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FOOTPRINT OF THE SIGMA PROTEIN

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Escherichia coli RNA Polymerase is a multi-subunit enzyme that catalyzes RNA synthesis, using DNA as a template. The sigma subunit of this enzyme plays an important role in the recognition of promoter sites on DNA. Using DNase I footprinting, we have found that in the absence of the other subunits, sigma binds specifically to the bacteriophage lambda P_R promoter DNA sequence. In the presence of the sigma subunit alone, a protective footprint encompassing the region between residue positions -41 and +17 was observed (where +1 is the transcription start site). The holoenzyme gave a footprint covering the same region. Thus not only does the sigma subunit interact with the DNA promoter site in the absence of the other components of RNA polymerase, but also the footprint of sigma is indistinguishable from that of the holoenzyme.

E. coli RNA polymerase (EC 2.7.7.6) is composed of four major subunits, α, β, β' and σ [1-3] with total molecular weight 449,068. The *core enzyme* consists of $\alpha_2\beta\beta'$ and catalyzes the elongation of RNA, using DNA as a template. The sigma subunit of the enzyme determines specificity for promoter sites on DNA [4, 5], at which transcription is initiated. Genetic and biochemical data indicate that two conserved DNA sequences, located at -10 and -35, are specifically recognized by RNA polymerase [6, 7, 8, 9]. As the binding of the enzyme to DNA is essential, the interactions of individual subunits of the enzyme with nucleic acids have been examined by several investigators [6, 10, 11, 12]. Most of these used filter-binding techniques, in which DNA did not bind to nitrocellulose filters, whereas DNA-protein complexes were retained. These analyses showed that the isolated β' subunit bound to DNA [10, 11]. Low concentrations of urea (2 M or less) dissociated the enzyme into $\alpha_2\beta'$ and $\alpha_2\beta$ complexes [12, 13, 14], each of which bound to DNA.

No interaction of DNA with sigma was observed in any of these studies [10, 13, 14]. However, the sigma factor of *B. subtilis* was shown to form a sigma-DNA complex with supercoiled DNA by Doi et al. [15], who also found that the free σ^{70} subunit of *E. coli* RNA polymerase associated with supercoiled plasmid DNA's [16]. These results suggested that sigma factor may have a direct role in binding the holoenzyme to the promoter. This supported previous hypotheses [5, 10, 17, 18] that the sigma subunit was involved in promoter selection through interaction with DNA, and led to unwinding of the DNA.

Although it is established that interactions between DNA and protein regulate gene expression, very few techniques are capable of directly probing these interactions. DNase I footprinting can probe protein-DNA association *in vitro* [19, 20, 21]. We have used DNase I and a 5'-labeled 171 base pair DNA fragment containing the bacteriophage lambda P_R promoter and part of the *cro* transcript sequence to observe a very distinct footprint due to free sigma^{70}. This footprint covers the same DNA region, -41 to +17, as the holoenzyme.

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ABBREVIATIONS: BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; DEAE, diethylaminoethyl.

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MATERIALS AND METHODS

MATERIALS

All reagents and solvents used were the purest available. Nanopure water (Barnstead) was used throughout the experiments. *E. coli* MRE 600 cells were purchased from Grain Processing Corp. Bactotryptone and yeast extract were from Difco. Ultrapure urea and acrylamide were from ICN. Soluble RNA, dithiothreitol, Tris base, sodium cacodylate, ethidium bromide, agarose, heparin, and DNase I were purchase from Sigma. Restriction enzymes BstY1 and Bsp1286 were obtained from New England Biolabs, polynucleotide kinase was from Promega. [γ-^{32}P]ATP was purchased from Amersham. Kodak XAR films were used with intensifying screens for autoradiography.

Buffers:
BstY1: 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 µg/ml BSA. Reaction carried out at 60 °C.
Bsp1286: 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM β–mercaptoethanol, 100 µg/ml BSA. Reaction carried out at 37 °C.
DNase I reaction buffer: 50 mM sodium cacodylate (pH 8.0), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 200 µg/ml BSA, 100 mM NaCl, 5% glycerol.
DNase I stop buffer: 1.5 M sodium acetate, 0.1 M Na_2EDTA, 100 µg/ml tRNA
Heparin solution: 1 mg/ml heparin in DNase I reaction buffer.
Sample application buffer: 80% deionized formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 50 mM tris-borate (pH 8.3), 1 mM Na_2EDTA.

