Immunochemical detection of multiple conformations within a 36 base pair oligonucleotide

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ABSTRACT

A 36 base pair chimeric oligonucleotide containing a central core of DNA duplex flanked by RNA/DNA hybrid at each end was synthesized. These distinct regions of the oligonucleotide adopt different conformations which were detected with antibody probes. Enzyme linked immunosorbent assays (ELISA) and a gel electrophoresis retardation assay were used to demonstrate the binding of antibodies which recognize B-DNA, Z-DNA and RNA/DNA hybrid. The DNA duplex core of this oligonucleotide adopts the B-conformation in 0.14 M NaCl. In high salt solution (4 M NaCl) the DNA core adopts the Z-conformation. The RNA/DNA hybrid at the ends of the oligomer adopt a conformation which is distinct from both B-DNA and A-RNA.

INTRODUCTION

The double helical structures of nucleic acids are generally grouped into three main categories termed A-form, B-form and Z-form. The A- and B-helices are the right-handed helical conformations which represent the average solution structures of RNA and DNA. The Z-form is a left-handed helix which occurs in nucleic acids with alternating purine-pyrimidine sequences under certain conditions of solvent composition or helical stress. A detailed review of nucleic acid conformation can be found in Saenger. While it is the base sequence or primary structure of nucleic acids that contains the genetic information, there is also information in the helix conformation, which is probably important for site specific interactions of some proteins with DNA and RNA. These protein-nucleic acid interactions are critical for regulation of gene expression, replication and packaging of the genetic material. It is possible that a small region of A- or Z-form helix within a DNA molecule that is mostly in the B-form could act as a signal for protein binding. For Z-DNA, there is evidence that suggests a role in recombination. Unusual structures such as cruciforms and bent helices might also constitute protein recognition sites. There is, therefore, great interest in the factors which influence nucleic acid conformation and the
recognition of nucleic acid helical conformation by proteins. Antibodies to nucleic acids are useful both as probes of conformation and as model systems for the recognition of conformation by proteins. Antibodies that recognize Z-DNA, double stranded RNA and RNA/DNA hybrids have been induced experimentally. In some cases monoclonal antibodies to these conformations have been prepared and their binding specificity characterized. Native DNA (B-DNA) is generally not immunogenic; however, autoantibodies present in the disease systemic lupus erythematosus (SLE) do bind to native DNA and monoclonal antibodies have been prepared from both humans and mice with lupus.

The most detailed information on the structure of nucleic acids has been obtained with relatively small oligonucleotides, which are amenable to study by X-ray diffraction with single crystals or by nuclear magnetic resonance (NMR) in solution. Immunochemical probes can detect structures in either oligonucleotides or in larger polymers, which may be more relevant to the structures found in biological systems. Antibodies can thus provide a link between physical studies and biological systems.

In this work the oligonucleotide (rA)$_{12}$(dGdC)$_6$(dT)$_{12}$ (the 36mer) was prepared and the helix conformation characterized by the binding of conformation-specific antibodies. This oligomer contains two segments of RNA/DNA hybrid. Hybrid duplexes are generally considered to be of the A-family, though some demonstrate features of a B-helix and, as this and some previous work indicate, they have unique characteristics as well. The central core of this oligomer is DNA duplex with the alternating dGdC sequence, which can form either a B- or a Z-helix. It is shown that these conformations do exist within this short oligonucleotide duplex; thus, this oligomer is a suitable system for studies of the hybrid helix geometry and the conformation of the helix at A-B and A-Z junctions.

