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CD of homopolymer DNA·RNA hybrid duplexes and triplexes containing A·T or A·U base pairs

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ABSTRACT

CD spectra and difference-CD spectra of (a) two DNA·RNA hybrid duplexes (poly[r(A)·d(U)] and poly[r(A)·d(T)]) and (b) three hybrid triplexes (poly-[d(T)·r(A)·d(T)], poly[r(U)·d(A)·r(U)], and poly[r(T)·d(A)·r(T)]) were obtained and compared with CD spectra of six A·U- and A·T-containing duplex and triplex RNAs and DNAs. We found that the CD spectra of the homopolymer duplexes above 260 nm were correlated with the type of base pair present (A·U or A·T) and could be interpreted as the sum of the CD contributions of the single strands plus a contribution due to base pairing. The spectra of the duplexes below 235 nm were related to the polypurine strands present (poly-[r(A)] or poly[d(A)]). We interpret the CD intensity in the intermediate 255-235 nm region of these spectra to be mainly due to stacking of the constituent polypurine strands. Three of the five hybrids (poly[r(A)·d(U)], poly-[r(A)·d(T)], and poly[d(T)·r(A)·d(T)]) were found to have heteronomous conformations, while poly[r(U)·d(A)·r(U)] was found to be the most A-like and poly[r(T)·d(A)·r(T)], the least A-like.

INTRODUCTION

The structure and function of DNA·RNA hybrids in biological processes have been much less well studied than those of DNA and RNA. Yet, hybrids are involved in the key biological processes of replication and transcription. (1) DNA·RNA hybrids are required as primers during replication of both procaryotic and eucaryotic genomes,[1,2] and are intermediates in the reverse transcription of retroviral RNA.[1-3] (2) Hybrids may also be intermediates during DNA synthesis by unique mitochondrial DNA polymerases that have a great deal of sequence homology with known reverse transcriptases.[4,5] (3) There is an exploitation of hybrids in genetic experiments with mRNAs being used as probes of gene location and size in viral and cloned DNAs,[6] and with DNA inhibitors being used to control translation.[7,8] (4) The lability of some hybrid sequences such as oligo[d(A)·r(U)] may help add to the dissociation of RNA messengers from their DNA templates during transcription.[9,10]

The secondary structure of a DNA·RNA hybrid was first investigated by Milman *et al.*,[11] who obtained fiber-diffraction data of fd phage DNA plus its

transcribed RNA. The authors found that the hybrid duplex had diffraction patterns characteristic of the A conformation (*i.e.* 11 base pairs per helical turn, C3'-*endo* furanose conformation[12]). Fiber data for poly[r(I)·d(C)][13] indicated that the hybrid was isostructural with its RNA analogue, poly-[r(I)·r(C)], and was in the A' conformation (12 base pairs per helical turn, C3'-*endo* furanose conformation[12]). From these data, it has been considered virtually canonical that all DNA·RNA hybrids have the secondary structure of the A family. Various studies of hybrids have now demonstrated that not all are A-form. Gray and Ratliff[14] have shown that CD spectra of poly[d(A-C)·r(G-U)] and poly[r(A-C)·d(G-T)] are intermediate to CD spectra of their respective DNA and RNA counterparts. A study by Zimmerman and Pheiffer[15] showed that the homopolymer hybrid poly[r(A)·d(T)] can have two distinct secondary structures (A' or B-like). The authors suggested that the B-like conformation found in wet fibers is actually heteronomous, *i.e.* the two strands of the duplex maintain different conformations.[15] Specifically, they suggested that the poly[r(A)] RNA strand has an A-like conformation, while the poly[d(T)] DNA strand has the sugar pucker of the B conformation. Arnott *et al.*[16] have proposed that the homopolymer hybrids poly[d(A)·r(U)] and poly[d(I)·r(C)] might also have heteronomous secondary structures. In both instances, the DNA strand has the B-form C2'-*endo* deoxyribose pucker, while the RNA strand has the A-form C3'-*endo* ribose pucker.[16]

Fiber diffraction techniques have led to a determination of the conformations of regular, triple-stranded complexes of homopolymer purines and pyrimidines (poly[r(U)·r(A)·r(U)], poly[r(U)·d(A)·r(U)], poly[d(C)·d(I)·d(C)], and poly[d(T)·d(A)·d(T)]).[17-19] The consensus concerning these triplexes is that they can form if the second polypyrimidine strand binds through Hoogsteen base pairing in the major groove of a Watson-Crick base-paired duplex.[20] All three strands of such triple-stranded structures have been shown to be A-form, albeit with reduced base tilt,[19] with the second polypyrimidine strand running antiparallel to the first polypyrimidine strand.[18] Raman spectroscopy of poly[r(U)·r(A)·r(U)] suggests that the conformation of ribose in the two pyrimidine strands may differ, with C3'-*endo* pucker in the central duplex and C2'-*endo* pucker in the third added strand.[21] Triplex structures composed of monomeric[22] or dimeric[23,24] rA or dA with poly[r(U)] can also form at high concentrations of salt.

Optical studies have demonstrated the formation of more unusual triple-stranded nucleic acids such as poly[d(T)·d(A)·r(U)],[25-27] poly[d(T)·r(A)·d(T)],[25] poly[d(T)·r(A)·r(U)],[25] poly[r(T)·d(A)·r(T)],[28] poly[r(5-ethyl-U)·r(A)·r(5-ethyl-U)],[29] poly[r(U)·r(2-amino-A)·r(U)],[30] poly[d(T-C)·d(G-A)·

d(C^+-U)],[26] and poly[d(T-C)•d(G-A)•d(C^+-T)].[31] Triplexes such as poly[r(I)•r(A)•r(I)][19] and poly[r(U)•r(A)•r(I)][32] exist since hypoxanthine has the same functional imino and carbonyl groups as uracil. Finally, it has been suggested that poly[r(5-bromo-U)•r(N6-methyl-A)•r(5-bromo-U)] adopts a novel base pairing scheme in which the N6-methyl group of the purine rotates to alternately hydrogen-bond with the C2-oxygen atoms of both polypyrimidines.[33]

CD investigations of hybrids and triplexes are sparse although CD spectral features of the B-, A-, and Z-forms of DNAs and RNAs have been delineated.[34-37] As mentioned above, Gray and Ratliff[14] have shown that CD spectra of poly[d(A-C)•r(G-U)] and poly[r(A-C)•d(G-T)] are unique, with bands inconsistent with either a pure B or a pure A conformation. CD and NMR studies of the hybrid oligo[d(T-C-A-C-A-T)•r(A-U-G-U-G-A)] and of its DNA analogue suggest that both duplex oligomers have B-form secondary structures at 5°C, although end effects may play an important structural role in the conformations of these relatively short oligomers.[38] A study of the hybrid copolymer poly[r(G)-d(C)] demonstrated that its CD spectrum has features of the Z-form at low [Na^+], those of the A-form at intermediate [Na^+], and those of the Z-form at very high [Na^+].[39] Several investigations of drug interactions with hybrids have also suggested that the conformations of some DNA•RNA hybrids are not A-form. For example, the antibiotic netropsin, which requires the B-conformation for binding to the minor groove, shows a higher degree of binding to poly[r(A)•d(T)] than to poly[r(A)•d(U)] (which has a CD spectrum unlike poly[r(A)•d(T)] and is more like that expected for an A-conformation).[40] However, a CD study of the binding of the anti-tumor agent CC-1065, which also requires the B-form, suggests that both poly[r(A)•d(T)] and poly[r(A)•d(U)] can be induced to assume the B-conformation.[41] Finally, it was demonstrated during a CD study of the effect of 2'-fluroribose substitution for ribose that the spectrum of poly[d(I)•r(C)] resembles spectra of its RNA analogue and poly[fluro-d(I)•r(C)], while the spectrum of poly[r(I)•d(C)] resembles spectra of its DNA analogue and poly[fluro-d(I)•d(C)].[42]

In this work, we have used CD spectroscopy to monitor the secondary structure of A•T and A•U duplex and triplex hybrids. An advantage of utilizing CD spectroscopy is its sensitivity to aspects such as base tilt, base stacking, and nearest-neighbor frequency.[34,43] However, the interpretation of CD spectra relies largely on empirical associations of spectra with known polymer conformations. We have assumed that the RNAs poly[r(A)•r(U)] and poly[r(U)•r(A)•r(U)] are A-form[12,17] and that the DNA poly[d(A)•d(T)] is close to the B-form,[44,45] (see conclusion no. 5), and we have relied on these

as references for the solution structures of the other polymers.

