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Binding of BAL 31 RNA Polymerase to PM2 DNA as Determined by Electron Microscopy and Protection against Restriction Endonuclease Cleavage

PAULINA BULL,* MAITE SUSAETA, BERNARDO GONZÁLEZ, AND ARTURO YUDELEVICH

Laboratorio de Bioquímica, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

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Specific binding sites of BAL 31 RNA polymerase on PM2 DNA have been mapped by protection against *Hinc*II and *Hind*III cleavage and by observation of enzyme-DNA complexes by electron microscopy. Nine specific binding sites were observed at map units 0.19, 0.20, 0.28, 0.54, 0.63, 0.65, 0.71, 0.72, and 0.75 by the first method. All these sites were confirmed by electron microscopy which, in addition, revealed another site at 0.05 map unit. Published nucleotide sequences of the region surrounding sites at 0.71 and 0.75 map units show the presence of consensus sequences for procaryotic promoters.

Bacteriophage PM2 belongs to a rather unique class of bacterial viruses characterized by the presence of a lipid-containing envelope (4, 13) and a closed circular duplex DNA structure in the mature particle (5). Its DNA has a molecular weight of about 6.3×10^6 and is one of the most tightly supercoiled DNA molecules known (16). Limited information is available on its gene structure and expression, despite the fact that the structure and physicochemical properties of the phage DNA have been well studied (5, 16), a restriction map has been constructed (2), and even small regions have been sequenced (12, 15).

The general purpose of our experiments was to locate and study RNA polymerase promoter sites on PM2 DNA. The interaction of BAL 31 RNA polymerase with PM2 DNA was studied by the following methods: (i) analysis of the digestion pattern of *Hinc*II- or *Hind*III-cleaved PM2 DNA after incubation with RNA polymerase, (ii) assessment of those DNA fragments that are retained on nitrocellulose filters after incubation with RNA polymerase, and (iii) location of the binding positions of RNA polymerase molecules on PM2 DNA by electron microscopy. Figure 1 shows a simplified restriction map of PM2, indicating the position of *Hinc*II and *Hind*III cleavage sites (2).

In order to use an homologous system, a new purification procedure for BAL 31 RNA polymerase was developed. This procedure, which includes Polymin P (BDH Chemicals, Poole, England) fractionation, DEAE-cellulose chromatography, and DNA-cellulose chromatography, is better in terms of yield and specific activity than that described previously (17).

We made use of the fact that a number of promoters recognized by *Escherichia coli* RNA polymerase contain one of the sequences cleaved by *Hinc*II (1, 6) and that at least some of them can be protected against *Hinc*II cleavage by the presence of RNA polymerase (1). RNA polymerase was incubated with supercoiled PM2 DNA, followed by digestion with *Hinc*II and agarose gel electrophoresis of the resulting DNA fragments. Both the effect of NaCl and the enzyme/DNA ratio were analyzed. The results are shown in Fig. 2. Using either a molar enzyme/DNA ratio of 4.5 and increasing salt concentrations or a fixed salt concentration (0.15 M NaCl) and increasing enzyme/DNA ratios resulted in alteration of the digestion pattern of PM2 DNA. This alteration consisted of the disappearance of some DNA fragments and the appearance of new ones. For instance, a new band of 1,400 base pairs (bp) was evident at an enzyme/DNA ratio of 4.5 or higher (Fig. 2, lanes 1 through 4, 8 and 9). This band may correspond to the sum of *Hinc*II fragments 9, 12, 11, and 7 (9-12-11-7) (Fig. 1), indicating RNA polymerase binding at sites located at 0.71, 0.72, and 0.75 map units (relative to the single *Hpa*II cut indicated in Fig. 1).

