CD of six different conformational rearrangements of poly[d(A-G)·d(C-T)] induced by low pH

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ABSTRACT

On the basis of circular dichroism (CD) data, we have now identified six different conformational states (other than the duplex) of poly[d(A-G)·d(C-T)] at pH values between 8 and 2.5 (at 0.01M Na^+; 20°C). Three of these structural rearrangements were observed as the pH was lowered from 8 to 2.5, and three additional rearrangements were observed as the pH was raised from 2.5 back to neutral pH. The major components of the six conformational states were defined using appropriate combinations of the CD spectra of the duplex, triplex, and denatured forms of this polymer, as well as the CD spectra of the individual single strands and their respective acid-induced self-complexes. Our results show that the acid-induced rearrangements of poly[d(A-G)·d(C-T)] include not only the poly[d(C^+-T)·d(A-G)·d(C-T)] triplex, but also include the poly[d(C-T)] loop-out structure and a self-complexed form of the poly[d(A-G)] strand that is pH-dependent.

INTRODUCTION

The current explosion of DNA sequence information has resulted in increasing evidence that simple, non-coding sequences of nucleic acids are involved in genetic control processes, although the mechanisms by which these sequences function are poorly understood. Polypurine·polypyrimidine stretches frequently flank transcribed eukaryotic genes and have been found to be hypersensitive to DNase I and single-strand-specific nucleases.[1-6] Hypersensitivity to these nucleases is induced by supercoiling and is also pH-dependent. The reason for this hypersensitivity is not known, although an altered structure in the polypurine·polypyrimidine region seems most likely.[1-6]

Several specific models have been proposed for such pH-dependent nuclease-sensitive structures. Pulleyblank et al.[3] interpreted their data with the plasmid pTC45 (containing a $d(A\text{-}G)_{45}\cdot d(C\text{-}T)_{45}$ insert) as indicating that the base pairing at pH 5 remains interstrand and that hypersensitivity to single-strand-specific nucleases is due to the formation of an alternating structure in which dA·dT Watson-Crick base pairs alternate with ^{syn}dG·dC^+ base pairs. To explain the S1-nuclease cleavage pattern observed in the 209bp-long poly-

purine•polypyrimidine sequence of a cloned fragment of the thyroglobulin gene promoter region, Christophe et al.[4] suggested that a triple-stranded structure is formed, with the excess polypurine being single-stranded. Another type of model was introduced by Lyamichev et al.[5] to explain the nuclease hypersensitivity of a $d(A\text{-}G)_{16}\cdot d(C\text{-}T)_{16}$ insert in plasmid pEJ4. They suggested that the $d(C\text{-}T)_{16}$ sequence upon protonation might form a self-complex of the loop-out type proposed by Gray et al.,[7] while the complementary $d(A\text{-}G)_{16}$ sequence remains essentially single-stranded. In an addendum to this work, Lyamichev et al.[8] favored the formation of a triplex structure, as in the model described by Christophe et al.[4] Our own preliminary studies with poly[d(A-G-G)•d(C-C-T)] have shown that structures containing $C\cdot C^+$ base pairs may be involved in the conformations of polypurine•polypyrimidine sequences at low pH.[9]

Synthetic poly[d(A-G)•d(C-T)] has been studied as a model polypurine•polypyrimidine sequence. Thiele et al.[10] observed that CD data from a titration of this polymer (at 0.15M NaCl, 25°C) showed significant hysteresis when the pH was lowered to 2.5 and then brought back to neutrality. On the basis of physicochemical studies and model building, Johnson and Morgan[11] proposed that poly[d(A-G)•d(C-T)] formed a tetra-stranded structure upon lowering the pH to below 6. However, Morgan and his coworkers later found that melting profiles, S1 nuclease digestion data, buoyant density measurements, and CD studies were inconsistent with the tetraplex model but were consistent with a model in which the duplex disproportionated at low pH to give a triple-stranded structure (poly[d(C^+-T)•d(A-G)•d(C-T)]) plus a polypurine self-complex.[12] The triplex required protonation of cytosine, but the polypurine self-complex was thought to be independent of pH since its Tm was unaffected by raising the pH from 5 to 8.[12] Poly[d(A-G)•d(C-T)] was in a buffer containing 0.2M or 0.5M NaCl for the studies by Morgan and coworkers.[11,12]

We have now performed detailed pH titrations of poly[d(A-G)•d(C-T)] between pH 8 and 2.5. Our data showed six different structural rearrangements for this polymer (at 0.01M Na^+) as the pH was lowered to 2.5 and then raised to 8. The acid-induced rearrangements included the triplex identified by Lee et al.,[12] the loop-out structure of the polypyrimidine strand described by Gray et al.,[7] and a self-complex of the polypurine strand that was pH-dependent.

MATERIALS AND METHODS

Poly[d(A-G)•d(C-T)] was synthesized and the poly[d(C-T)] strand was iso-

lated as previously described.[13,14] The polymers were dialyzed first against 0.01M EDTA, 0.5M NaCl, and 0.01M Na^+(phosphate), second against 0.01M NaCl and 0.01M Na^+(phosphate), and finally against 0.01M Na^+(phosphate), all at pH 7 or 8. The oligomer $d(A\text{-}G)_{30}$ was synthesized by the phosphite triester method using a Beckman Model 1 DNA synthesizer. It was purified on a 20% polyacrylamide gel, electroeluted, and ethanol-precipitated. The oligomer was then resuspended in 0.01M Na^+(phosphate), pH 7.8. For one experiment, a mixture of $d(A\text{-}G)_n$ (where $10 < n < 30$) was dialyzed as described for the polymers. Extinction coefficients (ϵ_{260}) used for poly[d(A-G)·d(C-T)] and poly[d(C-T)] were 5700 and 7500 $L \cdot mol^{-1} \cdot cm^{-1}$, respectively.[15,12] The extinction coefficient for $d(A\text{-}G)_{30}$ was assumed to be 9500 $L \cdot mol^{-1} \cdot cm^{-1}$, the same as for poly[d(A-G)].[12]

UV absorption spectra were measured with a Cary-Varian Model 118 spectrophotometer. Circular dichroism measurements were made using a Jasco J500A circular dichrometer calibrated with d-10-camphorsulfonic acid (Aldrich Chemical Co.) to give a value within 2% of 0.336 deg ellipticity at 290.5nm for a 0.1% (w/v) solution.[16] The ratio of the magnitudes of the peaks at 192.5nm and 290.5nm was -2.05 ±0.03, close to the value of -2.00 obtained by Chen and Yang.[16] CD spectra were measured using a spectral band width of 1nm, a time constant of 1s, and a scan speed of 10nm/min. Digitized data were obtained every 0.1nm with a Jasco DP-500N data processor, the data were smoothed by a sliding 7-point third order polynomial, and every tenth point was transferred to a Hewlett Packard 9816S computer. The data were then smoothed by a sliding 13-point quadratic-cubic function.[17] CD spectra per mole of nucleotide monomer are plotted as $\epsilon_L - \epsilon_R$ in units of $L \cdot mol^{-1} \cdot cm^{-1}$. Sample temperatures during the optical measurements were controlled using a Neslab programmable circulating water bath. The reported temperatures are accurate to within ±0.5°C.