We obtained absorption and CD spectra for two double-stranded and three triple-stranded homopolymer hybrids at pH 7.0 and various concentrations of Na^+. The duplex hybrids investigated were poly[r(A)·d(U)] and poly[r(A)·d(T)], while the triplex hybrids studied were poly[r(U)·d(A)·r(U)], poly-[r(T)·d(A)·r(T)], and poly[d(T)·r(A)·d(T)]. We compared the hybrid spectral data with data for six duplex and triplex RNAs and DNAs. RNAs and DNAs used for comparison were poly[r(A)·r(U)], poly[r(U)·r(A)·r(U)], poly[d(A)·d(U)], poly[d(U)·d(A)·d(U)], poly[d(A)·d(T)], and poly[d(T)·d(A)·d(T)].

METHODS

Single-stranded homopolymers (mol. wt. > 100,000 g) were obtained from either Sigma (poly[r(A)], poly[d(A)], poly[r(U)], and poly[d(T)]) or P-L Biochemicals (poly[d(U)]). All polymers were dialyzed into phosphate buffer (pH 7.0) as described previously[46] to a final Na^+ concentration of 0.100 M. Extinction coefficients at 257 nm (ε_{257}) of poly[r(A)] and poly[d(A)] were taken to be 10,000[47] and 9,650[78] $L \cdot mol^{-1} \cdot cm^{-1}$, respectively, while the ε_{260} values of poly[r(U)], poly[d(U)], and poly[d(T)] were taken to be 9,430,[47] 9,900,[49] and 8,140[48] $L \cdot mol^{-1} \cdot cm^{-1}$, respectively, at 0.100 M Na^+, 20°C.

The duplex and triplex homopolymers were obtained by mixing the single-stranded constituents in phosphate buffer (pH 7.0) at the cation concentrations noted in Table I. An exception was poly[r(T)·d(A)·r(T)] which was synthesized as described below. The polypyrimidine and the polypurine solutions were adjusted to equal concentration (±0.25%), as determined by their absorption spectra and the above extinction coefficients. Polymer concentrations in terms of nucleotides were *ca.* 8.5×10^{-5} $mol \cdot L^{-1}$. The two solutions were mixed to yield nine samples with pyrimidine percentages of 100, 80, 67, 60, 50, 40, 33, 20, and 0%, to ± 0.5%. Samples were allowed to stand at room temperature for 4-5 h to allow sufficient time for duplex or triplex formation. An exception was poly[r(A)] plus poly[r(U)] mixing in phosphate buffer ($[Na^+]$ = 0.100 M, pH 7.0) where a period of 24 h was needed to effect formation of the duplex, since it is formed by disproportionation of triplex and free poly[r(A)].[47] To avoid precipitation of poly[r(A)] at salt concentrations > 1.0 M,[25] mixtures containing poly[r(A)] were first made with phosphate buffer ($[Na^+]$ = 0.100 M, pH 7.0), to which buffered, concentrated solutions of Na_2HPO_4 (plus CsCl in the case of poly[d(T)·r(A)·d(T)]) were then added so that the final pH value was 7.0. Consistent with published reports, we were unable to generate poly[d(A)·r(U)],[25,50] or poly[d(U)·r(A)·d(U)].[49]

Absorption spectra of each of the nine samples in an experiment were

recorded at 20°C ±0.5° in a Cary-Varian Model 118C recording spectrophotometer. Mixing curves (not shown) for the RNA, DNA, and hybrid homopolymers were generated by plotting the ε_{260} of each of the nine samples as a function of the mole fraction of polypyrimidine. Proof that the stoichiometry (polypyrimidine:polypurine) of the samples was either 1:1 or 2:1 was obtained from the mixing curves by noting the mole fraction of polypyrimidine (either 0.50 or 0.67) that yielded the greatest hypochromicity at 260 nm.

For mixtures of (a) poly[r(A)] plus poly[r(U)] at both 0.100 and 1.01 M Na^+, (b) poly[d(A)] plus poly[d(U)] at 0.88 M Na^+, and (c) poly[d(A)] plus poly[d(T)] at 1.11 M Na^+, both duplexes and triplexes could be formed depending on the stoichiometry of the mixtures. In these cases, only the triplex could be detected at 260 nm. To distinguish the duplex forms, mixing curves were also plotted at an isosbestic wavelength of the 1:1 mixture and the free polypurine. (In the above cases, the isosbestic wavelengths used were 278.5 nm, 279 nm, and 275.3 nm, respectively.) At this wavelength, a breakpoint in the new mixing curves was detected at a mole fraction of 0.50 pyrimidine, proving that we obtained the duplex as well as the triplex form. The ε_{260} values for each of the duplexes and triplexes are given in Table I.

Poly[r(T)·d(A)·r(T)] was made as follows. Ribothymidine triphosphate (5-methyl UTP) was synthesized from rTMP using the procedure of Ott *et al.*[51] Poly[r(T)] was synthesized on a poly[d(A)] template using 75 A_{262} units of rTTP, 45 A_{260} units of poly[d(A)] (average length = 1,000 nucleotides), and *E. coli* RNA polymerase under previously described conditions.[14] After deproteinization, 76 A_{260} units of the hybrid complex were isolated and dialyzed in phosphate buffer (0.010 M [Na^+], pH 7.0). Computer-aided curve-fitting of the absorbance spectrum of a sample at 60°C (using 60°C spectra of poly[d(A)] and poly[d(T)] as references) indicated that the ratio of poly[d(A)] to poly[r(T)] was 1:1). However, the hybrid complex in 0.100 M Na^+ (phosphate buffer, pH 7.0) could bind a 1/4 nucleotide equivalent of free poly[d(T)], showing that free poly[d(A)] was present and that the hybrid complex was not double-stranded, but rather triple-stranded as described earlier.[28] Thus, at 0.100 M Na^+, the samples of poly[d(A)] plus synthesized poly[r(T)] contained 75% poly[r(T)·d(A)·r(T)] and 25% poly[d(A)]. To process the absorption and CD spectra, we assumed the ε_{260} value of poly[r(T)·d(A)·r(T)] to be equal to that of poly[d(T)·d(A)·d(T)], which is 5300[25] at 1 M Na^+.

