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Analysis of a curved DNA constructed from alternating dA_n:dT_n-tracts in linear and supercoiled form by high resolution chemical probing

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Abstract

Complex of osmium tetroxide and bipyridine (Os,bipy), $KMnO_4$, and diethyl pyrocarbonate (DEPC) were used to probe curved DNA at single nucleotide resolution. The DNA was constructed from repeated dA_n:dT_n-blocks with dATATA and dAGAGA interblock sequences. The DNA was probed in the linear and supercoiled form at various salt concentrations. While all purines were available for DEPC attack, the thymines within the blocks were resistant to chemical probing by $KMnO_4$ and Os,bipy. Only the 3′-flanking dTs were available for modification. The thymines within dTC and dTA sequences showed modification indicating that these thymines display an unstacked structure allowing both probes to attack. Under destabilizing conditions, at low ionic strength and superhelical stress, considerable unstacking was observed. We found experimental indications that under these destabilizing conditions unpaired regions might appear, probably within the dATATA sequence.

Keywords: Curved DNA; dA_n-tracts; Chemical probing

1. Introduction

The structural polymorphism of DNA contributes to many regulatory processes [1,2]. The homogeneous B-form DNA structure is modified by elements of site-directed curvature and flexibility which contribute substantially to the binding affinity and specificity of some regulatory and other proteins.

Although the nature of DNA curvature has been extensively investigated, open questions remain. Macroscopic DNA curvature has been primarily associated with the presence of properly phased stretches of adenines (dA_n-tracts, [3]).

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Short (4–9 bp) runs of deoxyadenines repeated with the helix screw (10.5 bp) produce a global curvature of the DNA double helix (for review see [4–8]). However, also non-dA_n sequence elements cause DNA curvature [9,10].

Experimental evidence has been accumulated to explain the behaviour of dA_n-tracts. NMR studies on double stranded oligomers containing repeated dA_n blocks have shown that the DNA adopts the B-form, however, local conformational deviations exist [11,12]. Distinctive properties of poly(dA).poly(dT) indicate that it has an unusual 'B′-form' structure slightly different from standard B-DNA. Its helical repeat 10.0 bp per turn in solution is smaller than that for standard DNA (10.5 bp/turn; [13,14]). The B′-form structure is formed by dA_n:dT_n-blocks when $n \geq 4$ [15,16]. Essential for the formation of the B′-form is the absence of a large exocyclic group (e.g. an NH_2 group) at the purine 2 position [17]. The dA:dT basepairs fold into the minor groove at position 2 of the purine to balance for the NH_2 groups at purine position 6 [17]. A major feature distinguishing this B′-form structure from a common B-form is a high propeller twist maximizing purine-purine stacking interactions and narrowing the minor groove [18–20]. Structural models for homopolymeric $(dA)_n$.$(dT)_n$ sequences have been developed [21,22] in which the minor groove contains a spine of hydration bridging bases of the opposite strands [21]. The presence of bifurcated hydrogen bonds [20] and pyrimidine methyl groups [23] are not essential for DNA curvature [24]. It is assumed that basepair stacking (mainly purine-purine) is the dominant driving force for the formation of the B′-form structure [17,20,22,24]. The stacking of the dA_n:dT_n-blocks is rather stable [25]. The B′-form structure is sufficiently different from B-form DNA that proteins recognize the difference [26–28].

The structure of dA_n-tracts and the molecular origin of their curvature is under intensive discussion (for review see ref. [29]). The degree of curvature is not substantially influenced by the flanking sequence elements in agreement with the curvature model of Haran et al. [30]. Also the unique hydration pattern of dA_n blocks is in agreement with the bending within the dA_n-tracts [31,32]. On the contrary, crystallographic data did not reveal bends within dA_n sequences of studied oligomers [33]. Thus these authors attributed the curvature to non-dA-tract sequences [33]. To resolve this controversy new experiments are necessary.

A specific B′ structure of dA_n:dT_n-blocks as well as distortions at the boundaries to common B-DNA can be detected by chemical structural probes. Chemical probing ($KMnO_4$, osmium tetroxide, pyridine complex (Os,py), hydroxyl radical and DEPC) of dA_n:dT_n-tracts has shown a characteristic pattern of modification on a single-nucleotide level within plasmid and kinetoplast DNA [34–38].

In this paper we have used thymine-specific (Os,bipy and $KMnO_4$) and purine-specific (DEPC) chemical probes to study the structure of curved DNA segment (due to repeated dA_n:dT_n-blocks) at single nucleotide resolution. In linear DNA we could see substantial Os,bipy and $KMnO_4$ modification of thymines within dATATA, dTATAT and dTCTCT sequences and the majority of 3′-terminal thymines of dT_n-tracts, while all other dT_n-block thymines resisted this probing attack. On the contrary, DEPC modification of dATATAs was weak compared to the strong reactivity with dA_n-block adenines (with the exception of the 3′-terminal dA) and dAGAGA purines. In supercoiled DNA we observed increased intensity of the probing pattern without major qualitative deviations from the pattern found in linear DNA. Substantial change has been noticed in superhelical DNA at low ionic strength. Under these conditions we found increased signals from dTATAT purines (by DEPC) and all dT_n-tract thymines (by $KMnO_4$ only).

2. Materials and methods

2.1. Plasmid DNA

pK5/6T217 is a recombinant 2328 bp derivative of pJW200 which contains an 80-bp insert at the *Cla*I site [15,39]. The plasmid pJW200 was constructed out of pBR322 by the deletion of the sequence in between two *Hae*II sites at 236 bp and 2352 bp [40]. The 80-bp insert is composed of

a repeated 21-bp-long $dATATAT_6AGAGAT_5$ segment [41]. All together it contains seven dA_n:dT_n-tracts (alternating $n = 5$ and $n = 6$) repeating with the turn of the double helix. The plasmid was isolated from chloramphenicol-amplified *Escherichia coli* K12 HB101 cells by detergent lysis [42] and two rounds of isopycnic centrifugation in CsCl in the presence of ethidium bromide. Recovered DNA was multiply extracted with cold butan-1-ol and extensively dialysed against 10 mM Tris·HCl buffer (pH 7.8), 0.1 mM EDTA. The DNA was sequenced as described in ref. [43]. We have found that an extra base pair appeared within the insert (see Fig. 2, 27th base pair from the left), probably due to recombination events that occurred in the host cells.