Unless otherwise specified, the pH titration of samples was carried out at 20°C in a starting buffer of 0.01M Na^+(phosphate). The samples were first centrifuged for 15 min at 15,600 g in an Eppendorf microcentrifuge. The pH was then lowered by the addition of small aliquots of dilute HCl and was raised with small aliquots of NaOH solutions. Spectra were corrected for dilution by HCl and NaOH, which was never more than 10%. The sample pH was measured directly before and after each set of optical measurements, using a Beckman Century SS-1 pH meter and a Beckman 5mm Futura Combination electrode. The reported pH values are accurate to within ±0.05 pH unit.

To determine the extent of depurination during the acid and back titra-

tions, triplicate samples of poly[d(A-G)•d(C-T)] at 0.01M Na^+(phosphate) were subjected to the extremes of pH encountered in the experiments. The samples were then precipitated with perchloric acid at 0°C and quickly passed through a 0.45μm Millipore filter. The extent of depurination was determined from the non-precipitable material in the filtrate. A standard for a completely depurinated sample was prepared by heating triplicate samples of poly[d(A-G)•d(C-T)] at pH 2.5 for 2.5 hours at 90°C. The extent of depurination in these samples was taken as 100%. When compared with this standard, the depurination of samples equivalent to those subjected to the most extreme conditions in the experiments was less than 2.5%.

The CD spectra of poly[d(A-G)•d(C-T)] obtained at pH values between 8 and 2.5 were analyzed by fitting each with a set of basis spectra, using a program based on linear-programming techniques (written by Dr. F.S. Allen, Chemistry Dept., Univ. of New Mexico, Albuquerque). The basis set for this analysis consisted of the CD spectra of (i) the duplex at pH 8.4, (ii) the triplex at pH 5.0, (iii) free $d(A-G)_{30}$ at pH 7.8, (iv) the $d(A-G)_{30}$ self-complex at pH 4.0, (v) free poly[d(C-T)] at pH 2.5, (vi) the poly[d(C-T)] self-complex at pH 5.1, and (vii) the denatured single-strands at pH 2.5. Each spectrum was represented by 31 data points (from 305 to 215nm at 3nm intervals). Spectra used in the analyses included those from two detailed acid titrations and two detailed back (alkali) titrations. Analyses of 3 of the 38 spectra from the acid titrations, and 9 of the 31 spectra from the alkali titrations were not included in the plots shown in Figures 6 and 7 either because the standard deviation of the fit to the measured spectrum was greater than 0.37 ϵ_L - ϵ_R ($L \cdot mol^{-1} \cdot cm^{-1}$) or because the sum of the unnormalized fractions of the spectral components fell outside the range 0.8 - 1.4. Spectra taken during the back titrations were probably less accurate because of the accumulation of errors due to dilution and manipulation. (The standard deviation of a fit was defined as $\sigma_{fit} = [\Sigma_\lambda(CD_m(\lambda) - CD_c(\lambda))^2/(N-1)]^{0.5}$, where $CD_m(\lambda)$ and $CD_c(\lambda)$ are the molar CD values of a measured and a calculated spectrum, respectively, at N wavelengths.)

The measurement error was estimated from the noise at each wavelength in measured CD spectra (combined with the noise of baselines). The range of noise at each wavelength, $CD_n(\lambda)$, was determined for spectra of poly[d(A-G)•d(C-T)] at three different pH values. One-half of $CD_n(\lambda)$ was taken as the maximum error at each wavelength. The measurement error of a spectrum was then estimated to be $[\Sigma_\lambda(CD_n(\lambda)/2)^2/(N-1)]^{0.5} = 0.14 \pm 0.04$ $L \cdot mol^{-1} \cdot cm^{-1}$.

RESULTS AND DISCUSSION

Circular dichroism data of poly[d(A-G)•d(C-T)] at 0.01M Na^+(phosphate), 20°C, indicated that the polymer could exist in six different conformational states between pH 8 and 2.5. In order to interpret the CD changes observed for poly[d(A-G)•d(C-T)] as the pH was lowered to 2.5 and then returned to 8, the CD changes of the individual strands as a function of decreasing pH were studied. We also measured the CD spectrum of the poly[d(C^+-T)•d(A-G)•d(C-T)] triplex. The next three paragraphs describe the spectra of these polymer conformations that were subsequently found to form during the acid-induced transitions of poly[d(A-G)•d(C-T)].

CD of the Poly[d(C^+-T)•d(A-G)•d(C-T)] Triplex

Various amounts of single-stranded poly[d(C-T)] were added to the duplex poly[d(A-G)•d(C-T)] at pH 7 (0.1M Na^+). Three days later, the pH of the samples was lowered to 6, and after three more days the pH was lowered to 5. CD measurements made during these intervals showed no significant changes at a given pH. The mixing curve at pH 5, shown in the inset to Figure 1, had a break point at about 33% poly[d(A-G)] and 67% poly[d(C-T)], confirming that a triplex was formed with a 1:2 purine:pyrimidine stoichiometry. The CD spec-

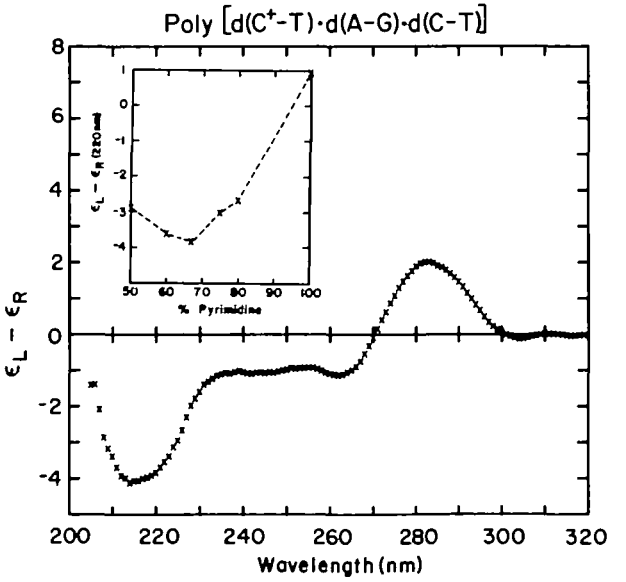


Figure 1. CD spectrum of the triplex, poly[d(C^+-T)•d(A-G)•d(C-T)] at pH 5, 0.1M Na^+(0.01M phosphate + 0.09M Cl^-), 20°C, formed by the addition of poly-[d(C-T)] to the duplex, as described in the text. Inset: the mixing curve for the duplex plus added poly[d(C-T)], at pH 5.

