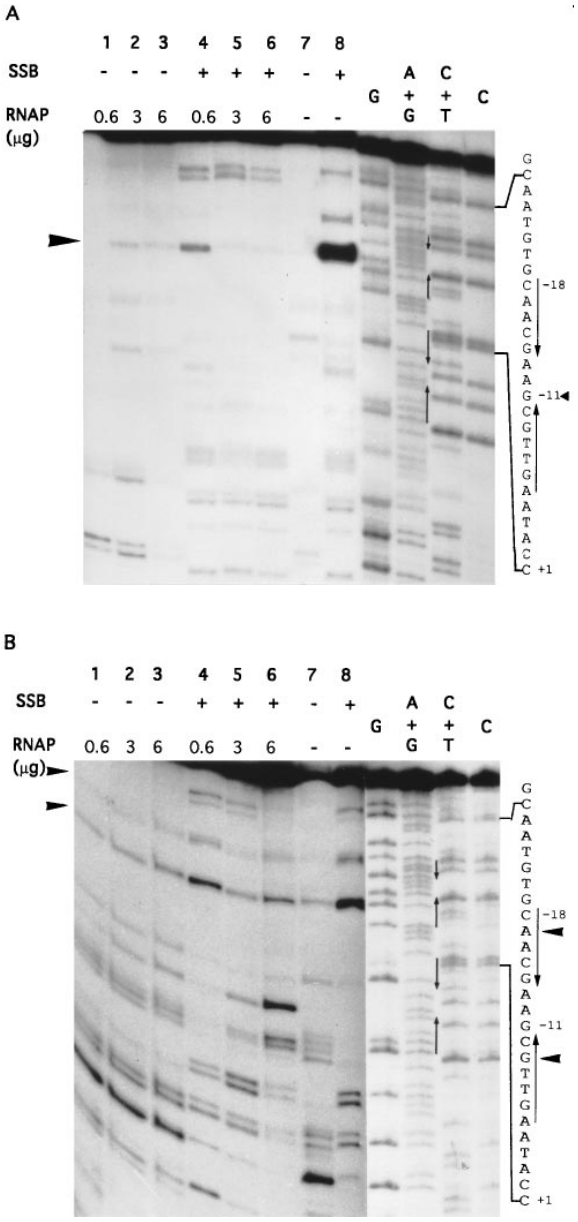

Figure 3. DNase I and NCS Footprinting of N4 Virion RNA Polymerase and EcoSSB Interactions at the Wild-Type Promoter P1 Template Strand

The 5′ end–labeled 136 base fragment was treated with DNase I (A) or NCS (B) in the presence of EcoSSB (at a 1:1 protein to DNA ratio [w/w]) or increasing amounts of virion RNA polymerase (or both). The sequence of the promoter is displayed to the right. The inverted repeats are marked by arrows both on the autoradiogram and next to the sequence. Large arrowheads indicate EcoSSB-induced DNase I or NCS cleavage sites. Conditions are described in Experimental Procedures.

at position −11 (Figure 3A, lane 7), indicating the presence of a preexisting hairpin structure. Surprisingly, upon addition of EcoSSB, the cleavage at position −11 is enhanced (Figure 3A, lane 8). Other cleavages occur at the ends of the promoter region. Addition of virion RNA polymerase results in the inhibition of EcoSSB-induced DNase I cleavage at the −19 to +12 region

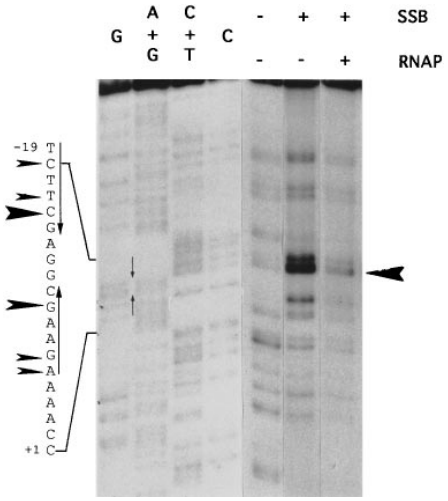

Figure 4. NCS Footprinting of N4 Virion RNA Polymerase and EcoSSB Interactions with a Single-Stranded DNA Fragment Containing the Wild-Type Promoter P2 Template Strand

The 5′ end–labeled 230 base fragment was treated with NCS in the presence of EcoSSB (EcoSSB to DNA ratio, 1:1 [w/w]), virion RNA polymerase (6 μg), or both. Other conditions were as described in Figure 3 and Experimental Procedures.