**MATERIALS AND METHODS**

**Antibody Preparation**

The anti-native DNA antibody H241 is a monoclonal autoantibody derived from an MRL-lpr/lpr 'lupus' mouse. This antibody recognizes double stranded DNA (B-DNA) with a preference for alternating dGdC sequences. The anti-Z-DNA antibody Z22 is a monoclonal antibody induced by immunizing mice with brominated poly(dGdC)-poly(dGdC). The preparation and characterization of this anti-Z-DNA antibody has been described. Gt4 is a polyclonal antibody from a goat immunized with poly(A)-poly(dT), which binds specifically to RNA/DNA hybrids. Each of the
three antibodies was immunospecifically purified, either from tissue culture fluid with a goat anti-mouse-Ig-Sepharose column, or, in the case of anti-RNA/DNA by dissociation from an antigen-antibody precipitate; as described. The enzyme-linked immunosorbent assay (ELISA) was used to detect the binding of antibodies to nucleic acids as previously described. Competitive assays were performed to compare the affinity of the antibodies to the oligonucleotides and various polynucleotide antigens. For each antibody, a polynucleotide antigen was coated onto the surface of the wells of an ELISA plate after the plate was treated with UV light. The antigens used for H241, Z22 and anti-hybrid antibodies were calf thymus DNA (Sigma), poly(dGme5dC) and poly(A)-poly(dT) respectively. The antibodies were incubated with the competitors at various concentrations for 1 hour before they were added to the ELISA plates. For the Z22 competition experiments, the NaCl concentration was 4 M for the incubation of antibody with competitor. All other steps in the Z22 experiment were performed in the presence of 20mM MgCl2, in which poly(dGme5dC) exists in the Z-form. The H241 and anti-hybrid experiments were performed with 10mM sodium phosphate, 140mM NaCl, pH 7.2 (PBS). Binding of antibody was detected by standard techniques using anti-mouse antibody for Z22 and H241, and anti-goat antibody for the anti-hybrid experiments, with alkaline phosphatase conjugated to each. After addition of p-nitrophenyl phosphate (Phosphatase Substrate, Sigma), the absorbance at 410 nm was monitored at ten minute intervals using a Dynatech MR600 Microplate Reader.

Electrophoresis

Electrophoresis of oligonucleotides was performed with 20% acrylamide gels (29:1 acrylamide/bisacrylamide). Denaturing gels contained 7M urea and were run with 90mM Tris-borate (pH 8.3), 1.25mM EDTA (TBE buffer). Non-denaturing gels were run with 90mM Tris-borate (pH 8.3), 5mM MgCl2 (TBM buffer) at 4°C.

A gel retardation assay was used to detect antibody binding. For these experiments, a 3.5% acrylamide stacking gel was poured on top of the 20% non-denaturing gel. Samples were prepared by incubating 0.5 ug of the oligonucleotide with varying amounts of the antibody in PBS buffer for 1 hour at 4°C prior to loading them onto the gel.

Oligonucleotide Preparation

Deoxyoligonucleotide (dGdC)6(dT)12 (the 24mer) was synthesized and purified as previously described. The concentration of oligonucleotides
was determined from the absorbance at 260 nm. For single stranded (dGdC)_6(dT)_{12} an extinction coefficient of 199,000 M^{-1}cm^{-1} (based on moles of oligonucleotide) was calculated from the coefficients of the constituent mono- and di-nucleotides^{16}. A duplex can form with base pairing of the alternating dGdC section. The cooperative melting of this oligomer was observed by monitoring the absorbance at 260nm as a function of temperature; the percent hypochromicity of duplex formation was determined to be 7%. Absorbance values were corrected for hypochromicity when calculating the deoxyoligonucleotide concentration. Oligo(rA)_{12} was purchased from Pharmacia and the extinction coefficient of 91,400 M^{-1}cm^{-1} (22.4 A_{260} units/mg) was used to determine concentrations.

