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Chemical probing of the homopurine · homopyrimidine tract in supercoiled DNA at single-nucleotide resolution

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Local structure of the homopurine · homopyrimidine tract in a supercoiled plasmid pEJ4 was studied using chemical probes at single-nucleotide resolution. The conformation of the homopyrimidine strand was probed by osmium tetroxide, pyridine (Os,py) while that of the homopurine strand was tested by diethyl pyrocarbonate (DEPC), i.e. by probes reacting preferentially with single-stranded DNA. At weakly acidic pH values, a strong Os,py attack on three nucleotides at the centre of the $(dC\text{-}dT)_{16}$ block and a weaker attack on two nucleotides at the end of the block were observed. DEPC modified adenines in the 5'-half of the homopurine strand. Os,py modification at the centre of the block corresponded to the loop of the hairpin formed by the homopyrimidine tract, while DEPC modification corresponded to the unstructured half of the homopurine strand in the model of protonated triplex H form of DNA.

DNA cleavage; Chemical modification; Supercoiled DNA; Homopurine · homopyrimidine tract

1. INTRODUCTION

The problem of the structure of homopurine · homopyrimidine tracts in supercoiled DNAs has attracted considerable attention in recent years [1–13]. On the basis of the results of 2D gel electrophoresis we have proposed a model of a protonated triplex H form [14] in which the homopyrimidine strand forms a hairpin, while half of the homopurine strand interacts with the hairpin to form a triplex (fig.1); the other half of the homopurine strand is unstructured. Quite recently we used chemical probes of the DNA structure [15] osmium tetroxide, pyridine (Os,py), specific for the homopyrimidine strand, and glyoxal, specific for the homopurine strand, to verify the proposed H structure [14]. Our studies of the recombinant plasmid pEJ4 (containing the 60-bp long homopurine · homopyrimidine tract from sea urchin *P. miliaris* histone gene spacer) showed that the chemical probes recognize an unusual protonated structure containing unpaired bases or non-Watson-Crick base pairs. At pH 5.6 the site-specific chemical modification occurred at or close to the middle of the homopurine · homopyrimidine tract, suggesting that a hairpin may be involved in the unusual structure. We used nuclease S1 to recognize and cleave regions made permanently single-stranded due to the chemical modification, i.e. the technique which was successfully applied in the osmium tetroxide probing of cruciform structures [16,17], B-Z junctions [18,19] and other unusual local DNA structures [20,21]. The mechanism of action of nuclease S1 is, however, not known in detail, and it has been shown [19,23] that this enzyme does not always recognize one or a few chemically modified nucleotides.

Recently another technique has been applied to cleave DNA at the site of the osmium modification [21–24] in which the enzymatic digestion is replaced by cleavage with hot piperidine and nucleo-

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tide sequencing is applied to recognize the osmium binding sites at nucleotide resolution.

To obtain a more detailed picture of the exposure of bases to the chemical probes we have applied this technique for the study of properties of the homopurine·homopyrimidine tract in the supercoiled pEJ4 plasmid. As a method for chemical cleavage of the DNA strand at the glyoxal-modified site is not available we have used in this paper in addition to Os,py another DNA probe [22,25–27], i.e. diethylpyrocarbonate (DEPC), specific for the homopurine strand, which has been shown to destabilize the DNA strand at the modification site, making it cleavable with hot piperidine [25]. The modification pattern we obtained with Os,py and DEPC at single-nucleotide resolution is in a very good agreement with the H form of DNA suggested earlier [14].

2. MATERIALS AND METHODS

The construction of recombinant plasmid pEJ4 (fig.2) carrying the histone gene unit h22 of sea urchin *P. miliaris* has been described [13]. DNA was isolated by the boiling method as previously described [28].

2.1. *Chemical modification of DNA*

The reaction was performed with 20 μg of supercoiled pEJ4 DNA and 1 mM OsO_4 (Fisher Scientific Co.), 2% pyridine (v/v), either in 100 mM Na-citrate buffer (pH 5.5) or in 10 mM Tris-HCl buffer (pH 8.5), 1 mM EDTA, 100 mM NaCl in a final reaction volume of 100 μl, 15 min at 26°C. The reaction was terminated by ethanol precipitation. More details are given in [15].