(Figure 3A, lanes 4–6) and enhancement of cleavages at the edges of this region. To rule out the possibility that the ends of the fragment were phasing the binding of EcoSSB, we analyzed the DNase I footprinting pattern on the longer (154 nt) EcoRI DNA fragment, in which the promoter resides 43 nt from the 3′ end. The same cleavage pattern was observed, with enhanced cleavage at position −11, upon EcoSSB binding (data not shown).

The specific footprinting pattern elicited by EcoSSB binding at the promoter region is not restricted to DNase I. Neocarzinostatin (NCS), which makes single-stranded breaks on the double-stranded DNA helix preferentially at thymidine residues (Poom et al., 1977), cleaves at regions of potential double-strandedness, suggesting the existence of a hairpin (Figure 3B, lane 7). These cleavages are enhanced upon EcoSSB binding, indicating that hairpin formation is stabilized (Figure 3B, lane 8). Upon addition of RNA polymerase, cleavages in the −20 to +9 region are reduced with enhancement of cleavage downstream of this region (Figure 3B, lanes 4–6). The region protected from DNase I cleavage by RNA polymerase was larger than that protected from NCS cleavage. This difference in cleavage pattern may reflect the ability of the smaller NCS probe to cleave where the larger DNase I molecule is sterically hindered by the presence of the virion RNA polymerase.

Similar results were observed with DNA containing promoter P2 (Figure 4). As in the case of P1, regions of potential double-strandedness in promoter P2 were preferentially cleaved by NCS upon the addition of EcoSSB. When both EcoSSB and the RNA polymerase were present, enhanced cleavages due to the presence of EcoSSB disappeared. The results of DNase I footprinting of promoter P2 are presented in Figure 5. Addition of EcoSSB results in changes in the cleavage pattern (as compared with naked DNA) only at those

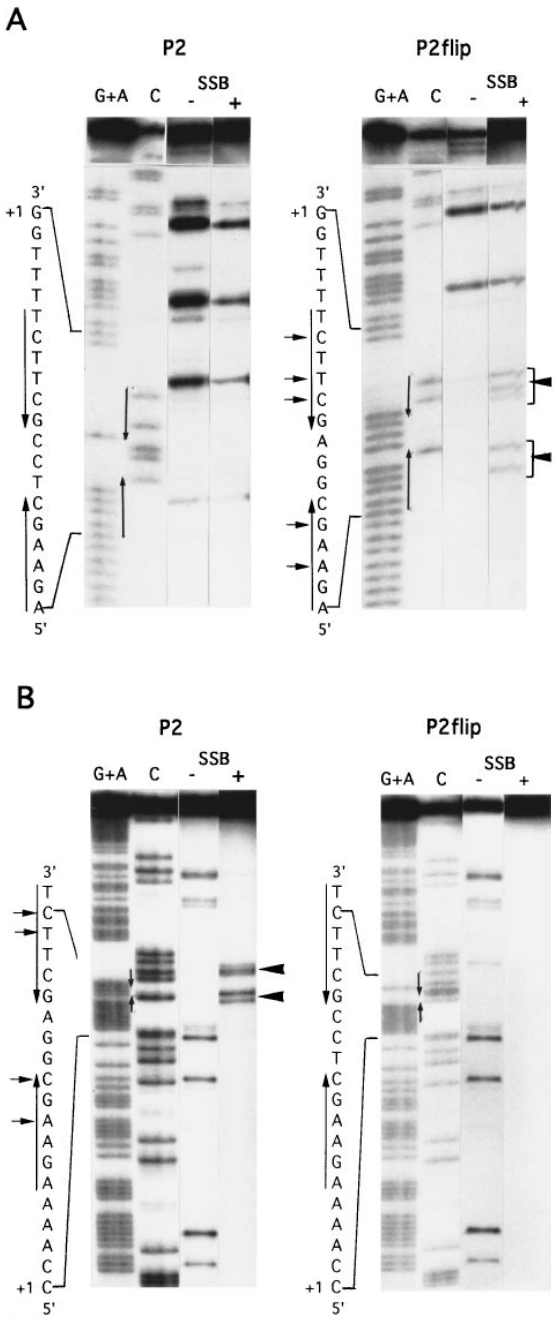

Figure 5. DNase I Footprinting of Promoters P2 and P2flip with EcoSSB Protein

Nontemplate (A) and template (B) strands are shown. The EcoSSB to DNA ratio was 1:1 (w/w). 5′ end–labeled 230 base fragments were treated with DNase I in the presence of EcoSSB (EcoSSB to DNA, 1:1 [w/w]). Arrows indicate EcoSSB-induced DNase I–hypersensitive sites. Other conditions were as described in Figure 3 and Experimental Procedures.

