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Circular Dichroism and Thermal Melting of Two Small DNA Restriction Fragments of the Same Molecular Weight†

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ABSTRACT: The thermal melting and circular dichroism of two 147 base pair restriction fragments of pBR322 have been studied. The fragment with the higher GC content, 12B, melts at a higher temperature than the other fragment, 12A, as expected. The melting temperatures are proportional to the logarithm of the concentration of NaCl or tris(hydroxymethyl)aminomethane (Tris) buffer, between 1 mM and 0.2 M added salt. In 1 mM Tris buffer, the melting temperatures of the two fragments are nearly equal. The circular dichroism spectra of fragments 12A and 12B in 0.2–10 mM Tris buffer are characteristic of B-form DNA. In 81% ethanol, the circular dichroism spectra of the two fragments are characteristic of A-form DNA. With 1 mM Tris buffer as the supporting electrolyte, fragment 12A exhibits a very sharp B → A transition, with a midpoint at 79% ethanol. However, a biphasic transition is observed for fragment 12B, with midpoints at 73% and 80% ethanol. This biphasic transition may represent the conversion of separate domains of fragment 12B from the B conformation to the A conformation; half of this fragment is much more GC rich than the other half. Methods are also described for preparing polymers of the 12A and 12B fragments.

X-ray diffraction studies of oriented DNA fibers have shown that DNA can adopt a number of different conformations depending on the degree of hydration and the concentration and type of counterions (Langridge et al., 1980a,b). Characteristic circular dichroism (CD)[1] spectra have been correlated with A-form, B-form, and Z-form DNA (Tunis-Schneider & Maestre, 1970; Pohl & Jovin, 1972; Ivanov et al., 1973, 1974). Small DNA restriction fragments have also been found to exhibit these characteristic spectra (Hillen & Wells, 1980; Hillen et al., 1981c; Klysik et al., 1981).

Transitions between A-form and B-form DNA depend on the relative humidity of the sample; in solution, dehydration is effected by adding ethanol or other organic solvent to aqueous solutions of DNA (Ivanov et al., 1973; Malenkov et al., 1975). As the ethanol concentration is increased, the CD spectrum characteristic of B-form DNA changes abruptly to one similar to that of RNA (Brahms & Mommaerts, 1964; Ivanov et al., 1973, 1974). Direct evidence that these circular dichroism changes correspond to the B → A conformational transition has been obtained by X-ray (Gray et al., 1979; Zimmerman & Pfeiffer, 1979) and Raman (Erfurt et al., 1975) measurements. The mechanism of the transition from B-form to A-form DNA has been suggested to be the gradual replacement by ethanol of the spine of water molecules in the minor groove of B-form DNA (Dickerson et al., 1982; Conner et al., 1982). The water molecules around the still hydrated phosphate groups are then proposed to form a network across the major groove, which is quite narrow in A-form DNA (Dickerson et al., 1982; Conner et al., 1982). Since the spine of water molecules down the narrow groove of B-form DNA is disrupted by G residues (Drew & Dickerson, 1981; Dickerson et al., 1982; Conner et al., 1982), fibers of GC-rich DNA are converted more easily into the A conformation than fibers of AT-rich DNA (Pilet & Brahms, 1973; Arnott & Selsing, 1974; Arnott et al., 1974; Pilet et al., 1975).

Previous studies of the ethanol-induced B → A transition in solution have found little or no dependence of the transition on the GC content of the DNA (Pilet & Brahms, 1972; Ivanov et al., 1974, 1983b; Hillen & Wells, 1980). However, most of these studies involved high molecular weight DNAs and/or sheared samples of calf thymus DNA, in which such a dependence might be averaged out. In one study involving DNA restriction fragments (Hillen & Wells, 1980), the midpoint of the B → A transition was found to occur at 72 ± 2% ethanol, independent of DNA sequence and/or molecular weight. However, in this study, the concentration of low molecular weight electrolyte apparently was not kept constant but varied with ethanol concentration. Unfortunately, the midpoint of the B → A transition is very sensitive to electrolyte concentration (Ivanov et al., 1973, 1974). Recently, Ivanov et al. (1983a) have found that the midpoint of the B → A transition of the decanucleotide $(CCCTGCAGGG)_2$ increased from 74% to 80% when the GC base pairs at residues 3 and 8 were replaced by AT base pairs. Hence, base pair sequence

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[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism; T_m, melting temperature; A, adenine; T, thymine; G, guanine; C, cytosine.

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can have a marked effect on the midpoint of the B → A transition in relatively small DNA fragments. Studies of the B → A transition are of interest because of hypotheses that the RNA polymerase (Ivanov et al., 1983b) and/or DNA regulatory proteins (Hillen & Wells, 1980) may bind to A-form DNA.

The double-stranded helical conformation of DNA can be disrupted into single-stranded random coils by heat. The stability of a given DNA can be characterized by a denaturation temperature, T_m, which is a function of base composition and solution ionic conditions. [For recent reviews of the thermal melting of DNA, see Wada et al. (1980), Record et al. (1981), and Gotoh (1983)]. Recently, thermal melting studies have been extended to short, sequenced DNA restriction fragments (Hillen et al., 1981a,b; Benight et al., 1981). Some of these fragments, ranging in size from 43 to 800 base pairs (bp), were found to melt in single domains, while others exhibited two or three subtransitions (Hillen et al., 1981a,b).