Digitized CD spectra of selected samples (100, 80, 67, 50, 33, and 0% polypyrimidine) at 20°C ± 0.5° were obtained with either a Cary Model 61 or a Jasco J500A circular dichrometer and processed with a Varian 620i or

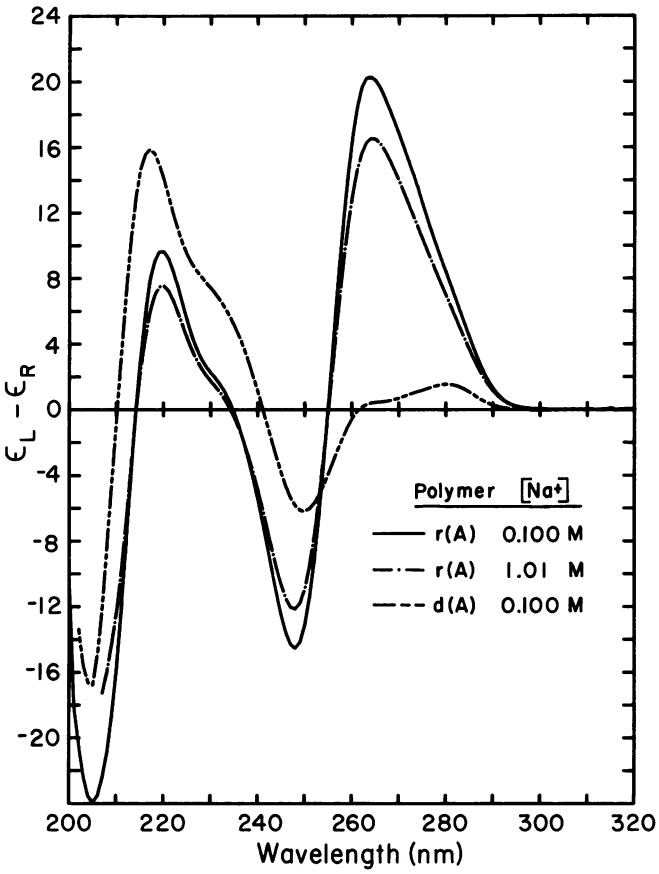

Figure 1. CD spectra of poly[r(A)] and poly[d(A)] at pH 7.0 (phosphate buffer) and the cation concentrations indicated.

Hewlett Packard 9816 computer, respectively. Both CD instruments were calibrated with *d*-10-camphorsulfonic acid (Aldrich Chemical Co.) as described earlier.[52] CD spectra were smoothed as in earlier work.[52] CD data are plotted as $\varepsilon_L - \varepsilon_R$ in units of L·(mol nucleotide)$^{-1}$·cm^{-1}. To generate the CD spectrum of poly[r(T)·d(A)·r(T)], the 25% contribution of free poly[d(A)] was subtracted from the molar spectrum of the poly[d(A)]-plus-poly[r(T)] complex, and the resulting spectrum was multiplied by 1.333.

RESULTS AND DISCUSSION

CD of Single-Stranded Homopolymers

The CD spectra of poly[r(A)] and poly[d(A)] from 320 to 205 nm are given in Fig. 1. Poly[r(A)] had a spectrum composed of four, large ($|\varepsilon_L - \varepsilon_R| \geq 8.0$

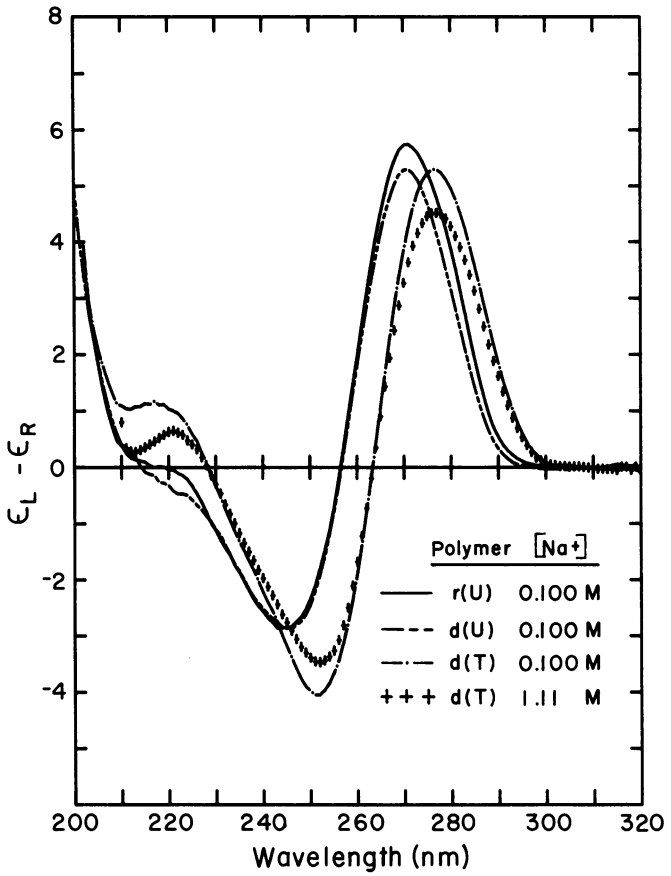

Figure 2. CD spectra of poly[r(U)], poly[d(U)], and poly[d(T)] at pH 7.0 (phosphate buffer) and the cation concentrations indicated.

$L \cdot mol^{-1} \cdot cm^{-1}$) CD bands, centered at 263, 247, 219, and 206 nm. The spectrum of poly[d(A)] had a compound band of small magnitude ($|\epsilon_L - \epsilon_R| \leq 3.0$ $L \cdot mol^{-1} \cdot cm^{-1}$) at 292-261 nm, a band of moderate intensity ($|\epsilon_L - \epsilon_R| = 6.0$ $L \cdot mol^{-1} \cdot cm^{-1}$) centered at 250 nm, a large, compound CD band centered at 218 nm, and a large band at 206 nm. Increasing the $[Na^+]$ from 0.1 to *ca.* 1.0 M had very little effect upon the CD spectrum of poly[d(A)] (not shown), but did slightly depress the CD bands of poly[r(A)] (Fig. 1). The CD spectra of both poly[r(A)] and poly[d(A)] showed a spectrum-wide increase in CD intensity and band complexity relative to that of their mononucleotide components due to the sugar-specific geometry of the stacked bases.[53,54]

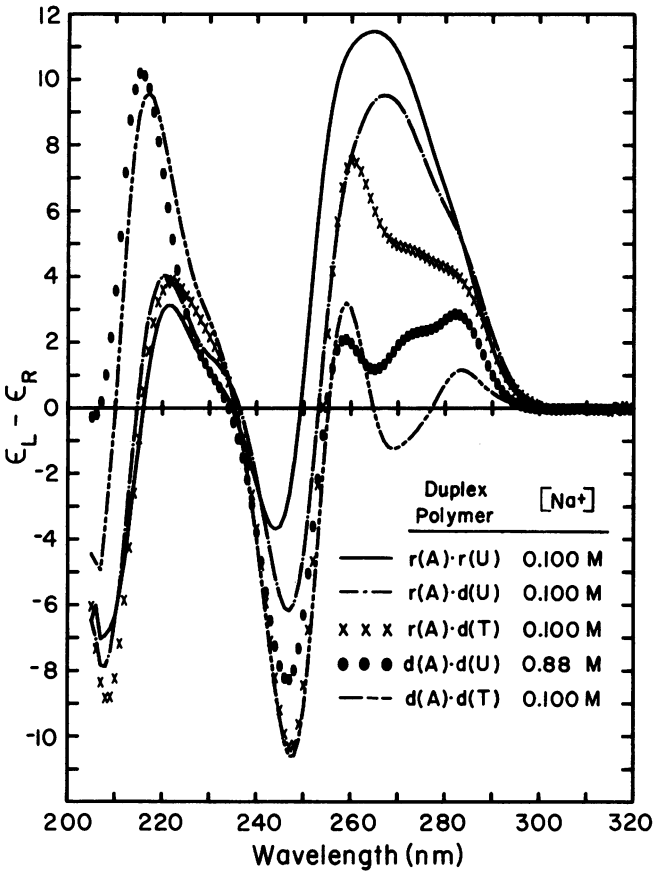

Figure 3. CD spectra of homopolymer duplexes at pH 7.0 (phosphate buffer) and the cation concentrations indicated.