Samples of DNA of defined superhelical density (σ) were generated according to Singleton and Wells [44].

Linear DNA was prepared by cleavage of the plasmid with either *Hae*III (upper strand probing) or *Sau*96I (bottom strand probing). DNAs were purified by phenol plus phenol/chloroform extraction and ethanol precipitation.

2.2. Chemical probing reactions

Either supercoiled or linear DNAs (3 μg) were reacted in 100 μl total volume in either sodium cacodylate buffer (pH 7.4), EDTA (for probing by $KMnO_4$) or Tris·HCl buffer (pH 7.8), EDTA (for probing by either Os,bipy or DEPC) plus NaCl in indicated concentrations.

2.2.1. OsO_4 reactions

Chemical modification was performed by adding OsO_4 and 2,2′-bipyridine to a final concentration of 1.0 mM/1.5 mM (supercoiled DNA) or 2.0 mM/3.0 mM (linear DNA) for 1 h at 26°C. After reaction OsO_4 was removed by CCl_4 extraction, ethanol precipitation and repeated pellet rinsing with 80% ethanol.

2.2.2. $KMnO_4$ reactions

Reaction proceeded after addition of $KMnO_4$ solution to a final concentration of 0.5 mM (supercoiled DNA) or 1.0 mM (linear DNA) for 10 min at room temperature. The reaction was terminated by addition of 10 μl of 1.4 M 2-mercaptoethanol. The DNA was ethanol precipitated and rinsed with 80% ethanol repeatedly.

2.2.3. DEPC reactions

DNA was reacted with 3 μl of DEPC (Serva) at room temperature for 30 min, with occasional mixing. Reaction was stopped by a quick ethanol precipitation and repeated 80% ethanol rinsing.

2.3. Mapping of chemically modified nucleotide residues

Linear DNAs were cleaved with a second restrictase, the supercoiled DNA was digested with a pair of restrictases selected to obtain fragments with one protruding and one blunt end. Fragments were separated by gel electrophoresis and radioactively [3′-^{32}P] labelled with Klenow fragment (Promega). Purified labelled fragments were backbone cleaved at sites of chemical modification by reaction with 1 M piperidine (Sigma) at 90°C. After extensive lyophilisation the products were separated on a denaturing sequencing gel containing 7 M urea, loaded on the gel next to the chemical sequencing reactions [43] derived from unmodified DNA fragment. Radioactive DNA fragments were observed by autoradiography at −70°C with intensifier screens and quantified by laser densitometry (Molecular Dynamics) of autoradiograms (see Fig. 1A). Baselines were calculated from the graphs (see Fig. 1B). Peak integrals (baseline subtracted) were calculated and used for analysis. For presentation, peak integrals were normalized to the peak of largest intensity in the given lane and sequence (set to 100%; see Fig. 2 and Fig. 4).

Each experiment was carried out several times (3–5 times). It is indicated in the figure legend when the data of several experiments were averaged.

3. Results

The strongly curved double-stranded DNA sequence 5′-d($ATATAT_6AGAGAAT_5ATATAT_6AGAGAT_5ATATAT_6AGAGAT_5ATATAT_6AGA$

GA)-3′ was cloned in the vector pJW200 yielding vector pK5/6T217. DNA fragments containing this sequence had been experimentally analyzed extensively in polyacrylamide gel electrophoresis [15] as well as by circularization [41]. The dT_5 and dT_6 blocks phase well with the helical repeat (10.5 bp) resulting in strong curvature [15,39,41]. This sequence has been sensitive to chemical probing (Os,py) under superhelical stress [25]. Its arrangement (repeated 21 bp long sequence) offers the particular advantage that the local chemical reactivity can be verified in the following repeats under identical sequence context conditions. This excludes local artefacts and increases considerably the data quality. This sequence arrangement allows us to study the properties of the dA_n:dT_n-blocks as well as of sequences of particular interest: alternating dAT and dAG stretches.

The DNA fragment is very AT-rich (90%) and easily destabilized by external conditions like low salt concentrations and superhelical stress. Thus, structural variations due to destabilizing conditions will appear in this insert first before other vector DNA is affected. This allows us to work at low chemical probe concentrations under single hit conditions. This is of importance when superhelical DNA is studied. The chemical probes give us direct access to nucleotide resolution which is required for the understanding of the sequence properties. The chemical probes we picked have the following properties.

Both $KMnO_4$ and complexes of osmium tetroxide attack the 5–6 double bond of thymine from above and below the base plane, hence reactivity does not require unpairing of the dA:dT base-pairs [45,46] and is probably reporting the degree of base stacking [47,48]. $KMnO_4$ and complexes of osmium tetroxide differ in the chemistry of their reaction with thymine and in the charge, size and shape of the molecule (the MnO_4^- anion is smaller than the bulky neutral Os,bipy complex). In our hands, $KMnO_4$ and Os,bipy were both strictly thymine-reactive in contrast to observations that these two compounds also react with cytosine or even guanine [34–37,49]. Instead of OsO_4 complexed with pyridine, we used Os,bipy and thus avoided modification bubble propagation which is observed in the presence of uncomplexed pyridine [50]. DEPC is the smallest probe and does not react very efficiently with double-stranded B-DNA [51]. In double-stranded DNA DEPC attacks both single-stranded and unstacked purines at the N_7 position of the purine ring [52–54] in contrast to DNA triple strands where the N_1 and N_3 atoms are attacked [55].

3.1. Specific base reactivity in linear DNA

The plasmid pK5/6T217 was linearized by restriction digest. The linear fragments were reacted with chemical probes under different buffer conditions and salt concentrations.

3.1.1. Os,bipy

The pattern of modification with Os,bipy did not depend on ionic strength from 5 mM Tris·HCl buffer (pH 7.8) up to 25 mM Tris·HCl buffer plus 0.1 M NaCl (see Fig. 1A) or even plus 0.5 M NaCl (data not shown). We did not detect modification of any cytosine or another base but thymine. Os,bipy preferentially attacked thymines of the dATATA, dTATAT and dTCTCT ('interblock') sequences in both DNA strands but hardly of the dTs within the dT_n-blocks (see Fig. 2). Within all interblock sequences of the bottom strand we noticed stronger modification of the first two 3′-end thymines and a weak modification of the third interblock thymine. In the upper strand we observed strong modification of both dATATA interblock thymines and a mild modification of the 3′-terminal thymine of the dT_5 and dT_6 blocks (see Fig. 2). In both strands some thymines in the vector regions 5′-adjoining the 80-bp insert were considerably modified.