In this report, thermal melting and circular dichroism measurements are extended to two 147 base pair (bp) restriction fragments generated by the restriction enzyme *Msp*I from the plasmid pBR322. These two fragments migrate separately on polyacrylamide gels and can be isolated and studied separately. Dimerizing the fragments increases the difference in their electrophoretic mobilities (Stellwagen, 1983b). The two fragments have also been studied by electric birefringence. Fragment 12B, the electrophoretically normal fragment, orients in an electric field by the expected induced dipole mechanism (Stellwagen, 1982). However, fragment 12A, the electrophoretically "anomalous" fragment, orients in the electric field by an apparent permanent dipole mechanism (Stellwagen, 1982). The rate of decay of the birefringence of the two fragments is nearly equal, indicating that the two fragments have nearly identical end to end lengths in solution. The results described below indicate that these two fragments differ in their thermal melting behavior. The two fragments also differ in the mechanism by which the B → A transition is induced by ethanol. A biphasic transition is observed for fragment 12B, possibly indicating that a hybrid B–A helix exists in solutions containing 73–80% ethanol.

Materials and Methods

Construction of Plasmids. Plasmids containing multiple copies of *Msp*I fragments 12A and 12B were constructed following the general procedure of Hartley & Gregori (1981). The reaction scheme is outlined in Figure 1. The plasmid pBR322 was first digested with *Msp*I at 37 °C, the sticky ends were filled by incubation at 4 °C with CTP, GTP, and DNA polymerase I, and the fragments were separated by electrophoresis on a 6% polyacrylamide gel. After the gel was stained with ethidium bromide, the bands were visualized with long-wavelength UV light and the bands corresponding to fragments 12A and 12B excised. The DNAs were recovered by crushing the gel slices and allowing them to stand overnight at room temperature in a 2-fold excess of T0.1E buffer. The supernatants were collected, and the DNAs were separately concentrated on small DEAE columns, eluted with 1.5 M sodium acetate plus 0.5 M Tris base at 60 °C, ethanol precipitated, rinsed with cold ethanol, redissolved in T0.1E buffer, and stored at −20 °C.

The isolated, filled 12A and 12B fragments were then ligated with *Eco*RI linkers, in order to facilitate recovery of the fragments from recombinant plasmids. *Eco*RI linker (d5′-GGAATTCC-3′) was phosphorylated by incubating with rATP and T4 polynucleotide kinase and was separately ligated

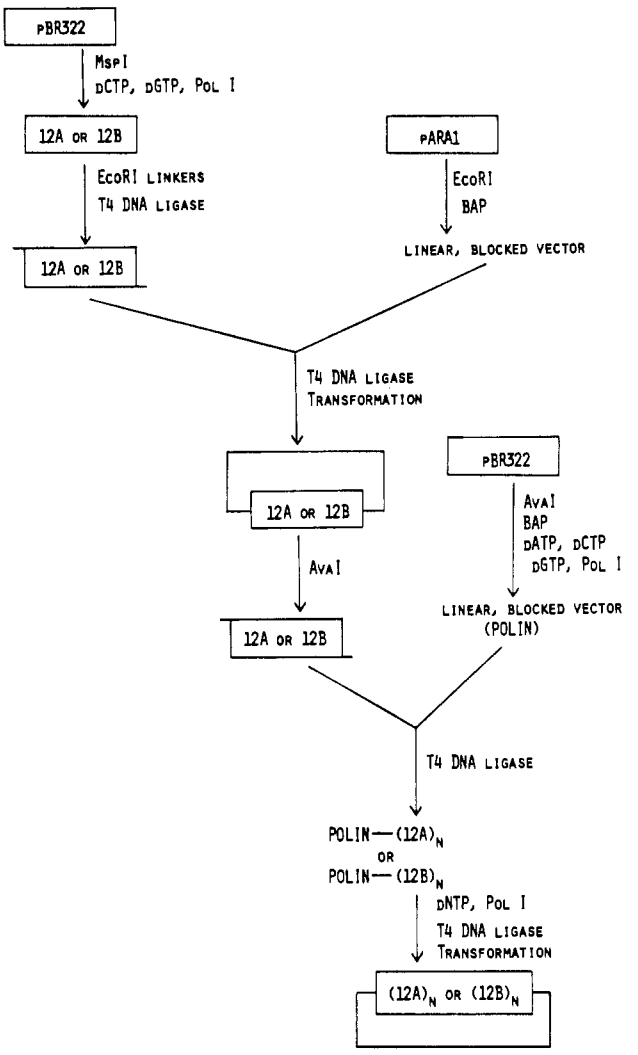

FIGURE 1: Schematic diagram of construction of plasmids containing polymerized 12A or 12B. The 12A and 12B fragments obtained from this polymerization scheme have the original *Msp*I site filled in and also contain *Eco*RI "sticky ends". For this reason, the actual molecular weight of each fragment is 153 bp, not including the four unpaired bases at each end.

to the 12A and 12B fragments by incubating with T4 DNA ligase overnight at 16 °C. After the ligase was inactivated by heating at 65 °C for 15 min, the 12A and 12B fragments were separately digested with *Eco*RI and ligated (overnight, 16 °C) to the vector, *Eco*RI cut, BAP'd pARA1 (Hartley & Gregori, 1981). The incubation mixtures contained 300 ng of vector, a 2–8-fold excess of the 12A or 12B fragments, and 1 unit of T4 DNA ligase in 30 μL of ligase buffer. The ligated vectors were then transformed into competent *Escherichia coli* HB101 cells (30 min, 0 °C) and spread on LB-TET plates (Morrison, 1979; Schlief & Wensink, 1981). After incubation for 2 days at 37 °C, transformants were found on all plates containing 12A– or 12B–vector ligation mixtures.

The transformants were screened for plasmids containing the 12A or 12B inserts by using the miniprep procedure of Holmes & Quigley (1981). The cells in a given colony were suspended in STET buffer and lysed with lysozyme in a boiling water bath. The nucleic acids were precipitated with 2-propanol, redissolved, phenol extracted to remove proteins, extracted with ether to remove the phenol, and ethanol precipitated. The DNA was then redissolved in T0.1E buffer containing RNase A and digested with *Eco*RI (37 °C, 2 h). The digestion products were radioactively labeled with [α-^{32}P]dATP by using *E. coli* DNA polymerase I (4 °C, 10 min)

and electrophoresed on a 1.2% agarose gel. All plasmids contained one *Eco*RI site, but only rarely was a plasmid found which contained two *Eco*RI sites flanking the 12A or 12B fragments. After 50 transformants were screened, five were located from which fragments 12A or 12B could be completely excised with *Eco*RI.

