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Protonated triplex DNA in *E. coli* cells as detected by chemical probing

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The triplex structure in vitro is well established; however, no direct evidence has been available concerning its existence in the cell. Using the direct chemical probing here we show that the triplex H structure can exist in *E. coli* cells at acidic intracellular pH values; this structure differs in some details from that observed in vitro.

Osmium tetroxide; Triplex DNA; *E. coli*; H-DNA conformer; Homopurine–homopyrimidine sequence; Intracellular plasmid; Intracellular pH

1. INTRODUCTION

Chemical probing of some polypurine–polypyrimidine tracts in recombinant plasmids yielded direct and compelling evidence [5–11] in favour of the model of intramolecular protonated triplex H-DNA [1–4] proposed by Lyamichev et al. H triplex is stabilized in vitro by negative supercoiling and acidic pH; at neutral pH under specific conditions [9,13] a different type of triplexes was observed. H triplex introduces a curvature [8,12] or a hinge into the DNA molecule. H-DNA might be specifically involved in a number of processes in vivo, including transcription, recombination, mutational changes, chromatin assembly, etc. [1]. But does the triplex structures exist in the cell? So far little is known about the DNA structure in situ, mainly due to lack of suitable techniques. Recently we proposed a method [14–17] for the analysis of local DNA structures in cells. This method is based on a chemical probe, osmium tetroxide, 2,2′-bipyridine (Os,bipy), which penetrates into the bacterial cell without disturbing its integrity and site-specifically modifies DNA bases accessible to the probe. By means of this probe we brought direct evidence of the existence of left-handed DNA in the cell [14,15] based on the site-specific modification of the DNA B–Z junctions. Recently Rahmouni and Wells [18] used the same approach to show variation in supercoiling between different loci of recombinant plasmids derived from pBR322. We used Os,bipy to study the local structure of $(dA–dT)_n$ segments [15,16] and we found that these segments can extrude cruciform structures in *E. coli* cells under some conditions.

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2. EXPERIMENTAL

E. coli cells containing pL153 (Fig. 1) were grown in the usual way and after plasmid amplification with chloramphenicol the cells were harvested, resuspended in 0.1 M buffer of a desired pH (between 4.0–7.6) and preincubated in the given buffer for 60 min at 37°C. The cells were then treated with 2 mM Os,bipy at 37°C for 30 min (if not stated otherwise), washed and DNA was isolated [14] by the boiling method. DNA Os,bipy modification in vitro [5,7] and in situ [14] was performed as described. pL153 DNA was cleaved with *Tth*111I or *Asp*I, labelled with [α-^{32}P]dTTP using the Klenow fragment and treated according to the standard protocol [20]. Intracellular pH measurement: cells were incubated with 100 μM fluorescein diacetate and the fluorescence intensity at 520 nm after excitation at 435 and 490 nm was recorded with a Perkin-Elmer MPF 3 spectrofluorimeter as described [21].

3. RESULTS AND DISCUSSION

We have shown [5,7] that osmium tetroxide, pyridine site-specifically modifies the centre of the homopyrimidine strand of the supercoiled pEJ4 DNA in vitro at acidic pH values up to pH 6.0 while no such modification occurs at pH 7.8. The presence of the triplex H-DNA in pEJ4 in vitro is manifested by a strong modification in the centre of the $(dC–dT)_n$ sequence and a weaker modification at the triplex boundary [7]. Osmium tetroxide, pyridine is a powerful probe of the DNA structure in vitro; on the other hand its applicability for the studies in situ is limited since it induces early lysis in *E. coli* cells [17]. Modification of the supercoiled pL153 DNA with Os,bipy at pH 5.0 in vitro produced a similar pattern (Fig. 2, lane 2) with stronger modification at triplex boundaries. This pattern differs from those observed in any of the local supercoil-stabilized structures (e.g. cruciforms and B–Z junctions) so far studied [16,22] and is thus sufficiently characteristic to be used for triplex identification in *E. coli* cells.