The reaction mixture for DEPC modification, consisting of 3–5 μg plasmid DNA, 2 μl of DEPC (Fluka), 50 mM Na-acetate buffer (pH 4.8), 1 mM EDTA in a final volume of 200 μl, was incubated at 25°C. The reaction was terminated by ethanol precipitation.

2.2. *Mapping of osmium binding sites*

After treatment with Os,py, DNA was linearized with *Hin*dIII. The 5′-ends were labelled with polynucleotide kinase [γ-^{32}P]ATP, DNA cleaved with *Eco*RI and loaded on 5% polyacrylamide gel. After the electrophoresis, the *Eco*RI-*Hin*dIII fragment was recovered, dissolved in 100 μl of 1 M piperidine, and incubated at 90°C for 30 min. Piperidine was evaporated under vacuum and the DNA was dissolved in formamide and loaded on the sequencing 6% polyacrylamide gel containing 7 M urea. To obtain the reference 'ladder' the uniquely labelled *Hin*dIII-*Eco*RI fragment of pEJ4 DNA was treated in accordance with the Maxam-Gilbert protocol [29] with the modification of Churpilo and Kravchenko [30] for cytosines, adenines and guanines. The gels were autoradiographed at −70°C using Orwo RX film.

2.3. *Mapping of DEPC binding sites*

After the reaction with DEPC DNA was digested by *Hin*dIII and 3′-end labelled. The following procedure was basically the same as with the osmium-labelled fragment.

3. RESULTS AND DISCUSSION

3.1. *Probing of the homopyrimidine tract with osmium tetroxide*

Plasmid pEJ4 at native superhelical density was treated with 1 mM OsO_4, 2% pyridine in 100 mM sodium citrate, pH 5.5, i.e. under conditions where the homopurine·homopyrimidine tract assumes an unusual structure [14,15]. Treatment of this plasmid with Os,py at pH 8.5 (i.e. under conditions unfavourable for the formation of the DNA H form) was used as a control.

The *Hin*dIII-*Eco*RI fragment (fig.2) was cleaved at the point of osmium modification by incubation with 1 M piperidine at 90°C. The sequencing gel is shown in fig.3. Modification of supercoiled pEJ4 DNA at pH 5.5 resulted in the appearance of two distinct modification regions (fig.3, lane 2): a strong one at the centre of the homopyrimidine tract and a weaker one at the tract boundary. The results of densitometric scanning of the sequencing gel in fig.2 show differences in the intensities of the bands, suggesting that the strongest attack of Os,py occurs at thymine 15. The neighbouring thymine bands are slightly less intensive. The intensities of bands of thymine 19 and 21 in the central region are about the same as those of thymines 794 and 795 at the boundary of the tract.

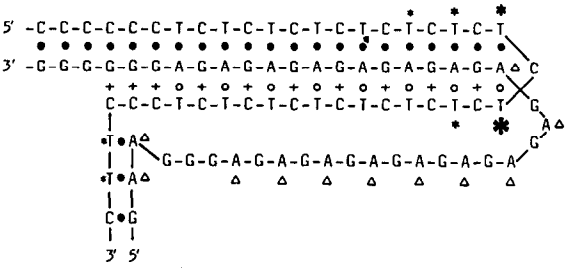

Fig.1. One of the two possible 'isomeric' forms which may exist in the structure of the model of the H form proposed by Lyamichev et al. [14]. The major element of the structure is the triple helix, which includes the Watson-Crick (•) duplex associated with the homopyrimidine strand by Hoogsteen base pairing (○,+) where the cytosines are protonated. The asterisks denote osmium modified thymine residues found in the 60-bp long homopurine·homopyrimidine tract of plasmid pEJ4 at acid pH. The open triangles denote adenines after reaction with DEPC.