Large-scale (1 L) preparations were made of these five plasmids, yielding 620–930 μg of each plasmid DNA. Portions of each of the plasmids were digested with *Alu*I to identify which contained fragment 12A and which contained fragment 12B [only fragment 12B contains an *Alu*I site (Sutcliffe, 1978)]. Portions of each of the plasmids were also digested with *Ava*I to see which contained the fewest number of *Eco*RI linkers flanking the 12A or 12B fragment. [The vector pARA1 has two *Ava*I sites flanking the *Eco*RI site of the insertion (Hartley & Gregori, 1981).] The two plasmids which generated the smallest 12A or 12B fragments when excised with *Ava*I were chosen for polymerization.

Five hundred micrograms of each of these plasmid DNAs was digested with 75 units of *Ava*I for 12 h at 37 °C (6× overcut). The progress of the digestion was followed by electrophoresis of aliquots of the digestion mixture on agarose gels. After completion of the digestion, the 12A and 12B fragments, now with *Eco*RI sites flanked by *Ava*I sticky ends, were separated by electrophoresis on a 34 × 40 × 0.6 cm polyacrylamide gel. The fragments were recovered from the gel as described above.

The 12A and 12B fragments with *Ava* sticky ends were then ligated into a POLIN vector, as described by Hartley & Gregori (1981). To prepare the vector, 50 μg of pBR322 was phenol extracted, ether extracted, ethanol precipitated, redissolved, and digested with *Ava*I (4 h at 37 °C, 8× overcut). The progress of the digestion was monitored on agarose gels. After completion of the *Ava*I digestion, the POLIN was reacted with bacterial alkaline phosphatase (BAP) (65 °C, overnight), phenol extracted to remove the enzymes, ether extracted to remove the phenol, and ethanol precipitated. After being redissolved, one end of the vector was filled by incubating with dATP, dCTP, dGTP, and DNA polymerase I (0 °C, 10 min). Only one end of the POLIN was filled by using this procedure because of the absence of dTTP. The reaction mixture was then phenol extracted to inactivate the enzyme, extracted with ether, ethanol precipitated, and redissolved in T0.1E buffer. A test with ligase showed that the POLIN was unable to self-ligate.

The POLIN vectors were then ligated separately to the 12A and 12B fragments with *Ava*I sticky ends. The reaction mixtures contained 400 ng of the POLIN in 50 μL of ligase buffer, ca. 1 μg of either the 12A or the 12B fragment, and 0.5 unit of T4 DNA ligase. The small volume of solution was designed to facilitate polymerization at the expense of ring closure. The reaction was allowed to proceed at 16 °C overnight, with periodic additions of more ligase. A ladder of bands representing the family of ligated products was obtained. The reaction was stopped by filling in the ends with DNA polymerase I and all four nucleotides, dATP, dCTP, dGTP, and dTTP (20 min, 4 °C). Each DNA reaction mixture was then extracted with phenol, extracted with ether, ethanol precipitated, and redissolved in 500 μL of ligase buffer. The POLIN–$(12A)_n$ or POLIN–$(12B)_n$ polymers were closed by blunt-end ligation overnight at 16 °C. The vectors were then transformed into competent *E. coli* HB101 cells, as described above.

Transformants were obtained with both POLIN–$(12A)_n$ and POLIN–$(12B)_n$ polymers. About 75 colonies were screened

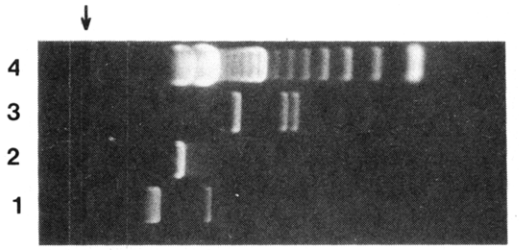

FIGURE 2: Partial digestion of plasmid P11 (containing fragment 12A) with *Eco*RI. Standards were the following: lane 1, uncut pBR322; lane 2, *Eco*RI-cut pBR322 (4363 bp); lane 3, *Bst*NI-cut pBR322 (1857, 1060, 928, and 383 bp) (from left to right); lane 4, digestion mixture after 17 h at 37 °C. The arrow indicates the origin of electrophoresis; the direction of migration is to the right. Similar partial digestion patterns are observed with plasmid PB20 (containing fragment 12B).

for each polymer; 90% were found to contain inserts. DNA minipreps, as described above, were used to find the plasmids containing the largest number of 12A or 12B inserts. These plasmid DNAs were then cut with *Bam*HI and *Pvu*II, and the portions of the molecule containing the *Ava*I site were compared in size by electrophoresis on agarose gels. One of the plasmids (P11) was estimated to contain about 12 copies of fragment 12A; another plasmid (PB20) was estimated to contain about 14 copies of fragment 12B. Large-scale (6 L) preparations were made of each of these plasmids, yielding 6–8 mg of DNA. The plasmids were stored in concentrated solution (ca. 1 mg/mL in T0.1E buffer) at −20 °C; they were stable for long periods of time.

