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Alternating d(G-A) sequences form a parallel-stranded DNA homoduplex

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The oligonucleotides d[(G-A)$_7$G] and d[(G-A)$_{12}$G] self-associate under physiological conditions (10 mM $MgCl_2$, neutral pH) into a stable double-helical structure (ps_{RR}-DNA) in which the two polypurine strands are in a parallel orientation in contrast to the antiparallel disposition of conventional B-DNA. We have characterized ps_{RR}-DNA by gel electrophoresis, UV absorption, vacuum UV circular dichroism, monomer–excimer fluorescence of oligonucleotides end-labelled with pyrene, and chemical probing with diethyl pyrocarbonate and dimethyl sulfate. The duplex is stable at pH 4–9, suggesting that the structure is compatible with, but does not require, protonation of the A residues. The data support a model derived from force-field analysis in which the parallel-stranded d(G-A)$_n$ helix is right-handed and constituted of alternating, symmetrical $G_{syn}\cdot G_{syn}$ and $A_{anti}\cdot A_{anti}$ base pairs with N1H...O6 and N6H...N7 hydrogen bonds, respectively. This dinucleotide structure may be the source of a negative peak observed at 190 nm in the vacuum UV CD spectrum, a feature previously reported only for left-handed Z-DNA. The related sequence d[(GAAGGA)$_4$G] also forms a parallel-stranded duplex but one that is less stable and probably involves a slightly different secondary structure. We discuss the potential intervention of ps_{RR}-DNA in recombination, gene expression and the stabilization of genomic structure.

Key words: DNA structure/homopurine sequences/Hoogsteen base pairing/pyrene fluorescence/vacuum UV CD

Introduction

The ability of certain DNA sequences to form parallel-stranded (ps) duplexes with A·T base pairing in which both strands have the same 5′→3′ orientation is now well established (reviewed in Jovin *et al.*, 1990; Jovin, 1991; Rippe and Jovin, 1992). Parallel-stranded DNA (ps-DNA) exhibits spectroscopic, thermodynamic and biochemical properties that differ from those of conventional antiparallel B-DNA and yet it is remarkably stable under physiological conditions. Interspersed d(G·C) base pairs can be incorporated into mixed d(A·T) sequences, but lead to a somewhat reduced stability of the ps duplex structure (Rippe *et al.*, 1990; Rentzeperis *et al.*, 1992). The question arises as to the existence of related parallel-stranded structures with other base pairing schemes. We were encouraged by initial model building studies indicating the stereochemical and energetic feasibility of parallel-stranded homopurine duplexes and undertook to generate and characterize them experimentally.

Polypurine·polypyrimidine regions are abundant in eukaryotic DNA. Alternating [d(G-A)·d(T-C)]$_{>20}$, for example, constitutes up to 0.4% of the total mammalian genome (Manor *et al.*, 1988) and has been ascribed functional roles in various biological processes in eukaryotic organisms (reviewed in Wells *et al.*, 1988; Paleček, 1991; Tripathi and Brahmachari, 1991; Yagil, 1991). Homopurine·homopyrimidine tracts are virtually absent from bacterial and phage genomes but are overrepresented in higher vertebrates (rodents and primates). They are located predominantly in non-coding regions, i.e. introns and the 5′/3′ flanking regions of many eukaryotic genes. Some of these sequences are highly conserved. Polypurine·polypyrimidine stretches in chromatin exhibit a hypersensitivity to single-strand specific nucleases, indicative of a non-B-DNA conformation. They have been implicated in transcriptional regulation, recombination and evolution.

The structural polymorphism of homopurine·homopyrimidine sequences is considerable. Upon negative supercoiling and/or at acidic pH, these tracts rearrange to form a pyrimidine·purine·pyrimidine triplex designated as H-DNA (Lyamichev *et al.*, 1986; Htun and Dahlberg, 1988; Shimizu *et al.*, 1989; Paleček, 1991) and can assume other non-B conformations (Parniewski *et al.*, 1989). In the triplex structure, one half of the pyrimidine strand is folded back on the duplex and forms Hoogsteen base pairs in the major groove; the complementary segment of the purine strand is looped out. In the presence of Zn^{2+} and at neutral pH, d(G-A)$_{22}$·d(T-C)$_{22}$ inserts in negatively supercoiled plasmids appear to adopt a triplex structure with purine·pyrimidine·purine triads (Bernués *et al.*, 1989; Lyamichev *et al.*, 1991). Another unusual homopurine DNA structure proposed in relation to chromosome telomere sequences and exhibited by other DNAs with runs of G (Sen and Gilbert, 1988; Sundquist and Klug, 1989; Williamson *et al.*, 1989) is a quadruplex stabilized by Hoogsteen base pairing between four guanine residues in alternating *syn* and *anti* conformations (Kang *et al.*, 1992; Smith and Feigon, 1992).

In this report we present experimental evidence and a model based on force-field calculations for a novel duplex form of DNA, referred to here as ps_{RR}-DNA, in which oligopurine strands are paired with each other in a parallel orientation under physiological conditions. We have analysed the formation, stoichiometry and stability of ps_{RR}-DNA by gel electrophoresis, UV absorption and vacuum UV circular dichroism. Chemical probing with diethyl pyrocarbonate and dimethyl sulfate supports the proposed base pairing scheme. In order to identify the relative orientation of the two strands

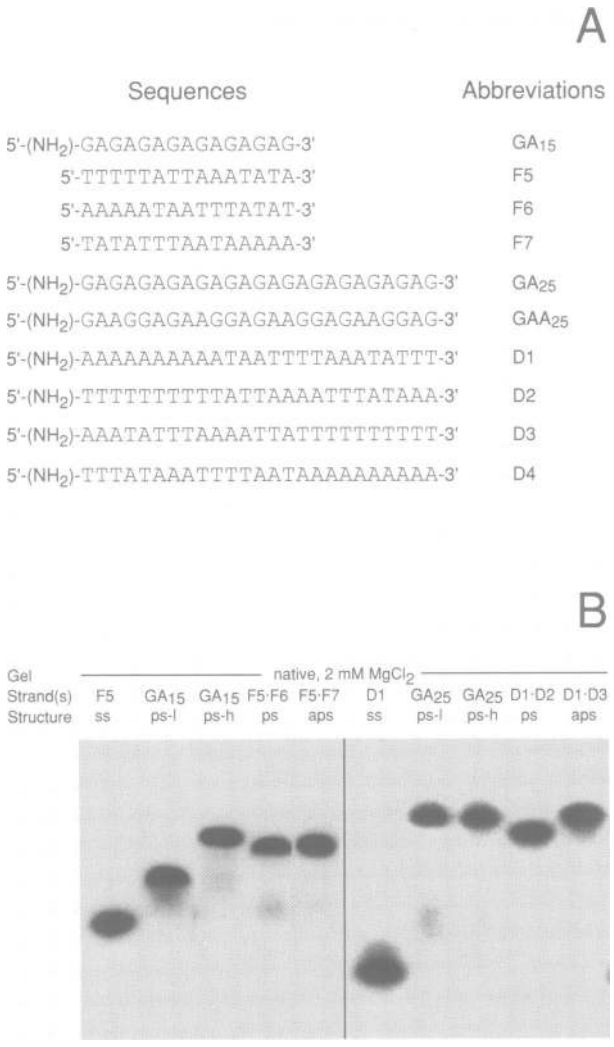

Fig. 1. Sequences and electrophoretic analysis of duplex formation. (**A**) Sequences and abbreviations of oligonucleotides used in this study. The designation (NH_2) indicates that these sequences were synthesized with (and also without) a 5′-terminal aminoalkyl group. (**B**) Electrophoretic mobility of the given strands on a polyacrylamide gel under native conditions. The abbreviations l and h indicate low (0.2 μM strands) or high (2 μM strands) concentrations, respectively, of the given GA oligonucleotide. The 15 nt and the 25 nt oligonucleotides were run on separate gels as indicated by the vertical line.

