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Comparison of the A-T rich regions and the Bacillus subtilis RNA polymerase binding sites in phage φ29 DNA

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ABSTRACT

By using a modification of the BAC spreading method for mounting the DNA for electron microscopy, partial denaturation maps of protein-free Ø29 DNA and of Ø29 DNA containing protein p3 were obtained. In Ø29 p3-DNA[1] the protein does not seem to influence the melting of the ends of the molecules.

The comparison of the partial denaturation map and the B. subtilis RNA polymerase binding sites indicates that five of the seven early promoters (A1, A2, A3, B2 and C2) are located in A-T rich DNA regions whereas the other two early promoters (B1 and C1) are located in less A-T rich sites.

INTRODUCTION

The double stranded DNA of B. subtilis bacteriophage Ø29 has a molar mass of $11.8 \pm 0.4 \times 10^6$ g mol^{-1} [1,2] and contains a protein covalently attached to the 5' termini[3-6]. The protein has been characterized as the product of cistron 3, p3[3], has a molar mass of 27,000 g mol^{-1} [7,8] and is needed for DNA replication[9-12]. The protein was visualized by electron microscopy at the ends of unit length DNA molecules[3].

Recently we located seven B. subtilis RNA polymerase binding sites on the DNA of bacteriophage Ø29[2]; their positions were determined with accuracies from ± 70 base pairs for the more precisely located binding sites to ± 180 base pairs for the least definite. The promoters were named A1, A2, A3, B1, B2, C1 and C2 corresponding to their location in the EcoRI fragments. The same seven binding sites were found regardless of the presence or absence of protein p3 in Ø29 DNA.

The relationship between RNA polymerase binding sites and partial denaturation maps has been investigated by different groups. In λ DNA[13-15] as well as in M13 DNA[16] it was found that RNA polymerase binding sites coincide fairly well with A-T rich

regions. In the present paper, we describe a precise partial denaturation map of protein-free Ø29 DNA and of Ø29 p3-DNA. Preliminary partial denaturation maps of Ø29 DNA were obtained previously[17,18]. The present map was obtained with a modification of the BAC technique[19] which allowed the visualization of even very small denaturation regions. We compare the partial denaturation map obtained here with the map of the B. subtilis RNA polymerase binding sites described previously[2].

MATERIALS AND METHODS

Preparation of Ø29 DNA

Ø29 DNA was prepared from purified phage[20] by extraction with phenol after treatment with proteinase K in the presence of 0.5% sodium dodecylsulfate[21]. No nicks were detected in the DNA, as determined by alkaline sucrose gradient centrifugation.

Ø29 p3-DNA was isolated from phage labelled with ^{3}H -uracil as described by Salas et al[3].

Partial denaturation of DNA

Ø29 DNA in a buffer containing 35 mM triethanolamine-HCl (pH 8), 50 mM KCl, 8 mM Mg-acetate and 3% formaldehyde was heated for 11 min at 68 ºC and immediately quenched in ice. For Ø29 p3-DNA the incubation buffer and the time were the same, but the temperature was 66 ºC. In some experiments, the Ø29 DNA was incubated with EcoRI[21] and the fragments were partially denaturated as described above for protein-free Ø29 DNA.

Specimen preparation for electron microscopy

The BAC technique[19] was used under the following conditions: to 250 µl of the partially denaturated DNA samples at a concentration of 0.5 µg/ml, 80 µl of 90% formamide in 180 mM Na-acetate (pH 8.5)[22] and 3 µl of a 0.2% BAC stock solution in formamide were added. This solution was spread over a hypophase of redistilled water. The DNA-BAC film was picked up with carbon coated grids, which were pretreated by floating for 15 min on droplets containing 30 µg/ml ethidium bromide; the excess ethidium bromide solution was removed by drying on a filter paper with or without passing briefly through redistilled water. In some experiments the DNA was spread over a hypophase containing 15 µg/ml

ethidium bromide and the film was picked up with carbon films with or without the above described pretreatment with ethidium bromide. After adsorption of the DNA the grids were washed by floating on redistilled water for 15 min. They were stained with uranyl acetate as described previously[19] and rotary shadowed with platinum-carbon.