Digestion of the Plasmids. Solutions containing about 1 mg of DNA were digested with *Eco*RI, using about 3000 units of enzyme in a total volume of 2 mL (4× overcut/h). Progress of the digestion was followed by electrophoresis on agarose gels. Because of the large number of copies of each fragment, each digestion required about 24 h at 37 °C. Partial digestion yielded a series of bands corresponding to multimers of fragment 12A or 12B, as shown in Figure 2. After digestion was complete, the reaction mixture was heated at 65 °C for 15 min to inactivate the enzyme, cooled, ethanol precipitated, and redissolved in T0.1E buffer, and the 12A or 12B fragments were separated by electrophoresis on 6% polyacrylamide gels. In some preparations, the digestion mixture was phenol extracted before the ethanol precipitation. The bands containing the 12A and 12B fragments were excised from the gel as described above and recovered either by electroelution or by diffusion into T0.1E buffer. The DNAs were concentrated on small DEAE columns as described above, ethanol precipitated, redissolved in T0.1E buffer, and stored at −20 °C. The DNA stock solutions (ca. 1–2 μg/mL) were stable for many months in the freezer; freezing and thawing the stock solutions had no effect on the physical properties of the DNAs.

Enzymes, Plasmids, and Other Chemicals. Plasmid pARA1 and competent HB101 cells were prepared by T. Gregori of the DNA Recombinant Core Laboratory here. Bacterial alkaline phosphatase (BAP) and *Bam*HI were obtained from Bethesda Research Laboratories (BRL). *E. coli* DNA polymerase I, *Alu*I, and *Pvu*II were obtained from New England Biochemicals (NEB). T4 DNA ligase, *Ava*I, and *Eco*RI were purchased from both BRL and NEB. *Msp*I either was obtained from NEB or was prepared by T. Gregori. T4 polynucleotide kinase was obtained from Boehringer-Mannheim. [α-^{32}P]dATP was purchased from Amhersham Corp. The *Eco*RI linkers were from Collaborative Research, Inc., lot 697-33. RNase A, β-mercaptoethanol, and Tris base (Trizma) were obtained from Sigma. The phenol was BRL

reagent grade, layered under 0.1 M Tris-HCl, pH 8.0. Tetracycline (TET) was Terramycin, from Pfizer. d-10-Camphorsulfonic acid was from Eastman Kodak. All other chemicals were reagent grade.

Buffers. Digestions with *Eco*RI, *Ava*I, *Alu*I, and *Pvu*II were carried out in a buffer containing 10 mM Tris-HCl, pH 7.4, 6 mM β-mercaptoethanol, 6 mM $MgCl_2$, 50 mM NaCl, and 100 μg/mL autoclaved gelatin. Digestions with *Bam*HI were carried out in the same buffer, but with 150 mM NaCl. For *Msp*I digestions, the NaCl concentration was 10 mM. The DNA polymerase I buffer contained 70 mM Tris-HCl, pH 7.4, 70 mM NaCl, and 10 mM $MgCl_2$. STET buffer contained 8% sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris-HCl, pH 8.0. Ligase buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP. The polynucleotide kinase buffer contained 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol. T0.1E buffer contained 10 mM Tris-HCl, pH 7.4–8.1, and 0.1 mM EDTA. The 1 mM Tris buffer used in the CD and melting experiments was 0.1 T0.1E (1 mM Tris-HCl, pH 8.0, and 0.01 mM EDTA). LB-TET plates were poured from a mixture containing 5 g of NaCl, 10 g of Bactotryptone (Difco), 5 g of yeast extract (Difco), 15 g of agar, and 15 mg of tetracycline per L.

Melting Experiments. All DNA absorption spectra were measured by using a Gilford System 2600 UV–vis spectrophotometer equipped with a Model 2527 thermal programmer. Thermal denaturation studies were also carried out with this instrument, using Teflon-stoppered 0.3-cm^3 quartz microcuvettes with 1-cm path lengths. The heating rate was 0.25 °C/min. Evaporation during the course of an experiment was negligible. No correction was made for the thermal expansion of the solvent; in many cases, thermal expansion nearly compensates the temperature dependence of the extinction coefficient (Albergo & Turner, 1981; Frier et al., 1981). Fragments 12A and 12B were always melted in separate cuvettes in simultaneous experiments, so that their melting temperatures would be directly comparable. Melting curves were followed at 282, 260, and 240 nm. Within experimental error, melting temperatures, defined as $[d(OD)/dT]_{max}$, were identical at all three wavelengths. Repeat experiments showed that the melting temperatures in solutions containing at least 1 mM NaCl or Tris buffer were reproducible to better than ±0.5 °C.

Solutions were prepared for melting experiments by direct dilution from the DNA stock solutions into the desired solvents. The solvents were prepared by dilution from stock buffer solutions. Except for studies of the concentration dependence of the melting temperature, most solutions had an optical density of 0.4–0.5. Concentrations were calculated from the optical density at 260 nm, using extinction coefficients calculated from the equation

$$\epsilon(\text{DNA}) = H_{AT}\epsilon(\text{A·T}) + H_{GC}\epsilon(\text{G·C})$$

where H_{AT} is the mole fraction of A + T residues and H_{GC} is the mole fraction of G + C residues (Gray & Tinoco, 1970). The extinction coefficients (per mole of phosphate) of fragments 12A and 12B were calculated to be 6550 and 6680 M^{-1} cm^{-1}, respectively, by using values of ϵ(A·U) = 6000 M^{-1} cm^{-1} and ϵ(G·C) = 7600 M^{-1} cm^{-1} (Allen et al., 1972).

Circular Dichroism Measurements. All circular dichroism (CD) measurements were made by using a Cary Model 60 recording spectropolarimeter equipped with a Model 6001 CD accessory. The calibration of the instrument was checked by using a 1 mg/mL solution of *d*-10-camphorsulfonic acid, taking $\Delta\epsilon$ = +2.37 at 290.5 nm (Chen & Yang, 1977). All spectra were measured in Teflon-stoppered quartz cuvettes with a 0.5-cm path length. The cell was also sealed with parafilm to prevent evaporation during the course of an experiment. All measurements were made at room temperature, 24 ± 1 °C. The scanning rate was 2.5 nm/min.