3.1.2. $KMnO_4$

The pattern of $KMnO_4$ probing had similar features as that of Os,bipy but it differed in a few important points (see Fig. 2). First of all, the modification was strongly dependent on ionic strength. It gradually increased from no modification at 5 mM cacodylate buffer, pH 7.4 (data not shown) to the degree of modification similar to that with Os,bipy at 25 mM cacodylate buffer plus 0.5 M NaCl (see Fig. 2). We observed further increase in chemical modification with increasing

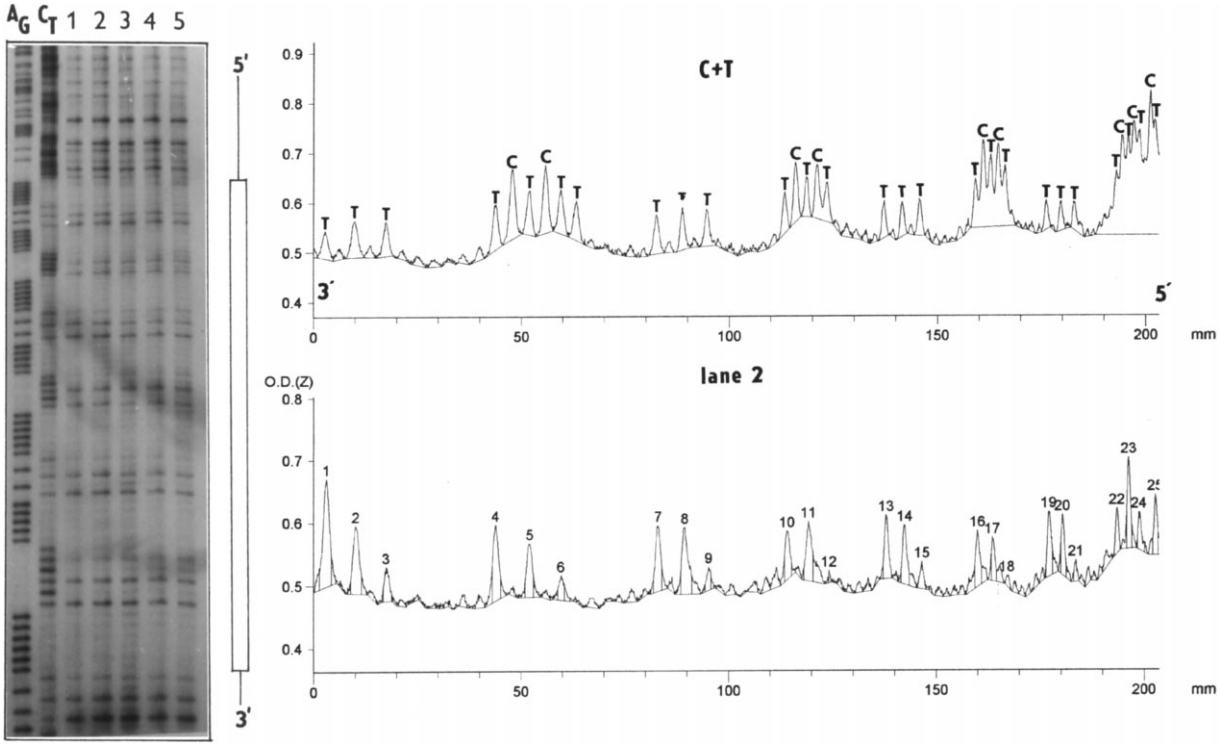

Fig. 1. The effect of ionic strength on the pattern of Os,bipy probing of curved DNA sequence in a linear fragment of pK5/6T217 plasmid. Bottom strand. (A) DNA was linearized with *Sau*96I, modified with Os,bipy in 1 mM EDTA and Tris·HCl (pH 7.8) buffer, plus NaCl in indicated concentrations: 5 mM (lane 1), 10 mM (lane 2), 25 mM (lane 3), 25 mM plus 25 mM (lane 4), and 25 mM plus 0.1 M (lane 5), respectively. Modified DNA was cleaved with *Rsa*I, the purified fragment was radioactively end-labelled, cleaved with hot piperidine and resolved on a sequencing gel together with A + G, and C + T sequencing reactions [43], respectively. The dried gel was subjected to autoradiography. The rectangle indicates the extent of the curvature-inducing segment. (B) Working laser densitometric tracing of the C + T sequencing reaction and lane 2, respectively, as used in further processing. The intensity peaks are numbered.

NaCl concentration up to 3.9 M (data not shown). The ionic strength dependence of $KMnO_4$ modification is documented in Fig. 3. This figure displays the modification of the 3′-end thymines of all dT_6-blocks in the ionic strength range from 5 mM cacodylate buffer (pH 7.4) up to 25 mM cacodylate buffer plus 0.5 M NaCl.

In the upper strand 3′-end thymine modification of the dT_5-blocks was similar to that of thymines in dATATA interblock. This similarity is in contrast to the Os,bipy modification pattern. However in contrast to the dT_5-blocks, the 3′-end thymines in all dT_6-blocks (in front of the purine dAGAGA interblock) were very strongly modified (4–5 times more than any other thymine, see Fig. 2).

In the bottom strand there was nearly homogeneous modification within all interblock sequences with the exception of the bordering sequences, which exerted substantially higher modification than the more internal dTATAT and dTCTCT motifs. This modification pattern agrees with that of Os,bipy. In general, we observed remarkable resistance of four and five 5′-thymines of the dT_5- and dT_6-blocks against the chemical attack by $KMnO_4$ and Os,bipy indicating stable stacking within the blocks.

3.1.3. DEPC

The purine-specific probe DEPC modified all adenines in the bottom strand (see Fig. 2). The insert does not contain dGs in the bottom strand.