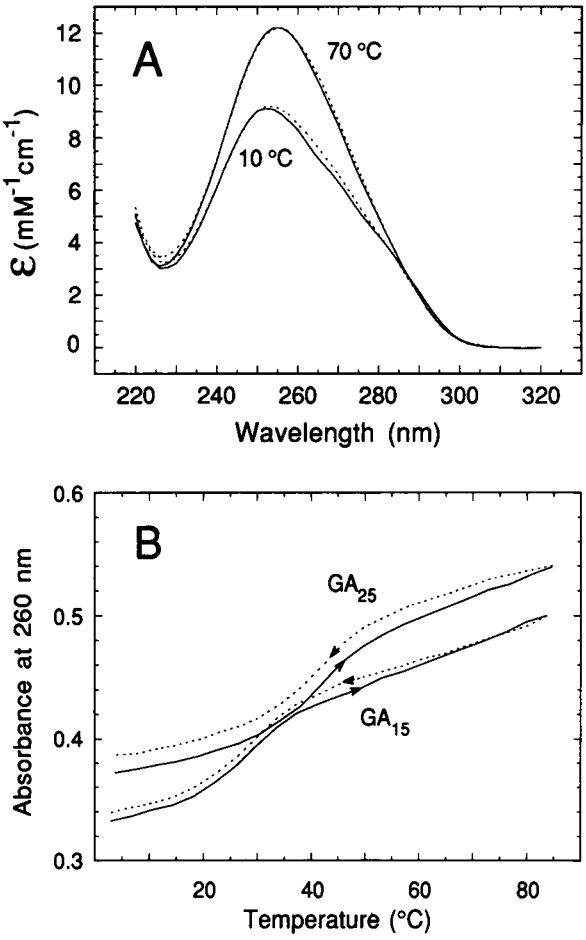

Fig. 2. (**A**) Ultraviolet absorption spectra of GA_{15} (solid line) and GA_{25} (dotted line) in the native state at 10°C and in the denatured state at 70°C. Solutions were in 10 mM $MgCl_2$ and 10 mM Na-cacodylate, pH 7.0. (**B**) Melting curves of GA_{15} and GA_{25} monitored by the absorbance at 260 nm. The ascending (solid) and descending (dotted) limbs of the curves are indicated by arrows. The heating and cooling rates were ~0.5°/min. The transitions were fully reversible; the observed slight upward displacement of the descending limbs of the curves is attributed to evaporation and condensation within the cuvette during the heating phase.

constituting the duplex, we have applied a new technique based on the relative monomer−excimer fluorescence of 5′-terminally located pyrene molecules. Finally we discuss biological processes in which the proposed structure may play a central role.

Results

Gel electrophoresis and UV spectroscopy

We synthesized the oligonucleotides GA_{15}, GA_{25} and GAA_{25} (Figure 1A) and compared them with the known AT-containing parallel-stranded (ps-D1·D2, ps-D3·D4, ps-F5·F6) and antiparallel-stranded duplexes (aps-D1·D3, aps-D2·D4, aps-F5·F7) (Rippe *et al.*, 1989; Jovin *et al.*, 1990; Rippe and Jovin, 1992). The generation and stability of the putative duplex structures were assessed by gel electrophoresis under native conditions in the presence of $MgCl_2$ (Figure 1B). GA_{15} and GA_{25} were less mobile than the corresponding single-stranded oligonucleotides but similar to the reference ps duplexes, from which we conclude that GA_{15} and GA_{25} form homoduplexes. The mobility of GA_{15} increased at low concentrations (0.2 μM strands; Figure 1B, lane GA_{15}, ps-l), probably due to partial dissociation of the duplex. High DNA concentrations (0.1 mM strands) did not result in the generation of more slowly migrating bands than those obtained at 2 μM (Figure 1B, lanes GA_{15}, ps-h, and GA_{25}, ps-h). Thus, the existence of higher order species such as intermolecular triplexes or quadruplexes could be excluded since these would have been retarded with respect to the duplexes (Pilch *et al.*, 1991; Sundquist and Klug, 1988). Intramolecular hairpins, e.g. foldback stuctures (Williamson *et al.*, 1989) with higher mobilities than the unstructured single-stranded species were likewise not apparent. The GA_{15} and GA_{25} bands showed very little fluorescence upon staining with ethidium bromide as compared with the AT-containing references, an observation in agreement with prior studies of purine

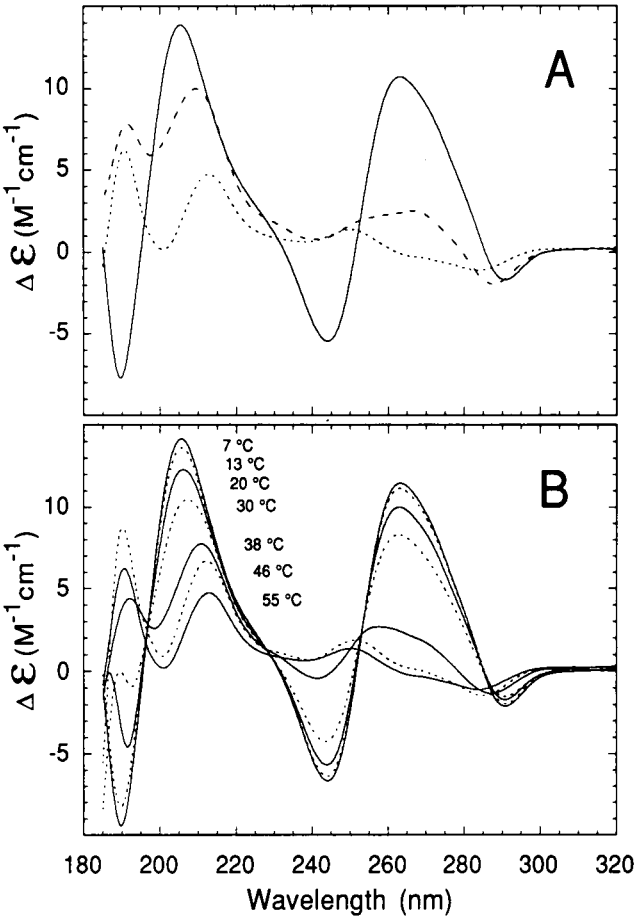

Fig. 3. Circular dichroism spectra of GA_{15}. (**A**) At 7°C without $MgCl_2$ (dashed line), and with 5 mM $MgCl_2$ at 7°C (solid line) and at 55°C (dotted line). (**B**) In 5 mM $MgCl_2$ at increasing temperatures.

polymers (Lee *et al.*, 1979, 1980). We conclude that intercalation in ps_{RR}-DNA is unfavourable and/or does not lead to the classical fluorescence enhancement of bound ethidium bromide.