The results for cells containing pL153 which were

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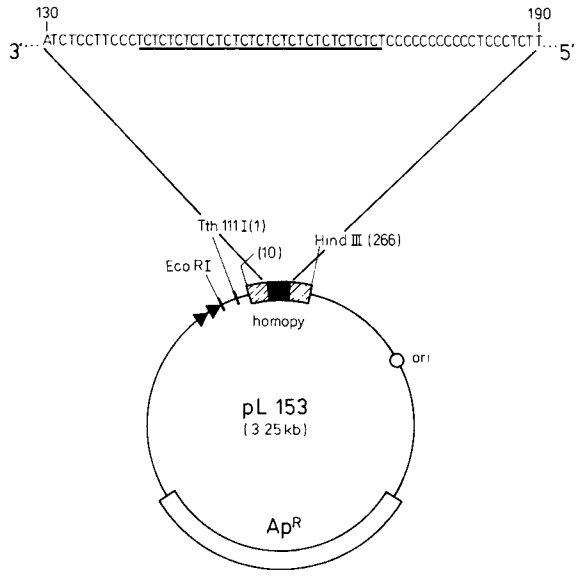

Fig. 1. Map of the plasmid pL153 containing a 256 bp-long fragment of the sea urchin *P. miliaris* histone gene spacer region recloned from pEJ4 [4] into pSP65CS [19] by ligating 265 bp *Asn*I-*Hin*dIII fragment (with filled-in *Asn*I site) into *Sma*I and *Hin*dIII cleaved pSP65CS.

treated with Os,bipy at pH 4.5, 5.0 and 7.6 are shown in Fig. 2. Treatment at pH 4.5 and 5.0 (lanes 1,4) resulted in patterns with strong modification in the centre of the homopyrimidine tract and a weaker one at the tract boundary, i.e. a pattern characteristic of triplex modification in vitro (lane 2). No such pattern was observed due to the cell treatment at pH 7.6 (lane 3); this treatment resulted in a weak, evenly distributed modification of thymines in the $(C\text{-}T)_{16}$ tract in good agreement with the result obtained in vitro with pEJ4 at pH 8.5 [7]. In this experiment cells were treated with Os,bipy for a relatively long period of time (30 min) to ensure modification of all available triplexes in the intracellular plasmids. However, further experiments showed no dependence of the modification patterns on time (Fig. 3, lanes 10–14) in the range of 5–60 min. This result shows that triplex modification in the cell produces a rapid snapshot of the DNA structure close to the situation in vivo. In vitro, at normal bacterial superhelix density, H-DNA was observed up to pH 6.0 [5]. In *E. coli* cells we observed modification patterns characteristic of H-DNA up to an external pH of 5.2 (Fig. 2, lanes 1,4 and Fig. 3, lanes 2,3) but not at pH 5.4 (Fig. 3, lane 4). Under the given conditions the intracellular pH (pH_{in}) did not differ very much from the external pH values; in 0.1 M sodium phosphate, pH 7.6, the pH_{in} remained neutral and in 0.1 M sodium acetate, pH 5.0 and 5.2, the pH_{in} was about 5.5 and 5.7, respectively. Thus at these external pH values the pH_{in} was well within the pH range where the triplex is formed in vitro. Our results thus suggest that the triplex structure can exist in *E. coli* cells depending on the intracellular pH.

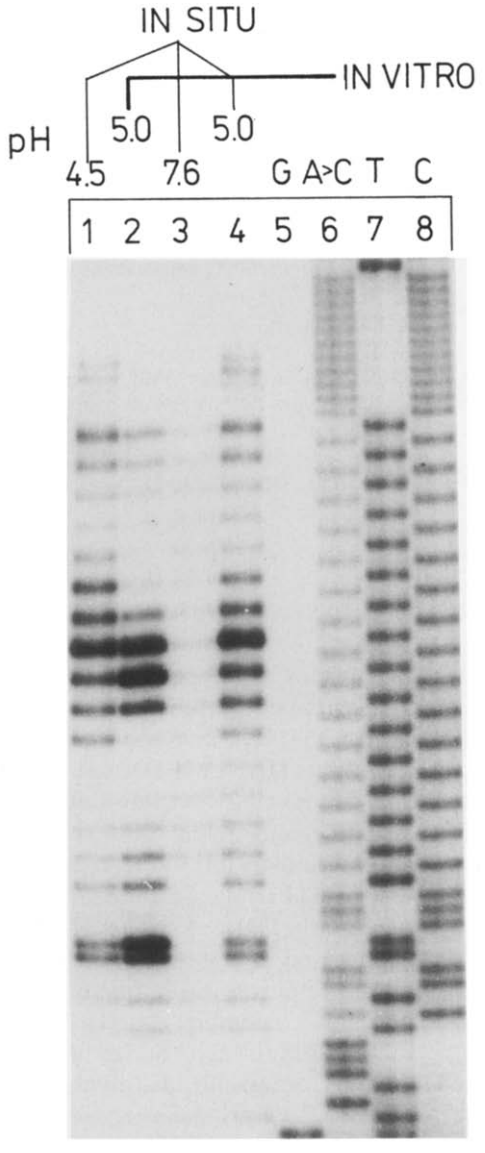

Fig. 2. Osmium binding sites in the homopurine–homopyrimidine region of pL153. *E. coli* JM109 (pL153) treated with Os,bipy at pH 4.5 (lane 1), pH 5.0 (lane 4) and pH 7.6 (lane 3). pL153 DNA modified in vitro at pH 5.0 (lane 2).