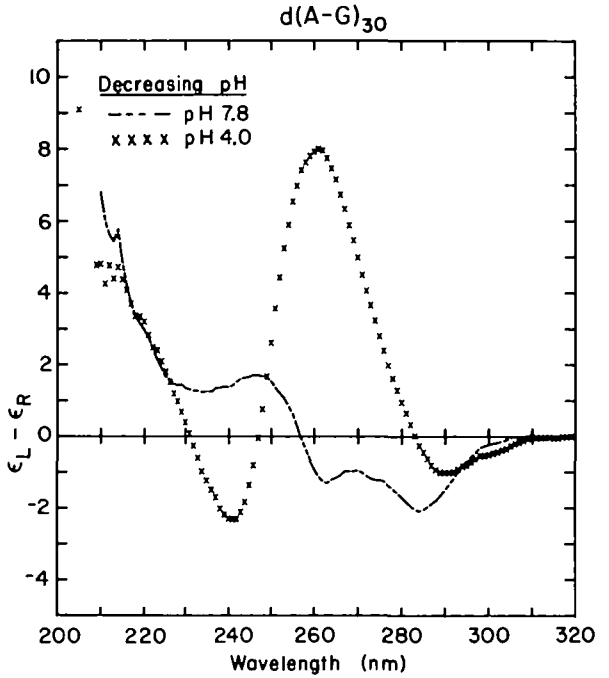


Figure 2. CD spectra of $d(A-G)_{30}$ (0.01M Na^+(phosphate), 20°C) at pH 7.8 (—·—·—) and in its self-complexed form at pH 4.0 (x x x).

trum of the mixture at the break point is also shown in Figure 1. This spectrum was similar to that of the mixture at pH 6. No significant changes were observed in the CD spectrum of the sample at pH 5 after it was kept for one month at 4°C. A generally similar spectrum down to 220nm has been published by Lee et al.[12] for the triplex, poly[d(C^+-T)•d(A-G)•d(C-T)]. A dominant feature of the CD spectrum of the triplex was a negative band at 215nm. The latter was a useful indicator of the presence of the triplex in samples of acidified poly[d(A-G)•d(C-T)]. The protonation of cytosine in the second poly[d(C-T)] strand is required to allow Hoogsteen pairing of the pyrimidines of this strand with the purines of the poly[d(A-G)] strand.[12]

CD of the Acid Self-Complex of $d(A-G)_{30}$

Circular dichroism data of $d(A-G)_{30}$ at 0.01M Na^+ showed that an acid-induced transition occurred as the pH was lowered from 7.8 to 4. The CD spectrum of the acid-induced self-complex at pH 4 included a large positive band at 261nm and a negative band at 241nm (see Figure 2). The CD spectrum of this oligopurine self-complex at pH 4 was similar in shape to that reported by

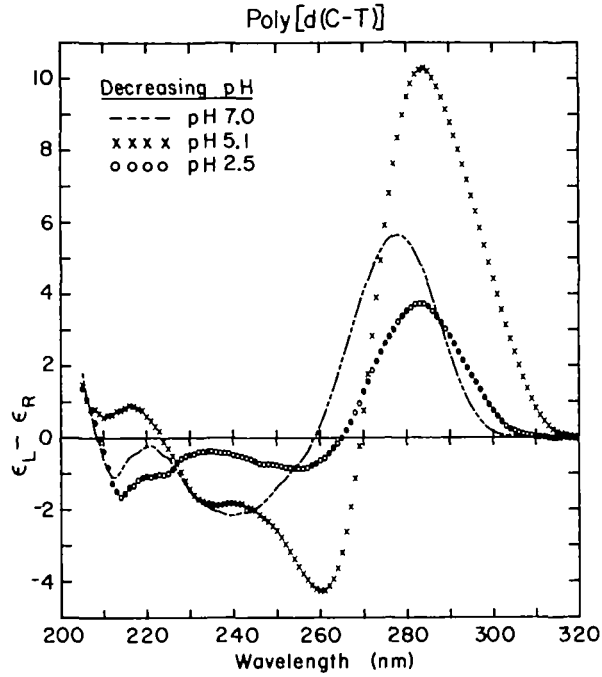


Figure 3. CD spectra of poly[d(C-T)] (0.01M Na^+(phosphate), 20°C) at pH 7.0 (——·——), in its self-complexed form at pH 5.1 (x x x), and in its protonated single-stranded form at pH 2.5 (o o o).

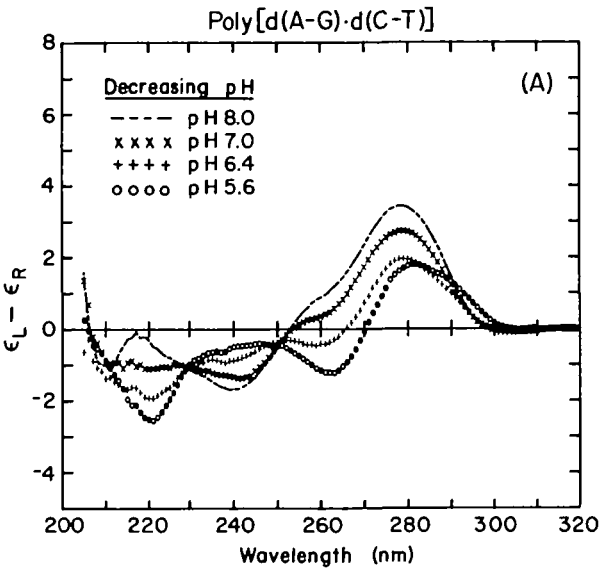
Lee et al.[12] for the polymeric self-complex (at 0.2M NaCl, 50mM Na^+(acetate), pH 5), although the CD bands were less in magnitude for the oligomeric self-complex (by 13% at 260nm and by 44% at 240nm). Lee et al.[12] concluded that the polypurine self-complex was not pH-dependent since its Tm was unaffected by raising the pH from 5 to 8. In a separate experiment, we increased the Na^+ concentration from 0.01M to 1M of a sample of $d(A\text{-}G)_n$ (where $10 < n < 30$) at neutral pH, and found CD spectral changes (with a midpoint at about 0.2M Na^+) similar to those we observed for $d(A\text{-}G)_{30}$ upon decreasing the pH (data not shown). Thus, a transition to the $d(A\text{-}G)_n$ self-complex could be induced by lowering the pH or raising the salt concentration.