The UV spectra of the ps_{RR}-DNAs (GA_{15} and GA_{25}) displayed a hypochromism characteristic of ordered DNA duplexes (24%; Figure 2A), at moderate temperatures and ionic strengths. The absorption maxima were at 253 nm (10°C, native state) and at 256 nm (70°C, single-stranded state). The divalent cation Mg^{2+} had a stabilizing influence that increased in the range 2–10 mM. The structures formed rapidly with no resolvable kinetics in the minute range. The absorbance changes upon thermal cycling were reversible (Figure 2B), indicative of a cooperative, rapidly equilibrating helix–coil transition. The melting points, T_m, of GA_{15}, GA_{25} and GAA_{25} were 25, 40 and 34°C, respectively, in 10 mM $MgCl_2$, 10 mM Na-cacodylate, pH 7.0, and at a strand concentration of 2 μM. The van't Hoff enthalpy, ΔH_{vH}, for the helix–coil transition of GA_{15} calculated from the shape of the transition curve (Ramsing *et al.*, 1989) was 210 kJ/mol with a corresponding entropy change ΔS of 0.60 kJ/mol/K. A similar value for ΔH_{vH} (220 kJ/mol) was obtained from the DNA concentration dependence of T_m, in accordance with the postulated duplex–coil transition mechanism.

Circular dichroism spectroscopy

The CD spectrum of GA_{15} confirmed the formation of secondary structure upon addition of 5 mM $MgCl_2$ at 7°C (Figure 3A). There was a strong positive peak at 263 nm, as in the spectra of poly[d(G-A)] (Lee *et al.*, 1979) and poly[d(G-A)$_{30}$] (Antano *et al.*, 1988), and a previously unreported negative peak at 190 nm in the vacuum UV region. Denaturation by heating to 55°C of GA_{15} led to the disappearance of the positive CD peak at 263 nm and to the inversion of the peak at 190 nm from negative to positive values (Figure 3A and B). The temperature dependence of the circular dichroism is displayed as a set of spectra in Figure 3B. It is apparent that formation of the GA_{15} duplex can be monitored by CD measurements; the observed T_m of 30°C agrees well with the value of 28°C estimated for these experimental conditions (5 mM $MgCl_2$, 20 μM strands) from the UV absorption data.

Pyrene monomer–excimer fluorescence

We determined the strand orientation in the duplex formed by the GA oligonucleotides with a new technique that exploits the fluorescent properties of the aromatic hydrocarbon pyrene. Upon excitation at 300–350 nm, pyrene can form a complex with another molecule in the ground state if the latter is in close proximity or rapidly diffusible during the exceptionally long-lived (τ generally > 100 ns) excited state (Birks, 1970). This excited state dimer (excimer) shows a broad unstructured fluorescence in the region 430–600 nm that is widely separated from the structured monomer emission at 380–400 nm. A pyrene molecule was attached to the 5′-end of the oligonucleotides via an aminoalkyl linker. It was anticipated that in a ps duplex with both 5′-ends in close proximity, the excimer fluorescence would be detected, whereas in an antiparallel conformation with the 5′-ends at opposite ends of the duplex, only the monomer fluorescence would appear. This expectation was fulfilled with the set of molecules studied here (Figure 4). Upon addition of 10 mM $MgCl_2$, excimer emission was observed with the pyrene-labelled GA_{25}, GA_{15} and GAA_{25} (Figure 4A and B) and with the ps, but not the aps, reference duplexes (Figure 4E). The addition of $MgCl_2$ to the single-stranded oligonucleotide D1 led only to a quenching of the pyrene monomer fluorescence (Figure 4D). The single-stranded D2, D3 and D4 also lacked excimer emission either in the presence or absence of $MgCl_2$ (Figure 4F). Denaturation (strand separation) by heating resulted in the loss of the excimer emission of all ps-DNAs in agreement with the predicted melting points, as shown for GA_{25} in Figure 4C. It should be noted that a particular virtue of the pyrene excimer procedure is that the aromatic groups on the two strands do not interact in the ground state and therefore do not perturb the monomer–duplex equilibrium.

The ionic conditions required for formation of ps_{RR}-DNA were explored using the pyrene fluorescence signals (Table I). The excimer emission indicative of the ps duplex of GA_{15} was observed in the pH range 4–9 (and in the presence of Mg^{2+}) but not at pH 12. Uni-univalent salts, specifically KCl, were much less effective than $MgCl_2$ in the stabilization of ps_{RR}-DNA. Thus, whereas the excimer–monomer emission ratio increased in solutions of KCl at concentrations >0.1 M and in the absence of divalent cations, it did not reach the value measured in 10 mM $MgCl_2$ even at the plateau levels of 0.8–1 M KCl. The