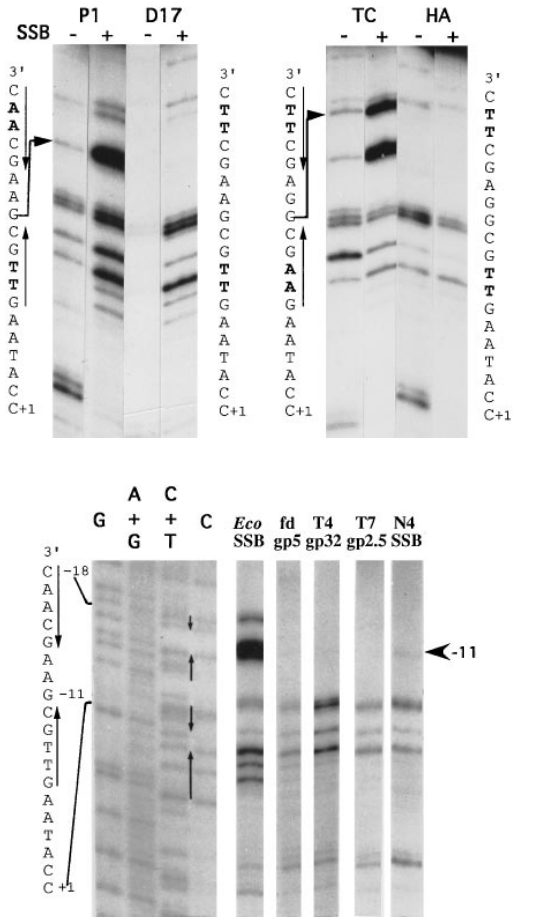

Figure 6. DNase I Footprinting of Promoter Mutants Lacking the Inverted Repeats with EcoSSB and of Wild-Type Promoter P1 with Different Single-Stranded DNA–Binding Proteins

(Top) Wild-type (P1 and TC) and mutant (D17 and HA) promoter template strands were used. The EcoSSB to DNA ratio was 1:1 (w/w). Larger closed arrowheads indicate the EcoSSB-induced DNase I–hypersensitive site at −11.

(Bottom) The 5′ end–labeled 136 base template strand fragment containing promoter P1 was used. Single-stranded DNA–binding proteins were present at a 1:1 protein to DNA ratio (w/w). Other conditions were as described in Figure 3 and Experimental Procedures.

sequences that encompass the inverted repeats (Figure 5B, left).

The enhancement of cleavages at the loop of the hairpin upon EcoSSB binding implies that, unexpectedly, EcoSSB does not melt but stabilizes the formation of a hairpin structure. The inhibition of cleavage upon N4 virion RNA polymerase binding indicates that it binds at the region defined as the promoter by sequence comparison and mutagenesis (Haynes and Rothman-Denes, 1985; Glucksmann et al., 1992). This binding results in protection of the hairpin from cleavage or, alternatively, in its melting. At this point, we cannot distinguish between these two possibilities.

The EcoSSB-Induced DNase I–Hypersensitive Site Is Dependent on the Presence of the Inverted Repeats

We have previously shown that the transcriptional activity of the early N4 promoters depends both on the presence of specific conserved bases and the inverted repeats (Glucksmann et al., 1992). Figure 6 (top) shows the results of DNase I footprinting experiments performed on DNA fragments containing the wild-type P1

promoter and its active derivative TC, compared with DNA fragments of the same size containing mutated, inactive promoters D17 and HA. The D17 and HA promoters contain changes at the two nonconserved bases of the inverted repeats, resulting in their disruption. Both D17 and HA DNAs lack the EcoSSB-induced DNase I–hypersensitive site present at position −11 in the wild-type promoters. The pattern of cleavage in the rest of the DNA fragments containing the mutant promoters is unchanged. These results indicate that the EcoSSB-induced DNase I cleavage is determined by the presence of the inverted repeats.