The pattern of DNA modified in vitro at pH 5.0 (Fig. 2, lane 2) differed in details from those modified at the acidic external pH in situ (Fig. 2, lane 4). While in situ the strongest attack of Os,bipy was at T157 (Figs 1–4), in vitro modification resulted in the strongest attack on T155 and on slightly less intensive T157. Around these two thymines there were two additional weaker modifications at T153 and T159. On the other hand in the samples modified in situ weak modification included not only T153 and T159 but also T151 and T161 (Fig. 2, lanes 1,4 and Fig. 3, lanes 2,3). Differences between modification in vitro and in situ were observed not only in the triplex loop (Fig. 4), but also at its junctions. T136 and T137 at the 3'-end of the homopyrimidine tract were more strongly modified in vitro (Fig. 2, lane

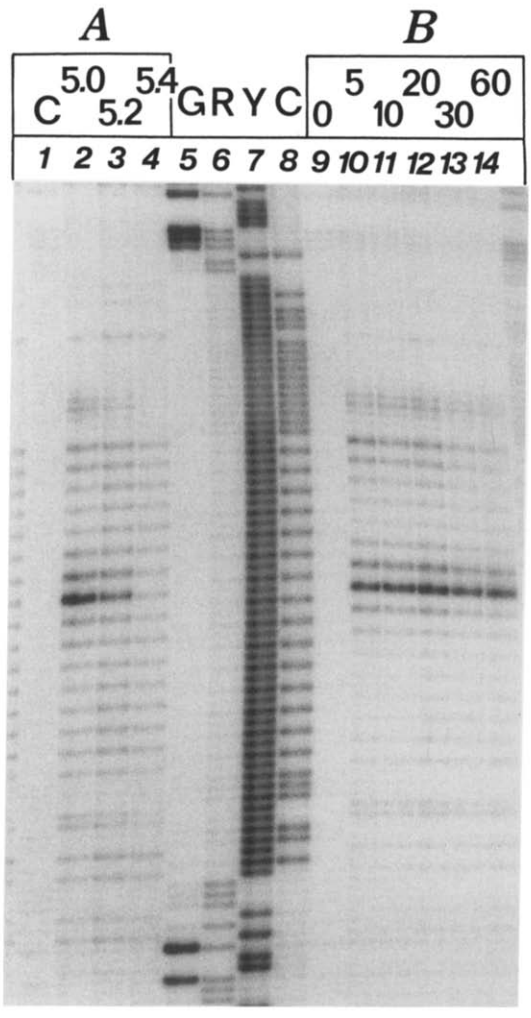

Fig. 3. Osmium binding sites in the homopurine–homopyrimidine sequence of pL153 present in *E. coli* cells. (A) pH dependence: cells were treated with Os,bipy at pH 5.0 (lane 2), pH 5.2 (lane 3) and pH 5.4 (lane 4). Lane 1, unmodified control. (B) Time dependence: cells were treated with Os,bipy at pH 5.0 for 0 (lane 9), 5 (lane 10), 10 (lane 11), 20 (lane 12), 30 (lane 13) and 60 min (lane 14). R, purine; Y, pyrimidine.