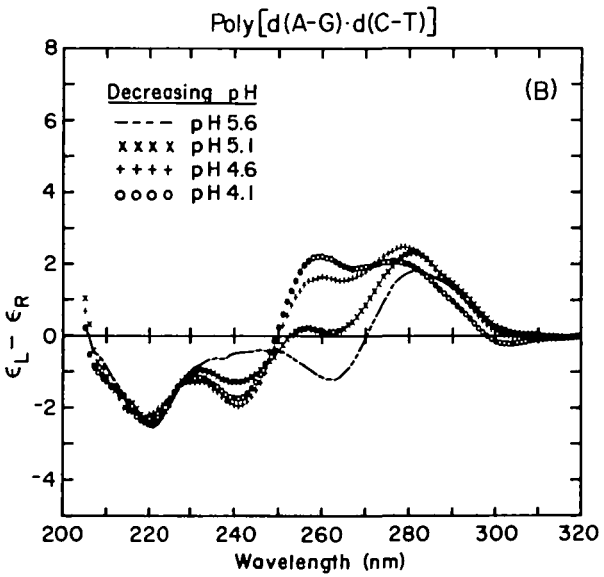
The structure of the acid-induced $d(A\text{-}G)_{30}$ self-complex is not known. On the basis of melting studies and model building of various polypurine DNA and RNA self-complexes, Lee et al.[18] proposed that such polypurine self-complexes might be tetra-stranded structures with paired guanines and with the intervening adenines possibly paired. However, the base pairing schemes proposed

by Lee et al.[18] do not take into account any protonation of the bases.

CD of the Acid Self-Complex of Poly[d(C-T)]

The CD spectral changes observed for poly[d(C-T)] at 0.01M Na^+(phosphate) as the pH was lowered from 7 to 5 included the formation of a large positive





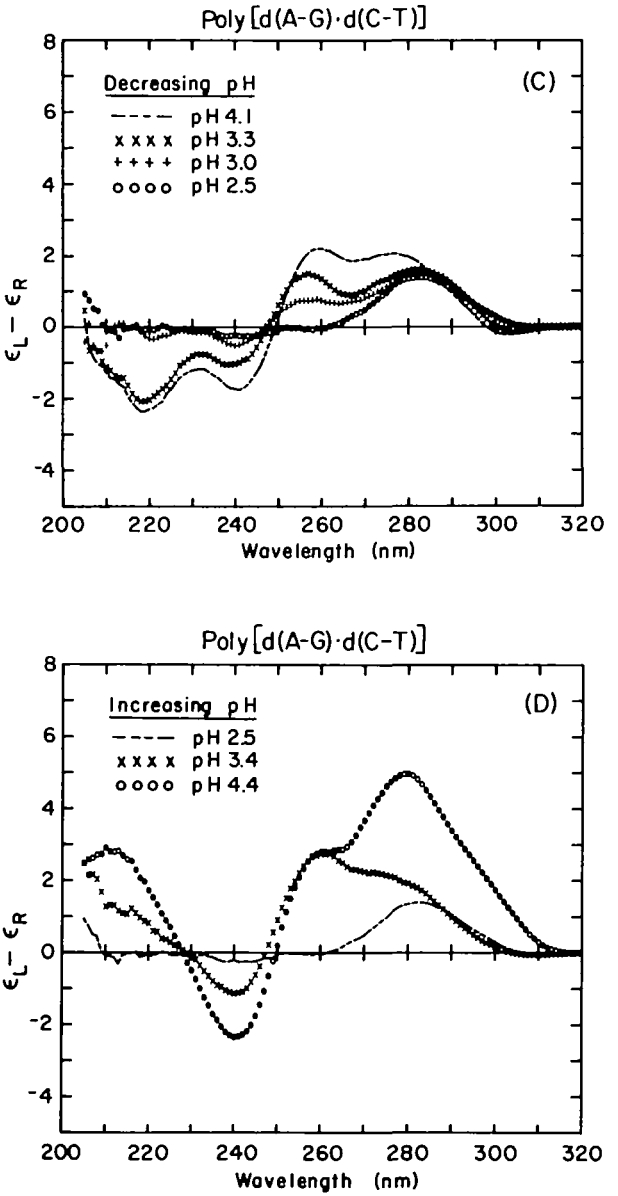


Figure 4. Representative CD spectra from a titration of poly[d(A-G)•d(C-T)] (0.01M Na^+(phosphate), 20°C): (A) from pH 8.0 to 5.6, (B) from pH 5.6 to 4.1, (C) from pH 4.1 to 2.5, and (D) from pH 2.5 back to 4.4.

band at 283.5nm and a negative band at 260nm (Figure 3). These spectral changes were also observed for this polymer at higher Na^+ concentrations (0.08M and 0.09M Na^+) and are characteristic of the protonated poly[d(C-T)] loop-out structure.[7,14,19] This structure consists of a core of stacked, hemiprotonated $C \cdot C^+$ base pairs with the intervening thymidyl residues looped out.[7,14,19,20] Below pH 4, poly[d(C-T)] (at 0.01M Na^+) was denatured to its protonated single-stranded form (Figure 3). The pH range over which the self-complex was present was smaller and the magnitudes of the CD bands of the self-complex were less for the polymer at 0.01M Na^+ than for the polymer at the higher Na^+ concentrations.[19]

Six Structural Rearrangements of Poly[d(A-G)•d(C-T)]

The acid-induced CD spectral transitions observed for poly[d(A-G)•d(C-T)] will first be described in qualitative terms. Representative CD spectra characterizing the pH-dependent rearrangements of the polymer at 0.01M Na^+ are shown in Figure 4. Quantitative analyses of such spectra using a spectral fitting program will be presented in the next section.

The first transition occurred between pH 8 and 5.6 and resulted in a generally decreased positive CD at wavelengths above 253nm, while distinct negative bands appeared at 263 and 220nm (Figure 4A). A negative band at shorter wavelengths was a dominant feature of the CD spectrum of the poly-[d(C^+-T)•d(A-G)•d(C-T)] triplex (Figure 1). Thus, the negative CD band that appeared at 220nm as the pH was lowered from 8 to 5.6 indicated that there was disproportionation of the duplex to give the triplex, which would have to be accompanied by the release of some polypurine strands. The absence of a positive CD band at 260nm indicated that the released poly[d(A-G)] was not self-complexed (Figure 2).

The second transition (pH 5.6 to 4.1) was characterized by an increase in the CD at about 260nm (giving a positive band) and the appearance of a distinct negative band at 240nm (Figure 4B). These changes were similar to those that occurred upon formation of the acid-induced self-complex of $d(A-G)_{30}$ (Figure 2). The magnitude of the negative CD band at 220nm did not change significantly, indicating that the triplex remained intact during this transition. Hence, we suspected that during this transition the polypurine strands that had been released during the first transition formed a pH-dependent self-complex that now co-existed with the triplex.