EcoSSB Does Not Induce a DNase I–Hypersensitive Cleavage on the Nontemplate Strand

Analysis of the interactions of EcoSSB with the nontemplate strand by DNase I footprinting (Figure 5A, left) indicates that no DNase I hypersensitivity was induced in the −18 to +1 region upon EcoSSB binding. In this case, the DNase I cleavages at the inverted repeats in the absence of EcoSSB disappeared upon EcoSSB binding. Similar results were obtained with promoter P1 nontemplate strand (data not shown).

Since the template– and nontemplate–strand hairpins differ only in sequence at the hairpin loops, the differential behavior toward EcoSSB binding must depend on the effect of loop sequences on the conformation or stability (or both) of the template– and nontemplate–strand hairpins. To test this hypothesis, we constructed a mutant promoter, P2flip, in which the sequences at the hairpin loops of the two strands of promoter P2 were exchanged. The interactions of the two strands of P2flip with EcoSSB were examined by DNase I footprinting (P2flip in Figure 5). As noted previously, the binding of EcoSSB to the template strand of P2 led to enhanced DNase I cleavages within the promoter inverted repeats (Figure 5B, left), while DNase I cleavages at the double-stranded stem of the P2 hairpin on the nontemplate strand were inhibited by EcoSSB (Figure 5A, left). In contrast, EcoSSB protected the template strand of mutant promoter P2flip from DNase I cleavage (Figure 5B, right), while enhanced cleavages at the inverted repeats on the nontemplate strand of P2flip were observed (Figure 5A, right). These results strongly suggest that the conformation of the hairpin is dependent on the base composition of the loop and determines how each promoter strand interacts with EcoSSB. The implications of these results will be discussed below.

The DNase I Cleavage Pattern at Position −11 Is Specific to EcoSSB

DNase I footprinting on the wild-type promoter P1 was carried out in the presence of different single-stranded DNA–binding proteins (fd gpV, T4 gp32, T7 gp2.5, and N4SSB). Only EcoSSB binding induces a DNase I–hypersensitive site at the loop of the hairpin of the wild-type promoter (Figure 6, bottom). These results correlate with the observation that only EcoSSB, and not other single-stranded DNA–binding proteins, is able to activate transcription at the N4 virion RNA polymerase promoters (Markiewicz et al., 1992). We suggest that the specificity of transcriptional activation by EcoSSB resides in its unique ability to stabilize a small DNA hairpin upon DNA binding to the promoter region, while other single-stranded DNA–binding proteins tested destabilize the promoter hairpin.

Discussion

Coliphage N4 virion-encapsulated, DNA-dependent RNA polymerase displays unique in vitro template requirements: all determinants of promoter recognition are present on the template strand where the enzyme recognizes specific sequences between −18 and +1, including a DNA hairpin centered at −12 (Glucksmann et al., 1992). How is this structure at the promoter formed in vivo? Since template supercoiling and EcoSSB are required (Markiewicz et al., 1992), we have proposed that supercoiling facilitates extrusion of the promoter hairpins. A promoter is maintained in an activated form through the binding of EcoSSB. Subsequently, virion RNA polymerase recognizes a hairpin structure and specific bases present at −18 to +1 on the template strand in the activated promoter (Glucksmann et al., 1992; X. D. et al., unpublished data). This model predicts extrusion of small hairpins at the promoters and persistence of such hairpins upon EcoSSB binding. However, it conflicts with present knowledge of the energetics of cruciform extrusion, as well as the properties of single-stranded DNA–binding proteins. Cruciform structures arising from small palindromes will occur only at very high, unphysiological, superhelical densities (Vologodskii, 1992). We have, however, recently detected supercoiling-dependent extrusion of hairpins at the N4 virion RNA polymerase promoters at physiological superhelical densities. Extrusion is dependent on the presence of Mg^{2+} and specific sequences at the template–strand hairpin (Dai et al., submitted).

