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Localization of melted regions in supercoiled DNA

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Diethyl pyrocarbonate (DEPC) was used as a probe of local denatured regions in ccDNA pAO3 plasmid. It was found that in native ccDNA molecules only adenosine residues in the loop of the cruciform structure react with DEPC. Denaturation of ccDNA is accompanied by the appearance of two short regions (20 bp long) at both borders of the cruciform structure. Further increase in the denaturation process is associated with considerable expansion of the region located to the left of the cruciform, while the cruciform structure itself and the denatured region located to the right of it disappear.

DNA, supercoiled; Melting pattern; DNA modification; Cruciform structure; Open region

1. INTRODUCTION

Negative supercoiling causes some regions in the DNA molecule to adopt structures that are different from the conventional B-form. These include the left-handed Z-DNA, cruciform and protonated structures [1–3]. The interest aroused by such alternative structures in supercoiled covalently closed DNA (ccDNA) is largely associated with their possible role in the functioning of DNA. Denatured regions whose formation is facilitated by negative supercoiling should also be classified with such alternative structures. However, we know far less about their formation in ccDNA than we do about the other alternative structures. We have recently [4] examined the formation of melted regions in ccDNA by a method based on gradient gel electrophoresis [5]. This approach enabled us to observe the behaviour of individual topoisomers, so that we could visualize the early melting pattern for each topoisomer and obtain certain quantitative characteristics of this stage in the melting of ccDNA. However, a number of important questions remained to be elucidated. First of all, it was essential that we localize the melted regions in ccDNA and understand the influence of the cruciform structure in the process of denaturation. These questions are addressed in the present paper.

We used the method of chemical modification, choosing diethyl pyrocarbonate (DEPC) as a probe. Earlier DEPC had been used with considerable success for detecting cruciform [7,8] and Z-form [6] segments in ccDNA. Since DEPC only very slightly modifies DNA bases in the B-form [9], it is a suitable agent for probing melted regions in ccDNA at the nucleotide level.

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Abbreviations: DEPC, diethyl pyrocarbonate; ccDNA, covalently closed supercoiled DNA

2. MATERIALS AND METHODS

DNA of the pAO3 plasmid was isolated as in [4] using a modified alkali extraction procedure followed by cesium chloride density-gradient centrifugation with ethidium bromide.

10 μg DNA in 200 μl TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) with different denaturant concentrations (a mixture containing 40% formamide and 7 M urea was regarded as 100% denaturant concentration) was incubated at 33°C with 2 μl DEPC (Fluka) for

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a specified time. The time was mostly 5 min, but to characterise the kinetics of ccDNA modification by DEPC we chose modification times of 2 and 20 min. After incubation, the reaction was stopped by adding 10 μl 2-mercaptoethanol, and then the unreacted DEPC and denaturants were removed by chromatography on a column with Toyo Pearl HW-50 gel. Thereafter, DNA was precipitated using 2 vols ethanol and cut by *Eco*RI restriction endonuclease. The resulting linear molecules were labelled at the 5′- or 3′-ends, after restriction by *Hpa*II, and a labelled fragment of 805 bp was isolated after polyacrylamide gel electrophoresis. The fragment was treated for 15 min with 10% piperidine at 90°C, the products of partial chemical degradation being then separated using 10% denaturing polyacrylamide gel electrophoresis. Meanwhile the same fragment of native DNA was sequenced by the Maxam-Gilbert method with some modifications [11]. Gel autoradiographs were analysed with the help of an Ultroscan LKB 2202 laser densitometer (LKB, Sweden).

3. RESULTS

A preparation of pAO3 ccDNA containing a set of topoisomers was treated with DEPC at 33°C in the presence of 20, 40 and 60% denaturants. These conditions almost completely encompass the range of early melting of the most supercoiled topoisomers carrying cruciform structures [4]. Fig.1 presents a gel autoradiograph corresponding to the *Eco*RI part of the 805 bp long segment of the *Eco*RI-*Hpa*II fragment (see section 2) which contains, apart from the main palindrome, the most AT-enriched region of pAO3 DNA [4,10,12]. Tracks 1–4 correspond to the Maxam-Gilbert sequencing ladders of the native DNA fragment, and 5–10 to the modification of ccDNA by DEPC. One can see that at the initial stage (track 5) the major sites of modification were three adenosine residues in the loop of the cruciform structure, consistent with published data [7,8]. The modification of bases in other sites increases at higher values of the denaturant concentration. At the same time, the degree of modification of nucleotides in the loop of the cruciform decreases.

Similar results were yielded by our analysis of the complementary strand (not shown). They demonstrate clearly that the temperature rise and increased denaturant concentration produce the same effect: greater modification outside the cruciform structure and weaker modification in their loop.