METHODS

RNA polymerase: The enzyme was purified from *E. coli* MRE600 cells according to the method of Burgess and Jendrisak [22] as modified by Lowe et al. [23]. RNA polymerase concentration was determined using $E_{280}^{1\%} = 6.2$ [23].
Sigma subunit: The sigma^{70} subunit was purified from an over-producer strain by the method of Gribskov and Burgess [24, 25]. The plasmid pMRG8 was a generous gift from the laboratory of R. R. Burgess. Sigma concentration was determined using $E_{280}^{1\%} = 8.4$ [23].

Both proteins were analyzed by denaturing SDS/8% polyacrylamide gel electrophoresis [26], as shown in Figure 1.
DNA: The bacteriophage lambda P_R promoter was obtained from plasmid pGW7 as described by Bernhard and Meares [27]. The plasmid was digested with BstY1 endonuclease and the 2400 basepair fragment was isolated using standard methods [28]. This fragment was 5'-end-labeled with polynucleotide kinase and [γ-^{32}P]ATP, and digested with Bsp1286 to yield a 171 base pair DNA fragment containing the lambda DNA sequence from base number 3435 to 3606. DNA concentrations were determined by measuring the absorbance at 260 nm (one A_{260} unit = 50 µg/ml).
DNase I footprinting: The method of Galas and Schmitz [19], as modified by Kovacic [29], was used. End-labeled DNA was added to 200 µl of DNase I reaction buffer. Either RNA polymerase or sigma was added (1:4 molar ratio of DNA to protein) and the final volume was adjusted to 210 µl. The final DNA, RNA polymerase, and sigma concentrations were 1.59×10^{-9} M, 6.4×10^{-9} M, and 6.31×10^{-9} M respectively. The mixture was incubated at 37 °C for 30 min, followed by addition of 2 µl of heparin solution and incubation for 5 min at 37 °C to eliminate nonspecific protein binding. Each sample was then treated with 0.04 units of DNase I for 5 min at 37 °C. The reaction was stopped by addition of 60 µl of DNase I stop buffer, followed by phenol extraction and ethanol precipitation [28]. The DNA pellet was rinsed with 70% ethanol to remove salts. The samples were dissolved in 10 µl of sample application buffer, heated at 100 °C for 3 min, and immediately chilled before loading onto a 5% polyacrylamide DNA-sequencing gel.
DNA gel electrophoresis: As marker DNA's, G and C lanes were prepared by the method of Maxam and Gilbert [30]. The gels were 0.4 mm thick, 40 cm long, with 5% acrylamide concentration [28]. Electrophoresis was carried out at 1500 volts for 3-4 hr. Autoradiography was carried out on the undried gels, with intensifying screens and Kodak XAR film at -80 °C overnight. Results are shown in Figure 2.

RESULTS AND DISCUSSION

Sigma^{70} was isolated from an overproducing strain as described by Gribskov [25]. The purification of the sigma protein is based on: (i) the size of the protein; (ii) its rapid and complete renaturation from guanidine hydrochloride; and (iii) its strong binding to DEAE cellulose. Figure 1 shows SDS gel analysis of the sigma preparation used in these experiments; the purity of the protein is >90%.

A 5'-labeled 171 base pair fragment containing 81 bases downstream and 90 bases upstream from the transcription start site of lambda P_R was used as a template for *in vitro* assay. RNA polymerase shows affinity for all regions of DNA [5, 11, 31, 32]. Nonspecific binding occurring at the ends of the DNA fragment is electrostatic in nature and is sensitive to polyanions including single stranded DNA. However, a highly stable complex is formed between holoenzyme and DNA promoter sites [5, 8, 9]. To avoid nonspecific binding, we used heparin, a polyanion which competes with the nucleic acid for binding proteins [29]. The formation of tight binding non-promoter complexes [31, 32] was eliminated by using high salt concentration. The sigma factor binds strongly to immobilized heparin [32]. Hence, in the presence of heparin, a footprint of either RNA polymerase or the sigma protein should represent a specific DNA-protein complex.

Many investigators have used DNase I footprinting to determine the position of RNA polymerase binding sites on various promoter-containing DNA fragments [33-39]. RNA polymerase has been found to protect the lambda P_R promoter between nucleotides -40 and +22 [34, 39], where +1 is the start site of transcription. On the T7 A3 and *lac* UV5 promoters, RNA polymerase protects a similar range of bases [33-38].