Micrographs were taken in a Jeol 100B electron microscope at 80 KV and at a magnification of 10,000. The magnification was determined with a Carbon Grating replica of 2160 lines/mm from Balzers Union (Liechtenstein).

Quantitative Analysis

After a six-fold enlargement of the electron micrographs, the contour lengths of the DNA molecules were measured with a SAC Digitizer. The data were processed in a PDP 11/45 minicomputer with a DOS/BATCH and the histograms were displayed in a model 31 Varian/Statos Plotter.

RESULTS

Modification of the BAC technique

With a combination of BAC spreading and either treatment of the grids with ethidium bromide and/or presence of ethidium bromide in the hypophase (see Materials and Methods) we obtained reproducibly good adsorption of DNA. Both single- and double-stranded DNA's were unaggregated and unfolded. Using grids pretreated with ethidium bromide, a hypophase containing ethidium bromide produced usually more uniform preparations than a hypophase of quartz redistilled water. For double-stranded DNA, the best results were obtained with ethidium bromide concentrations between 10 and 20 µg/ml in the hypophase. With concentrations above 15 µg/ml the molecules frequently showed either curled and/or a supertwisted appearance. The best unfolding of single-stranded DNA was obtained with a hypophase containing 10 to 15 µg/ml of ethidium bromide, whether carbon films pretreated or untreated with ethidium bromide were used. The length of the double-stranded filaments depended very much on the hypophase used. On a hypophase of redistilled water Ø29 DNA had a contour length of 6.94 $\pm$ 0.17 µm (from 41 molecules) whereas on a hypo-

phase containing 15 μg/ml of ethidium bromide it increased to 9.68 ± 0.30 μm (from 43 molecules).

Partial denaturation maps

Figure 1 shows that the denaturation bubbles in partially denatured Ø29 DNA molecules appear to be asymmetrically distributed. One end of the DNA molecule is open whereas the other end remains closed in most cases. The molecules were oriented by considering the closed double-stranded end to belong to the left side of the genetic map (see below). In the few molecules observed with two open ends, the less open one was oriented to the left side. Fig. 2a represents the histogram obtained from 70 Ø29 genomes with an average degree of denaturation of 19%. The DNA stretches with separated strands were considered to correspond to A-T rich regions[23]. To correlate the A-T rich regions of Ø29 DNA with the genetic[24], physical[21] and transcription maps[2], the DNA was treated with the restriction endonuclease EcoRI and the fragments obtained were partially denatured. Fragments EcoRI A, B and C were analyzed and compared with the map of the intact genome. Fig. 3a shows a fragment EcoRI A where one half of the filament has large denatured regions. Comparing the partial denaturation map of the EcoRI A fragments with that of the whole DNA (Fig. 2a and b), it can be seen that there is a good correspondence between the larger denatured region of the fragment (Fig. 3a) and the left part of the genome. Using this criterion to orient fragment EcoRI A (50.5 units), the main denaturation sites in the fragment (Fig. 2b) coincides with the peaks in the left half of intact DNA (Fig. 2a). The histogram was obtained from 42 EcoRI A fragments with an average extent of denaturation of 35%.

The orientation of fragment EcoRI C was unequivocal. One side of the molecule was always open up to approximately one third of the total length of the fragment (Fig. 3c). Most likely this side corresponds to the right open end of the whole genome (Fig. 1). This is confirmed when the histogram obtained from 75 EcoRI C fragments with an average extent of denaturation of 47% (Fig. 2d) is compared with that of the right side (9.7 map units) in full length DNA (Fig. 2a).

