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FOOTPRINT OF THE SIGMA PROTEIN: A RE-EXAMINATION

Arthur Wellman and Claude F. Meares*

Chemistry Department, University of California, Davis, CA 95616

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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, Utpala Ramesh and Claude F. Meares [(1989) Biochem. Biophys. Res. Comm. 160, 121-125] reported that in the absence of the other subunits, sigma binds specifically to the bacteriophage lambda P_R promoter DNA sequence. We are unable to reproduce that result.

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 the specificity for promoter sites on DNA [4, 5, 6, 7, 8, 9], at which transcription is initiated. Genetic and biochemical data indicate that two conserved DNA promoter sequences, located at -10 and -35 relative to the start of transcription, are specifically recognized by RNA polymerase [6, 7, 8, 9, 10, 11, 12, 13].

In the absence of the other subunits, the sigma factor of *B. subtilis* was shown to form a sigma-DNA complex with supercoiled DNA by Doi et al. [14], who also found that the free sigma subunit of *E. coli* RNA polymerase associated with supercoiled plasmid DNA's [15]. Recently Ramesh and Meares [16] attempted to footprint the sigma protein on promoter DNA, and reported a very distinct footprint due to free sigma, covering the same DNA region as the holoenzyme.

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

*Author to whom correspondence should be addressed.

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

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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 purchased from Sigma. Restriction enzymes BstY1 and Bsp1286 were obtained from New England Biolabs, Bgl II and EcoR1 were from Boehringer Mannheim, as were polynucleotide kinase and BSA. NTP's were from Pharmacia. [γ-^{32}P] and [α-^{32}P] ATP were purchased from Amersham. DEAE cellulose disks were from Whatman (DE-81, 2.5 cm). Kodak XAR films were used for autoradiography.

Buffers:

All buffers used in the previous study [16] were used, as well as the following.

RPase Assay Buffer: 44 mM Tris-HCl (pH 7.9), 8 μM BSA, 1.1 mM DTT, 11 mM $MgCl_2$, 220 mM NaCl, 1.1 mM EDTA (pH 8.0), 1.1 mM K_2HPO_4 (pH 7.5), 0.22 mM C, G, UTPs, 0.05 mM ATP, 1 μCi [γ-^{32}P] ATP.

Assay Stop Solution: 2.5% SDS, 250 mM Sodium Pyrophosphate.

Low Salt DNase I reaction buffer: 12 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaCl, 67 μM DTT, 1 μM BSA, 5% glycerol.

Low Salt DNase I Stop Buffer: 95% Deionized Formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue, 0.05% xylene cyanol.

METHODS

RNA polymerase: The enzyme was purified from *E. coli* MRE600 cells as described previously [16].

Sigma subunit: The sigma^{70} subunit was purified from an over-producer strain by the method of Gribskov and Burgess [17, 18] as well as purified directly from holoenzyme by similar methods [19]. Plasmid pMRG8 was a generous gifts from the laboratory of R. R. Burgess. Sigma concentration was determined using $E^{1\%}_{280}$ = 8.4 [20].

All proteins were analyzed by denaturing SDS/8.75% polyacrylamide gel electrophoresis [21], as shown in Figure 1.

DNA: The bacteriophage lambda P_R promoter was obtained from plasmid pGW7 as described by Ramesh and Meares [16] and from plasmid pNL23 (a gift from the laboratory of John Hearst) as follows. pNL23 was digested with Bgl II, 5'-end-labeled with polynucleotide kinase and [γ-^{32}P]ATP, and then cut either with Bsp1286 to yield the 171 base pair fragment, or EcoR1 to yield the 355 base pair fragment which was then gel purified [22].

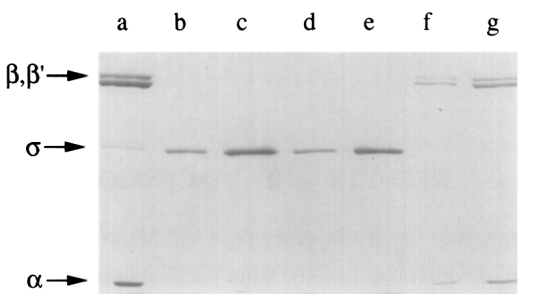

Figure 1. 8.75% SDS/polyacrylamide gel of proteins used in this study. Lane a: RNA polymerase. Lane b: sigma^{70} isolated from RNA polymerase (0.5 μg). Lane c: sigma^{70} isolated from RNA polymerase (4 μg). Lane d: sigma^{70} isolated from the overproducer (0.5 μg). Lane e: sigma^{70} isolated from the overproducer (4 μg). Lane f: core enzyme. Lane g: RNA polymerase.

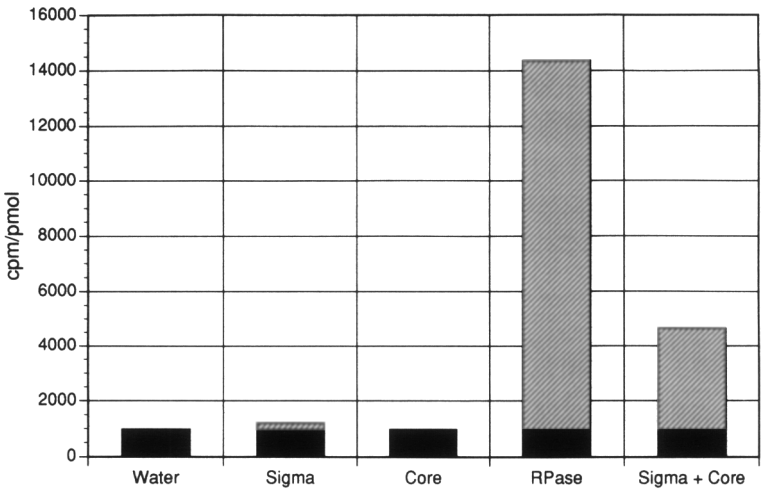

Figure 2. Activity assay of the proteins used in this study. See METHODS for details.