The third transition (pH 4.1 to 2.5) resulted in an overall reduction in magnitude of the CD at wavelengths below about 285nm (Figure 4C). The only significant CD band at the end of this transition was a positive one between

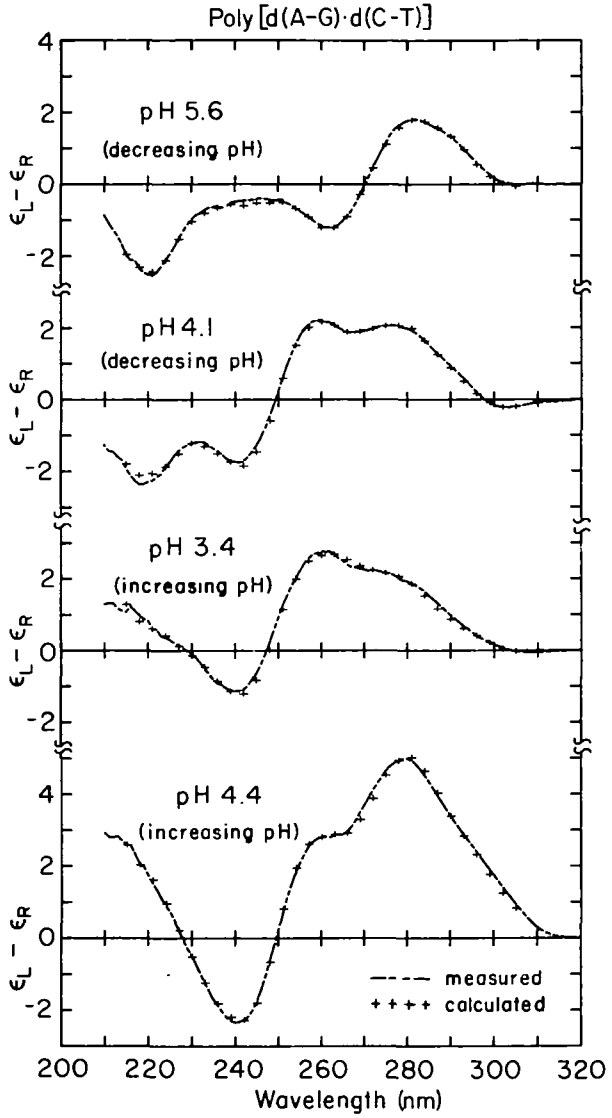


Figure 5. Examples of the spectral fits from the quantitative spectral analyses described in the text; calculated (+ + +) and measured (——·——) CD spectra for poly[d(A-G)·d(C-T)] at the various pH values.

263 and 300nm. At the end of this transition the CD bands at 260 and 220nm were lost, most likely due to the disruption of both the poly[d(A-G)] self-complex and the triplex. The UV absorption data showed increasing hyperchro-

micity especially between 300 and 250nm, with greater than 30% hyperchromicity at 260nm (data not shown). These UV absorption and CD spectral changes indicated that at pH 2.5 the polymer had denatured into the protonated single strands.

The fourth transition was observed as the pH was increased from 2.5 to 3.4 (Figure 4D). During this transition, the positive CD at 260nm increased with a concomitant increase in the negative CD at 240nm. These changes were evidence for the formation of some poly[d(A-G)] self-complex (Figure 2). However, the absence of a negative CD band at 220nm suggested that the triplex had not formed to any significant extent.

A fifth transition occurred as the pH was increased from 3.4 to 4.4 (Figure 4D). A large positive CD band appeared at 280nm, which was consistent with the formation of the poly[d(C-T)] self-complex (Figure 3). The CD of the poly[d(C-T)] self-complex is characterized by a negative band at 260nm as well as by a large positive CD band at long wavelengths. However, the CD at 260nm did not change significantly during the transition from pH 3.4 to 4.4 (Figure 4D) and suggested that the negative 260nm band from formation of the poly[d(C-T)] self-complex was compensated by a positive 260nm CD band from formation of additional poly[d(A-G)] self-complex (Figure 2). The addition of more poly-[d(A-G)] self-complex would also explain the further increase in the negative CD band at 240nm during this transition (Figure 4D). Thus, at the end of the fifth transition, the protonated self-complexes of the individual strands of poly[d(A-G)•d(C-T)] appeared to coexist in solution.

A sixth transition (not shown) occurred as the pH was raised from 4.4 to 6.2 and was characterized by a decrease in the positive CD between 270 and 250nm, indicating the disruption of some of the poly[d(A-G)] self-complex. A quantitative analysis (below) showed that the duplex polymer started to form during a back titration over this range.

Spectral features of the duplex poly[d(A-G)•d(C-T)] were generally present for samples as the pH was raised from 6.2 to 8 (data not shown). As will be shown below, the triplex was also present as a component of neutralized samples that had been exposed to low pH. The triplex present in these samples was disrupted and more duplex was formed when the samples were heated to 80°C for 30 min, cooled, and allowed to reanneal at 4°C overnight; these changes were evidenced by an increase in the positive CD above 255nm and a loss of the negative 220nm CD band (not shown).

The CD spectra of samples representative of the six structural rearrangements of poly[d(A-G)•d(C-T)] showed no significant changes when measured again after being stored for at least two days at 4°C.

Quantitative Analysis of CD Spectra of Poly[d(A-G)•d(C-T)]

The qualitative analyses just described for the CD spectra of poly[d(A-G)•d(C-T)] as a function of pH suggested that these spectra might be quantitatively analyzed using simple combinations of the CD spectra of the duplex, triplex, and denatured forms of this polymer, in addition to the CD spectra of the individual strands and their respective acid-induced self-complexes, each at a single pH value (see Materials and Methods). Figure 5 shows the spectral fit of these seven components to four representative CD spectra of poly[d(A-G)•d(C-T)] measured at the end of the first, second, fourth, and fifth transitions, respectively. The standard deviations of 43 of 69 fits were within the estimated measurement error of 0.14 ϵ_L - ϵ_R. Of the remaining 26 fits, 23 had standard deviations between 0.14 and 0.37 ϵ_L - ϵ_R. We considered these fits to be very good, especially since pH-dependent variations in the spectra of the components were neglected. The closeness of the fits strongly suggested that the basis set of seven components was sufficient to describe all the rearrangements of poly[d(A-G)•d(C-T)] at pH values between 8 and 2.5.

Analysis of samples at decreasing pH. Figure 6 shows the spectral contributions of each of the structures included in the basis set to spectra of poly[d(A-G)•d(C-T)] obtained during acid titrations from pH 8 to 2.5. As the pH was lowered from 8 to about 5.5, the fraction of duplex decreased while the amount of triplex and free poly[d(A-G)] increased. Between pH 5.5 and 4, the fraction of free poly[d(A-G)] decreased and that of its self-complex increased, while the amount of the triplex remained fairly constant. Thus, the acid titration of poly[d(A-G)•d(C-T)] (at 0.01M Na^+) was characterized by (1) the formation of the triplex and its presence down to pH 3.5 and (2) the release of a significant amount of the polypurine strand, which formed its own self-complex below pH 5.5. Below pH 3, the denatured single strands were predominant.