Single-stranded DNA–binding proteins function in replication and recombination, in part, by destabilizing secondary structures present on single-stranded DNA (Chase and Williams, 1986). EcoSSB activates virion RNA polymerase transcription on single-stranded templates. We have used this property to study the interaction of EcoSSB with single-stranded templates. Contrary to expectations, binding of EcoSSB to single-stranded, promoter-containing fragments elicited a distinct cleavage pattern by DNase I and NCS. DNase I cleaved promoter P1 hairpin at the stem–loop junction, while cleavages at promoter P2 are located in the stem. The difference in cleavage patterns might be due to sequence differences (the P2 inverted repeats contain a run of five purines or pyrimidines, while the P1 inverted repeats contain both pyrimidines and purines) that are reflected in structural differences in the minor groove or to the different lengths of the stems (5 bp in P1 and 6 bp in P2) (Drew, 1984). The EcoSSB-induced cleavage pattern was dependent on the presence of the inverted repeats. Addition of virion RNA polymerase protected the promoter region from cleavage. These results are consistent with the following scenario: EcoSSB does not melt the preexisting hairpin structure, but rather stabilizes the P1 and P2 hairpins. Promoter P3, which contains the most stable hairpin (7 bp stem; Dai et al.,

submitted), is not activated by EcoSSB (Figure 2B, right). In contrast, no DNase I–hypersensitive sites were observed at the inverted repeats on the nontemplate strand upon the addition of EcoSSB. Instead, cleavages that occur at the inverted repeats in the absence of EcoSSB disappeared when EcoSSB was added. We suggest that the binding of EcoSSB to the nontemplate strand erases a preexisting hairpin structure and that the two strands do not yield similar hairpin conformations.

Why are the small template–strand hairpins at the N4 virion RNA polymerase promoters resistant to melting by EcoSSB? Thermal denaturation experiments indicate that these hairpins are unusually stable. The T_m of template–strand hairpins is 5°C to 9°C higher than the T_m of nontemplate–strand hairpins, with promoter P3 hairpin being the most stable (Dai et al., submitted). Unusual hairpin stability is provided, in part, by loop sequences. Recent nuclear magnetic resonance spectroscopic studies indicate that the template–strand hairpins adopt a highly stacked structure (Hirao et al., 1994; M. Greizerstein et al., unpublished data).

Single-stranded DNA–binding proteins are required for DNA replication, repair, and recombination (Chase and Williams, 1986). These proteins are present in high concentrations in vivo and bind nonspecifically to single-stranded DNA (Chase and Williams, 1986). Surprisingly, only EcoSSB can activate virion RNA polymerase promoters on supercoiled templates, indicating that activation does not simply entail stabilization of a single-stranded region at the promoter (Markiewicz et al., 1992). The DNase I cleavage pattern obtained upon EcoSSB binding was restricted to EcoSSB; no other single-stranded DNA–binding protein elicited the specific cleavage. Moreover, EcoSSB is unique among single-stranded DNA–binding proteins tested in that it does not destabilize the hairpin present at the promoter on the template strand. The persistence of a DNA structure after EcoSSB binding is not unprecedented. EcoSSB plays an essential role in the formation of the structure required for the binding of the dnaG protein to the origin of replication in phages fK and G4 (Sims et al., 1980), where three hairpins are present. Binding of EcoSSB induces a change in conformation that is revealed through an altered pattern of cleavage by different nucleases (Benz et al., 1983; Hirao et al., 1990; Sun and Godson, 1994). The binding site size of EcoSSB, which exists as a tetramer, is dramatically affected by solution variables such as salt and ranges from approximately 35 to 65 nt per tetramer (Lohman and Ferrari, 1994). Under our experimental conditions (50 mM NaCl and 10 mM Mg^{2+}), EcoSSB binds in the $(SSB)_{56}$ (to 56 nt) or $(SSB)_{65}$ (to 65 nt) binding mode. The isolation of EcoSSB mutants that are able to bind DNA but are deficient in N4 transcriptional activation might allow the identification of EcoSSB determinants required for activation.

Recent evidence indicates that transcriptional activators enhance transcription initiation at E. coli promoters by making direct contact with the α or σ subunits of E. coli RNA polymerase (reviewed by Busby and Ebright, 1994). At present, we have no evidence of specific protein–protein interactions between EcoSSB and N4 virion RNA polymerase. Therefore, we propose that EcoSSB acts as a transcriptional activator of N4 virion RNA polymerase solely by providing the proper DNA structure, a stabilized DNA hairpin, at the promoter. In this context, EcoSSB belongs to a growing family of "architectural" proteins that provide the correct DNA topology to the transcriptional machinery (Wolfe, 1994), such as the integration host factor (IHF), MerR, and upstream binding factor (UBF) proteins. IHF binds to a specific sequence and bends the template at the *nifH* promoter to allow productive contacts between the regulatory protein NIFA and σ^{54} holoenzyme (Hoover et al., 1990). MerR binds between the −10 and −35 regions of MerR-activated promoters in the absence of Hg^{2+}; in its presence, MerR elicits a DNA conformational change that allows RNA polymerase transcription initiation (Ansari et al., 1992, 1995). UBF, a high mobility group box factor, binds to the upstream control element and core sequences of the rRNA promoter, leading to formation of a 180 bp turn that is probably responsible for recruitment of the RNA polymerase I–specific TATA box–binding protein complex (Bazett-Jones et al., 1994).