Oligo(rA)_{12} was ligated to the 5' end of (dGdC)_6(dT)_{12} to make the 36mer, (rA)_{12}(dGdC)_6(dT)_{12}. The procedure for this reaction was adapted from that used by Selsing and Wells^{17}. Before the ligation step the deoxyoligonucleotide was phosphorylated with T4 polynucleotide kinase (Pharmacia). Following the reaction the kinase was inactivated by heat in a boiling water bath for 15 minutes. Oligo(rA)_{12} was added in a 1:1 molar ratio to the deoxyoligomer. The ligation was catalyzed with T4 DNA Ligase (Pharmacia). The buffer for both the kinase and ligase reactions contained 20mM Tris-HCl (pH 8.0), 10mM MgCl_2, 50mM NaCl, 5mM dithiothreitol and 6% polyethylene glycol (PEG 8000, Sigma). In a typical reaction the enzymes were added at one enzyme unit per nanomole of oligonucleotide. For the kinase reaction the sample was incubated at 37°C for four hours and the ligation was done at 4°C for 16 hours. The final reaction mixture contained mainly the ligated oligonucleotide along with ATP and some unligated oligo(rA)_{12} and (dGdC)_6(dT)_{12}. The product was purified by chromatography on NACS-20 (Bethesda Research Laboratories) with a 0.5-1.0M KCl gradient, kept at 80°C in a jacketed column (6 cm by 0.5 cm). ATP was eluted in 0.5M KCl and three peaks were resolved by the gradient. The peaks were identified by electrophoresis as (in order of elution) (dGdC)_6(dT)_{12}, oligo(rA)_{12} and (rA)_{12}(dGdC)_6(dT)_{12}.

RESULTS
Preparation and Characterization of (rA)_{12}(dGdC)_6(dT)_{12}

The deoxyoligonucleotide (dGdC)_6(dT)_{12} was synthesized as described^{13}. The alternating dGdC section of this oligomer can base pair to form a duplex with oligo (dT)_{12} tails on each 3' end. The oligoribonucleotide (rA)_{12} was ligated to the DNA with T4 DNA ligase to form
a 36 base pair self-complementary oligonucleotide. The 24mer and 36mer were analyzed by electrophoresis under denaturing conditions (Figure 1) with Msp I restriction fragments of pBR322 as size markers. The purified 36mer ran as a single band in a denaturing gel indicating that the NACS-20 chromatography completely separated the ligated and unligated oligomers in the reaction mixture.

Electrophoresis of the ligated oligomer under non-denaturing conditions resolved two bands (Figure 2). Since the sample contains only one
Figure 3. Competitive ELISA of the binding specificity of H241. The coating antigen was native (double stranded) calf thymus DNA and the antibody was incubated with the following competitors before being added to the plate: calf thymus DNA (—□—), poly(A)-poly(dT) (—△—), (dGdC)\textsubscript{6}(dT)\textsubscript{12} (—■—) and (rA)\textsubscript{12}(dGdC)\textsubscript{6}(dT)\textsubscript{12} (—▲—).

species under denaturing conditions, the two bands seen in the non-denaturing gel are taken to represent different forms of the ligated oligonucleotide. Figure 2 also demonstrates that the relative amount of the two conformers is dependent on the strand concentration. Oligomer samples were prepared at concentrations ranging from 0.4 to 16 micrograms per ml, denatured by placing the mixture in a boiling water bath for 5 minutes and then reannealed by slowly cooling the samples to 0°C. The oligonucleotides were then loaded onto a non-denaturing gel. The relative amounts of the two bands depended on the sample concentration such that the slower migrating band was favored at high oligonucleotide concentrations.