Analysis of samples at increasing pH. An analysis similar to that shown in Figure 6 was carried out for CD spectra of poly[d(A-G)•d(C-T)] obtained during back titrations from pH 2.5 to 8. Figure 7 shows the decrease in the fraction of the single strands (top panel) and the concomitant increases in the fractions of the self-complexes of poly[d(A-G)] and poly[d(C-T)] (middle panel) as the pH was raised from 2.5 to 4.5. The formation of the poly[d(A-G)] self-complex preceded the formation of the poly[d(C-T)] self-complex. Between pH 4.5 and 6, free poly[d(A-G)] appeared while the fraction of the poly[d(A-G)] self-complex decreased. Coinciding with the presence of free poly[d(A-G)], the duplex also appeared in this pH range. The triplex was

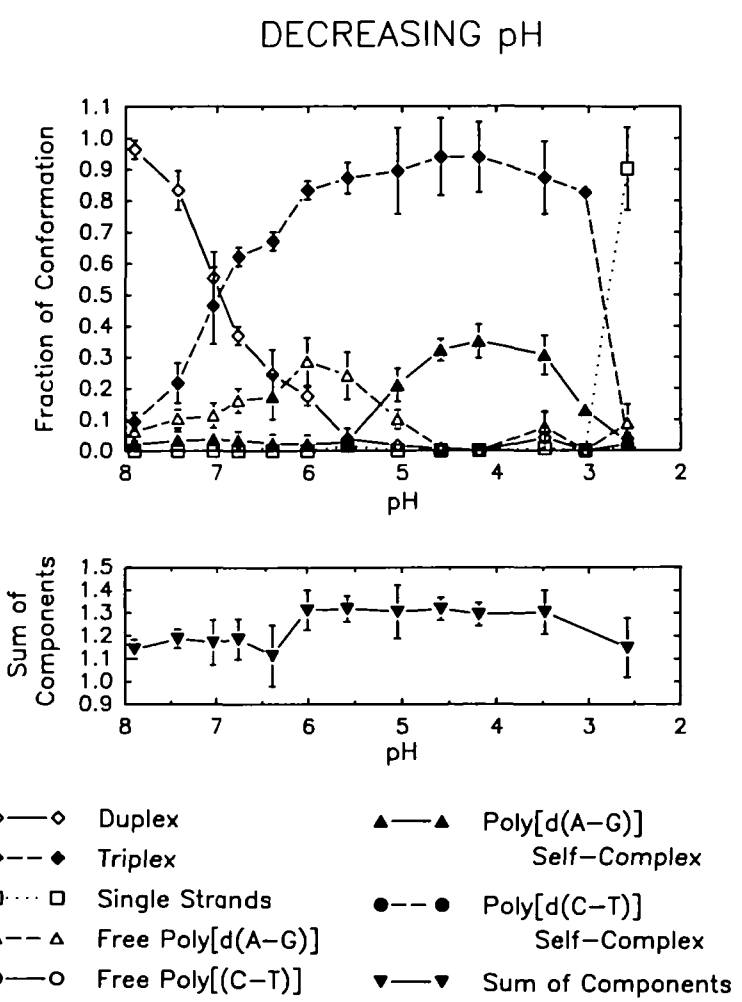


Figure 6. The contributions of 5 structures to the CD spectra of poly[d(A-G)•d(C-T)] measured as the pH was lowered from 8 to 2.5 (0.01M Na^+). Contributions by free poly[d(C-T)] and the poly[d(C-T)] self-complex were omitted for clarity; the sum of these contributions ranged from 0 to 0.12 and averaged 0.02. The symbols with error bars represent the average of two or more analyses for samples within ±0.2 pH units of the average. The error bars indicate ± one standard deviation. The lower panel in this and the following figure shows the sum of all seven components as a function of pH.

formed only as the poly[d(C-T)] self-complex was finally dissociated above pH 6. There were large variations in the amounts of the duplex and the triplex in different samples of poly[d(A-G)•d(C-T)] that were neutralized, as shown by

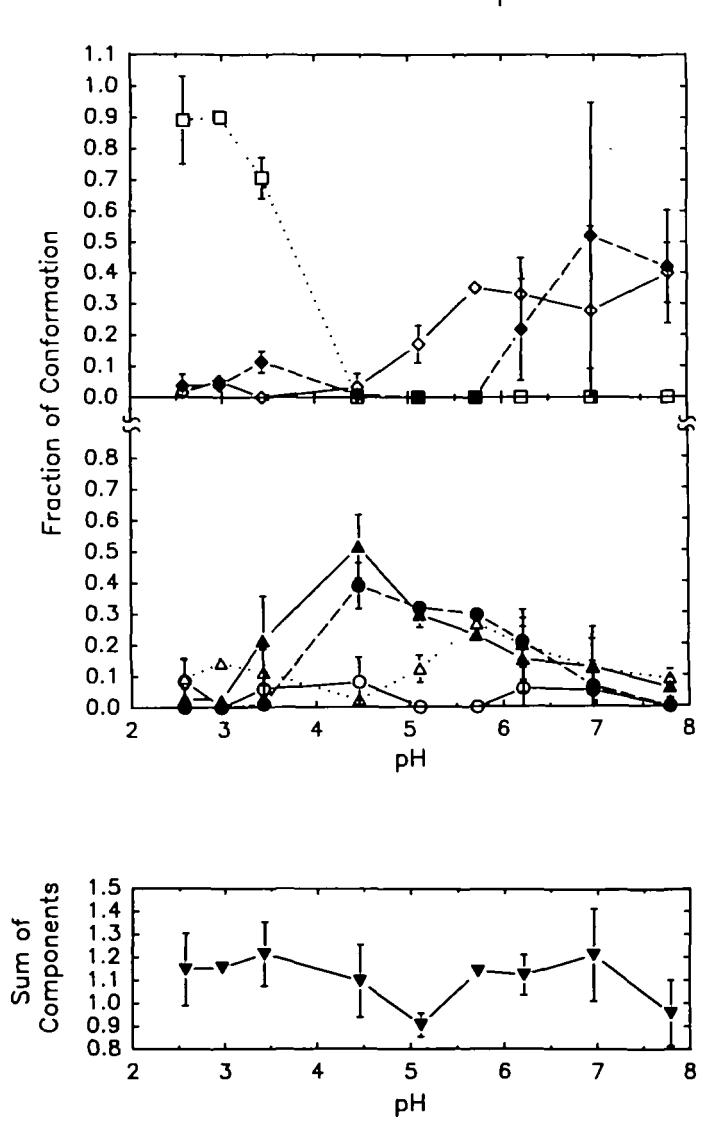


Figure 7. The contributions of 7 structures to the CD spectra of poly[d(A-G)•d(C-T)] measured during back titrations from pH 2.5 to 8 (0.01M Na^+). The symbols with error bars represent the average of two or more analyses for samples within ±0.2 pH units of the average, with the exception of the ones for pH 7, which were within ±0.4 pH unit. The error bars indicate ± one standard deviation.