The modification of pAO3 ccDNA at different stages of melting is more graphically illustrated in fig.2, which shows the autoradiograph densitometry results. Fig.2a demonstrates the modification of ccDNA without denaturants: the A residues in the loop of the cruciform structure are the most preferred modification sites in the initial DNA. In fig.2b (20% denaturants) one observes a noticeable increase in modification of the two regions bordering on the cruciform structure. A further increase in the degree of denaturation (fig.2c) is associated with considerable

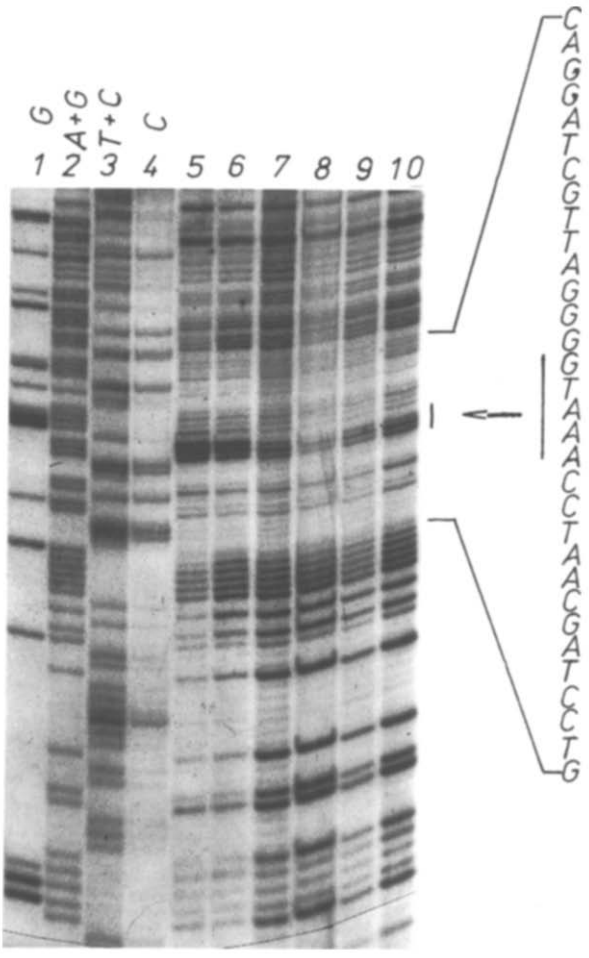

Fig.1. DEPC modification of ccDNA pAO3 at 33°C, top strand. Tracks: (1–4) part of the Maxam-Gilbert sequencing ladders of the *Eco*RI/*Hpa*II 805 bp fragment; (5–10) DEPC modification under different conditions [(5) no denaturants, 5 min; (6) 20% denaturants, 5 min; (7) 40% denaturants, 5 min; (8) 60% denaturants, 5 min; (9) 40% denaturants, 2 min; (10) 40% denaturants, 20 min]. The sequence of the palindrome region is shown on the right. The arrow indicates the loop of the cruciform.