Antibody Binding Experiments

Competitive ELISA experiments were used to measure the binding of H241, Gt4 and Z22 to the oligonucleotides. Calf thymus DNA in solution clearly competed with the coated antigen for H241 binding but poly(A)-poly(dT) did not (Figure 3); thus, H241 can distinguish between the B-DNA helix and the RNA/DNA hybrid helix. The oligonucleotides \((dGdC)\textsubscript{6}(dT)\textsubscript{12}\) and \((rA)\textsubscript{12}(dGdC)\textsubscript{6}(dT)\textsubscript{12}\) both competed for H241 binding (Figure 3). Since H241 binds to the ligated oligomer but not to poly(A)-poly(dT) one can conclude that the antibody binds to the central DNA section of the oligomer and not to the hybrid sections at the ends.
Figure 4. Competitive ELISA of the binding specificity of the anti-RNA/DNA hybrid antibody Gt4. The coating antigen was poly(A)-poly(dT). The antibody was incubated with the following competitors before being added to the plate: calf thymus DNA ( ), poly(A)-poly(dT) ( ), and (rA)$_{12}$(dGdC)$_{6}$(dT)$_{12}$ ( ). Poly(A)-poly(dT) was an effective competitor for the binding of Gt4 while calf thymus DNA was not (Figure 4). The oligonucleotide (rA)$_{12}$(dGdC)$_{6}$(dT)$_{12}$ competed for the anti-hybrid antibody (Figure 4), whereas (dGdC)$_{6}$(dT)$_{12}$, even at a concentration of 100 micrograms/ml did not (data not shown).

Competition experiments with Z22 were performed in a buffer which contained 4M NaCl and 20mM MgCl$_2$. Poly(dGme$_5$dC), which adopts the Z-conformation under these conditions, served as a positive control and clearly competed for the antibody (Figure 5). Neither calf thymus DNA nor poly(A)-poly(dT) adopt the Z-conformation under these conditions and consequently they were not bound by Z22. Both (dGdC)$_6$(dT)$_{12}$ and (rA)$_{12}$(dGdC)$_6$(dT)$_{12}$ were bound by Z22 (Figure 5) indicating that the (dGdC)$_6$ segment can adopt a Z-conformation.

Antibody binding to oligonucleotides can also be detected by a gel retardation assay. Antibody (IgG) will pass through a 3.5% acrylamide stacking gel but not through a 20% gel. When antibody and oligonucleotide are run together, bound oligomers stop at the 20% gel whereas unbound oligonucleotides enter this gel. Staining of the nucleic acids with ethidium bromide demonstrates the presence of bound and free forms of the oligonucleotides. Figure 6 shows the results of a gel retardation experiment in which H241, Z22 and Gt4 were tested with the (dGdC)$_6$(dT)$_{12}$ alone, a mixture of the (dGdC)$_6$(dT)$_{12}$ with oligo(rA)$_{12}$, and the ligated

![Graph](image-url)
Figure 5. Competitive ELISA with the anti-Z-DNA antibody Z22. The coating antigen was Z-form poly(dGme3dC) and the competitors were: poly(dGme3dC) (——○——), poly(A)-poly(dT) (——△——) and \((rA)_{12}(dGdC)_6(dT)_{12}\) (——△——).

Figure 6. Gel retardation assay of antibody binding to oligonucleotides. The lanes contain: a) the 24mer alone, b) the 24mer + oligo(rA)\(_{12}\), c) the 24mer with anti-hybrid antibody, d) the 24mer + oligo(rA)\(_{12}\) with anti-hybrid antibody, e) the 24mer with H241, f) the 24mer + oligo(rA)\(_{12}\) with H241, g) the 24mer with normal rabbit IgG, h) the 24mer + oligo(rA)\(_{12}\) with normal rabbit IgG, i) the 36mer with Z22 (low salt conditions), j) the 36mer with anti-hybrid (4:1), k) the 36mer with the anti-hybrid (2:1), l) the 36mer with H241 (4:1), m) the 36mer with H241 (2:1), and n) the 36mer alone. Each lane contained 0.5 microgram of oligonucleotide and 8 micrograms of antibody for an antibody/oligonucleotide duplex ratio of duplex ratio of 1.7 for the 24mer alone and 2.5 for the other samples.
Each of these oligonucleotide samples contains a segment of (dGdC)$_6$ duplex and, as expected, H241 bound to all three. The anti-hybrid antibody bound to (rA)$_{12}$(dGdC)$_6$(dT)$_{12}$ and to the mixture of (dGdC)$_6$(dT)$_{12}$ with oligo(rA)$_{12}$ but not to (dGdC)$_6$(dT)$_{12}$ alone. Z22 did not bind to any of these oligonucleotides under these conditions.