Changes in the CD spectra of pyrimidine mononucleotides upon polymerization are not as great as those for the purine mononucleotides.[53,54] Furthermore, pyrimidines have less tendency than purines to stack in solution.[55-57] An exception is poly[r(T)], which shows a very large (100%) hyperchromicity upon hydrolysis to mononucleotides.[58] The CD spectra of poly[r(U)], poly[d(U)], and poly[d(T)] are shown in Fig. 2. All three polypyrimidines had two CD bands of moderate magnitude above 230 nm, with very slight differences between poly[r(U)] and poly[d(U)] in the intensity of the two bands at 270 and 245 nm. The spectrum of poly[d(T)], with CD bands centered at 276 and 252 nm, was redshifted by 6-7 nm relative to the spectra of poly[r(U)] and poly[d(U)]. Although the CD spectra of poly[r(U)] and

poly[d(U)] from 230 down to 210 nm had magnitudes of $\leq$ 1.0 $L \cdot mol^{-1} \cdot cm^{-1}$, poly[d(T)] had a distinct CD band of small intensity at *ca.* 220 nm. Increasing the [Na^+] from 0.1 to *ca.* 1.0 M slightly depressed the CD bands of all three polypyrimidines, as typified by changes shown in Fig. 2 for poly[d(T)].

CD of Duplex Homopolymers

Fig. 3 shows CD spectra of the double-stranded polymers, which for convenience can be divided into three wavelength regions; 300-255 nm, 255-235 nm, and 235-205 nm. Above 255 nm, the spectrum of poly[r(A)·d(U)] was the most similar both qualitatively and quantitatively to that of the RNA (A-form), while the spectrum of poly[r(A)·d(T)] was intermediate in both shape and magnitude to that of the RNA and the two DNAs (B-form). In this wavelength region, the magnitude of the CD bands of the homopolymers depended on whether the attendant purine strand was poly[r(A)] or poly[d(A)].

In the 255-235 nm region, the spectrum of poly[r(A)·d(U)] was intermediate to that of the RNA and the two DNAs, while the spectrum of poly[r(A)·d(T)] was virtually identical to that of its DNA analogue, poly[d(A)·d(T)]. However, both hybrids actually underwent smaller CD changes on duplex formation than did the RNA or DNAs, as will be discussed below.

The most informative region of the CD spectra of the duplexes was below 235 nm. In this region, the spectra of the hybrids were quite similar to that of the RNA and thus easily distinguishable from the spectra of the DNAs; in essence, the CD spectra of the duplexes in this region were correlated with the presence of poly[r(A)] or poly[d(A)] strands. This should not have been surprising since the CD spectra of the isolated polypyrimidines were small in this region in comparison with the spectra of poly[r(A)] and poly[d(A)]. As a result, the spectra of the duplexes at wavelengths < 235 nm were related in both magnitude and shape to the spectra of the free polyadenosine strands [r(A) or d(A)]. Specifically, poly[d(A)·d(T)] and poly[d(A)·d(U)] had CD bands at *ca.* 217 nm of slightly more than one-half the magnitude of this band in the spectrum of free poly[d(A)], while poly[r(A)·d(U)], poly[r(A)·d(T)], and poly[r(A)·r(U)] had CD intensities at *ca.* 220 nm of a little less than one-half that of the CD of poly[r(A)]. Furthermore, poly[d(A)] and the two DNAs had low-wavelengths crossover points at *ca.* 210 nm, while poly[r(A)], the RNA, and the two hybrids had low-wavelength crossover points at *ca.* 215 nm. These facts implied that the poly[r(A)] and poly[d(A)] strands retained much of their original structure in the duplexes.

To aid in the study of the secondary conformations of the duplexes, we subtracted the average of the CD spectra of the single-strand constituents

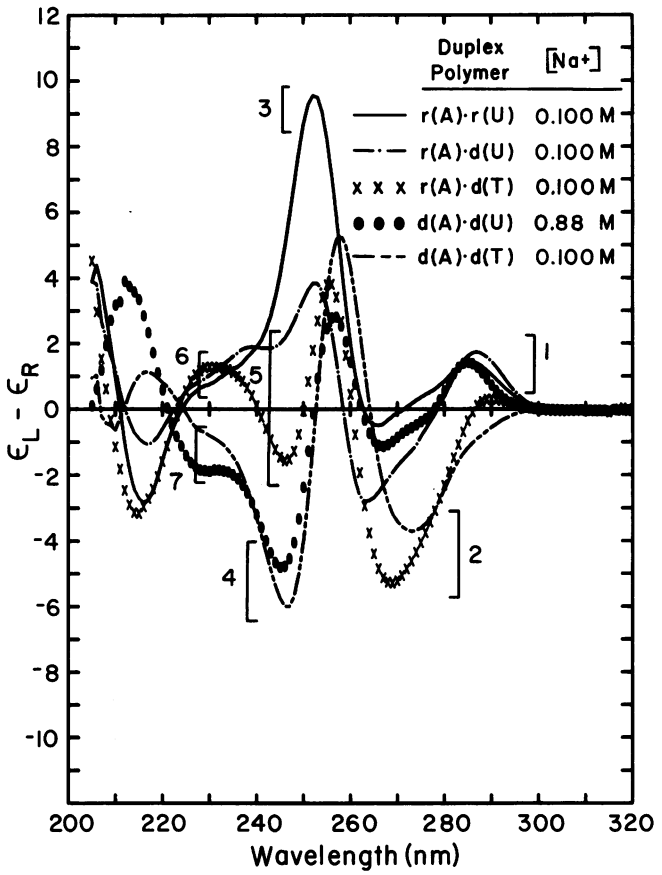

Figure 4. Difference-CD spectra for the double-stranded polymers whose CD spectra are shown in Figure 3. The spectra were calculated by subtracting the average of the CD spectra of the single-stranded homopolymer constituents from the CD spectrum of each duplex.

from the CD spectrum of each duplex (at the same salt concentration). From such difference-CD spectra, we derived changes in the CD of the single strands plus the CD arising from interactions that took place on formation of the double-stranded structures. Those changes are shown in Fig. 4. Two wavelength regions, 300-260 nm and 250-240 nm, were especially helpful in understanding the CD of the five duplex homopolymers.

Despite great variance in CD magnitude and spectral shape from 300-255 nm for the different duplexes (Fig. 3), all the poly[U]-containing duplexes had a positive difference-CD band at 300-280 nm (Fig. 4, bracket 1), while the two poly[T]-containing duplexes had a negative difference-CD band from

290-265 nm (bracket 2). We concluded that the CD spectra of the duplex polymers from 320-260 nm was correlated more with the type of base pair present (A·U or A·T) and with the conformations of the single strands rather than with the secondary structure of the resulting duplex. In effect, at wavelengths $\geq$ 260 nm, the CD spectrum of a given duplex was determined principally by whether the polypurine strand was ribo- or deoxyribo- and was modified by whether the polypyrimidine strand contained U or T.