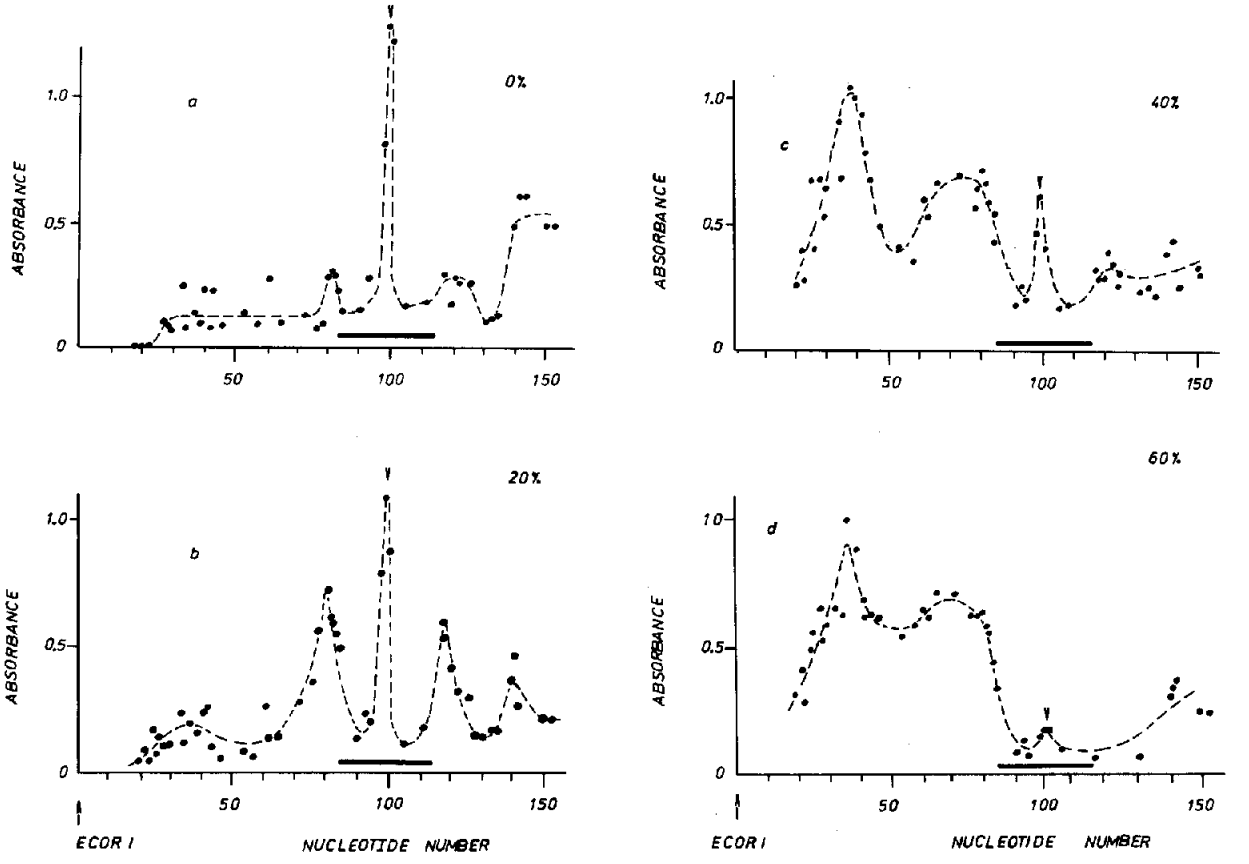

Fig.2. Densitometry results of tracks 5–8 in fig.1. (a) No denaturants; (b) 20% denaturants; (c) 40% denaturants; (d) 60% denaturants. The position of the modified A nucleotides from the *Eco*R1 site is shown above the *X*-axis.

expansion of the region located to the left of the cruciform, and the appearance of a strongly modified region of about 20 bp on its left. Note the concomitant drop in intensity of the peak corresponding to modification of the region to the right of the cruciform and the cruciform loop (cf. fig.2b and c). At 60% denaturant only the region on the cruciform's left is completely modified, while the palindromic sequence itself is not affected (see fig.2d).

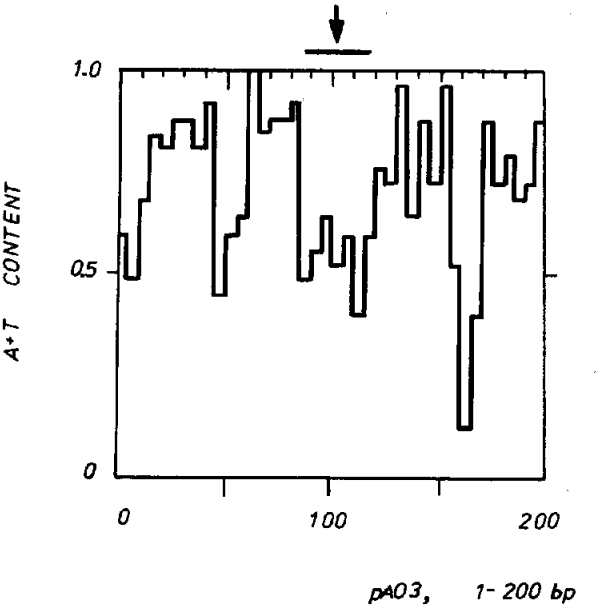

Fig.3. Histogram of AT distribution in the most AT-enriched region of pAO3 DNA (window size 5 bp). The bar above indicates the location of the major palindrome of this DNA. The arrow indicates the position of the loop in the cruciform structure.

4. DISCUSSION

The data in fig.2 present a complete picture of the modification of pAO3 ccDNA, reflecting its early melting pattern. The process starts with the formation of two short segments, about 20 bp long, the melted regions then expanding to about 80 bp at a later stage. Meanwhile, it follows from our earlier estimates based on gradient gel electrophoresis data [4] that the size of the denatured region is about 90 bp under complete topological relaxation. This value is close to that cited earlier for 60% denaturants, when the complete denaturation of topoisomers does not yet occur. This is one of the facts that warrant the use of DEPC as a probe in the study of ccDNA melting.

It is interesting to juxtapose the data on ccDNA melting with the distribution of AT pairs in this region of pAO3 DNA. Fig.3 shows a histogram of the AT content for the part of pAO3 DNA that contains its most AT-enriched regions and the palindromic sequence (see also [4]). Comparison of the histogram with the data in fig.2a–d shows that the sites of denaturation correspond to the most AT-enriched regions of pAO3 DNA. However, the melting of ccDNA does not start with the most AT-enriched regions but, rather, with two short melted segments forming at the cruciform borders (see fig.2b). The segment on the left is in a very AT-rich area, and subsequently its size grows. Meanwhile, the segment on the right, which has a slightly high GC content, subsequently renatures along with the disappearance of the cruciform structure. These data demonstrate that the complex pattern of pAO3 DNA early melting is determined by the presence of the cruciform structure, while its disappearance changes the melting topography.

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