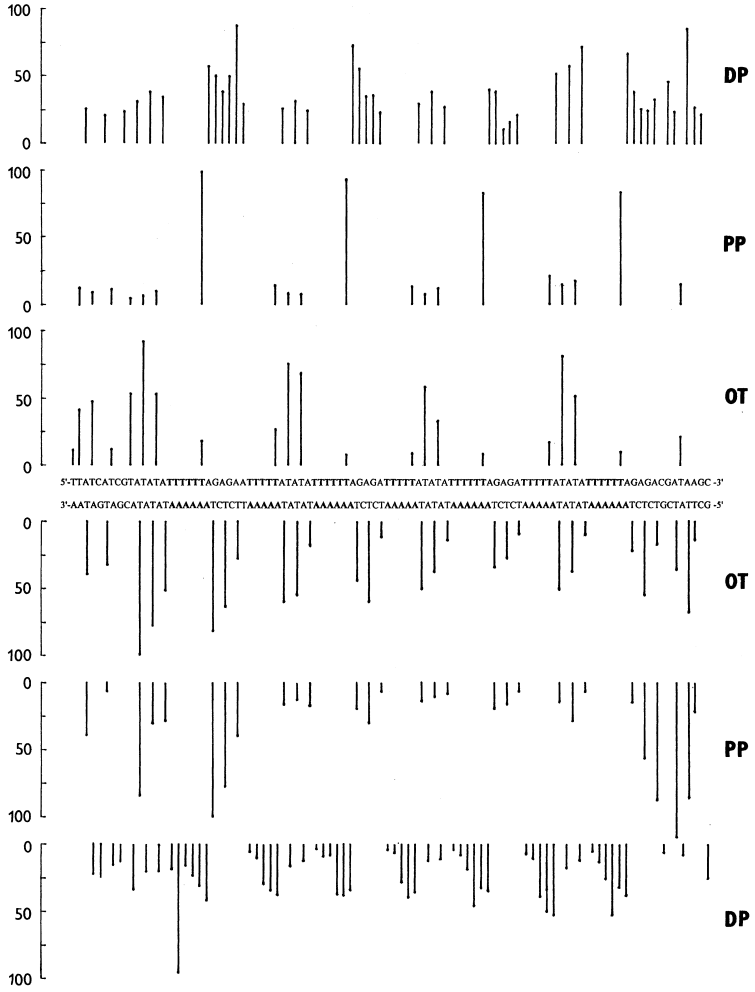

Fig. 2. Quantitative comparison of Os,bipy (OT), $KMnO_4$ (PP), and diethyl pyrocarbonate (DP) modification of linear DNA fragments (275 and 274 bp, respectively) of pK5/6T217 plasmid within its curved DNA sequence in 25 mM buffer (Tris–HCl for OT and DP, cacodylate for PP), 1 mM EDTA, 0.5 M NaCl. DNA was linearized with *Hae*III (upper strand), or *Sau*96I (bottom strand); modified with the probe of choice and cleaved with *Ban*I (upper strand) and *Rsa*I (bottom strand), respectively. Purified fragments were radioactively end-labelled, cleaved with hot piperidine and resolved on sequencing gels. The dried gels were subjected to autoradiography and quantified by laser densitometry. The baseline was determined (see Fig. 1B) and subtracted. Intensity of modification within each strand with any probe has been normalized against the most intensively modified base per lane and the results of at least three normalized experiments have been averaged and illustrated (standard error within the values is $\leq 10\%$). dA_n-tracts within the sequence are highlighted in bold.

The first three (for dA_5) or four (for dA_6) 5′-end adenines in the dA_n-blocks were modified about 3- to 4-fold stronger than the following two adenines in the blocks. These two 3′-end adenines were as weakly modified as the interblock dAs in dTATAT. All purines were modified in the upper strand. The modification pattern showed roughly homogeneous adenine modification in dATATA interblocks and heterogeneous modification of purines in dAGAGA. In dAGAGA strong modification was observed for the 5′-end adenine with a significant decrease towards the 3′-end dAs.

DEPC modification of both strands was independent of ionic strength in the range from 5 mM

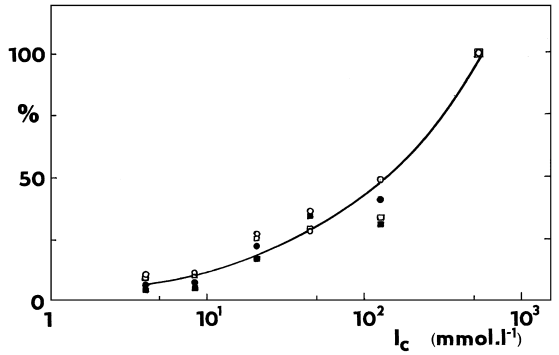

Fig. 3. Ionic strength dependence of the $KMnO_4$ modification intensity of the curved DNA sequence (upper strand) as part of a linear DNA fragment (275 bp) of the pK5/6T217 plasmid. DNA was linearized with *Hae*III, modified with $KMnO_4$ in 1 mM EDTA and 5 mM, 10 mM, 25 mM cacodylate buffer (pH 7.4), or 25 mM cacodylate buffer plus 25 mM, 0.1 M, 0.5 M NaCl, respectively, and cleaved with *Ban*I. Purified fragments were radioactively end-labelled, cleaved with hot piperidine and resolved on a sequencing gel. The dried gels were subjected to autoradiography and quantified by laser densitometry. The baseline was determined and subtracted. Modification of 3′-terminal thymines of all four dT_6 blocks has been normalized against the modification at the highest ionic strength (25 mM buffer plus 0.5 M NaCl; 100%) in each dT_6-stretch. These thymines are depicted as ■, ●, □, ○, from 5′ to 3′ end (see Fig. 2), the ionic strength (I_c) is expressed in logarithmic scale.

Tris·HCl buffer up to 25 mM Tris·HCl buffer plus 0.5 M NaCl (data not shown).

3.2. The influence of superhelicity

To analyze the influence of superhelicity on the chemical modification we performed experiments with plasmids of native superhelicity (i.e. $\sigma = -0.055$) as well as more and less negative superhelicity. While the increase ($\sigma = -0.08$) of negative σ amplified modification, its decrease ($\sigma = -0.03$) suppressed modification quite substantially (data not shown). The intensity of the chemical modification of supercoiled DNA (at native σ) was substantially higher than that of the same DNA in the linearized state with both, Os,bipy and $KMnO_4$. Therefore, for experiments with supercoiled DNA we reduced the concentration of the chemical reagents Os,bipy and $KMnO_4$.

As for linear DNA, Os,bipy modification was nearly ionic strength independent within the middle and higher concentration range (i.e. from 25 mM Tris·HCl buffer (pH 7.8), 2.5 mM EDTA up to 25 mM Tris·HCl buffer (pH 7.8), 2.5 mM EDTA plus 0.5 M NaCl; data not shown). Only at low ionic strength (5 and 10 mM TE buffer (pH 7.8)) the intensity of modification was increased by up to one third.