In contrast to the difference-CD bands in the 300-260 nm wavelength region, difference-CD bands from 250-240 nm had intensities and shapes that appeared reflective of the secondary structure of the duplexes. Bracket 3 shows that the RNA duplex had by far the largest positive difference-CD band, which mirrored the greater breadth of the CD band of poly[r(A)·r(U)] when compared with the same band for the other duplexes. Bracket 4 demonstrates that the two DNAs clustered together in this region and had large, negative difference-CD bands. Bracket 5 shows that the hybrids, poly[r(A)·d(U)] and poly[r(A)·d(T)], held difference-CD intensities intermediate to those of the DNAs and the RNA, despite the fact that poly[d(A)·d(T)] and poly[r(A)·d(T)] had the same CD intensity at 248 nm. We also noted that at wavelengths $\leq$ 230 nm, the difference-CD spectra of the duplexes clustered with respect to whether they contained poly[r(A)] (bracket 6) or poly[d(A)] (bracket 7).

The 250-240 nm region of the difference-CD spectra of the duplexes appeared to result from increased stacking of the polypurine strands. For example, the CD at 248 nm of poly[d(A)·d(T)] and poly[d(A)·d(U)] (Fig. 3) was more negative than the average of the single-stranded components (Fig. 4). Since the CD of the duplexes below 235 nm indicated that the overall conformations of the free poly[A] strands had changed little on becoming part of the duplexes, and since a narrow 248 nm band was unique to the poly[A] strands, we concluded that poly[d(A)] increased its stacking within the DNA duplexes. This was in agreement with Greve *et al.*,[59] who studied the CD of poly[d(A)·d(T)] and poly[d(A-T)] and suggested that the magnitude of this band represented the extent of base stacking.

The difference-CD of poly[r(A)·r(U)] (Fig. 4) showed a large, positive band at 250-240 nm due to the increase in breadth and magnitude of the positive CD band at 292-249 nm (Fig 3). Such an increase in the CD band on duplex formation could have resulted from increased stacking of poly[r(A)] in the duplex. The idea that poly[r(A)] increased its stacking in the RNA is not inconsistent with our conclusion above that increased stacking of the poly[d(A)] strand led to an increased negative CD in the 250-240 nm region,

since poly[d(A)] does not share with poly[r(A)] the large, positive CD band.

The CD of poly[r(A)·d(T)] from 250-240 nm was relatively close to the average of its constituent homopolymers (Fig. 4). This fact, along with the characteristic poly[r(A)] CD bands evident below 235 nm in Fig. 3, implied that poly[r(A)] and poly[d(T)] changed their respective secondary structures relatively little upon formation of the hybrid duplex. Thus, our results for poly[r(A)·d(T)] agreed with the finding of Zimmerman and Pheiffer[15] that the two separate strands of the duplex hybrid have different secondary conformations in wet fibers, and as such constitute a heteronomous duplex.

The constituent poly[r(A)] and poly[d(U)] strands also seemed limited in their conformational changes on forming poly[r(A)·d(U)]. Both the CD spectrum and the difference-CD spectrum from 280-240 nm were less positive for poly[r(A)·d(U)] than for poly[r(A)·r(U)]. Since the CD of the duplexes below 235 nm indicated only small conformational changes in the poly[r(A)] strand, and since poly[r(U)] and poly[d(U)] (Fig. 2) had virtually the same CD, we attribute the spectral differences between the RNA and poly[r(A)·d(U)] to be mainly due to structural differences between poly[r(U)] and poly[d(U)] in the respective duplexes. Both netropsin and CC-1065 require the B-form to bind to nucleic acids, and both bind poly[r(A)·d(U)].[40,41] Poly[d(U)] may allow the hybrid to retain some aspects of the B-form in the duplex helical structure such as base tilt or a less shallow minor groove, as is true of poly-[r(A)·d(T)].[15] Thus, poly[r(A)·d(U)] was probably heteronomous in solution.

CD of Triplex Homopolymers

CD spectra of the six triple-stranded homopolymers are shown in Fig. 5. Four of the spectra correspond to adding another polypyrimidine strand to the duplexes [r(A)·r(U)], [r(A)·d(T)], [d(A)·d(U)], and [d(A)·d(T)]. Two triple-stranded polymers, poly[r(U)·d(A)·r(U)] and poly[r(T)·d(A)·r(T)], do not correspond to available duplexes, and an additional poly[d(U)] strand could not be added to poly[r(A)·d(U)] to form the expected triplex.

Three wavelength domains characterize the spectra in Fig. 5; 300-255, 255-235, and 235-205 nm. Triplexes composed of one polypurine and two polypyrimidine strands are generally thought to be A-form.[19] However, CD bands in the three wavelength regions did not always offer a pattern of shapes and intensities like that of poly[r(U)·r(A)·r(U)], a model A-form triplex.[17,19] Above 255 nm, spectra of poly[r(U)·d(A)·r(U)] and poly[d(U)·d(A)·d(U)] resembled the triplex RNA spectrum, while spectra of poly[d(T)·d(A)·d(T)] and poly[r(T)·d(A)·r(T)] had compound CD bands of similar shape and magnitude to those of the duplex DNA, poly[d(A)·d(T)]. The spectrum of the third hybrid

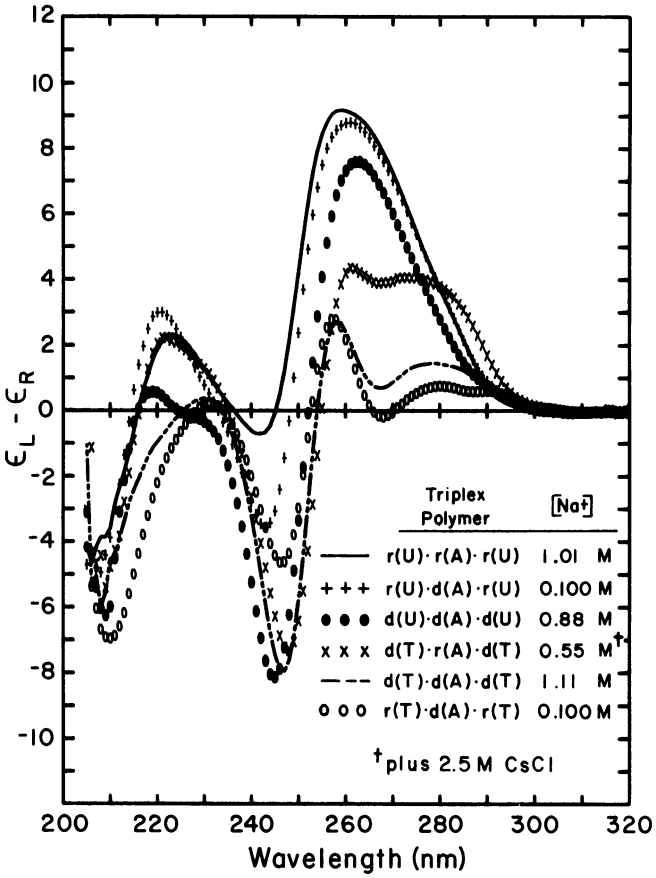

Figure 5. CD spectra of homopolymer triplexes. The spectra of the six triple-stranded polymers were obtained at pH 7.0 (phosphate buffer) and at the cation concentrations indicated. Note that poly[d(T)•r(A)•d(T)] was formed in 2.5 M CsCl and 0.275 M Na_2HPO_4, pH 7.0.

triplex, poly[d(T)•r(A)•d(T)], had a band shape and intensity intermediate to the CD bands of the other two triplex hybrids. On the other hand, in the 255-235 nm wavelength domain, the CD of poly[d(T)•r(A)•d(T)] was like that of the DNAs, while the spectra of poly[r(U)•d(A)•r(U)] and poly[r(T)•d(A)•r(T)] were intermediate in magnitude to those of the triple-stranded RNA and DNAs. Below 235 nm, CD spectra of all six triplexes were very similar, although poly[d(T)•d(A)•d(T)] and poly[r(T)•d(A)•r(T)] differed from the others in having their lowest wavelength crossover point redshifted by 10-12 nm.