**DISCUSSION**

**The 36mer Forms a Hairpin Loop**

The sequence of the 36mer is self-complementary and can form two different base paired structures: a duplex structure or a single stranded loop structure (Figure 7). These structures will be in equilibrium and the relative amount of each conformer will depend on the total strand concentration. In non-denaturing gel electrophoresis this oligomer migrated in two bands. When samples were reannealed at high concentrations the slower migrating form predominated whereas at low concentrations both forms were present. Based on the concentration dependence of the two bands the slower migrating band was identified as the duplex form of the 36mer and the faster band as the loop. Both forms of the 36mer were present in the gel retardation experiments and the preference for binding to the duplex structure provides insight into the binding interaction, as will be discussed below.

**The Alternating dGdC Segment is B-Form in Low Salt**

In the current work H241 was used as a marker of the B-DNA conformation. Both the ligated and unligated oligonucleotides contain twelve base pairs of alternating dGdC sequence which, under low salt conditions, forms a strong binding site for H241. Both the competitive ELISA and gel retardation assays demonstrated the binding of H241 to these oligomers.

H241 showed a strong preference for binding to the duplex form of the 36mer. This may be due to a reduction in the size of the binding site in the loop form. The most likely structure for the loop has a bend at the center of the oligomer with some of the G-C base pairs broken. This reduces the number of DNA/DNA base pairs in the stem region for the antibody to bind. Any distortion of the helical conformation is likely to reduce the binding site further. Previous studies with H241 binding to oligonucleotides have shown that the optimal binding site is (dGdC)$_4$ or longer with binding reduced to (dGdC)$_3$ and no binding to (dGdC)$_2$. For a B-form helix the most stable loop contains 4-5 nucleotides. If the loop contains four unpaired bases, as drawn in figure 7, then the stem would contain only a (dGdC)$_2$ stretch and would present a very poor binding site for H241.
As expected, the anti-hybrid antibodies bound only to oligonucleotides containing an RNA/DNA hybrid segment. In both the competitive ELISA experiments and the gel electrophoresis retardation experiments, Gt4 bound to the ligated oligomer but not to (dGdC)_6(dT)_{12}. The anti-hybrid antibody, at a high ratio of antibody to oligonucleotide, formed a complex which was retarded by the stacking gel. This probably results from the binding of more than one antibody per duplex and linking of oligonucleotides together to form high molecular weight complexes. Linking of oligonucleotides requires two binding sites on both the antibody and the oligomers. The ligated oligomer has two regions of hybrid per duplex and the antibody also contains two binding sites. Fab fragments produced by papain digestion of the antibody have only one binding site and therefore cannot crosslink oligonucleotides. In gel retardation experiments using Fab fragments the complexes were not retarded by the stacking gel even at very high ratios of Fab to oligonucleotide (data not shown).

As with H241, the anti-hybrid antibody showed a preference for binding to the duplex form of the 36mer over the loop. It is unlikely that
loop formation disrupts the binding site for Gt4 since the hybrid helix is in the stem of the loop. A more reasonable explanation is the bivalent binding of the antibody to the two hybrid regions of one oligonucleotide duplex, which would be energetically more favorable than monovalent binding or bivalent binding to two different oligomers. The energetics of bivalent binding to one oligomer depend on the distance between the sites and the flexibility of the hinge region which connects the two binding sites of the antibody. In previous work it was suggested that the minimal chain length for bivalent antibody binding to DNA was 35-40 base pairs\textsuperscript{19}. The footprinting experiments of Runkel and Nordheim suggest bivalent binding of Z22 to a segment as small as 22 base pairs\textsuperscript{20}. It is reasonable then that the anti-hybrid antibody can bind the two ends of the 36mer to form the energetically favored bivalent complex.