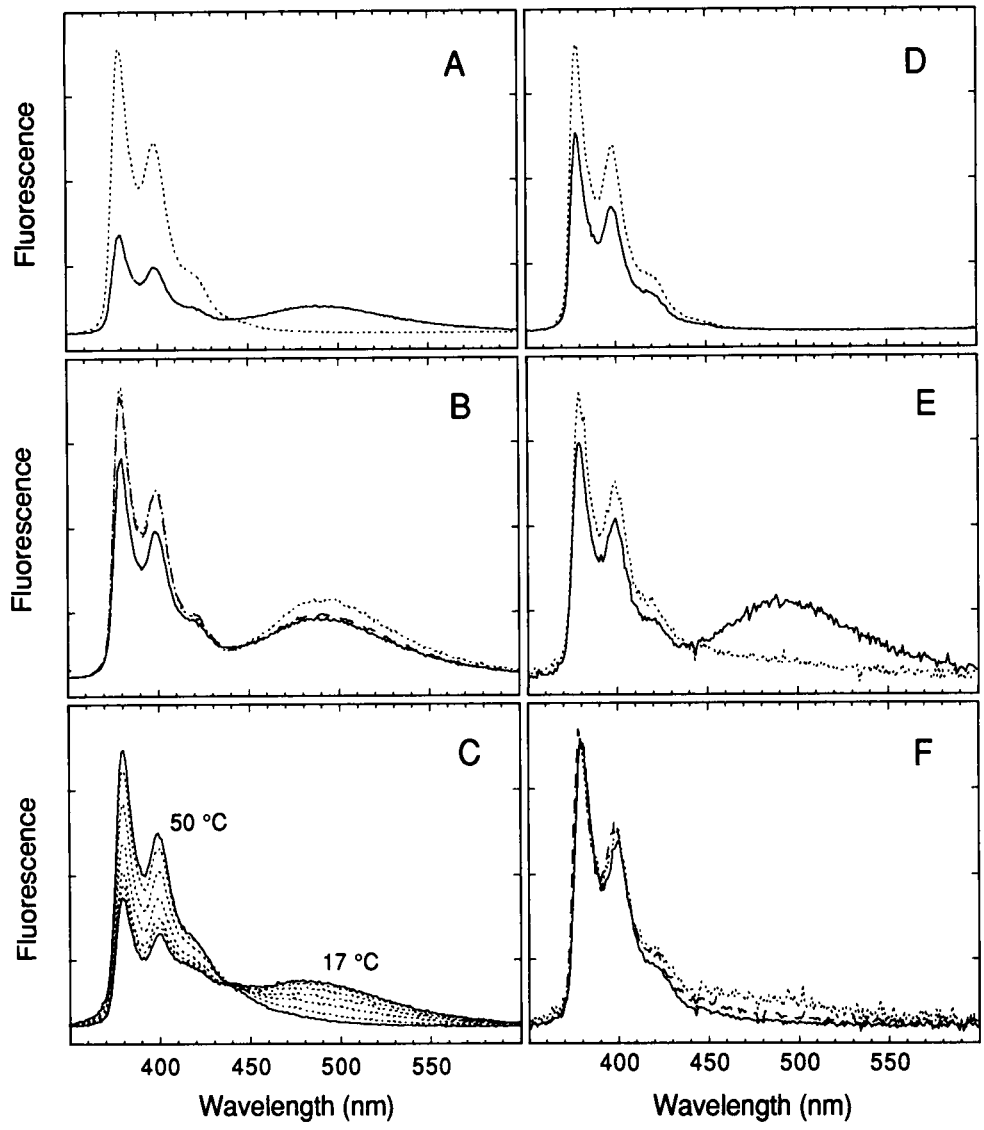

Fig. 4. Fluorescence emission spectra of pyrene-labelled oligonucleotides. (**A**) GA_{25} in 10 mM $MgCl_2$ (solid line) and without $MgCl_2$ (dotted line). (**B**) GA_{25} (solid line), GAA_{25} (dashed line) and GA_{15} (dotted line), in 10 mM $MgCl_2$. (**C**) Melting of GA_{25} in 12 mM $MgCl_2$. The fluorescence spectra were recorded at 17, 22, 27, 32, 37, 43 and 50°C. (**D**) Oligonucleotide D1 without $MgCl_2$ (dotted line) and with 10 mM $MgCl_2$ (solid line). Note that addition of $MgCl_2$ led not to a fluorescence signal indicative of excimer formation, but rather to a quenching of the monomer fluorescence. (**E**) Duplexes ps-D3·D4 (solid line) and aps-D2·D4 (dotted line) in 2 mM $MgCl_2$. (**F**) D2 (dotted line), D3 (dashed line) and D4 (solid line) in 2 mM $MgCl_2$. The spectra in (F) were normalized to the respective maximum intensities for comparison.

possibility that a multimeric structure was being mediated by hydrophobic interactions between the pyrene groups and/or the alkyl linker was excluded by the lack of a significant change in the excimer–monomer ratio upon addition of the non-ionic detergent polyoxyethylene lauryl ether (Table I).

Modification with diethyl pyrocarbonate (DEPC) and dimethyl sulfate (DMS)

To elucidate the type of base pairing involved in the ps duplex structure adopted by GA_{15}, GA_{25} and GAA_{25}, we used the chemical probes diethyl pyrocarbonate (DEPC) and dimethyl sulfate (DMS). DEPC carbetoxylates N7 of dA residues (and dG to a lesser extent). It reacts much more readily with single-stranded DNA than with double-stranded B-DNA or ps-DNA with dA·dT base pairing (Klysik *et al.*, 1990) and has been used to detect Z-segments within circular plasmids, cruciform loops and single-stranded regions in the H-DNA triplex (reviewed in Paleček, 1991). DMS methylates N7 of guanine under the conditions used, with no significant differences between a single-stranded oligonucleotide and a B-DNA duplex. The N7 group is unreactive to DMS if it participates in hydrogen bonding, as for example in a Hoogsteen base pair (Sen and Gilbert, 1988; Sundquist and Klug, 1989). The DEPC or DMS modified bases can be detected with single nucleotide resolution by cleaving the backbone of the ^{32}P-labelled DNA with piperidine and analysing the reaction products on a sequencing gel.

Since the formation of secondary structure was dependent on the presence of the divalent cation (see above), we studied the effect of $MgCl_2$ on the DEPC and DMS modification of the GA_{25} oligonucleotide. The reactions were analysed at 5 min intervals over a period up to 30 min and representative data are shown in Figure 5. The reactivities of the dA and dG residues with DEPC were uniformly lowered in the presence of 10 mM $MgCl_2$ (Figure 5A and B), whereas with DMS only a slight

Table I. Formation of parallel-stranded duplexes by pyrene-labelled oligonucleotides monitored by the excimer/monomer fluorescence ratio[a]

DNA	pH[b]	Additions[b]	Eximer/monomer fluorescence ratio (475 nm/377 nm)	
			$-MgCl_2$	$+MgCl_2$
GA_{15}	7		0.01	0.34
	4	10 mM Na-acetate	0.05	0.31
	9	10 mM Na-borate	0.01	0.42
	12	10 mM NaOH	0.02	0.02
	7	0.4% Brij-35[c]	0.01	0.28
	7	0.04 M KCl	0.02	
	7	0.1 M KCl	0.05	
	7	0.2 M KCl	0.12	
	7	0.4 M KCl	0.19	
	7	0.6 M KCl	0.22	
	7	0.8, 1.0 M KCl	0.23	
GA_{25}	7		0.01	0.37
GA_{25}	7	0.05 M NaCl	0.14	0.37
GA_{25}	4	10 mM Na-acetate	0.08	0.38
GAA_{25}	7		0.01	0.28
ps-D3·D4	7			0.34
aps-D2·D4	7			0.02

[a]The temperature was 7°C. The quantum yield of pyrene was dependent on (i) the base sequence at the 5′-end of the oligonucleotide, (ii) the presence of $MgCl_2$ (10 mM), (iii) the salt concentration, and (iv) the pH. Thus, we used the ratio of the excimer fluorescence at 475 nm to the monomer fluorescence at 377 nm to compare the relative extent of excimer formation, assuming that the monomer and the excimer were equally quenched.
[b]All solutions at pH 7 contained 10 mM Na-cacodylate.
[c]Brij-35 (Pierce) is the non-ionic detergent polyoxyethylene lauryl ether.

reduction in the modification of G was observed (Figure 5B). The $MgCl_2$ dependent change in reactivity with both chemicals was quantified by determining the integrated optical density per given band from the autoradiographs of the modification reactions (Figure 5B). From these values the mean relative decrease in reactivity upon addition of Mg^{2+} was: DEPC reaction, 60% for A and 50% for G (excluding the terminal G1 residue, which showed little change); DMS reaction, 20% for G. The DMS results suggest that the involvement of the N7 of guanine in hydrogen bonding is unlikely. The DEPC data reflect the general reduction in reactivity for this reagent consequent to formation of secondary structure (Klysik *et al.*, 1990). We interpret the larger effect upon the relative (and to an even greater degree, absolute) extent of A modification as compatible with, albeit not confirmatory of, Hoogsteen base pairing (see Discussion).

Base pairing and model structure

A sequence (prototypic for ps_{RR}-DNA) was derived from stereochemical modelling and force-field calculations (Figures 6 and 7). The G·G and A·A self-pairs with a *trans* orientation of the glycosidic bonds were chosen so as to maintain the two-fold symmetry axis parallel to the helical axis. This led us to specify the *syn* conformation for deoxyguanosine (symmetrical N1H...O6 hydrogen bonds) and the *anti* conformation for deoxyadenosine (symmetrical N6H...N7 hydrogen bonds). We note that A engages in Hoogsteen base pairing in three other known ps helical duplexes: a complex of CpA with proflavine (Westhof *et al.*,