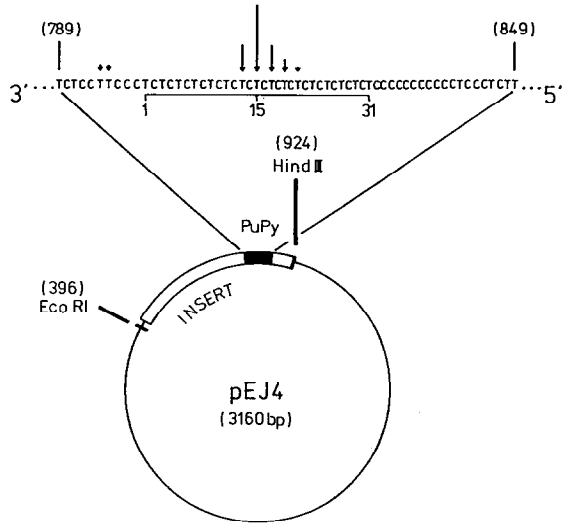

Fig.2. Map of the plasmid pEJ4 (a recombinant 3160 bp plasmid that contains a 509-bp long fragment of sea urchin *P. miliaris* histone gene spacer region, cloned in the polylinker of the pUC19 vector [13], showing the nucleotide sequence of the 60-bp long homopurine·homopyrimidine tract in the insert; relative positions of the targets of restriction endonucleases used in the study are also indicated. The arrows denote the Os,py-hypersensitive sites in the homopyrimidine strand in pEJ4 DNA at native superhelical density in 100 mM sodium citrate, pH 5.5, at 26°C. The lengths of the vertical arrows represent the relative intensities of the bands on the sequencing gel (fig.3) obtained by densitometric scanning. The homopyrimidine sequence starts at position 789 and ends at position 849. The $(dC\text{-}dT)_{16}$ block within this sequence is underlined; the first thymine (T1) in this block corresponds to the position 799 of the whole molecule and the last one (T31) to that of 829.

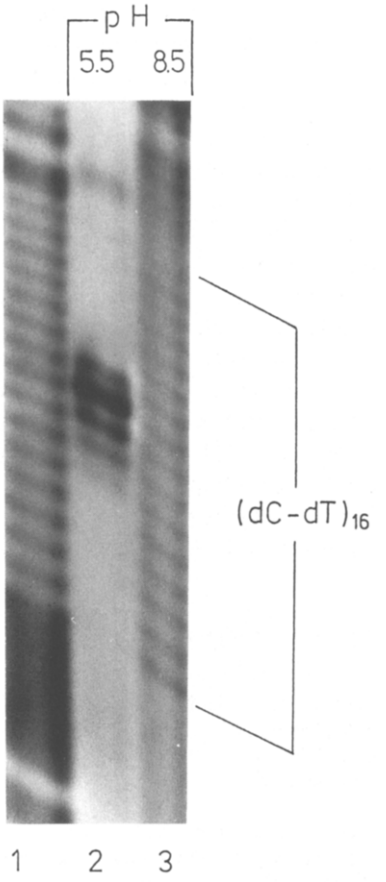

Fig.3. Mapping of the 'pyrimidine' strand by Os,py. Samples of supercoiled pEJ4 plasmid were reacted with 1 mM OsO_4, 2% pyridine, cleaved with *Hin*dIII and 5'-ends were labelled using polynucleotide kinase. After digestion with *Eco*RI, the *Eco*RI-*Hin*dIII fragment was recovered from acrylamide gel and treated with 1 M piperidine (30 min, 90°C), which was evaporated before loading of the sample on the sequencing gel. DNA modified in 100 mM sodium citrate, pH 5.5 (lane 2), and in 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.5 (lane 3). Lane 1 shows sequencing cytosine reactions of the OsO_4-unreacted *Eco*RI-*Hin*dIII fragment, labelled by polynucleotide kinase at the *Hin*dIII site. The bracket indicates the $(dC\text{-}dT)_{16}$ block. The 5'-end is at the bottom.