Solutions were prepared for CD measurements by direct dilution from the DNA stock solutions into the desired solvent. In the solutions containing ethanol, the DNA was first diluted with the calculated amount of water, enough T0.1E was added to bring the buffer concentration of the final solution to the desired value (usually 1 mM), and then the calculated volume of 95% ethanol was added with vigorous mixing. The absorption spectrum of the solution was then measured and the exact DNA concentration calculated from the optical density at 260 nm, assuming the extinction coefficients to be independent of alcohol concentration. No significant light scattering effects were exhibited by the ethanol–water solutions; the optical density at 320 nm was always ≤0.02, and no CD ellipticity was observed in spectral regions which exhibited no optical absorption. Within experimental error, the optical densities of the ethanol–buffer–DNA solutions agreed with the values calculated from the dilution factor and the concentration of the DNA stock solutions. The optical density ratios observed at wavelengths of 230:260:280 nm were approximately constant and equal to those observed in aqueous solution. The only exception was a solution of fragment 12A in 85% ethanol, which exhibited an optical density of 0.07 at 320 nm and significantly lower 230:260:280 ratios. This solution was omitted from the results presented below.

DNA solutions with optical densities between 0.6 and 1.2 at 260 nm were typically used for the CD measurements. The ellipticity of the CD peak at 270 nm was independent of DNA concentration in this range, as previously observed (Brahms & Mommaerts, 1964). Drifts in the base-line position were compensated by zeroing the base line between 320 and 330 nm, as previously described (Hanlon et al., 1975). No time-dependent effects were noted in either the optical density or the circular dichroism of solutions containing ethanol, in contrast to other reports (Hillen & Wells, 1980). No difference was observed in the absorption spectra measured before or after the CD measurements.

The circular dichroism, $\Delta\epsilon$, was calculated from the measured ellipticity, θ, by using the equation

$$\Delta\epsilon = \epsilon_L - \epsilon_R = \frac{\theta[\epsilon(260)]}{33l[\text{OD}(260)]}$$

where l is the path length in centimeters, ϵ(260) is the extinction coefficient, calculated as described above, and OD(260) is the measured optical density.

Miscellaneous Methods. The electrophoresis procedures used in this work have been described in detail previously (Stellwagen, 1983a,b). All polyacrylamide gels were preelectrophoresed at least 2 h before samples were applied. Transformation techniques are described by Morrison (1979). Other techniques are described by Schleif & Wensink (1981).

Results and Discussion

Circular Dichroism in Aqueous Solution. Fragments 12A and 12B exhibited typical B-conformation circular dichroism (CD) spectra, as shown in Figure 3. The average value of $\Delta\epsilon$ observed at 270 nm was 2.8 ± 0.1 for fragment 12B and 2.7 ± 0.3 for fragment 12A in Tris buffer solutions ranging from 1 to 10 mM. These values are similar to those observed by Hillen et al. (1981c) for restriction fragments from the *lac* operon ranging in size from 43 to 360 bp. The amplitude of the peak at 270 nm is thought to be related to the duplex winding angle (Ivanov et al., 1973; Anderson & Bauer, 1978;

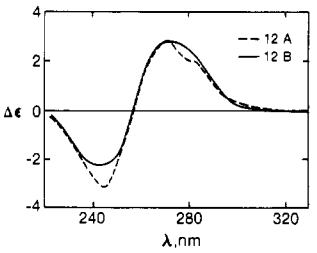

FIGURE 3: Circular dichroism spectra of fragments 12A and 12B in 1 mM Tris buffer, pH 8.0.

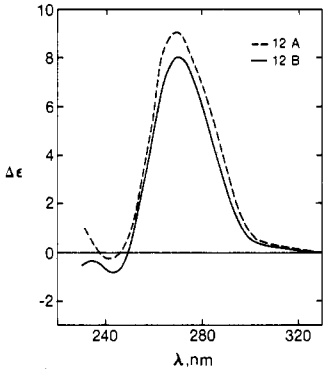

FIGURE 4: Circular dichroism spectra of fragments 12A and 12B in 81% ethanol plus 1 mM Tris buffer, pH 8.0.

Chan et al., 1979; Baase & Johnson, 1979; Johnson et al., 1981) and base pair twist (Johnson et al., 1981). A 10% increase in $\Delta\epsilon(270)$ was observed in 0.2 mM Tris buffer, possibly due to a decrease in winding angle at this low buffer concentration.

Circular Dichroism in 81% Ethanol. Fragments 12A and 12B exhibited typical A-conformation CD spectra in 81% ethanol, as shown in Figure 4. The spectra are similar to those reported by Hillen & Wells (1980) for seven small restriction fragments in 80% ethanol, and by others (Ivanov et al., 1973, 1974; Girod et al., 1973; Gray et al., 1979) for higher molecular weight DNA molecules in 80% ethanol. The B → A transition in ethanol and other organic solvents is an intramolecular transformation (Zavriev et al., 1978; Potaman et al., 1980; Ivanov et al., 1983b) governed by the activity of water in the mixed solvent (Malenkov et al., 1975; Gray & Ratliffe, 1975). X-ray (Zimmerman & Pheiffer, 1979; Gray et al., 1979), Raman (Erfurth et al., 1975; Martin & Wartell, 1982), and infrared (Pilet & Brahms, 1973) studies, as well as CD measurements (Tunis-Schneider & Maestre, 1970; Ivanov et al., 1973, 1974; Malenkov et al., 1975), have established that the conformation of DNA in 70–80% ethanol is the same as that of the A conformation in films and fibers at 75% relative humidity. The nonconservative nature of the CD spectrum of the A conformation has been attributed to the distance of the DNA base pairs from the helix axis (Moore & Wagner, 1973).