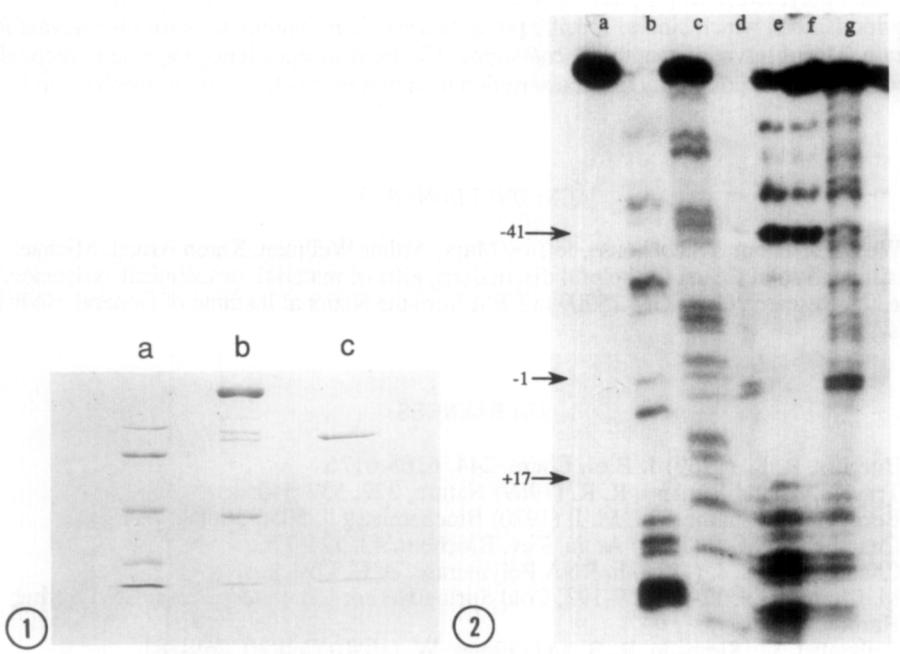

Figure 1. SDS/polyacrylamide gel electrophoresis of RNA polymerase and Sigma. Lane a: molecular weight markers (97 kDal, 66 kDal, 42 kDal, 30 kDal, and 21 kDal). Lane b : 8 μg of *E. coli* RNA polymerase. Lane c: 5 μg of free sigma (molecular weight 70 kDal).

Figure 2. DNase I footprints of the template strand of lambda P_R promoter DNA. Lane a: uncleaved DNA. Lane b: marker lane cleaved at G residues. Lane c: marker lane cleaved at C residues. Lane d: DNA (control) digested with 0.1 units DNase I. Lane e: DNase I (0.04 units) digest of 1.6 x 10^{-9} M DNA, pre-incubated with 6.4 x 10^{-9} M RNA polymerase. Lane f: DNase I (0.04 units) digest of 1.6 x 10^{-9} M DNA, pre-incubated with 6.3 x 10^{-9} M sigma^{70}. Lane g: DNA (control) digested with 0.04 units DNase I. The residues are numbered relative to the start site of transcription.

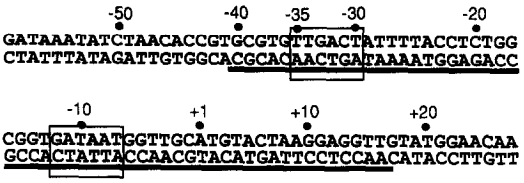

Figure 3. Promoter and transcript sequence of lambda P_R DNA. The solid line shows the region of the bottom strand protected by RNA polymerase and sigma. The -35 and -10 regions of the promoter are boxed.

Our footprinting results with the RNA polymerase holoenzyme and lambda P_R promoter (Figure 2) show a footprint between nucleotides -41 and +17, in good agreement with the other investigations. But we have found that an essentially identical footprint is produced by the sigma subunit *alone*, under identical conditions. The template sequence is displayed in Figure 3.

The DNase I digestion was carried out at 37 °C, under conditions where it is known that the RNA polymerase holoenzyme unwinds a segment of the DNA helix and separates the strands to form an open complex [34, 37]. The core enzyme, $\alpha_2\beta\beta'$, is incapable of unwinding the DNA helix [11]. Thus the sigma protein *by itself* could be capable of recognition and unwinding of the DNA at the promoter, a function previously ascribed only to the holoenzyme [17, 40].

Studies of the mechanism of transcription initiation by eukaryotic RNA polymerase II [41-44] and RNA polymerase III [45] have shown that promoter sites are first recognized by transcription factors, which bind to specific promoters and form binding sites for the relevant RNA polymerase. The observation that the *E. coli* sigma^{70} factor is independently capable of recognizing its promoter raises the possibility that transcription initiation occurs by a similar mechanism in *E. coli*.

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