With a high salt concentration (Fig. 2, lane 4) or a high enzyme/DNA ratio (lane 9), new fragments of 1,720, 1,190, 1,120, 810, and 700 bp appeared. The molecular weight of these fragments corresponds to the sum of the weights of *Hinc*II fragments 4-9-12-11, 6-15-8, 10-13-6, 11-17, and 13-6 respectively, indicating possible binding sites at 0.19, 0.2, 0.27, 0.28, and 0.65 map units (Fig. 1). The binding sites found by using high ionic strength tended to concentrate in two major areas of the PM2 genome which comprise the region from *Hinc*II-4 to *Hinc*II-7, (0.65 to 0.75 map units) and a second area from *Hinc*II-10 to *Hinc*II-8 (0.19 to 0.28 map units). These results indicate two types of interaction between RNA polymerase and PM2 DNA, the first detected at low ionic strength and high enzyme/DNA ratios and the second detected at high salt concentrations.

Three types of control experiments were performed to determine the binding specificity. First, core RNA polymerase, in molar enzyme/DNA ratios of 8 or 12.5 with 0.1 or 0.15 M NaCl, did not yield new fragments. Therefore, protection against *Hinc*II digestion is conferred exclusively by the holoenzyme. Furthermore, the holoenzyme denatured by heating to 100°C is not effective in protecting against *Hinc*II digestion. Finally, serum albumin or core RNA polymerase at molar ratios of protein/DNA of 16.5, 30, and 61 produced no appreciable distortion in the *Hinc*II restriction pattern, eliminating the possibility of a nonspecific effect due to high protein concentrations. It should be also stated that no protection was observed with either linear or relaxed PM2 DNA.

Similar binding experiments of BAL 31 RNA polymerase to PM2 DNA were carried out, followed by *Hind*III digestion (data not shown). Dependence between ionic strength and the protection effect was also observed in this case. A new fragment of 2,650 bp, corresponding to the sum of the molecular weights of *Hind*III-2 and *Hind*III-5, was observed

* Corresponding author.

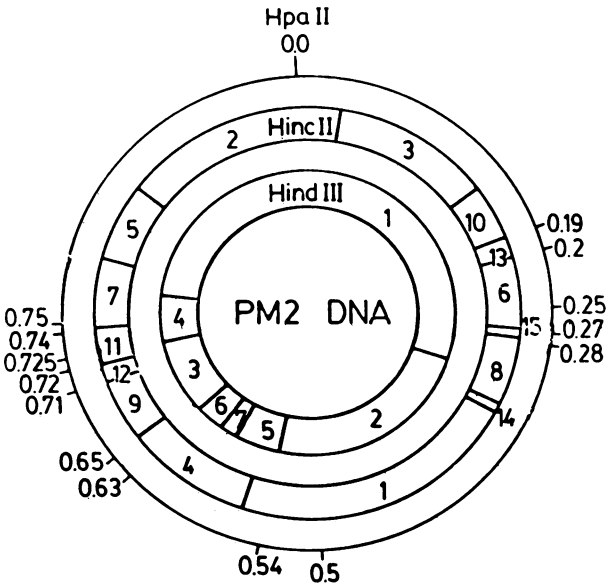

FIG. 1. Physical map of PM2 DNA. The restriction sites for *Hin*dIII and *Hin*cII are indicated as found previously (2). The positions of BAL 31 RNA polymerase-binding sites obtained by protection against restriction enzymes are shown. Sites are shown relative to the single *Hpa*II cut. The numbers outside the circle are map units; the two concentric circles within show the positions of *Hin*cII and *Hin*dIII fragments.

at low ionic strength, indicating that BAL 31 RNA polymerase was bound at 0.54 map units. When the concentration of NaCl was raised to 0.15 M, three new fragments were produced, indicating binding at 0.63 and 0.72 map units (Fig. 1). Simultaneous binding in a DNA molecule at 0.63 and 0.72 map units occurred only if high ionic strength and a high molar enzyme/DNA ratio were used simultaneously. Again, core or denatured RNA polymerase and serum albumin did not protect PM2 DNA from *Hin*dIII digestion.