Titration of Fragments 12A and 12B with Ethanol. The transition between the A and B conformations of fragments 12A and 12B is shown as a function of ethanol concentration in Figure 5. For fragment 12A, the ellipticity of the 270-nm CD peak decreased in 60% and 70% ethanol. For fragment 12B, no decrease in $\Delta\epsilon(270)$ was observed at these ethanol concentrations, although lower ellipticities were observed at ethanol concentrations below 50%. A decrease in the amplitude of the 270-nm peak is commonly observed at ethanol concentrations below the cooperative transition region (Girod et al., 1973; Usatyi & Shlyakhetenko, 1974; Gray & Ratliffe, 1975; Gray et al., 1978; Vorlichova et al., 1982). Historically,

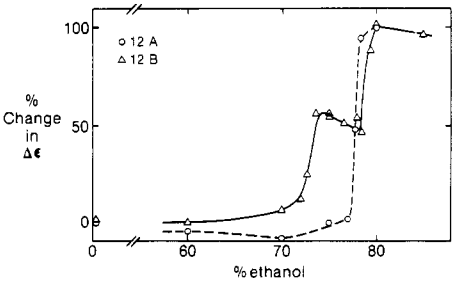

FIGURE 5: Titration of fragments 12A and 12B with ethanol. The amplitude of the ellipticity at 270 nm, $\Delta\epsilon(270)$, is plotted as a function of ethanol concentration. All solutions also contained 1 mM Tris buffer, pH 8.0. (O) Fragment 12A; (Δ) fragment 12B.

this decrease has been attributed to formation of the C conformation of DNA (Tunis-Schneider & Maestre, 1970; Girod et al., 1973; Brahms et al., 1973; Pilet & Brahms, 1973; Champeil & Brahms, 1974). However, recent Raman (Fish et al., 1983), X-ray (Zimmerman & Pheiffer, 1979; Gray et al., 1979; Chen et al., 1983), and CD (Baase & Johnson, 1979) studies have shown that DNA does not exist in the C conformation under these conditions.

The decrease in the amplitude of the 270-nm peak with increasing ethanol concentration below the cooperative transition region may indicate the unwinding or relaxation of a somewhat coiled-coil DNA conformation. Lee et al. (1981), studying the linking number of covalently closed, circular DNAs in solutions containing ethanol, found that the DNA molecules were progressively unwound by increasing concentrations of ethanol and other organic solvents.

For fragment 12A, the transition between the B conformation and the A conformation is very abrupt, the major portion of the transition occurring within a concentration interval of 0.5% ethanol. The midpoint of the transition occurs at 78.9% ethanol, significantly higher than the average concentration of 72–74% observed by others (Ivanov et al., 1973, 1974; Hillen & Wells, 1980) using 0.2–0.6 mM NaCl or sodium phosphate as the supporting electrolyte. The higher transition midpoint observed in 1 mM Tris buffer indicates that Tris buffer is "B-philic" (Ivanov et al., 1983a,b), i.e., stabilizes the B conformation in preference to the A conformation. Higher concentrations of Tris buffer, e.g., 10 mM, cause the B → A conformational transition of fragment 12A to occur at ethanol concentrations above 85% (data not shown), again indicating that Tris buffer stabilizes the B conformation.

The B → A conformational transition of fragment 12B is biphasic, as also shown in Figure 5. The midpoint of the first transition occurs at an ethanol concentration of 74.2%, while the midpoint of the second transition occurs at about 80.0% ethanol. Between ethanol concentrations of 74.8% and 79.5%, the amplitude of the 270-nm peak is essentially constant, or decreases slightly. A biphasic transition induced by ethanol has not been observed previously, either because it was not present in the restriction fragment being studied (compare fragment 12A) or because the samples being studied were sheared fragments of calf thymus or other large DNAs (Ivanov et al., 1973, 1974, 1983a) in which such a multiphasic transition would have been obscured.

The relative amplitude of the 270-nm peak in the plateau region suggests that the 12B fragments have about 50% A and 50% B character. In Figure 6, a comparison is made of the CD spectrum observed in 77.7% ethanol (about the midpoint of the plateau region) with a spectrum calculated from the average CD of fragment 12B in 1 mM Tris buffer and in 81% ethanol (from Figures 3 and 4). The close agreement between the calculated and observed spectra suggests that fragment

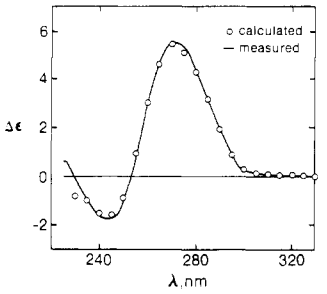

FIGURE 6: Comparison of the measured CD spectrum of fragment 12B in 77.7% ethanol (—) with the spectrum calculated by assuming 50% A-form DNA and 50% B-form DNA (O). The calculated spectrum is the average of the 12B spectra presented in Figures 3 and 4.

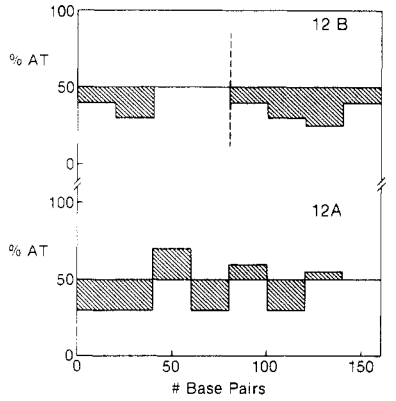

FIGURE 7: Schematic block diagram of base sequences of fragments 12B and 12A. For each block of 20 bp in each fragment, the average percent AT is plotted as a function of the location of that block in the sequence. The average percent AT is 39% for the whole 12B fragment and 47% for the whole 12A fragment. If fragment 12B is divided in half at the dashed line, the average percent AT of the left half is 42%, and the average percent AT of the right half is 32%.

12B has about equal A and B conformational character in these ethanol solutions. Either the 12B fragments have a conformation intermediate between that of the A-form and B-form DNA or else the individual DNA molecules must be half in the A conformation and half in the B conformation under these conditions.