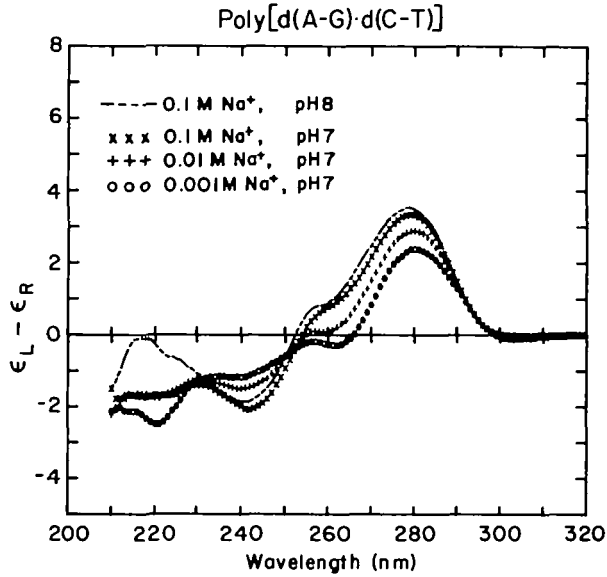


Figure 8. CD spectra at 20°C of poly[d(A-G)•d(C-T)] at pH 8, 0.1M Na^+(phosphate) (——·——); and at pH 7, 0.1M Na^+(phosphate) (x x x), 0.01M Na^+(phosphate) (+ + +), and 0.001M Na^+(phosphate) (o o o).

the standard deviations (denoted by the error bars) for these two components between pH 6 and 8. In summary, the back (alkali) titration was characterized by (1) rearrangements involving the self-complexes of the individual strands up to a pH of about 6 and (2) the absence of the triplex below pH 6 due to the sequestering of the individual strands into their respective acid self-complexes. The structural rearrangements that appeared during the back titration were not simply a reversal of those that occurred during the acid titration.

For spectra analyzed from both the acid and alkali titrations, the sum of the component fractions generally exceeded 1.0 (bottom panels of Figures 6 and 7). This was not unexpected, since the fractions relied on having an accurate (or at least a consistent) set of molar CD spectra for the individual components. The magnitudes of the molar CD spectra relied, in turn, on molar extinction coefficients, whose accuracy was uncertain. The molar CD spectrum of the triplex, in particular, appeared to be too small since the analyzed fraction of this component was sometimes as high as 0.94, although the triplex could not have constituted more than 0.75 of any sample. Thus, the high sum of components from analyses of spectra measured during the acid titration (bottom panel of Figure 6) was partly due to the unreasonably high fraction of

the triplex component (top panel of Figure 6). It was also possible that the fractions of free and self-complexed poly[d(A-G)] were overestimated since the CD spectrum of the self-complex of the oligomer $d(A-G)_{30}$ (Figure 2) had a lesser magnitude than that reported for the self-complex of poly[d(A-G)][12]. In summary, the absolute contributions of the different components at a given pH (shown in the top panels of Figures 6 and 7) cannot be taken to be correct. Nevertheless, we believe that the relative contributions from a given component at different values of pH were approximately correct since the spectral fits (Figure 5) were within or close to the error of measurement and all of the components involved appeared to be accounted for.

Salt-dependence of pH-induced Structural Rearrangements of Poly[d(A-G)•d(C-T)]

At pH 8, the CD spectra of samples of poly[d(A-G)•d(C-T)] that were dialyzed into 0.001M, 0.01M, or 0.1M Na^+(phosphate) showed no significant differences (data not shown) and were similar to the one shown in Figure 8 for the polymer at pH 8, 0.1M Na^+(phosphate). However, the CD spectra for the polymer dialyzed into buffers at pH 7 had a 220nm negative band that increased and a CD above 255nm that decreased with decreasing ionic strength (Figure 8). These spectral changes were similar to those observed during the first spectral transition of poly[d(A-G)•d(C-T)] at 0.01M Na^+(phosphate) (Figure 4A) and indicated increased disproportionation of the duplex to the triplex plus free polypurine at the lower salt concentrations at pH 7. It was interesting that the formation of the triplex, which requires the protonation of cytosine, occurred at neutral pH in samples of the unmethylated poly[d(A-G)•d(C-T)]. Lee *et al.*[21] reported that the methylated analogue poly[d(A-G)•d(me^5C-T)] forms a triplex at pH values as high as 8, but they did not detect the formation of the triplex in the unmethylated polymer at pH values above 6.

At the higher salt concentration of 0.1M Na^+ (0.01M Na^+(phosphate) + 0.09M NaCl), no extensive denaturation to the single-strands was observed as the pH was lowered from 8 to 2.5. CD spectral changes (not shown) were consistent with there being three transitions: (1) the disproportionation of the duplex to the triplex plus free polypurine, (2) the formation of a polypurine self-complex, and (3) the disruption of the polypurine self-complex while the triplex was still stable at pH 2.5. On raising the pH back to 8, the CD spectra indicated that the polymer went through the same three transitions it did upon lowering the pH, although the presence of a negative 220nm CD band was evidence for the presence of the triplex even above neutral pH.

At the higher Na^+ concentration (0.1M Na^+), the positive, long-wavelength CD band characteristic of the protonated polypyrimidine self-complex was not

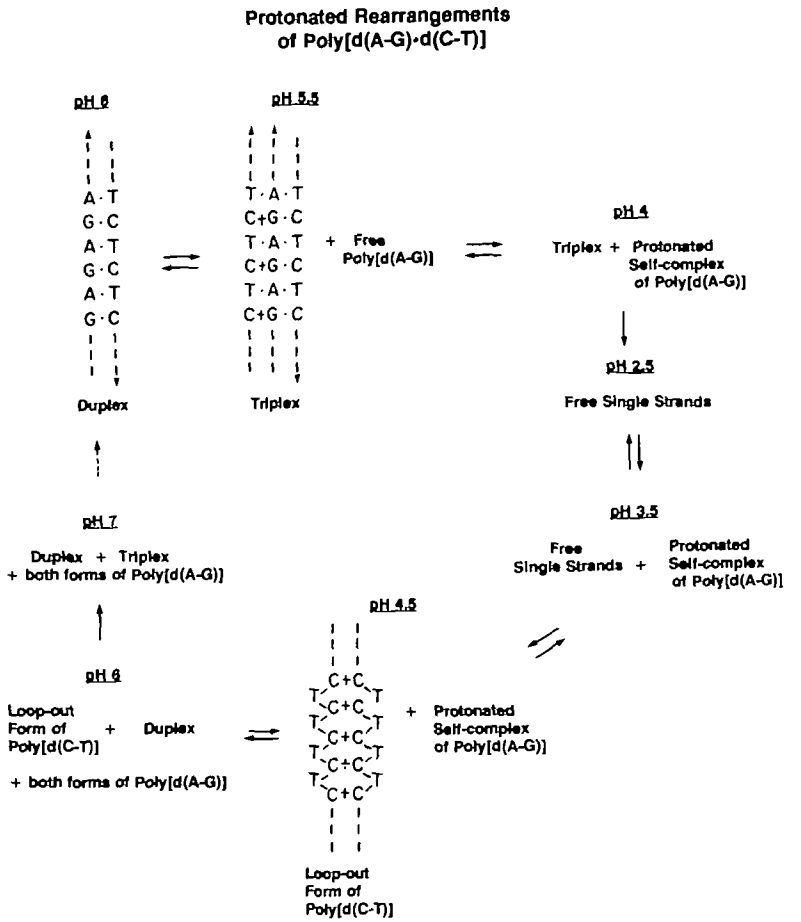


Figure 9. A schematic representation of the major pH-dependent transitions of poly[d(A-G)·d(C-T)] and the resulting structures that form at a cation concentration of 0.01M Na^+. The dashed arrow between pH 7 and 8 in the back titration is to indicate that samples were not completely reversed to the duplex unless they were heated to above the Tm of the triplex (see text).