Activity Assay: The activity (ability to produce RNA *in vitro*) of the various samples was determined according to the method of Lowe et al. [20]. Sigma samples were assayed with core enzyme to determine their activity, as well as alone for control. Briefly, 1-5 pmol of protein (or water for control) and 3.5 μg of whole pNL23 (3.2 kb) were added to 90 μl RPase Assay Buffer and the volume made up to 100 μl. The reaction was incubated for 30 min at 37 °C. The reaction was stopped with 20 μl Assay Stop Solution. 100 μl of the mixture was then spotted onto DEAE cellulose disks. The disks were then washed five times for 5 min each in 5% (w/v) Na_2HPO_4, then twice for 2 min each in water, ethanol and ether. The disks were allowed to dry and then counted. The results are shown in Figure 2.
DNase I footprinting, high salt: Footprinting in the presence of high concentrations of NaCl was performed as previously described [16].
DNase I footprinting, low salt: The method of Negaard and Hoffman [23] was used. Labeled DNA was incubated with proteins as above in Low Salt DNase I Reaction Buffer in a total volume of 15 μl. 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 0.001 units of DNase I (in 1 μl) for 30 sec at room temperature. The reaction was stopped by addition of 6 μl of Low Salt DNase I Stop Buffer and immediately boiled for 5 min. The reactions were then chilled and loaded onto a 6% sequencing gel.
DNA gel electrophoresis: The gels were 0.4 mm thick, 40 cm long, with 6% acrylamide concentration [24]. Electrophoresis was carried out at 1500 volts for 3-4 hr. Autoradiography was carried out on the dried gels using Kodak XAR film at -80 °C. Results on the 355 bp fragment are shown in Figure 3.

RESULTS AND DISCUSSION

In an effort to reproduce the results of Ramesh and Meares, several different preparations of sigma were used, one of which was isolated from purified holoenzyme as described above. Each preparation was tested for its purity, activity, and ability to produce a footprint on the P_R promoter. All preparations were pure by SDS/PAGE. By itself, no sigma sample showed any significant activity above background, nor did any sample show a footprint. However, when added to core enzyme, both activity and the ability to produce a footprint were regenerated.

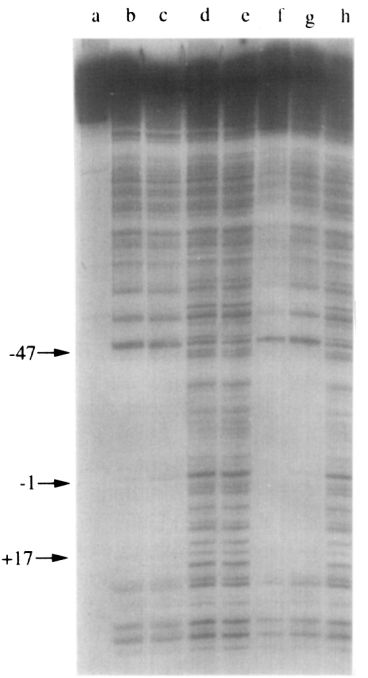

Figure 3. Low Salt DNase I footprint of the template strand of lambda P_R promoter DNA. The concentration of the 355 bp DNA fragment was 6.7 x 10^{-9} M in all lanes. Lane a: uncleaved DNA. Lane b: Digest of DNA preincubated with 6.7 x 10^{-8} M holoenzyme with 6.7 x 10^{-8} M sigma^{70} added. Lane c: Digest of DNA preincubated with 6.7 x 10^{-8} M holoenzyme. Lane d: Digest of DNA preincubated with 2.x 10^{-7} M sigma^{70}. Lane e: Digest of DNA preincubated with 6.7 x 10^{-8} M core. Lanes f,g: DNA preincubated with 2 x 10^{-7} M sigma^{70} and 6.7.x 10^{-8} M core, digested for 15 sec and 30 sec, respectively. Lane h: DNA (control) digested with 0.001 units DNase I. The residues are numbered relative to the start site of transcription.

After failure to reproduce the footprint using the high salt conditions of Ramesh and Meares, the low salt conditions and variations were also explored. Both the 171 bp fragment and the 355 bp fragment were used, yielding identical results. The pH was varied from 6.8 to 8.8, the concentration of protein was varied from 1.17 x 10^{-10} M to 7 x 10^{-6} M, while the DNA concentration ranged from 1.17 x 10^{-10} M to 7 x 10^{-9} M. The heparin addition was titrated from 2 μg to none. NaCl concentrations were varied from 5 mM to 250 mM. Temperature was varied from 4 °C to 65 °C, while time of incubation was varied from 10 sec to 3 hr. While several of these conditions showed a holoenzyme footprint, none were able to produce a sigma footprint.

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