There is some experimental evidence which suggests that a DNA conformation might exist which is intermediate between A-form and B-form DNA. The CD spectrum measured for poly[d(AC)]·poly[r(GU)] in 20 mM sodium phosphate buffer resembles that of fragment 12B in Figure 6 (Gray & Ratliffe, 1975). In addition, Arnott et al. (1983) have recently observed a fibrous form of poly[d(A)]·poly[d(T)] with a heteronomous secondary structure. Under dehydrating conditions, one chain, probably poly[d(A)], appears to be in the A conformation, while the other chain, probably poly[d(T)], appears to have a conformation characteristic of B DNA.

However, it seems more likely the plateau region observed for fragment 12B in solutions containing 73–79% ethanol indicates that this fragment has a hybrid A–B duplex structure in these solutions. The AT and GC base pairs are not evenly distributed throughout the length of fragment 12B, as shown in a schematic diagram of the sequence in Figure 7. If fragment 12B is divided in half at the dashed line, the AT content of the left half is 42%, while the AT content of the right half is 32%. The right half of this fragment also contains a run of six consecutive C (or G) residues. Since GG/CC stacks are "A-philic" (Ivanov et al., 1983a,b), it is possible that the right half of this fragment might be converted to the A conformation before the left half. The cooperativity length

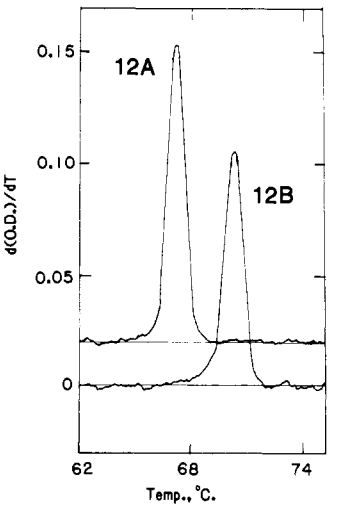

FIGURE 8: Typical thermal melting curves of fragments 12A and 12B in 10 mM Tris buffer, pH 8.0. The derivative of the optical density–temperature curve, $d(OD)/dT$, is plotted as a function of temperature. The melting temperature is defined as $[d(OD)/dT]_{max}$. The melting curve of fragment 12A is displaced vertically for clarity. The melting temperatures are 67.3 and 70.4 °C for fragments 12A and 12B, respectively.

for the B → A transition, i.e., the length of the segments into which the DNA is divided at the half transition point, has been estimated to be about 10 bp (Ivanov et al., 1974, 1983a,b; Minyot et al., 1978; Minchenkova & Zimmer, 1980). Therefore, it would be possible for the two halves of fragment 12B to undergo separate transitions and for fragment 12B to have a hybrid A and B duplex structure in the plateau region.

Block copolymers containing both A and B conformations of DNA have been prepared (Selsing et al., 1978; Selsing & Wells, 1979; Haasnoot et al., 1983). From computer modeling (Selsing et al., 1979) and from high-resolution 1D- and 2D-^{1}H NMR studies (Haasnoot et al., 1983), it appears that the junction region between blocks of DNA in the A conformation and in the B conformation is very small, perhaps involving only one base pair. The differences in base tilt of A-form DNA and B-form DNA would result in a bend of about 26° at the junction (Selsing et al., 1979). If fragment 12B does have a hybrid A–B structure in solutions containing 73–79% ethanol, it should have a bent conformation. If so, its behavior in an electric field ought to be very different from that of A-form or B-form DNA. Therefore, it may be possible to follow the transition from B form to B–A hybrid and then to A-form DNA with transient electric birefringence experiments, which are very sensitive to molecular length and the mechanism of interaction of the polyelectrolyte with the electric field (Fredericq & Houssier, 1973). Such experiments are currently being undertaken in this laboratory.

Thermal Melting Studies. The helix–coil transition of fragments 12A and 12B was studied as a function of electrolyte concentration. A typical derivative plot of the thermal melting curve in T0.1E buffer is shown in Figure 8. The derivative curves of both fragments are sharp and nearly symmetrical, indicating that each fragment melts as a single domain under these conditions. The melting temperature and shape of the melting curves are unchanged if the added electrolyte is 10 mM NaCl instead of 10 mM Tris buffer (T0.1E). The melting temperature of fragment 12B is higher than that of fragment 12A, as expected because of the higher GC content of fragment 12B (Marmur & Doty, 1962). The melting temperature of each fragment is independent of whether the transition is followed at 282, 260, or 240 nm. Since the helix–coil transition of AT base pairs has an isosbestic point at 282 nm (Blake &

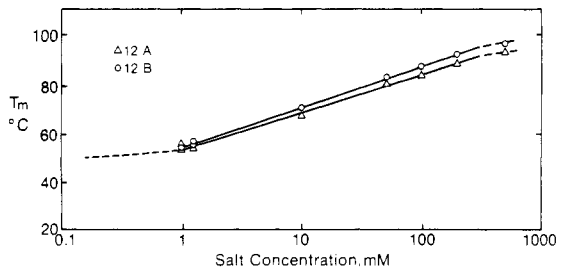

FIGURE 9: Dependence of the melting temperatures, T_m, of fragments 12A and 12B on the logarithm of the concentration of added NaCl or Tris buffer. Melting temperatures in 1 mM salt and below were obtained in solutions containing various concentrations of Tris buffer, pH 8.0. Melting temperatures in 10 mM salt were identical for solutions containing 10 mM Tris buffer, pH 8.0 (T0.1E), or 10 mM NaCl plus 0.2 mM Tris buffer, pH 8.0. Solutions of higher salt concentration contained the indicated concentration of NaCl plus 0.2 mM Tris buffer, pH 8.0.

Lefoley, 1978), thermal melting at this wavelength reflects only the melting of the GC base pairs. The lack of dependence of the melting temperatures on wavelength indicates that the AT and the GC base pairs melt at the same temperature, again indicating that each fragment melts primarily as a single domain.