In the whole concentration range measured the pattern of modification was very similar to that of linear DNA (see Fig. 4). The only difference appeared for the 3′-end thymines of the dT_6-blocks: a new modification appeared for the second to last 3′-end thymine (see Fig. 4). However, the intensity of the 3′-end thymines modification was substantially smaller compared to that of the dATATA stretches.

$KMnO_4$ probing showed a bimodal pattern of modification. At low ionic strength (5 and 10 mM cacodylate buffer (pH 7.4)) we observed strong modification of all thymines (with decreasing intensity from the 3′- to the 5′-end; Fig. 5, lanes 2, 3). With increasing buffer concentration (25 mM cacodylate; lane 4) the chemical modification decreased steeply, but at higher salt concentration (25 mM buffer plus 0.1 M NaCl) the modification gradually started to increase with ionic strength (Fig. 5, lane 6). Above 0.1 M NaCl the reactivity for the first 3′-thymine of the dT_6-tract became dominant (lane 7). The modification intensity was about constant from the 3′- to the 5′-end of the insert. The modification pattern at higher salt concentrations was similar to that of linear DNA.

Also with DEPC we observed a strong dependence of the modification on ionic strength when the DNA was supercoiled. We noticed intensive modification of all purines of the bottom strand. The highest modification was observed for dAs within the dTATAT interblocks and 5′-end adenines of $dA_{5,6}$-blocks at very low ionic strength (up to 10 mM TE buffer (pH 7.8); see Fig. 6, lanes 2, 3). The intensity of the modification within $dA_{5,6}$-blocks decreased from the 5′- to the 3′-end. With increasing ionic strength (at and above 25 mM TE buffer (pH 7.4)) the intensity of the modification dropped substantially and resumed the pattern typical for linear DNA – i.e. low modification within dTATATs, negligible

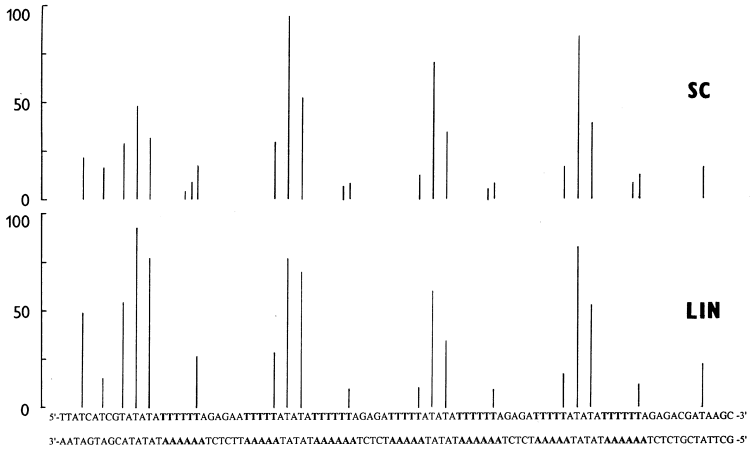

Fig. 4. Quantitative comparison of Os,bipy modification of linear (LIN) and native supercoiled (SC) pK5/6T217 plasmid within the curved DNA sequence. Results are shown only for the upper strand. Linearized (obtained by digestion with *Hae*III) and supercoiled DNA were modified with Os,bipy in 25 mM Tris·HCl buffer (pH 7.8), 2.5 mM EDTA plus 0.5 M NaCl. Modified supercoiled DNA was cleaved with *Hae*III and *Ban*I and further dealt with as in Fig. 1A (normalization and processing of the results as in Fig. 2). The results have been averaged of six (LIN) and four (SC) experiments, respectively. dA_n:dT_n-tracts within the sequence are highlighted in bold. Intensity of modification is expressed in relative values for each DNA form, with 100% for the most intensively modified base within the insert.

modification of the 3′-dAs and increasing modification towards the 5′-end of the $dA_{5,6}$-blocks (Fig. 6, lane 4). Further increase of the ionic strength (up to 25 mM TE buffer plus 0.5 M NaCl) exerted no significant effect on the pattern or the intensity of the chemical modification (see Fig. 6, lanes 5–7). The upper strand modification pattern showed a similar dependence on ionic strength, though less pronounced (data not shown).

The results obtained with $KMnO_4$ and DEPC at low ionic strength suggest the presence (partly and/or transiently) of single-stranded structures formed due to the high dAT content and negative supercoiling stress.

4. Discussion

Tracts of dA_n:dT_n ($n \geq 4$) adopt a B′-form structure different from standard B-DNA. dA_n:dT_n-tracts had been probed with chemical structural probes ($KMnO_4$, Os,py, DEPC, hydroxyl radical) within common B-DNA sequences in plasmids [34–36,38] or as a sequence element in kinetoplast DNAs [37]. Differences in nucleotide composition of flanking sequences conferred different sensitivity of the bases in dA_n:dT_n-tracts to the attack by chemical probes [34–37]. We analyzed a DNA sequence with two sequence motifs (dATATA and dAGAGA) flanking regularly repeated dA_5:dT_5- and dA_6:dT_6-blocks [15,41]. This sequence arrangement allowed us to compare the properties of dA_n:dT_n-blocks with those of other sequences of particular interest.

In the following we discuss and explain the observed modification pattern by the properties of both the DNA sequence and the chemical probes.

4.1. Linear DNA

In the linear plasmid analyzed here we observed strong $KMnO_4$ modification of the 3′-terminal thymines of dT_n blocks when followed by the 3′-flanking polypurine sequence (dAGAGA) and a low modification for the 3′-flanking dATATA motif. In both cases the Os,bipy modification of 3′-end thymine was small (see Fig. 2). The other dTs of dT_n-stretches showed considerable resistance to modification. The 5′-terminal dTs of the $dT_{5,6}$-blocks remained unmodified by both probes.