Using difference-CD calculations, we obtained additional data for four of the six triplexes, as shown in Fig. 6. Specifically, we subtracted 2/3

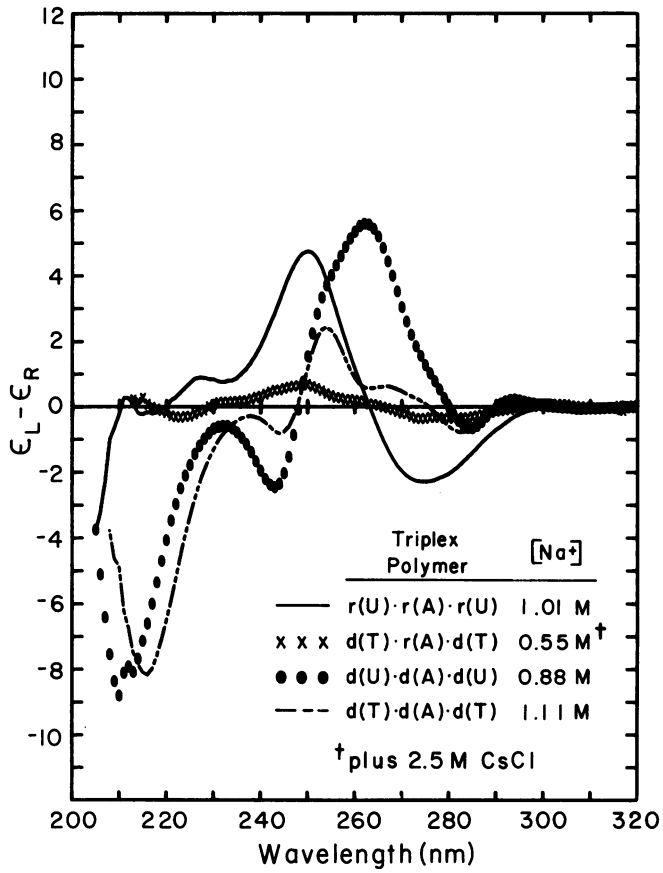

Figure 6. Difference-CD spectra for four triple-stranded polymers. The spectra were calculated by subtracting 2/3 of the spectrum of the corresponding duplex plus 1/3 of the spectrum of the appropriate polypyrimidine from the CD spectrum of a given triplex.

of the spectrum of the corresponding duplex plus 1/3 of the spectrum of the appropriate polypyrimidine from the CD spectrum of a given triplex in the same environment. This gave the CD changes of the third strand and its interactions with the duplex upon formation of the triple-stranded polymer, plus the CD resulting from any conformational changes in the duplex.

For poly[d(U)•d(A)•d(U)], the difference-CD spectrum revealed bands peculiar to CD spectra of A-form nucleic acids,[35] reflecting the many structural changes in poly[d(A)•d(U)] and poly[d(U)] that apparently accompanied a B- to A-like conformational transition. The difference-CD spectrum of poly-[d(T)•d(A)•d(T)] was similar to that of poly[d(U)•d(A)•d(U)], especially in

having a large negative CD at low wavelengths; however, there were differences in band shape and intensity from 280-262 nm. Poly[r(U)·r(A)·r(U)] had a somewhat conservative difference-CD spectrum with bands centered at 274 and 250 nm, while the spectrum of poly[d(T)·r(A)·d(T)] had very small difference-CD bands centered at same wavelengths and of the same sign.

Both the magnitude and position of the bands in the CD spectrum of poly-[r(U)·r(A)·r(U)] and their similarity to the magnitude and position of CD bands of poly[r(A)·r(U)] were consistent with an A-form polymer. Difference-CD spectra of both poly[r(A)·r(U)] and poly[r(U)·r(A)·r(U)] show positive bands at *ca.* 250 nm, which could have resulted from progressively increased stacking of poly[r(A)] in the duplex and triplex RNAs. This interpretation is supported by a Raman study[21] that detected increased stacking of poly-[r(A)] in the triple-stranded RNA relative to its stacking in the duplex.

Poly[r(U)·d(A)·r(U)] bore great similarity to poly[r(U)·r(A)·r(U)] in CD band shape, position, and intensity, and thus, was the most A-like of the hybrid triplexes. We deduced that the poly[d(A)] strand must have been forced to assume structural features of the A-form by the two poly[r(U)] strands. Conversely, poly[d(U)·d(A)·d(U)] had a CD band at 262 nm of slightly lower intensity than poly[r(U)·d(A)·r(U)], a CD band at 245 nm of the same intensity as poly[d(T)·d(A)·d(T)] and poly[d(T)·r(A)·d(T)], and a crossover point at 225 nm unlike four of the other five triplexes. CC-1065 will bind strongly to poly[d(U)·d(A)·d(U)] and weakly to poly[r(U)·d(A)·r(U)],[41] implying that the two differ considerably in secondary structure. Thus, while the conformation of poly[r(U)·d(A)·r(U)] was almost identical to that of the A-form RNA triplex, poly[d(U)·d(A)·d(U)] was not completely A-like.

Since the difference-CD spectrum of poly[d(T)·r(A)·d(T)] showed little change of the constituent heteronomous duplex and single strand on formation of the triple-stranded structure, the overall conformation of the triplex itself was likely heteronomous. This implied that poly[d(T)] was stacked equivalently as a single-stranded polymer and in the triplex at 3.05 M.

The CD spectra of poly[d(T)·d(A)·d(T)] and poly[r(T)·d(A)·r(T)] showed great similarity to one another and thus likely had very similar conformations. Netropsin and CC-1065 bind strongly to poly[d(T)·d(A)·d(T)] and may recognize, as suggested by Krueger *et al.*,[41] structural features of triplexes held in common with the B-form such as base tilt and vertical rise per residue. The existence of the low-wavelength, negative band in the CD spectra of poly[d(T)·d(A)·d(T)] and poly[r(T)·d(A)·r(T)], and in the difference-CD spectrum of poly[d(T)·d(A)·d(T)] was the only suggestion that these polymers had

A-like character. We concluded that of all the triple-stranded polymers, poly[d(T)•d(A)•d(T)] and poly[r(T)•d(A)•r(T)] were the least A-like.

CONCLUSIONS

Our conclusions concerning the A•U and A•T duplex and triplex hybrids were based on analyses of both CD spectra and the difference-CD spectra, and are enumerated below. Table I summarizes our interpretation of the solution conformations of the various homopolymer duplexes and triplexes.

1. CD bands of the duplex spectra below 235 nm gave a clear indication that the poly[r(A)] and poly[d(A)] strands had the same conformations when paired in the duplexes as when free in solution, except for minor changes (*e.g.* in base stacking) that affected band intensities.