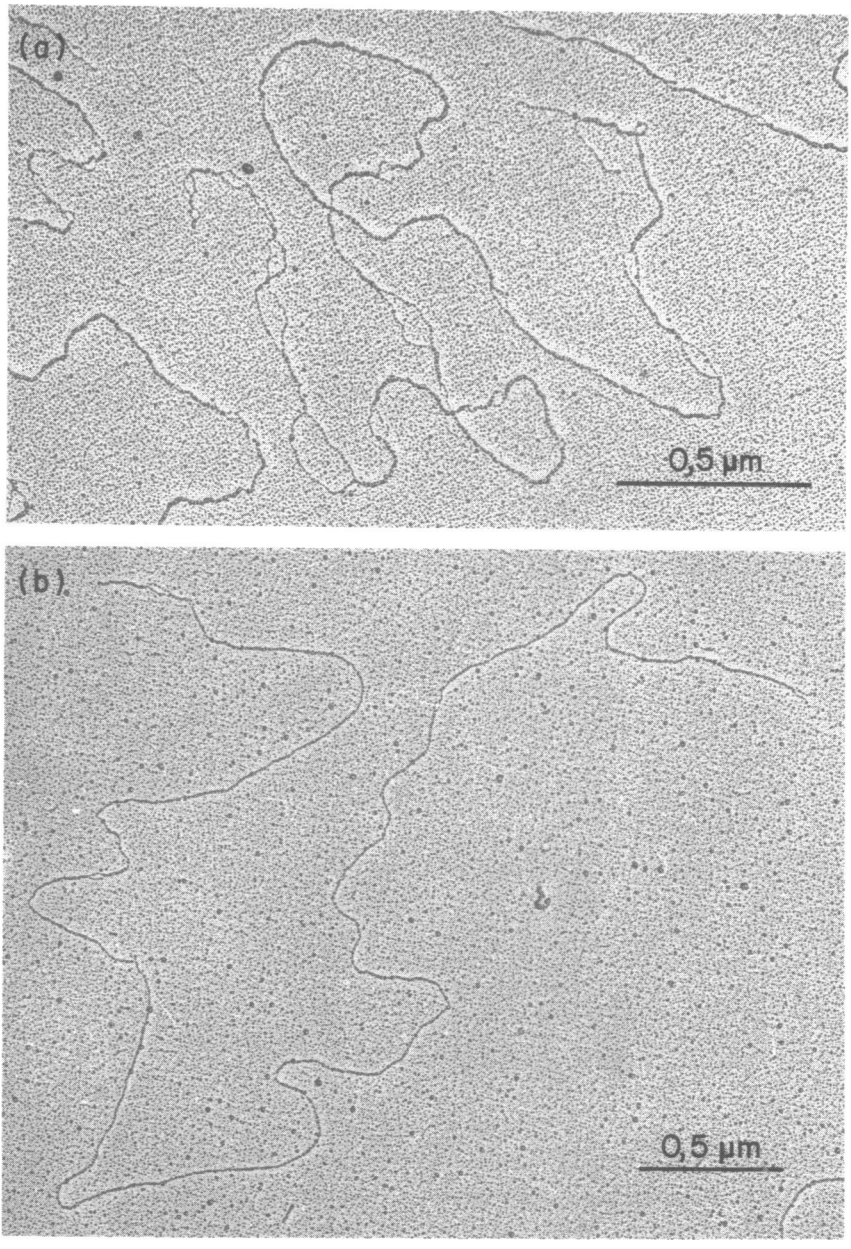

Fig. 1. Electron micrographs of partially denaturated Ø29 DNA molecules. a) Proteinase K-treated Ø29 DNA spread into water. b) Ø29 p3-DNA spread into a hypophase containing 15 µg/ml ethidium bromide. In both cases the DNA-BAC film was picked up with carbon support films pretreated with ethidium bromide.