observed before or after exposure to low pH. To determine if the stability of the triplex at 0.1M Na^+ at very low pH was the reason the polypyrimidine self-complex did not form, the pH of a sample of poly[d(A-G)·d(C-T)] at 0.01M Na^+ was lowered to 2.5, resulting in denaturation of the polymer. The salt concentration was then increased to 0.1M Na^+, and the pH was raised to 8. CD spectral changes characteristic of the poly[d(C-T)] self-complex were then observed during the back titration for the polymer at the increased ionic

strength (0.1M Na^+) (data not shown). Thus, denaturation of the polymer was necessary for the formation of the polypyrimidine self-complex. Further evidence that disruption of the triplex was required in order to obtain the polypyrimidine self-complex came from melting and reannealing experiments performed with samples of poly[d(A-G)•d(C-T)] at pH values below 5, at both 0.01M and 0.1M Na^+ (data not shown). Briefly, these experiments showed that the protonated polypyrimidine self-complex was formed upon reannealing to 20°C if the polymer at the highest temperature (usually about 85°C) was denatured, as evidenced by the UV absorption and CD spectra.

SUMMARY AND CONCLUDING REMARKS

Six different structural rearrangements of poly[d(A-G)•d(C-T)] at 0.01M Na^+ were observed as the pH was lowered from 8 to 2.5 and then raised back to 8. These six rearrangements could be interpreted in terms of the pH-dependent formation of the triplex and self-complexes of the individual strands. A schematic representation of these pH-induced transitions of poly[d(A-G)•d(C-T)] is shown in Figure 9. The pH values correspond approximately to the end of the spectral transitions described above. However, each structure occurred over a range of pH values as can be seen in Figures 6 and 7. Figure 9 shows that denaturation of the triplex upon lowering of the pH from 4 to 2.5 (at 0.01M Na^+) was a prerequisite for the formation of the protonated polypyrimidine self-complex when the pH was raised back up to 4.5.

The six structural rearrangements described here for poly[d(A-G)•d(C-T)] occurred in the linear DNA polymer. Some studies on the pH- and supercoiling-induced nuclease hypersensitivity of cloned polypurine•polypyrimidine sequences have been interpreted in terms of an altered conformation that consists of the triplex with the excess polypurine segment being essentially single-stranded.[4,8] Our finding that the polypyrimidine self-complex, in addition to the polypurine self-complex, can be formed after denaturation of the polymer raises the possibility that local denaturation (which is favored in plasmids under torsional strain) might lead to the formation of the self-complexes of the individual strands. The actual altered conformation induced by supercoiling and/or acid pH of the cloned, torsionally constrained polypurine•polypyrimidine sequences remains to be determined. Nevertheless, our data with the linear polypurine•polypyrimidine model sequence poly[d(A-G)•d(C-T)] clearly shows that the conformational polymorphism of this polymer, as illustrated in Figure 9, includes not only the poly[d(C^+-T)•d(A-G)•d(C-T)] triplex, but also the protonated poly[d(C-T)] loop-out structure and a self-complex of the poly[d(A-G)] strand that is pH-dependent.

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REFERENCES

1. Elgin, S.C.R. (1984) Nature 309, 213-214.
2. Cantor, C.R. and Efstratiadis, A. (1984) Nucl. Acids Res. 12, 8059-8072.
3. Pulleyblank, D.E., Haniford, D.B., and Morgan, A.R. (1985) Cell 42, 271-280.
4. Christophe, D., Cabrer, B., Bacolla, A., Targovnik, H., Pohl, V., and Vassart, G. (1985) Nucl. Acids Res. 13, 5127-5144.
5. Lyamichev, V.I., Mirkin, S.M., and Frank-Kamenetskii, M.D. (1985) J. Biomol. Struct. Dyn. 3, 327-338.
6. Fowler, R.F. and Skinner, D.M. (1986) J. Biol. Chem. 261, 8994-9001.
7. Gray, D.M., Vaughan, M., Ratliff, R.L., and Hayes, F.N. (1980) Nucl. Acids Res. 8, 3695-3707.
8. Lyamichev, V.I., Mirkin, S.M., and Frank-Kamenetskii, M.D. (1986) J. Biomol. Struct. Dyn. 3, 667-669.
9. Gray, D.M., Morgan, A.R., and Ratliff, R.L. (1978) Nucl. Acids Res. 5, 3679-3695.
10. Thiele,D., Sarocchi, M-T., Guschlbauer, W., Lezius, A., and Marck, C. (1973) Mol. Biol. Rep. 1, 155-160.
11. Johnson, D. and Morgan, A.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1637-1641.
12. Lee, J.S., Johnson, D.A., and Morgan, A.R. (1979) Nucl. Acids Res. 6, 3073-3091.
13. Leslie, A.G., Arnott, S., Chandrasekaran, R., and Ratliff, R.L. (1980) J. Mol. Biol. 143, 49-73.
14. Brown, D.M., Gray, D.M., Patrick, M.H., and Ratliff, R.L. (1985) Biochemistry 24, 1676-1683.
15. Wells, R.D., Larson, J.E., Grant, R.C., Shortle, B.E., and Cantor, C.R. (1970) J. Mol. Biol. 54, 465-497.
16. Chen, G.C. and Yang, J.T. (1977) Anal. Lett. 10, 1195-1207.
17. Savitzky, A. and Golay, M.J.E. (1964) Anal. Chem. 36, 1627-1639.
18. Lee, J.S., Evans, D.H., and Morgan, A.R. (1980) Nucl. Acids Res. 8, 4305-4320.
19. Gray, D.M., Ratliff, R.L., Antao, V.P., and Gray, C.W. (1987) in DNA and Its Drug Complexities, Vol 2 from the Proceedings of the Fifth Conversation in Biomolecular Stereodynamics, Sarma, R.H., and Sarma, M.H. (Eds.), Adenine Press, New York (to be published).
20. Sarma, M.H., Gupta, G., and Sarma, R.H. (1986) FEBS Lett. 205, 223-229.
21. Lee, J.S., Woodsworth, M.L., Latimer, L.P.J., and Morgan, A.R. (1984) Nucl. Acids Res. 12, 6603-6614.