Modification of supercoiled pEJ4 DNA at pH 8.5 (fig.3, lane 3) produced uniform modification of thymine residues in the homopyrimidine tract, the bands being substantially weaker than those resulting from the modification at pH 5.5 in the central region (fig.3, lane 2).

The modification pattern obtained after treatment of supercoiled pEJ4 DNA with Os,py at pH 5.5 (fig.3, lane 2) is in excellent agreement with the spatial organization of the homopyrimidine strand in the model of DNA H form (figs 1,2). The strongest modification occurs in the loop of the hairpin (T13, T15 and T17), while the more weakly modified region might correspond to the junction between H and B form DNA.

The uniform modification of the $(dC\text{-}dT)_{16}$ segment at pH 8.5 (fig.3, lane 3) does not indicate that this segment assumes the usual B form under the given conditions. Further work will, however, be necessary to elucidate the question of local structures of homopurine·homopyrimidine tracts in supercoiled DNAs at neutral and slightly alkaline pH values.

3.2. *Probing of homopurine strand with DEPC*

Fig.4 shows the results of modification of the

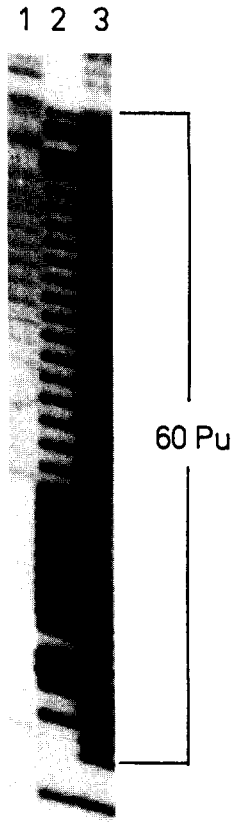

Fig.4. Mapping of the 'purine' strand by DEPC. Samples of supercoiled pEJ4 DNA were reacted with DEPC in 50 mM natrium acetate buffer, pH 4.8, 1 mM EDTA (lane 1), cleaved with *Hin*dIII and 3′-ends were labelled using Klenow fragment of DNA polymerase I. The *Eco*RI-*Hin*dIII fragment was treated as in fig.3. Guanine (G) and guanine + adenine (G + A) sequence reaction products of the DEPC-unreacted *Eco*RI-*Hin*dIII fragment are separated in lanes 2 and 3, respectively. The bracket denotes the 60-bp long homopurine·homopyrimidine tract. The 5′-end is at the top.

purine strand of $(dG\text{-}dA)_{16}$ stretch by DEPC. One can see that only the 5′-half of the purine strand is modified. This result is in excellent agreement with the proposed H form DNA [14], showing at the same time that of two possible isomeric forms of the H form the one with the triplex at its 3′-end predominates (fig.1). These data are consistent with results reported by Evans and Efstratiadis [8] and with our data on other plasmids [31]. The data in figs 1,3 and 4 also show that chemical modification is not restricted to the $(dG\text{-}dA)_{16}$ tract. In both cases some bases in the duplex adjacent to the single-stranded region are modified. This is most probably due to the presence of structural distortions at the junction between the H and B form detected also by Os,py (fig.3). It has been shown [26,27] that DEPC selectively reacts with bases in the cruciform loop without unwinding the stem or reacting with bases in the four-way junction. This result makes rather improbable the possibility that the weak modification of bases (fig.4) is induced secondarily due to the unwinding effect of DEPC. On the other hand the cruciform modification [26,27] was carried out at neutral pH, while in this paper we used slightly acidic pH to modify pEJ4 DNA. Basically the same is true of the results obtained with Os,py. Further work would thus be necessary to elucidate the question of the B-H junction.

4. CONCLUSION

The results obtained strongly support the model of H form DNA suggested [14] for homopurine·homopyrimidine tracts in supercoiled DNAs at slightly acid pH values. What is the biological role of the H form? Does this local structure exist in the cell? It now appears that it will be possible to attempt to answer such questions, since a technique for studying local changes in DNA structure of the cell has recently become available [32,33].

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