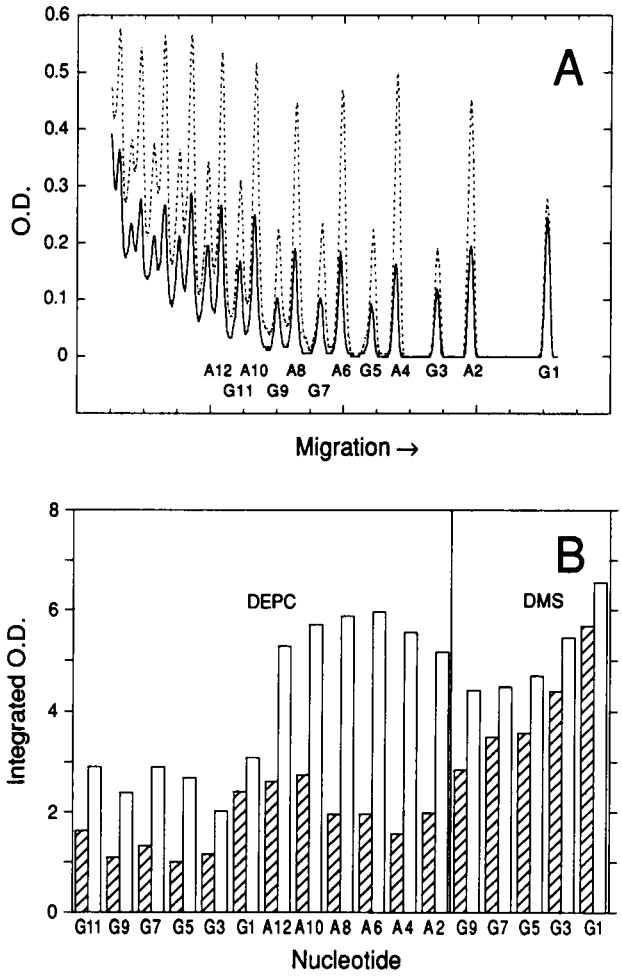

Fig. 5. Chemical modification of GA_{25} with diethyl pyrocarbonate (DEPC) and dimethyl sulfate (DMS). (**A**) Densitometric scans of an autoradiograph of the DNA sequencing gel on which the reaction products from the DEPC reaction after 30 min incubation time with 10 mM $MgCl_2$ (solid line) and without $MgCl_2$ (dotted line) were analysed. The optical density (O.D.) of the bands is plotted versus the electrophoretic migration. (**B**) Integrated optical density of well resolved bands from the DEPC and DMS (after 10 min incubation time) reactions. The hatched columns refer to the reaction in 10 mM $MgCl_2$ and the unfilled columns to the one without $MgCl_2$. The numbering of dA and dG residues is from the 5′-end.

1980), protonated poly(rA) (Rich *et al.*, 1961), and complexes of poly(rU) with poly(rA) bearing bulky substituents in the 2-position of the base (Hakoshima *et al.*, 1981).

The stacking geometries between the AG and GA steps are very distinct and are probably reflected in a differential stability: there is a very good overlap between A and G in the AG step (Figure 7B) but little stacking between G and A in the GA step (Figure 7C). Thus, the helical repeat is best described as a dinucleotide unit with a helical twist of 90°, corresponding to 8 bases per turn, and an average helical rise of 6.8 Å. The adenine nucleotides have standard conformations with the *gauche*(−), *gauche*(−) conformations for the phosphodiester and the *gauche*(+) conformation for the C4′−C5′ bond. However, the guanine nucleotides are in a Z-like conformation with both the P-O5′ and C4′−C5′ bonds in the *trans* orientation. From the minimized structures we derive C1′−C1′ distances of 13.6 Å for d(G·G) and 11.0 Å for d(A·A); the C1′−C1′−N angles are 28° and 11°, respectively.

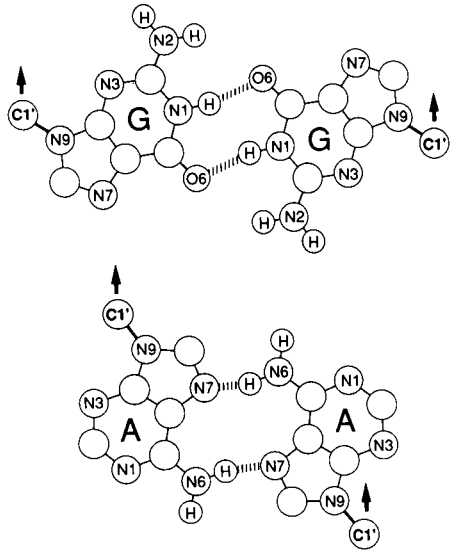

Fig. 6. Base-pairing scheme for the proposed parallel-stranded duplex structure. The $G_{syn}\cdot G_{syn}$ and $A_{anti}\cdot A_{anti}$ base pairs are shown as projections derived from positions 5 and 6 of the minimized $d(AG)_5$ duplex structure displayed in Figure 7.

Although it would break the two-fold symmetry, a mixture of *anti* and *syn* conformations for a homopurine base pair is also possible (with N1H...N7 and N2H...O6 for the $G_{syn}\cdot G_{anti}$ base pair and with N6H...N1 and N7...HN6 for the $A_{anti}\cdot A_{syn}$ base pair). Such base pairs may be adopted by the GAA_{25} oligonucleotide that lacks a strict alternation of G and A but still forms a ps duplex, albeit with reduced stability.

The possibility of A·G base pairing was also considered. Two theoretical models incorporating A·G base pairs in a parallel duplex can be built. The first requires protonation of all adenines on one strand and presents the alternating pairings N6H...O6/N1H$^+$...N7 and O6...HN6/N1H...N7 with all bases in *anti*. Since secondary structure formation was also observed in the neutral to basic pH range (7–9) (see above), this possibility can be excluded. In the second model, all the bases are in the *syn* conformation in one strand and in the *anti* conformation in the other strand; base pairing is via N6H...O6/N1...HN1 hydrogen bonds. In such a structure, the N7 atoms of all bases point to the solvent and one would not expect the strong observed dependence on Mg^{2+} in chemical probing of the adenines, although stacking could afford some protection. Furthermore, both models would lead to severe clashes in the case of the GAA_{25} oligonucleotide, since in that instance one would have to accommodate G·G and A·A oppositions with improper orientations of donor and acceptor atoms. We conclude that only the proposed model with A·A and G·G self-pairs (Figures 6 and 7) is able to explain all the experimental data. It is also the only model able to accommodate a lack of strict purine alternation, which makes it particularly attractive in terms of biological implications.

Discussion

A parallel-stranded homoduplex formed by purine oligonucleotides

We have demonstrated in this study that the oligonucleotides $d[(G\text{-}A)_{7,12}G]$ and $d[(GAAGGA)_4G]$ self-pair under physiological conditions to form a ps duplex designated as ps_{RR}-DNA. The model proposed for the helical structure (Figures 6 and 7) incorporates a dinucleotide repeat consisting of $G_{syn}\cdot G_{syn}$ and $A_{anti}\cdot A_{anti}$ base pairs.

Polyacrylamide gel electrophoresis established that the oligonucleotides GA_{15}, GA_{25} and GAA_{25} adopt a duplex conformation in the presence of $MgCl_2$ (Figure 1B). There was no evidence for triplexes or quadruplexes under the given experimental conditions, and it should be noted that the sequences lack the runs of G required for the formation of G4 quartets and related structures. Our results are consistent with those of two other studies showing that the oligonucleotides $d(G_3A_4G_3)$ (Pilch *et al.*, 1991) and d(GAAGAGAG) (J.W.Lown, personal communication) migrate as duplexes in Mg^{2+}-containing gels. In the latter case quadruplexes were not observed by NMR analysis even at high DNA concentration (2 mM strands, 5 mM $MgCl_2$). The divalent cation Mg^{2+}, but neither Na^+ nor K^+, was effective in stabilizing the dimeric form of d(GAAGAGAG), a feature shared by the oligonucleotides studied here (Table I).