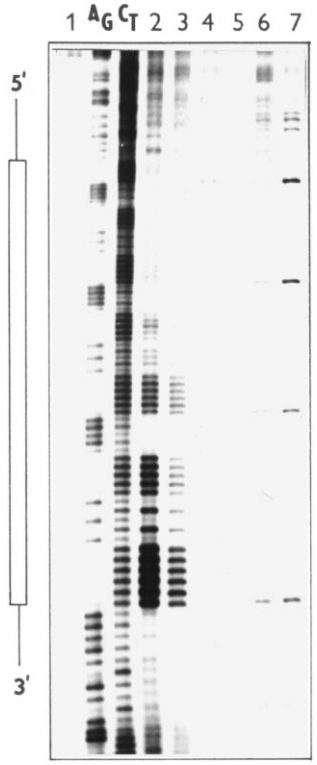

Fig. 5. The effect of ionic strength on the pattern of $KMnO_4$ probing of the curvature-prone sequence in the native supercoiled plasmid pK5/6T217. Upper strand. DNA was modified with DEPC in 1 mM EDTA and cacodylate buffer (pH 7.4) plus NaCl in concentrations as indicated: 5 mM (lane 2), 10 mM (lane 3), 25 mM (lane 4), 25 mM plus 25 mM (lane 5), 25 mM plus 0.1 M (lane 6), and 25 mM plus 0.5 M (lane 7), respectively. Control (unmodified) sample (lane 1) was incubated at the same ionic conditions as in lane 6. DNA was cleaved with *Sau*96I and *Rsa*I, and further dealt with as in Fig. 1A. The rectangle indicates the extent of the curvature-inducing segment.

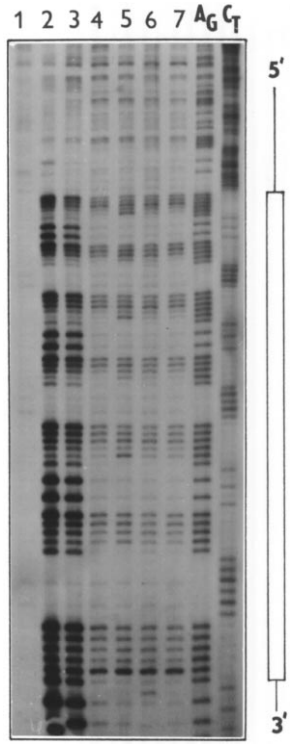

Fig. 6. The effect of ionic strength on the pattern of DEPC probing of the curvature-prone sequence in the native supercoiled plasmid pK5/6T217. Bottom strand. DNA was modified with DEPC in 1 mM EDTA and Tris·HCl buffer (pH 7.8) plus NaCl in concentrations as indicated: 5 mM (lane 2), 10 mM (lane 3), 25 mM (lane 4), 25 mM plus 25 mM (lane 5), 25 mM plus 0.1 M (lane 6), and 25 mM plus 0.5 M (lane 7), respectively. Control (unmodified) sample (lane 1) was incubated at the same ionic conditions as in lane 6. DNA was cleaved with *Hae*III and *Ban*I and further dealt with as in Fig. 1A. The rectangle indicates the extent of the curvature-inducing segment.

This is consistent with the finding [56] that the 3′-terminal thymine (flanked by an adenine) was the most flexible thymine in a dT_n-tract. Thus, most of the dTs in the blocks stack well with no access for both chemical probes. Obviously, at the 3′-end of the block the dT stacks on dA in a way which allows for the chemical attack by the anionic MnO_4^- and − to a much lesser extent − by the bulky Os,bipy. This might be an indication for a B′- to B-form junction structure which is particularly available for $KMnO_4$ attack when the polypyrimidine stretch dT_6 is followed by a polypurine stretch dAGAGA (see Fig. 7A). However, it might also display the stacking properties of alternating dTA and dTC sequences. The alternating sequences adopt stacking structures which allow for a chemical attack of the dTs by both probes.

The pattern and intensity comparison of the thymine modification within dAT-sequences outside and inside the inserted curved segment (see Figs. 1A, 2 and 4) indicates that the modification of interblock dATATA sequences is due to the partial unstacking of the dAT-sequences and that

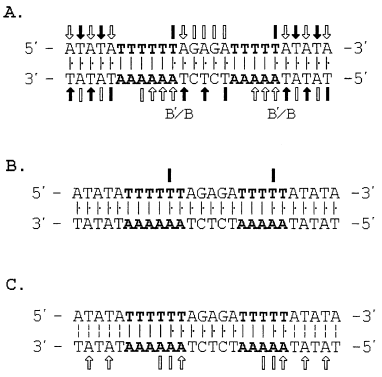

Fig. 7. A scheme of chemical probing within a repetitive motif of curved sequence in a linear pK5/6T217 plasmid (A) and the changes in the probing pattern due to superhelix stress (B) plus low ionic strength ($\leq$ 10 mM buffer; *c*). Vertical arrows and oblongs stand for strong and medium modification of the bases, with full (Os,bipy) and empty (DEPC) symbols, respectively. Interrupted bars (¦) denote substantially unstacked or partly unpaired bases, in contrast to moderately unstacked (|·), or normally paired (|) bases. (A) The structure of linear curved insert does not depend on ionic strength. Within the flanking dAGAGA and dATATA sequences unstacked regions appear as illustrated by strong Os,bipy (see also [58]) and DEPC probing (similarly with $KMnO_4$, not shown). The same is observed for 5′-terminal thymines of dT_n-tracts, well manifested at dTs 3′-flanked by dAGAGA, probed with $KMnO_4$ (Fig. 2). The rest of dT_n blocks stand stacked, suggesting the presence of B′ to B-DNA junctions (B′/B). (B) This scheme highlights only nucleotide modifications in addition to the probing pattern shown in the scheme (A). In supercoiled DNA at higher ionic strength, the structure is more accessible to probing attack, as compared to linear DNA but the pattern hardly changes. The only exception is the 3′-penultimate thymines in dT_n-tracts which are now moderately accessible to Os,bipy attack, suggesting further opening of these tracts at the 3′-end. (C) The scheme denotes only substantial increase in nucleotide modification in comparison to the scheme (B) pattern. Combined action of *supercoiling and low ionic strength* (below 10 mM buffer) leads to significant unstacking and/or partial unpairing of dATATA sequences (see also [59]) as detected by increased accessibility to DEPC attack. The dA_n blocks become more labile as illustrated by increased DEPC modification of 5′-end adenines. The same can be inferred from $KMnO_4$ probing of dT_n-tracts which is detected in all thymines (see Fig. 5). These structural changes are not large enough to be detectable by Os,bipy probing.

the modification is not strongly influenced by the presence of neighbouring dT_n-blocks.

The DEPC modification was strong within the $dA_{5,6}$-blocks of the inserted DNA (see Fig. 2), however, becoming weaker towards the 3′-ends. The DEPC modification pattern of dA_n-tracts in linear DNA (see Fig. 2) indicates that the B′-form structure of the adenines in $dA_{5,6}$-blocks is sufficiently open to allow for an attack by the smaller probe DEPC (see Fig. 7A).