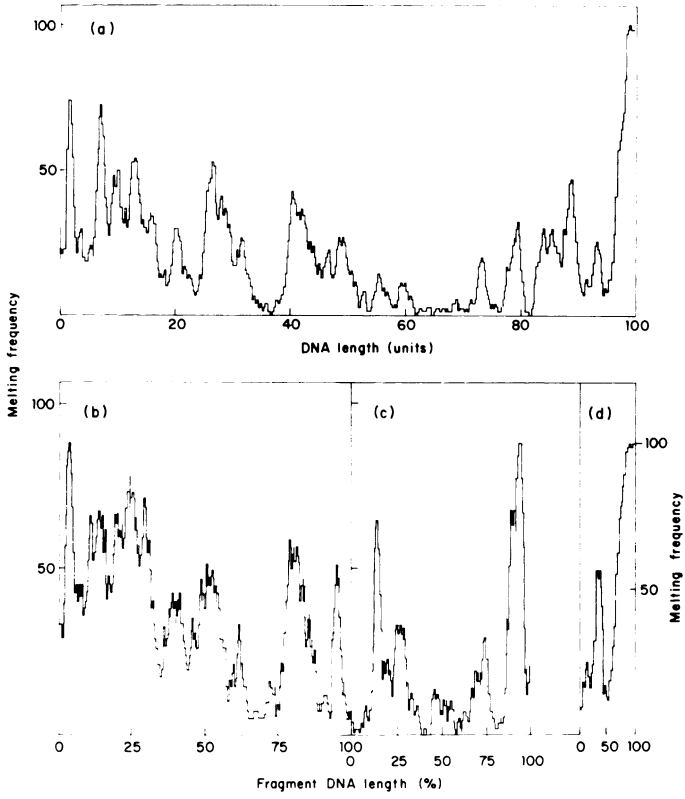

Fig. 2. Histograms of partially denaturated proteinase K-treated Ø29 DNA and fragments EcoRI A, B and C. a) 70 Ø29 genomes analyzed with a denaturation degree of 19%. b) 42 EcoRI A fragments analyzed with a denaturation degree of 35%. c) 56 EcoRI B fragments analyzed with a denaturation degree of 17%. d) 75 EcoRI C fragments analyzed with a denaturation degree of 47%. The contour length of intact Ø29 DNA was 7.45 ± 0.44 μm and that of fragments EcoRI A, B and C was 4.34 ± 0.56 μm, 2.52 ± 0.10 μm and 0.83 ± 0.13 μm, respectively.

Since it is known that EcoRI fragment A contains the left side of the genetic map and fragment EcoRI C the right end[21] we conclude from the unambiguous fitting of the partial denaturation maps of the EcoRI fragments A and C with that of the whole DNA that the left end of the map of the whole DNA in Fig. 2a must correspond to the left end of the genetic map. Accordingly we named the left end of the genome as "A" (Fig. 1 and Fig. 2a)