2. Difference-CD spectra, changes in the CD of the single-stranded homopolymer constituents on formation of the duplexes (Fig. 4), revealed a region of the CD spectrum to be extremely sensitive to base composition, 300-260 nm. Specifically, above 260 nm, the difference-CD of a homopolymer duplex was correlated with its constituent base pair (A•U or A•T) irrespective of the sugar (ribose or deoxyribose) present in either strand. This implied that in this wavelength region, the CD spectra of homopolymer RNA, DNA, and hybrid duplexes were principally a product of the secondary structure of the component single-strands and the type of base pair they formed.

The difference-CD results for the duplexes revealed a region at 250-240 nm that was apparently sensitive to the secondary structure of the duplex polymers. That is, the difference-CD spectra were divided into three groups containing (a) the RNA, (b) the two hybrids, and (c) the two DNAs. In part, this division resulted from differences in some aspect of conformation such as stacking of poly[r(A)] and poly[d(A)], with the CD of duplexes containing poly[r(A)] being more positive and the CD of duplexes containing poly[d(A)] being more negative than the average CD of their respective single-strands.

3. CD spectra of the double- and triple-stranded hybrids generally differed from the spectra of the non-hybrid DNA and RNA polymers. These differences reflected the type of base pairing (and cross-strand interactions) as well as the conformations of the individual strands. We found that the conformation of a DNA•RNA hybrid in solution can not always be determined from a cursory examination of its CD spectrum. For example, although the CD spectrum of poly[r(A)•d(U)] resembled that of poly[r(A)•r(U)], there were significant differences in the magnitudes of the CD bands above 235 nm that could be interpreted in terms of structural differences in the poly[d(U)] and

Table I.
Solution Conformations of Homopolymer Duplexes and Triplexes from Their CD Spectra.

Polymer	Conformation[a]	Cation Concentration[b]	ε_{260}[c]
Duplexes			
1. poly[r(A)•r(U)]	A	0.100 M Na^+	6680
2. poly[r(A)•d(U)]	heteronomous	0.100 M Na^+	7190
3. poly[r(A)•d(T)]	heteronomous	0.100 M Na^+	6280
4. poly[d(A)•d(U)]	B	0.88 M Na^+	6550
5. poly[d(A)•d(T)]	B	0.100 M Na^+	6150
Triplexes			
1. poly[r(U)•r(A)•r(U)]	A	1.01 M Na^+	5510
2. poly[d(T)•r(A)•d(T)]	heteronomous	0.55 M Na^+ plus 2.50 M Cs^+	5710
3. poly[r(U)•d(A)•r(U)]	A	0.100 M Na^+	5830
4. poly[r(T)•d(A)•r(T)]	least A-like	0.100 M Na^+	5300
5. poly[d(U)•d(A)•d(U)]	not fully A-like	0.88 M Na^+	6020
6. poly[d(T)•d(A)•d(T)]	least A-like	1.11 M Na^+	5140

[a] "Heteronomous" means that CD characteristics of the separate strands are maintained in the CD spectrum of a hybrid.
[b] Na^+ was added as Na_2HPO_4, except in the case of poly[d(T)•r(A)•d(T)] where Cs^+ was added as CsCl buffered with Na_2HPO_4 so that the final pH was 7.0.
[c] Extinction coefficients ($L \cdot mol^{-1} \cdot cm^{-1}$) determined from our mixing curves and the known values for the homopolymers (see Methods). In the case of poly[r(T)•d(A)•r(T)], the ε_{260} was assumed to be equal to that of poly-[d(T)•d(A)•d(T)], for which we used a published value (Ref. 25).

poly[r(U)] strands. This result helped lead us to the conclusion that the solution structure of the poly[r(A)•d(U)] hybrid was actually heteronomous.

4. Hybrids and triplexes in solution do not necessarily hold the A secondary conformation as is often assumed in the literature. In fact, of the two duplex and the three triplex hybrids, only poly[r(U)•d(A)•r(U)], appeared to have a conformation congruent with its RNA analogue. Excepting poly[r(U)•d(A)•r(U)], the hybrids investigated here appeared to have solution

structures which were either heteronomous (poly[r(A)•d(U)], poly[r(A)•d(T)], and poly[d(T)•r(A)•d(T)]) or at least intermediate to those of the A- and B-forms (poly[d(U)•d(A)•d(U)]). In addition, we found that the spectrum of the DNA poly[d(T)•d(A)•d(T)], like that of poly[r(T)•d(A)•r(T)], had only one feature indicative of A-like character, a negative band at low wavelengths.

Our conclusion concerning the heteronomy of poly[r(A)•d(T)] is in agreement with Zimmerman and Pheiffer's interpretation of X-ray diffraction data of wet fibers of this polymer.[15] Our data, in particular the fact that the CD spectrum of the hybrid retained features of an A-form polypurine strand, appear to be in conflict with an NMR-derived model that employs C1'-*exo* puckers (similar to C2'-*endo*) on both strands of the hybrid.[60] However, the intrinsic form of the sugars is not directly detected by CD measurements.

5. We found no evidence to conclude that poly[d(A)•d(T)] was heteronomous in solution at 20°C as has been suggested in a previous fiber-diffraction study.[61] That is, the poly[d(A)] strand of the duplex maintained the CD bands at both long and short wavelengths of the free polymer, which differed from those of poly[r(A)]-containing duplexes. Our results were in keeping with NMR results[44,62,63] which indicated that the poly[d(A)] of poly[d(A)•d(T)] was still in a B-like conformation, not A-form. However, other evidence suggests that the secondary structure of this polymer may be very sensitive to solution conditions. A study by Thomas and Peticolas[64] showed that conformational changes could be induced in poly[d(A)•d(T)] by variations in temperature and salt concentration. A separate Raman investigation[65] found that the poly[d(A)] strand took on bands associated with the A-form at 0°C. Wartell and Harrell[45] have observed the 816 cm^{-1} A-form band in Raman spectra of the polymer, but noted that the intensity of the band was not as great as expected for a heteronomous model containing 50% C3'-*endo* sugar. In sum, CD, NMR, and Raman spectroscopy indicate that the conformation of poly-[d(A)•d(T)] in solution at 20°C is not heteronomous. The heteronomy of the polymer may only be evident in fibers.