DEPC probing of homopurine stretches either outside or within the inserted segment (see Fig. 2) suggests that the dAGAGA sequences are open to chemical attack, too.

DEPC reactivity of dA_n-tracts studied before showed that for most dA_n-blocks DEPC reacted significantly with all but the 3′-end adenine while some dA_n-blocks exerted the highest reactivity at the next to 3′-end adenine [34]. The observed decreasing DEPC modification within dA_n-tracts agrees with the hydroxyl radical reactivity results showing progressive decrease from the 5′ to the 3′ end in dA_n-blocks [38]. The reduced hydroxyl radical reactivity within dA_n-tracts was interpreted as being due to the narrow minor groove which is a structural property of the B′-form.

Our results obtained with Os,bipy and $KMnO_4$ agree with those found in other sequences. In naturally occurring dA_n:dT_n-tracts a similar pattern of modification was reported. dT_n-blocks (including the 3′-terminal thymine) displayed resistance while $d(TA)_n$ sequences showed hyperreactivity with the osmium probe in both linear and supercoiled plasmid [35]. Our $KMnO_4$ modification results agree with those of McCarthy et al. [34,37]. We observed good coincidence for the effect of flanking nucleotide(s) on the modification of the 3′-terminal thymine in the dT_n-blocks: McCarthy et al. [34,37] found substantial modification for a 3′-flanking polypurine tract ($dAGA_n$, [34]) in contrast to no modification with a framing guanine [37].

The strong chemical accessibility of the 3′-end thymine in the dT_6-block (dT followed by dAG) compared to the dT_5-block (dT followed by dAT) cannot be explained on a dinucleotide step level. Obviously, the B′–B-form junction is additionally influenced by the DNA block structure.

4.2. Ionic strength dependence in linear DNA

In contrast to DEPC and Os,bipy, modification with $KMnO_4$ exerted a strong dependence on

ionic strength (see Fig. 3). The relative differences of base specific $KMnO_4$ modification remained. The gradual increase of modification intensity (from nearly none at low buffer concentration) with increasing ionic strength (see Fig. 3) can be explained by the electrostatic repulsion between the polyanionic DNA and the anionic oxidant permanganate [57]. The higher NaCl concentration shields the repulsive phosphate groups (the Debye length is reduced) and thus facilitates an approach of the negatively charged MnO_4^- molecule to its target thymine 5–6 double bond.

The different probing qualities of $KMnO_4$ and Os,bipy make them a helpful couple for the structural analysis of DNA. The small probe permanganate can detect even minor distortions, however, since it carries the same charge as DNA, hardly at low ionic strength. Os,bipy being too bulky to detect minor distortions, can be used in a wide range of ionic conditions for a quantitative analysis, as its reactivity is ionic strength independent.

4.3. Supercoiled DNA

Low ionic strength, higher temperatures and negative supercoiling destabilizes the stacking and pairing of the bases and increases local distortions of the DNA. Especially at low ionic strength (5 and 10 mM TE, or cacodylate buffer), due to superhelix stress the unstacking of thymines within $dT_{5,6}$-stretches increased to an amount that all thymines became accessible to $KMnO_4$ attack (Fig. 5, lanes 2, 3) although this unstacking was still not detectable for the bulky Os,bipy complex (see Fig. 4). Under these conditions the small DEPC molecule induced a strong modification at the 5′-end adenine in all $dA_{5,6}$-blocks, decreasing towards the 3′-end of the block (see Fig. 6, lanes 2, 3). Strong modification of adenines within dTATAT sequences (the amount being similar to that of 5′-terminal adenine in $dA_{5,6}$-tracts) suggests substantial unstacking within these sequences due to the destabilizing conditions (see Fig. 7C. While within dTATAT sequences we assume unstacking, unwinding or even unpairing of bases under destabilizing conditions, our data indicate that superhelix stress at low ionic strength does not open the dA:dT base pairs in $dA_{5,6}$:$dT_{5,6}$-blocks. These tracts, though partly unstacked as indicated by $KMnO_4$ and DEPC probing, remain basepaired since they are resistant to Os,bipy probing (see Fig. 7C).

With increasing ionic strength (at 25 mM buffer and higher) all three probes displayed the behaviour we observed for linear DNA (see e.g. Fig. 6, lanes 4–7; Fig. 7B). This result suggests that the hypersensitive structure is induced due to the concerted action of superhelix strain and low ionic strength.

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References

[1] A.A. Travers, Annu. Rev. Biochem. 58 (1989) 427–452.
[2] R.E. Harrington, I. Winicow, in: W.E. Cohn, K. Moldave (Eds.), Progress in Nucleic Acids Research and Molecular Biology, vol. 47, Academic Press, San Diego, 1994, pp. 195–270.
[3] J.C. Marini, D.D. Levene, D.M. Crothers, P.T. Englund, Proc. Natl. Acad. Sci. USA 79 (1982) 7664–7668.
[4] S. Diekmann, in: F. Eckstein, D.M.J. Lilley (Eds.), Nucleic Acids and Molecular Biology, vol. 1, Springer, Berlin, 1987, pp. 138–156.
[5] D.M. Crothers, T.E. Haran, J.G. Nadeau, J. Biol. Chem. 265 (1990) 7093–7096.
[6] E. Paleček, Crit. Rev. Biochem. Mol. Biol. 26 (1991) 151–226.
[7] E. Trifonov, Trends Biochem. Sci. 16 (1991) 467–470.
[8] S. Diekmann, A.A. Travers, in: D.M.J. Lilley, H. Heumann, D. Suck (Eds.), Structural Tools for the Analysis of Protein-Nucleic Acid Complexes, Birkhauser Verlag, Basel, 1992, pp. 63–79.
[9] I. Brukner, S. Susic, M. Dlakic, A. Savic, S. Pongor, J. Mol. Biol. 236 (1994) 26–32.
[10] M. Dlakic, R.E. Harrington, J. Biol. Chem. 270 (1995) 29945–29952.
[11] A. Kintanar, R.E. Klevit, B.R. Reidl, Nucl. Acids Res. 15 (1987) 5845–5862.
[12] M. Katahira, H. Sugeta, Y. Kyogoku, S. Fujii, R. Fujisawa, K. Tomita, Nucl. Acids Res. 16 (1988) 8619–8632.
[13] L.J. Peck, J.C. Wang, Nature 292 (1981) 375–378.