Anti-hybrid antibodies recognize unique features of the RNA/DNA helix. In general hybrid helices adopt an A-helical conformation\textsuperscript{21,22,23}. It is interesting that Raman spectral studies of \textit{r(GCG)d(CGC)} assigned an A-like geometry to this hybrid molecule but the spectrum differed from both the A-RNA and A-DNA spectra, indicating unique helical characteristics for the hybrid\textsuperscript{10}.

Poly(A)-poly(dT) is unique among RNA/DNA hybrids in that it may adopt a B-conformation. The conformation of this polymer is particularly relevant to the current work since the anti-hybrid antibody was prepared by immunization with poly(A)-poly(dT), and since the hybrid regions of the oligonucleotide model system have the base sequence (rA)\textsubscript{12}(dT)\textsubscript{12}. In the earliest fiber diffraction studies on poly(A)-poly(dT) the hybrid adopted and A-helix at low humidity but a B-helix at high humidity\textsuperscript{24}. From these data a model of the solution state was proposed in which the overall conformation was B-like but the sugar pucker was mixed (C3'endo for the poly(A) strand and C3'exo for the poly(dT)). More recently, a similar model was proposed based on Raman spectroscopy in solution and measurements of proton exchange rates\textsuperscript{25}. The overall shape of the helix is like the B-helix but the phosphodiester backbones contain features of both A- and B-helices.

The immunochemical data on RNA/DNA hybrids presented previously\textsuperscript{9,26} and in this work are consistent with a unique conformation for the hybrid in solution which is present in both poly(A)-poly(dT) and in hybrids with other base sequences. Purified anti-hybrid antibodies such as Gt4 react only with hybrid helices (whether they have A-dT, I-dC or mixed sequence base pairs) but not with B-DNA or A-RNA, suggesting unique conformational features for
the hybrid. On the other hand, unabsorbed sera of animals immunized with poly(A)-poly(dT)\(^9\) or mixed sequence hybrids\(^{27}\) also contain populations of antibodies that cross-react with A-form double stranded RNA. Some anti-poly(I)-poly(C) antibodies cross-react with poly(A)-poly(dT)\(^{26}\). These data suggest that all RNA/DNA hybrids share some conformational features that distinguish them from RNA and DNA, and that all hybrids, including poly(A)-poly(dT) have some features of the A-helix.

**In High Salt the Alternating dGdC Segment Adopts the Z-conformation**

Z22 is a highly specific probe for the Z-conformation\(^{14}\). The antibody was prepared by immunization of mice with brominated poly(dGdC) which retains the Z-conformation under physiological conditions. This antibody binds to poly(dGBr\(^5\)dC), poly(dGme\(^5\)dC) in 20mM MgCl\(_2\) and poly(dGdC) in 4M NaCl. Chemical footprinting experiments have demonstrated the binding of Z22 to specific Z-forming sites within highly supercoiled plasmids\(^{20}\). Thus this antibody specifically recognizes the Z-form helix, as seen in its binding to the high salt form of poly(dGme\(^5\)dC) but not to either calf thymus DNA or poly(A)-poly(dT) (Figure 5). The binding of Z22 to both the ligated and unligated oligonucleotides indicates that the (dGdC)\(_6\) segment can adopt the Z conformation even in the presence of the hybrid duplex on either side.

**A Model System For Conformational Junctions**

The 36mer has been shown to adopt different conformations in different sections of the molecule. Other systems containing regions of RNA/DNA hybrid have been studied. The oligomer \([r(GCG)d(TATAGGC)]_2\) has been crystallized and studied by X-ray diffraction\(^{23}\). Although this molecule contains both DNA duplex and RNA/DNA hybrid, the helix was A-form throughout. Selsing and Wells prepared block polymers which contained regions of DNA duplex and regions of RNA/DNA hybrid\(^{17}\). This work indicated different conformations for the helices and a model was proposed for an A-B junction which involved a bend in the helix. The 36mer may be a suitable model system for more detailed conformational studies to test this model. Since the 36mer contains Z-helix under high salt conditions it may also contain an A-Z junction.

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