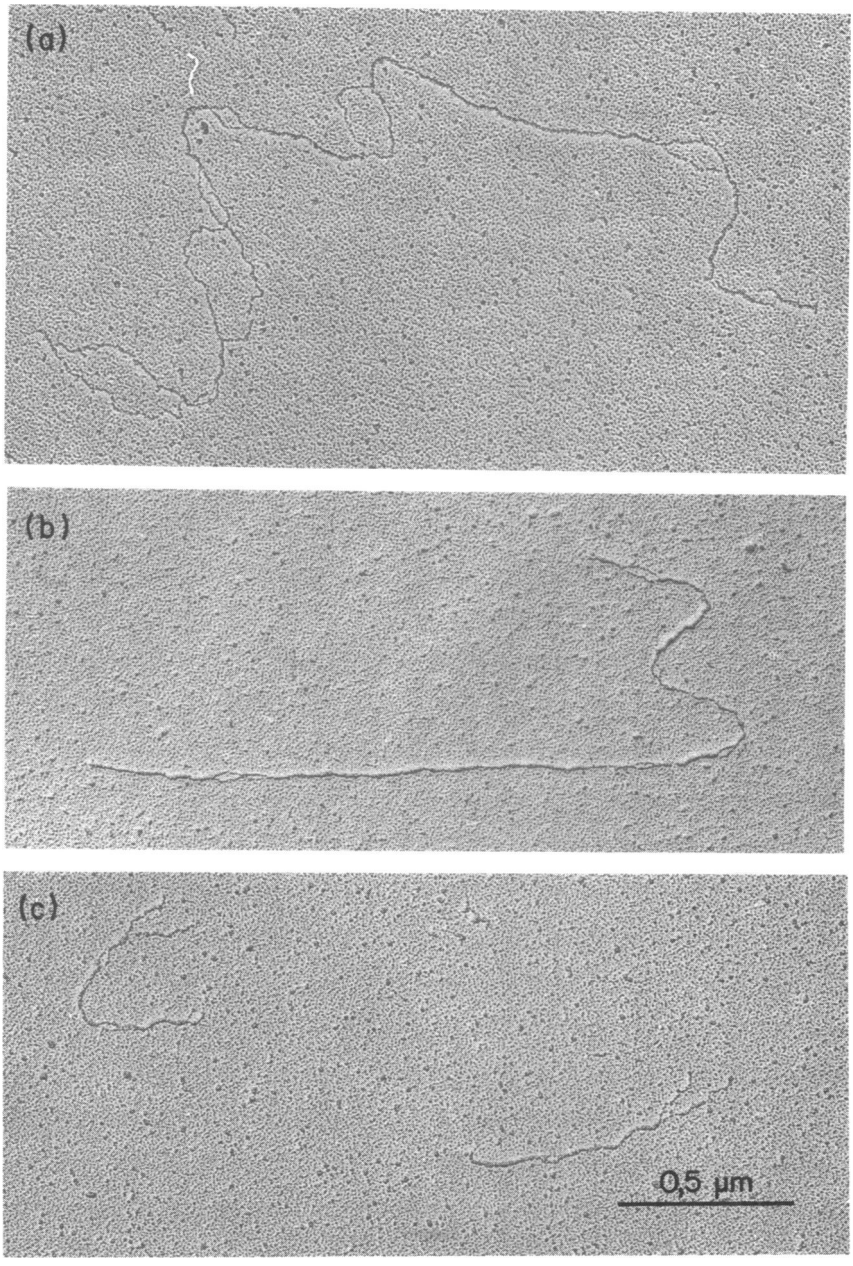

Fig. 3. Electron micrographs of partially denaturated Ø29 DNA fragments EcoRI A (a), EcoRI B (b) and EcoRI C (c) spread under the conditions of Fig. 1a.

and the right end as "C".

We also studied the partial denaturation of the EcoRI B fragment (Fig. 3b) located between positions 50.5 to 81.5 (31 map units long) from the left end of Ø29 DNA[21]. The histogram shown in Fig. 2c was obtained from 56 EcoRI B filaments with an average extent of denaturation of 17%. The four main areas of denaturation fitted unambiguously with the corresponding region in the full length genome (Fig. 2a).

The partial denaturation of Ø29 DNA with protein p3 covalently attached to the 5' termini[3] was also studied to see whether the protein has an influence on the melting behavior of the molecule. Under the conditions described in Materials and Methods, we obtained an average extent of denaturation of 4%, probably due to the fact that the DNA was heated to 66º instead of 68 ºC. Under these conditions the "C" end (right end) was always open, and the "A" end (left end) remained closed (Fig. 1b). The comparison of the partial denaturation map based on the analysis of 43 Ø29 p3-DNA molecules (Fig. 4) with that obtained with Ø29 DNA without p3 (Fig. 2a) indicated that the location of the A-T rich