The existence of a regular structure excluding hairpins, loops or bulges was also demonstrated by chemical probing with DEPC and DMS; the modification patterns with these reagents were uniform over the entire sequence (Figure 5). A novel technique was devised based on the monomer–excimer fluorescence exhibited by molecules end-labelled with pyrene, with which it was established that the two identical strands of the ps_{RR}-DNA duplex are oriented parallel to each other, i.e. have the same 5′→3′ polarity (Figure 4B, Table I). The formation of secondary structure required the divalent cation Mg^{2+} and was rapid; there were no detectable kinetic phenomena in the minute range. This finding is compatible with evidence from proton NMR for relatively rapid exchange between the single-stranded states and the self-complexes of $d(A\text{-}G)_4$ and $d(G\text{-}A)_4$ at low temperatures and in the presence of 5 mM $MgCl_2$ (Feigon *et al.*, 1990).

In numerous publications Lee and coworkers (Lee *et al.*, 1979, 1980; Lee, 1990) demonstrated that poly[d/r(GGA)], poly[d/r(G-A)], poly[d/r(GAA)], poly[d(G-n^{2}A)] and poly[d(I-A)] form secondary structures stabilized by divalent cations. The authors assumed that a quadruplex was involved. Our model for the ps homopurine duplex provides an alternative structural basis for interpreting their polymer data.

Helical conformation and base pairing of ps_{RR}-DNA

The inversion of the positive peak at 190 nm in the CD spectrum of GA_{15} upon addition of $MgCl_2$ (Figure 3) is unusual and in dramatic contrast to the observed changes upon formation of conventional B-DNA. A negative peak in the vacuum UV has been reported previously only for left-handed Z-DNA (Riazance *et al.*, 1987); we conclude that ps_{RR}-DNA shares some essential structural elements with this DNA conformation. Unfortunately, the relative contributions of the *syn* glycosyl configuration of dG and the dinucleotide repeat unit to the characteristic CD spectrum of Z-DNA are as yet unknown. These two features are shared by our proposed model for ps_{RR}-DNA and we suggest that the negative peak in the vacuum UV may reflect these properties rather than helical handedness. In support of this possibility are negative peaks at 195 nm in the CD

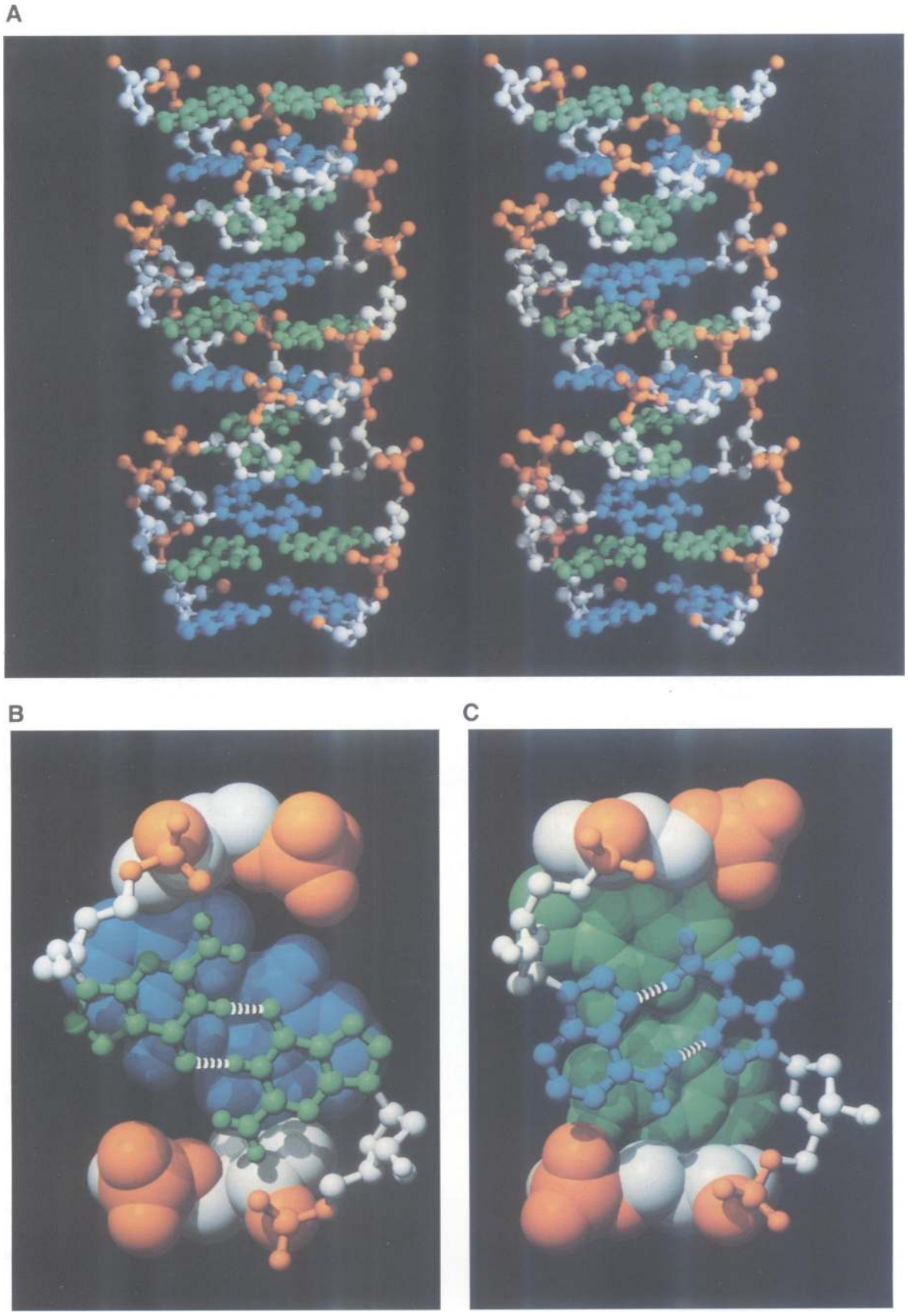

Fig. 7. Model of the parallel-stranded helix (ps_{RR}-DNA) adopted by oligonucleotides with the alternating sequence $d(AG)_5$. (**A**) Stereo view of the helix. (**B**) An AG dinucleotide section is shown with the upper d(G·G) base pair (towards the viewer) as a ball and stick model; probable hydrogen bonds appear as broken white lines. (**C**) GA dinucleotide with the upper d(A·A) base pair as a ball and stick model. Colour coding: sugars, white; phosphates (including O3′ and O5′), red; adenine, cyan; guanine, blue.

spectra of the nucleotide monomers 5′-GMP (Sprecher and Johnson, 1977) and 3′-GMP; the intensity of the latter is 20% higher. NMR data at slightly alkaline pH (Son *et al.*, 1972) demonstrate that ~50% of 5′-GMP and 70% of 3′-GMP are in the *syn* conformation. In a single-stranded oligonucleotide with conventional stacking, dG residues adopt the energetically favored *anti* conformation, to which we attribute the positive peak at 190 nm in the spectra of GA_{15} without $MgCl_2$ and in the melted state at 55°C.