The nitrocellulose filter binding assay has shown to be very useful in studying protein-DNA interactions; free DNA passes through the filter, but DNA to which protein is bound is retained (7). Filter binding assays showed that only preincubation with holoenzyme produced filter-retainable complexes after *Hin*dIII digestion. Furthermore, *Hin*dIII fragments 1, 2, and 3 and a fragment of 2,650 bp (Fig. 1) were resistant to heparin challenge (8), suggesting that they contain strong binding sites (data not shown).

The binding of BAL 31 RNA polymerase to PM2 was visualized by electron microscopy. Figure 3 shows a typical electron micrograph of BAL 31 RNA polymerase molecules bound to PM2 DNA. PM2 DNA was digested either with *Pst*I or with *Acc*I, which produce a single cut, in order to have a reference point. The histogram was obtained after orientating the linear molecules by the criteria proposed by Rassart et al. (10). A total of 520 DNA molecules were analyzed, to which 723 BAL 31 RNA polymerase molecules were bound. Six preferred binding sites were localized, most of which closely coincide with those detected with the other techniques; in addition, a new binding site at 0.05 map unit was detected. Moreover, all the binding sites detected were located in fragments which were retained in the nitrocellulose filter binding assay.

The DNA conformation seems to be critical for RNA polymerase binding. We could only detect RNA polymerase binding sites with supercoiled DNA; linear or relaxed closed circular DNA is not protected from digestion by RNA polymerase at any of the locations shown for supercoiled DNA. Similarly, in electron microscopy experiments, very few RNA polymerase molecules were bound when PM2 DNA was digested prior to incubation with RNA polymerase. These findings agree with in vitro transcription experiments done by Zimmer and Millette (18) and Richardson (11), which found a marked decrease in transcription of PM2 DNA when using closed relaxed DNA compared with supercoiled DNA. This would indicate that in this case, the topology imposed by negative supercoiling is required to generate the structure which allows interaction between promoter and RNA polymerase. Similar observations have been made with *E. coli* RNA polymerase, whose binding efficiency is strongly influenced by the degree of DNA supercoiling (3).

We have detected specific RNA polymerase-binding sites in the DNA of phage PM2 in a homologous system, i.e., by using the enzyme purified from the host BAL 31. These sites presumably correspond to true promoters, since binding and therefore protection from cleavage is observed only when native holoenzyme is used. Important evidence indicating that at least some of these binding sites are true promoters is obtained from examining the nucleotide sequence which is available for certain regions of PM2 DNA. *Hin*dIII-4 of PM2 DNA has been sequenced (12, 15), as well as the right end of *Hin*dIII-3 (12). Both of these fragments contain *Hin*cII sites that can be protected from cleavage by *Hin*cII by preincubation with PM2 RNA polymerase. *Hin*dIII-4 has a *Hin*cII site at 0.75 map unit. Examination of the nearby sequences shows that the sequence TATTAT is located 15 residues away from the *Hin*cII recognition site and that a sequence similar to TATAAT is 5 residues away from the *Hin*cII site located at 0.71 map units.