[14] D. Rhodes, A. Klug, Nature 292 (1981) 378–380.
[15] S. Diekmann, Nucl. Acids Res. 15 (1987) 247–265.
[16] S. Diekmann, E.v. Kitzing, in: W.K. Olson, M.H. Sarma, R.H. Sarma, M. Sundaralingam (Eds.), Structure and Expression, vol. 3, Adenine Press, New York, 1988, pp. 57–67.
[17] S. Diekmann, E.v. Kitzing, L. McLaughlin, J. Ott, F. Eckstein, Proc. Natl. Acad. Sci. USA 84 (1987) 8257–8261.
[18] D.G. Alexeev, A.A. Lipanov, I.Y. Skuratovskii, Nature 325 (1987) 821–823.
[19] M. Coll, C.A. Frederick, A.H.J. Wang, A. Rich, Proc. Natl. Acad. Sci. USA 84 (1987) 8385–8389.
[20] H.C.M. Nelson, J.T. Finch, B.F. Luisi, A. Klug, Nature 330 (1987) 221–226.
[21] V.P. Chuprina, Nucl. Acids Res. 15 (1987) 293–311.
[22] E.v. Kitzing, S. Diekmann, Eur. Biophys. J. 15 (1987) 13–26.
[23] S. Diekmann, EMBO J. 6 (1987) 4213–4217.
[24] S. Diekmann, J. Mazzarelli, L.M. McLaughlin, E.v. Kitzing, A.A. Travers, J. Mol. Biol. 225 (1992) 729–738.
[25] E. Paleček, E. Makaturová-Rašovská, S. Diekmann, Gen. Physiol. Biophys. 7 (1988) 379–393.
[26] S. Diekmann, D.A. Zarling, Nucl. Acids Res. 15 (1987) 6063–6074.
[27] D.A. Zarling, S. Diekmann, C.J. Calhoun, in: W.K. Olson, M.H. Sarma, R.H. Sarma, M. Sundaralingam (Eds.), Structure and Expression, vol. 3, Adenine Press, New York, 1988, pp. 87–96.
[28] S. Diekmann, L.W. McLaughlin, J. Mol. Biol. 202 (1988) 823–834.
[29] W.K. Olson, V.B. Zhurkin, in: R.H. Sharma, M.H. Sharma (Eds.), Biological Structure and Dynamics, vol. 2, Adenine Press, New York, 1996, pp. 341–370.
[30] T.E. Haran, J.D. Kahn, D.M. Crothers, J. Mol. Biol. 244 (1994) 135–143.
[31] K.J. Breslauer, Curr. Opin. Struct. Biol. 1 (1991) 416–422.
[32] S.C. Harvey, M. Dlakic, J. Griffith, R. Harrington, K. Park, D. Sprous, W. Zacharias, J. Biomol. Struct. Dyn. 13 (1995) 301–307.
[33] R.E. Dickerson, D. Goodsell, M.L. Kopka, J. Mol. Biol. 256 (1996) 108–125.
[34] J.G. McCarthy, L.D. Williams, A. Rich, Biochemistry 29 (1990) 6071–6081.
[35] J.K. Sullivan, J. Lebowitz, Biochemistry 30 (1991) 2664–2673.
[36] J.G. McCarthy, A. Rich, Nucl. Acids Res. 19 (1991) 3421–3429.
[37] J.G. McCarthy, C.A. Frederick, A. Nicolas, Nucl. Acids Res. 21 (1993) 3309–3317.
[38] A.M. Burkhoff, T.D. Tullius, Cell 48 (1987) 935–943.
[39] S. Diekmann, FEBS Lett. 195 (1986) 53–56.
[40] S. Diekmann, J.C. Wang, J. Mol. Biol. 186 (1985) 1–11.
[41] L. Ulanovsky, M. Boder, E.N. Trifonov, M. Choder, Proc. Natl. Acad. Sci. USA 83 (1986) 862–866.
[42] R. Bowater, F. Aboul-ela, J.A. McClellan, A.I.H. Murchie, D.M.J. Lilley, Methods Mol. Genet. 1 (1993) 241–266.
[43] A.M. Maxam, T. Gilbert, Methods Enzymol. 65 (1980) 499–560.
[44] C.K. Singleton, R.D. Wells, Anal. Biochem. 122 (1982) 253–257.
[45] H. Hayatsu, T. Ukita, Biochem. Biophys. Res. Commun. 29 (1967) 556–561.
[46] S. Neidle, D.I. Stuart, Biochim. Biophys. Acta 418 (1976) 226–231.
[47] D.M.J. Lilley, J.A. McClellan, in: R.H. Sarma, M.H. Sarma (Eds.), Structure and Expression, vol. 2, Adenine Press, New York, 1987, pp. 73–93.
[48] S. Sasse-Dwight, J. Gralla, J. Biol. Chem. 264 (1989) 8074–8081.
[49] S.A. Akman, J.H. Doroshow, M. Dizdaroglu, Arch. Biochem. Biophys. 282 (1990) 202–205.
[50] R. Bowater, F. Aboul-ela, D.M.J. Lilley, Biochemistry 30 (1991) 11495–11506.
[51] L. Ehrenberg, I. Fedorcsak, F. Solymosy, in: W.E. Cohn (Ed.), Progress in Nucleic Acids Research and Molecular Biology, vol. 16, Academic Press, New York, 1976, pp. 182–262.
[52] W. Herr, Proc. Natl. Acad. Sci. USA 82 (1985) 8009–8013.
[53] P.M. Scholten, A. Nordheim, Nucl. Acids Res. 14 (1986) 3981–3993.
[54] J.C. Furlong, D.M.J. Lilley, Nucl. Acids Res. 14 (1986) 3995–4007.
[55] D. Mendel, P.B. Dervan, Proc. Natl. Acad. Sci. USA 84 (1987) 910–914.
[56] V. Lyamichev, Nucl. Acids Res. 19 (1991) 4491–4496.
[57] U. Hänsler, S.E. Rokita, J. Am. Chem. Soc. 115 (1993) 8554–8557.
[58] J.A. McClellan, E. Paleček, D.M.J. Lilley, Nucl. Acids Res. 14 (1986) 9291–9309.
[59] J.A. McClellan, D.M.J. Lilley, J. Mol. Biol. 197 (1987) 707–721.