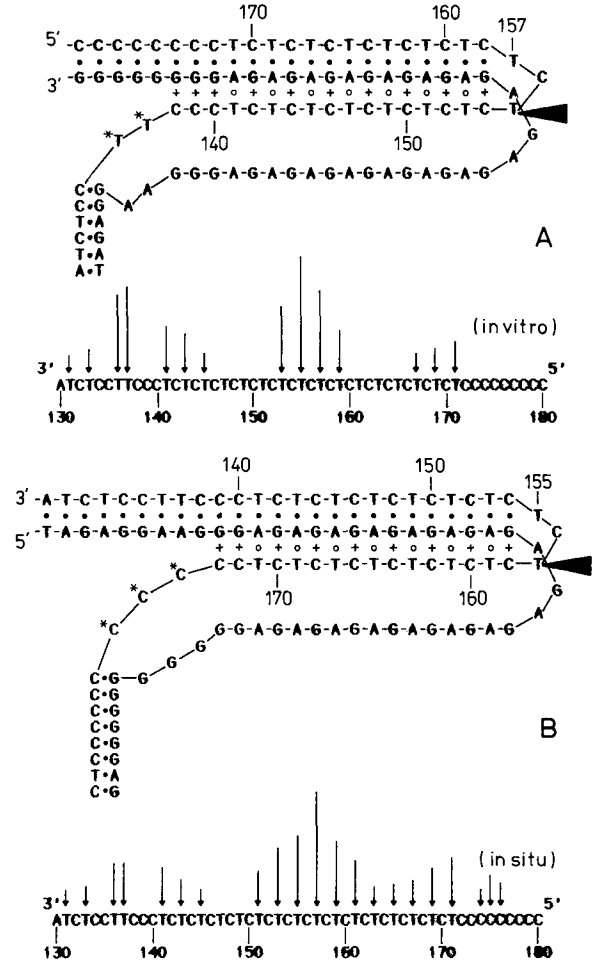

Fig. 4. (A) H-y3 and (B) H-y5 conformers [23] of the H-DNA triplex [4] and the Os,bipy modification of bases in the polymyrimidine tract of the pL153 insert (A), in vitro and (B), in situ. The lengths of the vertical arrows in the nucleotide sequence represent the relative intensities of the bands on the sequencing gel (Fig. 2) obtained by densitometric tracing, (A) after Os,bipy treatment of the supercoiled plasmid pL153 DNA in vitro (Fig. 2, lane 2) and (B) after treatment of cells with Os,bipy at external pH 5.0 (Fig. 2, lane 4). The main element of both H conformers is a triple helix [4] which includes the Watson-Crick duplex (.) associated with the homopyrimidine strand by Hoogsteen base pairing (o, +) where cytosines are protonated. The triangle shows the strongest modified base in the triplex and the asterisks denote the modification at the B–H junctions.

2) than in situ (Fig. 2, lanes 1,4 and Fig. 3, lanes 2,3). Three cytosines C174–176 at the 5′-end junction were in contrast more strongly modified in situ. These results suggest that the triplex structure in the cell might be somewhat perturbed with more thymines around the centre of the homopyrimidine tract available for the Os,bipy attack and that the duplex-triplex junction at the 5′-end is more open than in vitro while the junction at the 3′-end is less accessible to the Os,bipy probe in the cell than in vitro. At present it is not possible to give a definite explanation for the differences in the details of the triplex structure in vitro and in situ, but one can imagine that in the complex cell environment DNA interactions with other substances and structures may result in the triplex structure perturbations; in addition existence of DNA domains with different superhelical densities [18,24] might influence the triplex structure.

It was shown [23] that H-DNA can exist in vitro in two conformers (Fig. 4) and a strong bias toward the H-y3 conformer (in which the 3′ half of the polypyrimidine repeat is the donated region) was observed at native superhelical density. At less negative superhelical density the H-y5 conformer prevailed. Taking into consideration that the B–H junction at the 5′-end is strongly modified in the cell (Figs 2–4) (modification of this junction is characteristic of the H-y5 conformer in vitro [23,25]) it is tempting to suggest that in the cell the equilibrium might be shifted in favour of the H-y5 conformer. Such a shift could be induced by the less negative DNA superhelical density in

the cell which is supposed [26] to be roughly half of that observed in isolated plasmids. Further work will be necessary to obtain details of H triplex structure and its interactions in the cell.

Direct chemical probing of the DNA structure at single nucleotide resolution represents a powerful technique of research into the DNA structure in situ. Recently Sasse-Dwight and Gralla [27] have shown that potassium permanganate can also react with DNA inside the bacterial cell. The base specificity of this probe is unfortunately about the same as that of Os,bipy. For further development of the direct chemical probing of the DNA structure in situ it would be desirable to find new probes with different base specificities and structural perferencies; e.g. a probe reacting with purine bases might provide information about the structural details of the triplex homopurine strand in situ.

In our experiments the cells were treated with Os,bipy in 0.1 M buffers at various pH (Figs 2,3) and the H-DNA modification pattern was observed up to pH 5.2. These conditions cannot be considered fully physiological for *E. coli* but at pH 5.0 [28] and pH 5.2 the cells grew. Transfer of *E. coli* from pH 6.9 to pH 4.3 resulted in an induction of acid shock proteins but no such induction occurred due to the transfer of cultures to pH 5.0, and no significant decrease of viability was observed. Considering the fact that treating cells for 5 min with Os,bipy was sufficient to obtain a modification pattern characteristic of H-DNA (Fig. 3, lane 10) we believe that this pattern may represent an indication that the H triplex DNA can exist in living cells. The recent results of indirect immunofluorescence microscopy [29,30] with acid-fixed and -unfixed cells, isolated mouse chromosomes suggested that the triplex structures might be an inherent feature of eukaryotic DNAs.

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