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REFERENCES

1. Kornberg, A. (1980) DNA Replication, pp. 37-38, 221-225, 231-259, 362-396, 596-601, W.H. Freeman and Company, San Francisco.
2. Kornberg, A. (1980) 1982 Supplement to DNA Replication, pp. 104-117, 190-194, W.H. Freeman and Company, San Francisco.
3. Lobel, L.I. and Goff, S.P. (1985) J. Virol. 53, 447-455.
4. Steinhilber, W. and Cummings, D. (1985) in Abstracts of the International Symposium on Achievements and Perspectives in Mitochondrial Research, Quagliariella, E., Slater, E.C., Palmieri, F., Saccone, C., Kroon, A.M. Eds., p. 235, Adriatica Editrice, Bari.
5. Michel, F. and Lang, B.F. (1985) Nature 316, 641-643.
6. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721-732.
7. Blake, K.R., Murakami, A., and MIller, P.S. (1985) Biochemistry 24, 6132-6138.
8. Blake, K.R., Murakami, A., Spitz, S.A., Glave, S.A., Reddy, M.P., Tso, P.O.P., and Miller, P.S. (1985) Biochemistry 24, 6139-6145.
9. Gilbert, W. (1976) in RNA Polymerase, Losick, R. and Chamberlin, M.J. Eds., Cold Spring Harbor Laboratory, New York, 193-205.
10. Martin, F.H. and Tinoco, I., Jr. (1980) Nucleic Acids Res. 8, 2295-2299.
11. Milman, G., Langridge, R., and Chamberlin, M.J. (1966) Proc. Natl. Acad. Sci. (USA) 57, 1804-1810.
12. Arnott, S., Hukins, D.W.L., Dover, S.D., Fuller, W., and Hodgson, A.R. (1973) J. Mol. Biol. 81, 107-122.
13. O'Brien, E.J. and MacEwen, A. (1970) J. Mol. Biol. 48, 243-261.
14. Gray, D.M. and Ratliff, R.L. (1975) Biopolymers 14, 487-498.
15. Zimmerman, S.B. and Pheiffer, B.H. (1981) Proc. Natl. Acad. Sci. (USA) 78, 78-82.
16. Arnott, S., Chandrasekaran, R., Millane, R. and Park, H.-S. (1986) J. Mol. Biol 188, 631-640.
17. Arnott, S. and Bond, P.J. (1973) Nature New Biol. 244, 99-101.
18. Arnott, S. and Selsing, E. (1974) J. Mol. Biol. 88, 509-521.
19. Arnott, S., Bond, P.J., Selsing, E. and Smith, P.J.C. (1976) Nucleic Acids Res. 3, 2459-2470.
20. Saenger, W. (1984) Principles of Nucleic Acid Structure, pp. 243-246, Springer-Verlag, New York.
21. O'Connor, T. and Bina, M. (1984) J. Biomolec. Struc. Dyn. 2, 615-625.
22. Gukovskaya, A.S., Gukovsky, I. Ya., Chikh, V.P., and Sukhorukov, B.I. (1980) Biopolymers 19, 453-468.
23. Wierzchowski, K.L., Zielenkiewicz, A., and Miller, P.S. (1984) Biopolymers 23, 2361-2382.
24. Loomis, R.E. and Alderfer, J.L. (1986) Biopolymers 25, 571-600.
25. Riley, M., Maling, B., and Chamberlin, M. (1966) J. Mol. Biol. 20, 359-389.
26. Morgan, A.R. and Wells, R.D. (1968) J. Mol. Biol. 37, 63-80.
27. Morgan, A.R. (1973) Can. J. Biochem. 51, 436-451.
28. Barszcz, D. and Shugar, D. (1968) Eur. J. Biochem. 5, 91-100.
29. Swierkowski, M. and Shugar, D. (1970) J. Mol. Biol. 47, 57-67.
30. Howard, F.B., Frazier, J., and Miles, H.T. (1976) Biochemistry 15, 3783-3795.
31. Lee, J.S., Johnson, D.A., and Morgan, A.R. (1979) Nucleic Acids Res. 9, 3073-3091.
32. Rainen, L.C., and Stollar, B.D. (1977) Biochemistry 16, 2003-2007.
33. Higuchi, S. (1985) J. Biomolec. Struc. Dyn. 2, 675-682.
34. Johnson, W.C., Jr. (1985) in Methods of Biochemical Analysis, 31, Glick, D., Ed., pp. 61-163, John Wiley & Sons, Inc., New York.
35. Steely, H.T., Jr., Gray, D.M., Lang, D., and Maestre, M. (1986) Biopolymers 25, 91-117.

36. Hall, K.B. and Maestre, M.F. (1984) Biopolymers 23, 2127-2139.
37. Hall, K.B., Cruz, P., Tinoco, I., Jr., Jovin, T.M., and van de Sande, J.H. (1984) Nature 311, 534-556.
38. Reid, D.G., Salisbury, S.A., Brown, Tom, Williams, D.H., Vasseur, J-J. Rayner, B., and Imbach, J-L. (1983) Eur. J. Biochem. 135, 307-314.
39. Wu, H.-Y. and Behe, M.J. (1984) Proc. Natl. Acad. Sci (USA) 81, 7284-7287.
40. Zimmer, C., Kakiuchi, N., and Guschlbauer (1982) Nucleic Acids Res. 10, 1721-1732.
41. Krueger, W.C., Li, L.H., Moscowitz, A., Prairie, M.D., Petzold, G., and Swenson, D.H. (1985) Biopolymers 24, 1549-1572.
42. Kakiuchi, N., Marck, C., Rosseau, N., Leng, M., De Clercq, E., and Guschlbauer, W. (1982) J. Biol. Chem. 257, 1924-1928.
43. Beychok, S. (1966) Science, 1288-1299.
44. Behling, R.W. and Kearns, D.R. (1986) Biochemistry 25, 3335-3346.
45. Wartell, R.M. and Harrell, J.T. (1986) Biochemistry 25, 2664-2671.
46. Gray, D.M., Liu, J.-J., Ratliff, R.L., and Allen, F.S. (1981) Biopolymers 20, 1337-1382.
47. Blake, R.D. and Fresco, J.R. (1966) J. Mol. Biol. 19, 145-160.
48. Bollum, F.J. (1966) in Procedures in Nucleic Acid Research, Cantoni G.L., and Davies, D.R., Eds., pp.577-583, Harper and Row, New York.
49. Zmudzka, B., Bollum, F.J., and Shugar, D. (1969) J. Mol. Biol. 46, 169-183.
50. Martin, F.H. and Tinoco, I., Jr. (1980) Nucleic Acids Res. 8, 2295-2299.
51. Ott, D.G., Kerr, V.N., Hansbury, E., and Hayes, F.N. (1967) Analytical Biochem. 21, 469-472.
52. Edmondson, S.P. and Gray, D.M. (1983) Nucleic Acids Res. 11, 175-192.
53. Cantor, C.R., Warshaw, M.M., and Shapiro, H. (1970) Biopolymers 9, 1059-1070.
54. Warshaw, M.M. and Cantor, C.R. (1970) Biopolymers 9, 1079-1103.
55. Sollie, T.N. and Schellman, J. (1968) J. Mol. Biol. 33, 61-77.
56. Ts'O, P.O.P. (1974) in Basic Principles in Nucleic Acid Chemistry, Vol. I, Ts'O, P.O.P., Ed., pp. 453-584, Academic Press, New York.
57. Haasnoot, C.A.G. and Altona, C. (1979) Nucleic Acids Res. 6, 1145-1149.
58. Shugar, D. and Szer, W. (1962) J. Mol. Biol. 5, 580-582.
59. Greve, J., Maestre, M.F., Moise, H., and Hosoda, J. (1978) Biochemistry 17, 887-893.
60. Gupta, G., Sarma, M.H., and Sarma, R.H. (1985) J. Mol. Biol. 186, 463-469.
61. Arnott, S., Chandrasekaran, R., Hall, I.H., and Puigjaner, L.C. (1983) Nucleic Acids Res. 11, 4141-4155.
62. Sarma, M. H., Gupta, G., and Sarma, R.H. (1985) in Fourth Conversation in Biomolecular Stereodynamics, Sarma, R.H., Ed., pp. 77-78, State University of New York at Albany, Albany.
63. Behling, R.W. and Kearns, D.R. (1985) Biopolymers 24, 1157-1167.
64. Thomas, G.A. and Peticolas, W.L. (1983) J. Amer. Chem. Soc. 105, 993-996.
65. Jolles, B., Laigle, A., Chinsky, L., and Turpin, P.Y. (1985) Nucleic Acids Res. 13, 2075-2085