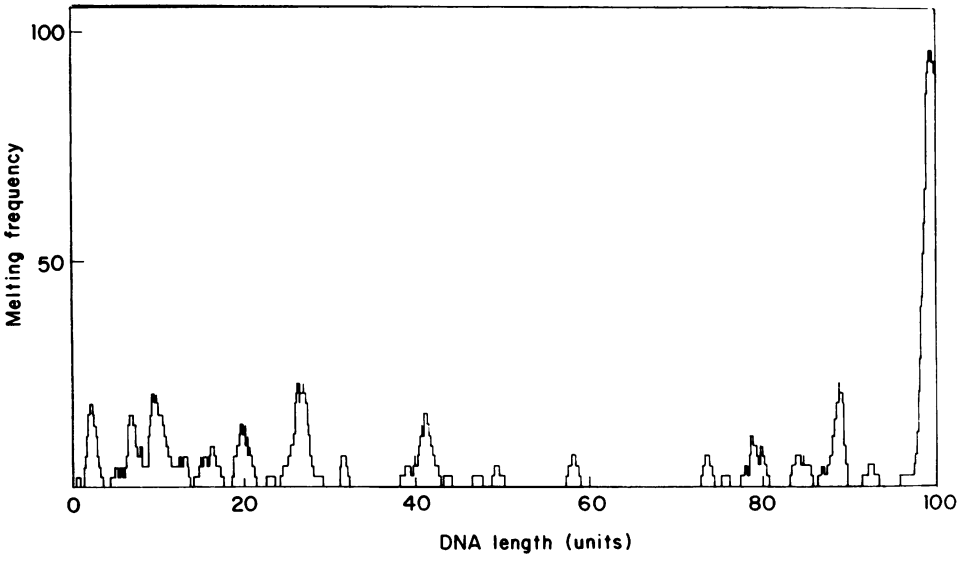

Fig. 4. Histogram of partially denaturated Ø29 p3-DNA. 43 molecules were analyzed and the denaturation degree was 4%.

sites are similar. The fact that the right end of the Ø29 genome is rich in A-T is confirmed and the presence of protein p3 clearly had no influence upon the melting behavior in this region.

Comparison of the partial denaturation map and the RNA polymerase binding sites of Ø29 DNA.

When double- and single-stranded DNAs were spread onto a hypophase of redistilled water, the contour length of single-stranded DNA (7.88 ± 0.71 µm from 98 molecules) was larger than that of double-stranded DNA (6.94 ± 0.17 µm). Therefore, to compare accurately the positions of the seven early promoters[2] with the partial denaturation map, we corrected the lengths of the single stranded segments to that of the double strands. The values shown in the histogram (Fig. 5a) are given in double-stranded units taking 100 as the total length of Ø29 DNA[2]. It should

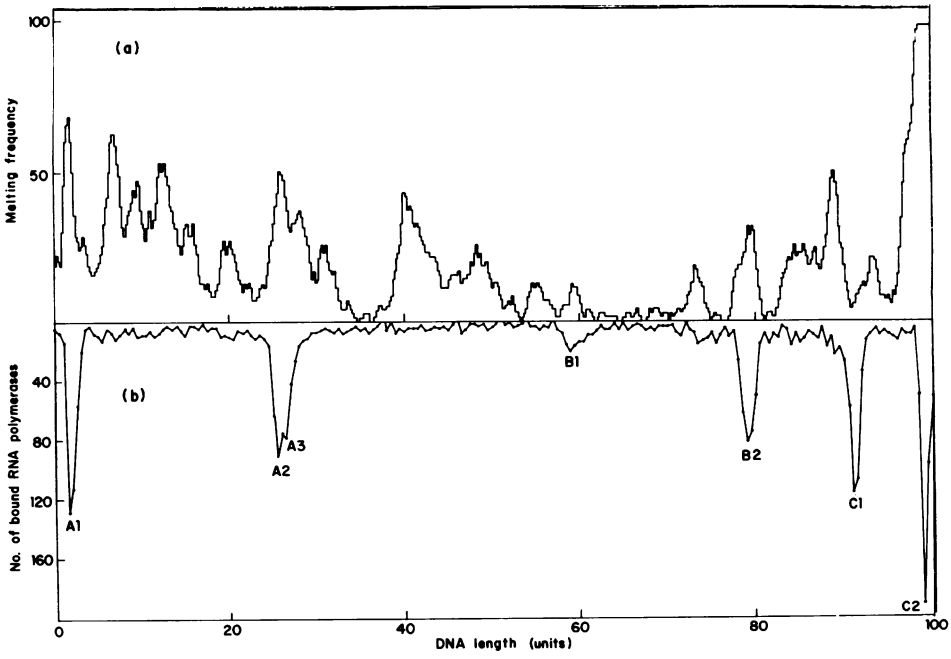

Fig. 5. Comparison of the histograms of partially denatured Ø29 DNA and B. subtilis RNA polymerase binding sites. a) Partial denaturation map of Fig. 2a corrected as described in the text. b) Histogram obtained from 3101 RNA polymerases bound to 426 Ø29 DNA molecules. The data were obtained from Fig. 2a and b and Fig. 6 from the work of Sogo et al[2].