As discussed earlier, G·A base pairing is unlikely because the GAA_{25} oligonucleotide apparently adopts a structure similiar to that of the strictly alternating oligonucleotides GA_{15} and GA_{25}. This finding is in agreement with the studies of poly[d/r(GGA)] and poly[d/r(GAA)] (Lee *et al.*, 1980) and supports the proposition that the secondary structure in ps_{RR}-DNA is established by G·G and A·A self-pairing. We sought to establish the hydrogen bonding interactions in the d(G·G) and d(A·A) base pairs with the reagents DEPC and DMS. There was a relative decrease in the reactivity of dA (60%) and dG (50%) with DEPC upon addition of $MgCl_2$. However, the stability and dynamics of the duplex during the time of reaction have to be considered in interpreting these data. Due to the relatively low ΔH_{vH} of ~310 kJ/mol determined for the helix–coil transition of GA_{25} in 10 mM $MgCl_2$, ~3% of the strands would be single-stranded at 25°C (15° below the optically determined T_m) and therefore readily accessible to DEPC. In addition, carbetoxylation of N7 in dA residues would lead to continuous displacement of the equilibrium toward the coil state, assuming that base modification disrupts the proposed duplex structure. As a consequence, the observed chemical reactivity for DEPC of both dA and dG may reflect the modification of single-stranded species, thereby masking the intrinsic changes in the accessibility of the N7 positions of the bases in the duplex.

Because of the higher reactivity of DMS, the reactions could be performed at 4°C and for shorter reaction times. At this temperature and in the presence of $MgCl_2$, the concentration of the oligonucleotide in the single-stranded state should have been negligible. Furthermore, protection of dG to reaction with DMS requires hydrogen bonding of N7, as in the case of G-quartet structures (Sen and Gilbert, 1988; Sundquist and Klug, 1989; Kang *et al.*, 1992; Smith and Feigon, 1992). Formation of a B-DNA duplex does not reduce the reactivity of DMS. Thus, the only slightly lowered DMS modification of ps_{RR}-DNA in the presence of $MgCl_2$ suggests explicitly that N7 of dG is not hydrogen bonded. Inasmuch as poly[d(I-A)] has properties similar to those of poly[d(G-A)] (Lee *et al.*, 1980), the N2 amino group of G is probably not involved in hydrogen bonding. These two findings support the $G_{syn}\cdot G_{syn}$ base pairing scheme with symmetrical N1H...O6 hydrogen bonds proposed in our model (Figures 6 and 7) and also found in the crystal structure of 8-bromoguanosine (Tavale and Sobell, 1970). The model is also in agreement with the observed lack of duplex formation at pH 12 (Table I) because N1 of G is deprotonated at this pH. The participation of G imino protons in hydrogen bonding has been demonstrated by proton NMR in the studies of $d(G\text{-}A)_4$ and $d(A\text{-}G)_4$ to which we have already alluded (Feigon *et al.*, 1990); the ionic conditions were very similar to those in our experiments.

The DEPC modification patterns are less definitive with respect to the participation of the N7 position of A in hydrogen bonding. The problem is inherent in that the formation of helical structure with N7 positioned in a groove by itself leads to reduced reactivity for DEPC as compared to the single-stranded state (Klysik *et al.*, 1990). However, there are numerous experimental results in support of the proposed *trans* A·A base pairing with N6H...N7 hydrogen bonds (Figures 6 and 7). (i) The ps duplexes formed by GA_{15} and GA_{25} are stable at pH 4 (Table I); at this pH, the A residues are partly protonated at the N1 position and are therefore incapable of forming N1...HN6 hydrogen bonds. We note that a ps double helix with the same A·A Hoogsteen base pair incorporated in our model is formed by poly[(rA)] (Rich *et al.*, 1961) and presumably by poly[(dA)] (Bollum *et al.*, 1964) under acidic conditions. The protonated N1 of A stabilizes poly[(rA)] by promoting hydrogen bonding of the free exocyclic amino hydrogens with phosphate oxygens. In addition, it has been demonstrated by CD measurements that a self-complex of $d(A\text{-}G)_{30}$ is induced upon lowering the pH to 4.0 (Antano *et al.*, 1988). The CD spectrum of this structure is very similar to those of poly[d(A-G)] at neutral pH (Lee *et al.*, 1979) and of the oligonucleotides we report in this study in the presence of $MgCl_2$. (ii) Methylation of adenine at N6 completely disrupts secondary structure in poly[d(G-m^6A)] (Lee *et al.*, 1980), providing another argument against A·A base pairing with N1...HN6 hydrogen bonds. (iii) The introduction of an amino group at the C2 of adenine has a stabilizing effect in poly[d(G-n^2A)] (Lee *et al.*, 1980). According to the structure proposed above (Figure 7) and in partial analogy with protonated poly[r(A)], the C2 of adenine would be positioned in the groove such that the exocyclic amino group could form an additional hydrogen bond with the opposite phosphate via a water molecule.

In the model of ps_{RR}-DNA displayed in Figure 7, two hydration sites are probably critical for the stabilization of the ps duplex. One is located between the N6 amino group of the adenine residues and an anionic oxygen atom of the symmetry-related adenine base (Westhof, 1990). The other site is located between the N2 amino group of the guanine base and its 3′-phosphate group, as observed in Z-DNA in which the guanine is also in the *syn* conformation (Wang *et al.*, 1979). Another feature common with Z-DNA is the placement of the Hoogsteen sites of the guanine bases toward the exterior of the helix, offering the possibility for interaction with Mg^{2+} ions.

Biological implications

Sequence relationships facilitating the formation of ps helical regions have been identified previously (Ramsing and Jovin, 1988; Jovin, 1991). A number of potential biochemical situations and models involving the interactions of ps sequences *in vivo* are discussed below and in more detail elsewhere (Rippe *et al.*, 1992).

Single-stranded $d(C\text{-}T)_n$ specific binding activities have been found in nuclear extracts of mammalian species including mouse, monkey and human (Yee *et al.*, 1991). The presence of such proteins as well as the formation of intramolecular H-DNA triplexes (Lyamichev *et al.*, 1986; Htun and Dahlberg, 1988; Shimizu *et al.*, 1989; Paleček, 1991) could render single-stranded regions of $d(G\text{-}A)_n$ and related sequences accessible for participation in the alignment and pairing of homologous DNAs during recombination or meiosis (Sen and Gilbert, 1988). A parallel-stranded

interaction of the looped out purine strand in H-DNA with another DNA or RNA purine strand of the same sequence is also of potential importance as it would stabilize the triplex structure. Such a phenomenon is possibly observed in the $d(AGGAG)_{28}$ repeat of the murine immunoglobulin IgA switch region (Collier *et al.*, 1988; Reaban and Griffin, 1990). Transcription of this region leads to a loss of ~13 superhelical turns and the generation of a structure sensitive to RNase H (Reaban and Griffin, 1990). It has been proposed (Reaban and Griffin, 1990) that a purine·pyrimidine·purine triplex is formed in the $(AGGAG)_{28}$ tract and stabilized by the $(AGGAG)_n$ RNA transcript as part of the mechanism of gene regulation [an error in the original paper has been corrected (Stavnezer, 1991)]. However, an alternative possibility would be that the transcript is incorporated into a ps RNA·DNA hybrid with G·G and A·A self-pairs stabilizing a more conventional pyrimidine·purine·pyrimidine H-DNA triplex. Such a triplex conformation is presumed to be adopted by this sequence upon negative supercoiling and in the absence of transcription (Collier *et al.*, 1988). In addition, it is noteworthy that at acidic pH, alternating d(C-T) forms an ordered structure, proposed to be a ps duplex with $C \cdot C^+$ base pairs and T·T bulges (Sarma *et al.*, 1986; Antano *et al.*, 1988). This offers the possibility that upon stabilization by low pH and negative supercoiling, two separate $d(A\text{-}G)_n \cdot d(C\text{-}T)_n$ tracts rearrange to form two ps duplex segments. The purines would be in the ps_{RR}-DNA conformation and the pyrimidines would form a ps duplex with $C \cdot C^+$ base pairs. Such a structure might explain the observed modification of an $(AG)_7N_{14}$-$(AG)_7$ insert by DEPC (reported as pattern A in Parniewski *et al.*, 1989).