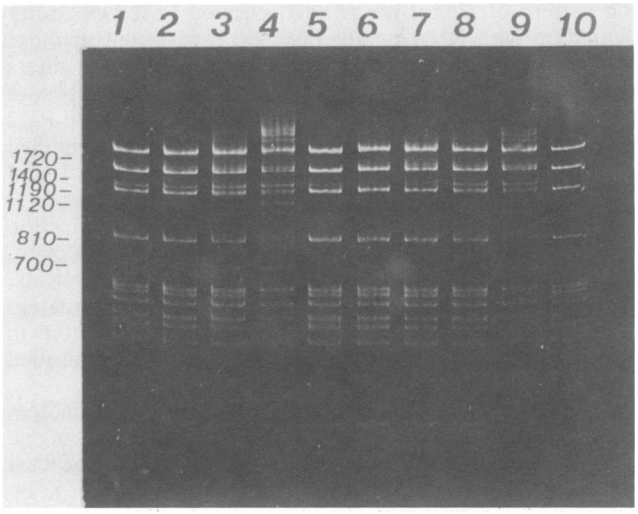

FIG. 2. Protection of *Hin*cII cleavage sites in PM2 DNA by BAL 31 RNA polymerase. Supercoiled PM2 DNA (5 μg) was incubated with 1.8 μg of BAL 31 RNA polymerase in the presence of 0 (lane 5), 0.05, 0.1, 0.15, and 0.2 M NaCl (lanes 1 to 4, respectively), or with 0 (lane 10), 0.35, 0.9, 1.8, and 2.7 μg (lanes 6 through 9, respectively) of BAL 31 RNA polymerase in 0.15 M NaCl. After 2 min of incubation at 28°C, the DNA was digested with *Hin*cII for 2 h at 37°C, and the digest was phenol extracted and precipitated with ethanol. The DNA samples were submitted to 5% polyacrylamide gel electrophoresis. Numbers to the left represent the size (in base pairs) of the new DNA fragments.

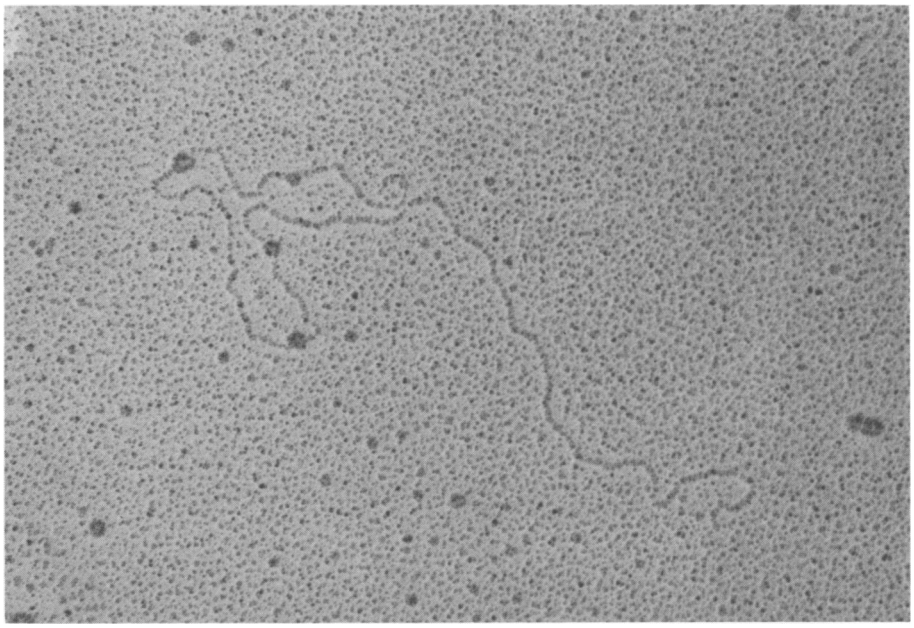

FIG. 3. Electron micrograph of PM2 DNA bound to BAL 31 RNA polymerase. Enzyme-DNA complexes were obtained by the procedure reported by Sogo et al. (14). BAL 31 RNA polymerase (15 μg) was incubated with 3 to 15 μg of PM2 DNA for 15 min at 30°C. DNA was then digested with *Pst*I or *Acc*I for 30 min at 37°C. After digestion, 0.1% glutaraldehyde was added. Unbound protein molecules were separated from the enzyme-DNA complexes by filtration through Sepharose 4B. Fractions containing the complexes were adsorbed to mica and processed by the method of Portmann and Koller (9). The mica sheets were rotary shadowed with Pt-Pd, and carbon replicas were prepared. Micrographs were taken in a JEOL electron microscope at 50 kV acceleration rates. Magnification, 30,000×.

In addition, preliminary experiments indicate that at least some of these sites are used for transcription in vitro (I. Urquiaga, unpublished results).

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