be pointed out that, although the mentioned correction is important, in our case the differences between the uncorrected and corrected maps are insignificant (compare Fig. 2a and Fig. 5a), probably due to the fact that the distribution of the partially denatured regions along the DNA molecule is more or less uniform. In the same histogram we included the B. subtilis RNA polymerase binding sites (Fig. 5b), obtained from the data of Fig. 2a and b and Fig. 6 from our previous work[2]. This allows us to compare directly the sites of A-T rich regions with the specific RNA polymerase binding sites (Fig. 5). It can be seen that five of the seven promoters, A1, A2, A3, B2 and C2, located at 1.7, 25.5, 26.7, 79.3 and 99.3 map units, are within A-T rich regions, whereas the other two binding sites, B1 and C1, located at 59.4 and 91.3 DNA length units are in regions of a clearly lower A-T content. There are also A-T rich regions which do not correspond

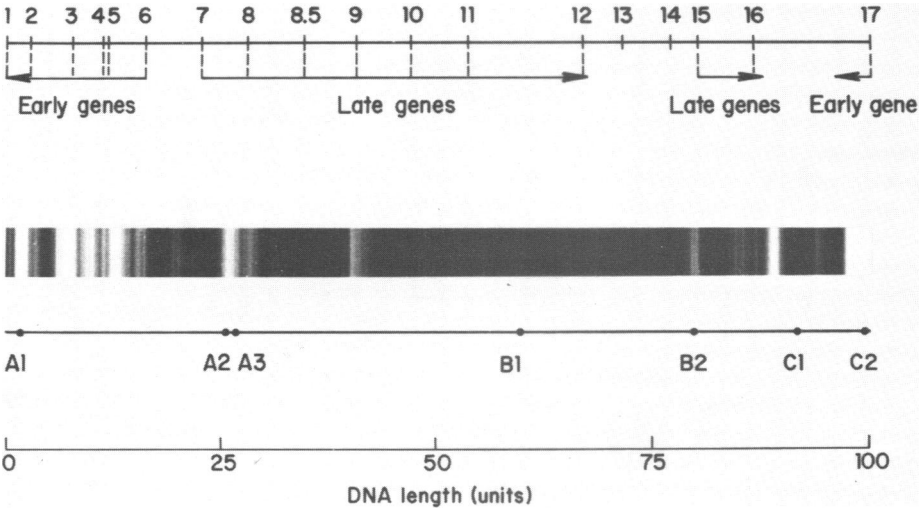

Fig. 6. Partial denaturation map and B. subtilis RNA polymerase binding sites in Ø29 DNA. I. Genetic map adapted from the works of Mellado et al[24] and Reilly et al[25]. The numbers indicate genes and the arrows below the genes indicate the direction of the transcription. II. The histogram of Fig. 5a was processed densitometrically to obtain the location and the size of the early melting regions. III. RNA polymerase binding map taken from the work of Sogo et al[2]. The B. subtilis RNA polymerase binding sites are indicated in the middle of the line.

to B. subtilis RNA polymerase binding sites. Fig. 6 summarizes these data and shows the genetic and partial denaturation map of Ø29 DNA as well as the B. subtilis RNA polymerase binding sites.