The sugar pucker of the model for ps_{RR}-DNA displayed in Figure 7 is C3′-*endo*. From this feature and the results obtained for the RNA purine polymers poly[r(GGA)], poly[r(GA)] and poly[r(GAA)] (Lee *et al.*, 1980), we surmise that an RNA oligomer may adopt a very similar structure, a possibility under current investigation. A ps-RNA duplex with G·G and A·A self-pairs might be involved in the dimerization of retroviral RNA genomes. These dimers are observed by electron microscopy and display an apparent parallel orientation of the two identical RNA strands (Kung *et al.* 1976). We note that such a structure could possibly be mediated by the RNA analogue of ps_{RR}-DNA, inasmuch as it would offer the means by which identical sequences could hybridize in a parallel orientation.

Materials and methods

Oligonucleotide synthesis and characterization

The oligonucleotides were synthesized, purified, labelled with ^{32}P and annealed as described previously (Rippe *et al.*, 1989). The purity of the isolated end-labelled oligonucleotides was established by electrophoresis under denaturing conditions (Rippe *et al.*, 1989). Oligonucleotides of the same length showed the same mobility and no contaminating more rapidly or more slowly migrating species were detected. Gel electrophoresis under native conditions was carried out at 7°C in 2 mM $MgCl_2$ as described previously (Rippe *et al.*, 1989). The samples were loaded in 15% Ficoll containing 10 mM $MgCl_2$. The molar extinction coefficient of the oligonucleotides GA_{15}, GA_{25} and GAA_{25} was taken to be 10.2 mM^{-1} (base) cm^{-1} at 255 nm and 21°C in 10 mM Na-cacodylate, pH 7.0 and was derived from the published value for poly[d(G-A)] (Lee *et al.*, 1980). It corresponded to 12.2 mM^{-1} (base) cm^{-1} at 255 nm and 70°C as extrapolated from the linear increase of absorbance above the melting point. The UV absorbance spectra and melting curves were recorded and analysed as described in Ramsing *et al.* (1989). The CD spectra were acquired in a 1 mm cell with a Jasco J-720 spectropolarimeter in 90% D_2O, 0.8 mM Na-cacodylate, pH 7.0, 0.8 mM NaCl and 2 mM Na-phosphate, pH 7.0.

Fluorescence measurements of pyrene-labelled oligonucleotides

The oligonucleotides for the pyrene labelling reaction were synthesized with a 5′-primary amino group using the Aminolink 2 reagent from Applied Biosystems. After deprotection they were purified by reversed phase HPLC. Then ~3 optical density (260 nm) units of the dried oligonucleotide with a 5′-primary amino group were dissolved in 50 μl of 0.2 M Na-borate pH 9.5, 50 μl H_2O, and 80 μl dioxane. To this solution 20 μl of a freshly prepared 10 mg/ml stock of succinimidyl-1-pyrenebutyrate (Molecular Probes) in dry dimethyl formamide were added and the solution was incubated overnight in the dark. Excess unreacted dye was then removed on a Sephadex G-25 column. The oligonucleotide with the attached pyrene was separated from the unreacted material by reversed phase HPLC on a Waters Delta Pak C18 300 Å, 3.9 mm × 15 cm, 5 μm column. The gradient used was 5 to 50% CH_3CN in 30 min followed by 50 to 80% CH_3CN in 5 min in 0.1 M triethylammonium acetate (TEAA), pH 7.0. The flow rate was 1.0 ml/min and the column was heated to 50°C to improve the resolution. Under these conditions the free oligonucleotide eluted at 24–25 min, the pyrene-labelled DNA at 33–34 min and the free pyrene butyrate at 37.5 min. The elution was monitored by UV spectroscopy from 220 to 450 nm in 2 nm steps with a Waters diode array detector. The collected fraction from the HPLC run was dried and then washed with 150 μl of 96% ethanol to remove any residual free dye and traces of the TEAA buffer. Complete labelling of the oligonucleotides corresponded to DNA/pyrene absorbance ratios per nucleotide of $A_{255}/A_{345} = 0.43-0.48$ for the GA sequences and $A_{264}/A_{345} = 0.35-0.36$ for the oligonucleotides D1, D2, D3 and D4. The fluorescence measurements were made with an SLM 8000 spectrofluorimeter at 7°C, unless otherwise noted, at a DNA concentration corresponding to an A_{260} of 0.1–0.2. All solutions were mixed at room temperature and then equilibrated to the temperature of the measurement for 10 min. Longer incubation times or heating before the measurement had no observable effect. The emission spectra were corrected by internal standards and the background was subtracted. Excitation was at 340 nm and the emission spectra were recorded in 1 nm steps from 350 to 600 nm in 5 × 5 mm quartz cuvettes. All solutions contained 10 mM Na-cacodylate, pH 7.0, in addition to the specified salt, except for the spectra recorded at other pH values.

Chemical modifications with DEPC and DMS

The ^{32}P-labelled GA_{25} oligonucleotide was incubated in 20 mM Na-cacodylate, pH 7.0, supplemented with either 10 mM $MgCl_2$ or with 1 mM EDTA. The modification reaction was performed in 10% (v/v) DEPC at 25°C and in 0.02% DMS at 4°C, respectively. Further treatment of the samples was as described previously (Klysik *et al.*, 1990). The autoradiographs from the sequencing gels were digitized with a CCD camera system and the images were processed according to Klysik *et al.* (1990). Integrated optical densities were calculated after subtraction of a baseline defined by the minima between successive A and G bands. Control experiments with the oligonucleotide $d(A_5GA_3GTAGT_4A_2GTAT_3)$ and its antiparallel B-DNA duplex confirmed that the slightly different salt conditions did not affect the inherent reactivity of the chemicals. In addition, it was demonstrated that the DMS reactivity was about the same with the single-stranded oligonucleotide and with the duplex.

Model structure

The model of ps_{RR}-DNA was first generated using FRODO (Jones, 1978) on a PS300 graphics system starting with standard DNA strands with either a C3′-*endo* sugar pucker or a C2′-*endo* sugar pucker. The construction was subjected to geometrical and stereochemical refinement using NUCLIN-NUCLSQ (Westhof *et al.*, 1985) with restraints on the base-pair geometry and enforcing the two-fold symmetry between the two strands. The C2′-*endo* pucker was found to be incompatible with the formation of the appropriate base pairs. The refined structure was then introduced into the molecular modelling program AMBER 3.0 (Weiner *et al.*, 1986) for energy minimization and molecular dynamics. Several parameters for the electrostatic interactions (Fritsch and Westhof, 1991) were tested. From the molecular dynamics simulations, average structures were obtained which were manually modified with FRODO, regularized with NUCLIN-NUCLSQ and subjected to further molecular dynamics simulations. Several cycles were needed before obtaining a satisfactory model with a behaviour similar to that of other DNA oligomers. The figure was generated by the SCHAKAL v86b program of Dr E.Keller, University of Freiburg, FRG.

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