The results presented here indicate that bacteriophage N4 exploits a novel specificity in EcoSSB. The recruitment of EcoSSB for activation of transcription at the N4 virion RNA polymerase promoters presents an appparent paradox: the use of a protein that binds nonspecifically to single-stranded DNA, but provides activation of transcription at specific sequences. In this context, protein HU, a small, basic, sequence-independent DNA-binding protein that plays a crucial role in phage Mu transposome assembly, has been shown to bind at a specific site in the Mu type 1 transposome structure (Lavoie and Chaconas, 1993). It remains to be seen whether other nonspecific DNA-binding proteins have analogous behavior and are exploited in a specific way in other biological systems.

Experimental Procedures

Materials

Most materials used have been described previously (Glucksmann et al., 1992). DNase I was obtained from Cooper Biochemicals, and NCS was a gift from I. H. Goldberg (Harvard University). EcoSSB and its antibodies and SSF were a gift of Dr. J. W. Chase (United States Biochemical Research Center). T4 gp32 and T4 gp32* were a gift of Dr. L. Gold (University of Colorado). T7 DNA-binding protein was a gift from P. Sadowski (University of Toronto). N4SSB was purified by G. Lindberg in this laboratory (Lindberg et al., 1989). The construction and characteristics of pBR-K, pBR-N, PM103, M13mp7–P2, M13mp7–TC, and M13mp7–HA, as well as the preparation and purification of template DNA fragments, have been described previously (Glucksmann et al., 1992). Mutant promoter P2flip was made using M13mp7–P2 as template and d(CTACTTTAAAGAG AAGAAGC**GGA**GCTTCTTTTGGATGAAGTA A) as primer.

Virion RNA Polymerase Transcription Assays

Standard transcription reaction conditions were used (Haynes and Rothman-Denes, 1985) with 5 μg of DNA and approximately 4.5 ng of virion RNA polymerase. The reactions were terminated by ethanol precipitation, and the products were run on 7 M urea–8% polyacrylamide gels or TCA precipitated and counted. The transcripts were visualized by exposure to X-ray film. When DNA-binding proteins or antibodies were used, the addition of RNA polymerase and nucleoside triphosphates was preceded by a 5 min preincubation at 37°C.

Footprinting Reactions

DNase I footprinting was performed as described previously (Hoess and Abremski, 1984). Single-stranded DNA fragments were isolated by restricting the M13mp7 viral strand containing N4 wild-type or mutant promoters with BamHI or EcoRI (Glucksmann et al., 1992). Each footprinting reaction included approximately 30 ng (0.7 pmol) of single-stranded, 5′ end–labeled DNA fragment. DNase I cleavage reactions (100 μl) contained increasing concentrations of purified virion RNA polymerase (0.6, 3, and 6 μg) and/or approximately 30 ng of purified EcoSSB or other single-stranded DNA–binding proteins (SSB to DNA ratio, 1:1 [w/w]). The proteins and DNA were preincubated for 15 min at 37°C in the presence of 1 mM GTP (initiating nucleotide) as described previously (Markiewicz et al., 1992). The reaction mixture was immediately transferred to 30°C and treated for 2 min with 20 ng of DNase I. The reaction was terminated by phenol extraction, and the DNA was ethanol precipitated, lyophilized, and counted. The DNAs were resuspended in 95% formamide, dye mix, boiled for 3 min, and run on an 8% or 12% polyacrylamide–7 M urea gel in TBE buffer. All the footprinting reaction products were run next to a Maxam and Gilbert sequencing ladder (Maxam and Gilbert, 1980) of the the same DNA template.

NCS footprinting reaction conditions were performed as described previously (Craig and Nash, 1984). Proteins and DNA were incubated for 15 min at 37°C and then treated with 10 μg of NCS for 10 min at room temperature. The reaction was terminated with phenol, ethanol precipitated, and loaded onto a gel as described above.

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