DISCUSSION

The BAC technique[19] as well as the recently described modification using anthrabis as spreading compound[26,27] has the advantage over other protein-free spreading techniques in that single- and double-stranded nucleic acids and nucleic acid-protein complexes can be visualized. In certain laboratories the BAC-technique has been found to be difficult to apply[27]. Recent results indicate[28] that at least some of these difficulties are due to unfavorable surface properties of the carbon support films which do not bind the DNA-BAC complex. As described in this paper a simple way to overcome this problem is to pretreat the carbon supports with ethidium bromide (30 µg/ml) prior to adsorption of the DNA and/or to add between 10 and 20 µg/ml of ethidium bromide in the hypophase. It is interesting to see that with the simultaneous interaction of BAC and ethidium bromide the length of double-stranded DNA (9.68 ± 0.30 µm) is significantly larger than when only BAC[19] or only ethidium bromide[29] are used, the lengths obtained in those cases being 6.94 ± 0.17 µm and 6.24 ± 0.30 µm (from 230 molecules)[30], respectively. A similar effect was described by Freifelder[31] with λ b2 DNA using the cytochrome C technique without and with the intercalating dye.

It is generally accepted[23] that the strand separated regions in a partially denatured DNA molecule correspond to sequences rich in A-T base pairs. Fragment EcoRI B of Ø29 DNA therefore appears to have the highest content in G-C. The positions from map units 33 to 37 from the left end appear also to be rich in G-C. The strand separation begins at the right end and consequently this side seems to be the richest in A-T sequences. Even at a partial denaturation of 19%, the left end of the molecule is not denatured. This suggests that the sequences close to the two ends are probably different, except for about 100 base pairs from each end which could not be recognized if they were denatured. Direct sequence analysis of the first 100 base pairs from each end will be needed to answer this question.

The protein covalently bound to the 5' termini of Ø29 DNA does not seem to interfere with the denaturation behavior since at an average denaturation degree of 4% the right end was already open when the DNA-protein p3 complex was used instead of protein-free DNA. The protein does not seem to favor to a high extent the degree of denaturation either since under the conditions described above, the left end of the Ø29 p3-DNA molecules still remained closed.

Five of the seven early Ø29 promoters are located in A-T rich regions. These data are similar to the ones obtained by other groups with λ DNA[13-15], M13-DNA[16] and with aroE-trKA-spc of the E. coli genome, transposon Tn3 and plasmid ColE1[32].In λ DNA, Vollenweider and Szybalski[15], found an imperfect quantitative correspondence for a few regions. Four of the five Ø29 promoters which lie in A-T rich regions, namely A1, A2, A3 and C2, are strong whereas the fifth, the B2 promoter, is salt-sensitive[2]. Two of the RNA polymerase binding sites (B1 and C1) are located in regions with a low A-T content. This is especially the case for the C1 promoter which is located at a site corresponding to a minimum of the partial denaturation map. Regarding the B1 promoter, when the extent of partial denaturation of the whole genome is 19%, the degree of denaturation corresponding to the EcoRI B fragment is about 6.5% and the position corresponding to map unit 60 begins to be open (Fig. 2a). When fragment EcoRI B was denaturated to 17% the peak corresponding to map unit 60 of the whole genome (Fig. 2c), is more clearly pronounced. It is noteworth that C1 is a strong binding site whereas B1 appears only under special conditions[2]. Therefore we have to conclude that, at least for B. subtilis RNA polymerase, promoters do not always lie in early melting DNA regions and that there is no correlation between the strength of a promoter and the local melting behavior as seen in the electron microscope. The nucleotide sequence and consequently the exact content of A-T in the Ø29 promoters and promoter-flanking regions is not yet known.

There are also A-T rich regions which do not correspond to B. subtilis RNA polymerase binding sites. This is also the case for T7[33,34] and λ[15] DNA where there are A-T rich regions which do not correspond to early promoters. It is possible that late

promoters lie in some of the remaining early melting regions. It is unlikely, however, that the A-T rich regions present in Ø29 DNA between positions 7 and 20 correspond to late promoters since there is no late transcription in that region (see Fig. 6).

From our data we conclude that 1) an A-T rich region defined by electron microscopy does not necessarily imply the existence of an RNA polymerase binding site; 2) RNA polymerase binding sites are not necessarily located in A-T rich regions; 3) binding of RNA polymerase to promoters which are present in A-T rich sites may be resistant or sensitive to salt[2], therefore there is no correlation between salt resistance of a promoter and its presence in an A-T rich DNA segment.

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