A summary of the melting temperatures observed for fragments 12A and 12B at various salt concentrations is given in Figure 9. At NaCl concentrations of 10 mM or higher, the average difference in melting temperature of the two fragments is 3.2 °C. If the melting temperatures of the two fragments are calculated from the equation given by Hillen et al. (1981b):

$$T_m = 40.6 + 49H_{GC} \qquad \text{(in 0.01 M Na}^+\text{)}$$

where H_{GC} is the mole fraction of G + C residues in the fragment, values of 66.6 and 70.5 °C are calculated for fragments 12A and 12B, respectively, in 0.01 M Na^+. The experimentally observed melting temperatures in 10 mM NaCl or 10 mM Tris buffer are 67.3 and 70.4 °C, respectively. Therefore, the thermal melting behavior of fragment 12B is typical of that observed for other small restriction fragments, while fragment 12A is somewhat more stable than expected. Melting temperatures calculated from equations for high molecular weight DNAs [e.g., see Frank-Kamemetskii (1971) and Gotoh & Tagashira (1981)] are higher than the values observed for restriction fragments (Hillen et al., 1981b).

Figure 9 also shows that the melting temperatures of fragments 12A and 12B are proportional to the logarithm of the salt concentration over a range extending from 1 mM to 0.2 M. This behavior is well-known for high molecular weight DNA (Dove & Davidson, 1962; Schildkraut & Lifson, 1965) and has also been observed for restriction fragments (Hillen et al., 1981a). The slopes of the straight lines in Figure 9 are 16.0 for fragment 12A and 16.5 for fragment 12B, similar to the values of 15.7–16.7 observed by Hillen et al. (1981a) for their small restriction fragments. The average slope observed for a variety of high molecular weight DNAs is 16.2 (Wada et al., 1980) or 17.5 (Mingot, 1981). The slopes of the straight lines in Figure 9 decrease at Na^+ ion concentrations greater than 0.2 M. This decrease in slope is accompanied by a broadening of the melting transition, as previously observed (Hillen et al., 1981a,b).

The enthalpy (ΔH) of the helix–coil transition can be calculated from an approximation of the van't Hoff equation (Riesner & Romer, 1973; Hillen et al., 1981b):

$$\Delta H = 4RT_m^2/\Delta T$$

where T_m is the melting temperature in degrees kelvin, R is the gas constant, and ΔT is the transition width, taken as the full width at half-height of the derivative of the melting curve. In 0.1 M NaCl, the enthalpy is calculated to be 917 kcal/mol for fragment 12A and 933 cal/mol for fragment 12B. These values are similar to the van't Hoff enthalpies of 817 kcal/mol calculated for a 101 bp restriction fragment and 1066 kcal/mol calculated for the melting of a 155 bp domain in a 301 bp restriction fragment (Hillen et al., 1981a).

If the enthalpy of the helix–coil transition is calculated from the calorimetric data of Marky et al. (1984) and the known base sequences of fragments 12A and 12B, values of 1184 and 1242 kcal/mol are calculated for fragments 12A and 12B, respectively. Comparing the van't Hoff enthalpies with the values calculated from the calorimetric data suggests that about 75% of the 12A and 12B fragments melt as a cooperative unit (Tsong et al., 1970; Patel et al., 1982). Since the melting transition did not appear to be biphasic (Figure 8), the low values obtained for the van't Hoff enthalpies of fragments 12A and 12B may indicate that the heating rate was too fast to obtain equilibrium melting curves. The transition widths decreased from 2.2 to 1.1 °C when the heating rate was decreased from 0.5 to 0.25 °C/min; still narrower melting curves may have been observed if it had been possible to use slower heating rates. The reverse melting curves were not followed, although for most of the solutions the optical densities at 260 nm returned to their original values after the solutions were quickly cooled.

When the helix–coil transition of fragments 12A and 12B was studied in Tris buffer solutions below 1 mM in concentration, the slope of the line describing the dependence of T_m on the logarithm of buffer concentration appeared to level off, as shown by the dashed line in Figure 9. The melting temperatures of fragments 12A and 12B became very similar, but the sharpness of the melting curves and the exact melting temperatures depended somewhat on experimental variables such as DNA concentration. Adding (4–8) $\times 10^{-5}$ M Na_2EDTA to the solutions did not decrease the melting temperatures significantly, although the melting curves were sharpened (data not shown). Circular dichroism spectra of fragments 12A and 12B in 0.2 mM Tris buffer showed that both fragments retained the native B-form conformation even at this low buffer concentration (see above). These results will be presented in more detail in a future communication.

Conclusions

The following conclusions may be drawn from the experiments described here:

(1) The thermal melting of fragments 12A and 12B follows the expected behavior in solutions containing at least 1 mM NaCl or Tris buffer. Fragment 12B has a higher melting temperature than fragment 12A, as expected because of its higher GC content. The melting temperatures of both fragments are proportional to the logarithm of the electrolyte concentration, between 1 and 200 mM added salt.

(2) The circular dichroism spectra of fragments 12A and 12B in 1 mM Tris buffer are typical of those observed for DNA in the B conformation.

(3) In 81% ethanol, the circular dichroism spectra observed for fragments 12A and 12B are typical of DNA in the A conformation.

(4) The transition between the B and the A conformations is monophasic for fragment 12A and biphasic for fragment 12B. The biphasic transition observed for fragment 12B may indicate that this fragment exists in a hybrid half-A, half-B conformation at ethanol concentrations between 73% and 80%.

Acknowledgments

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Characterization of a Crude Selective *Pol*I Transcription System from *Tetrahymena pyriformis*†

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ABSTRACT: A crude in vitro transcription system which selectively transcribes DNA fragments containing the promoter region of the *Tetrahymena pyriformis* rRNA gene has been prepared from *T. pyriformis*. The system requires both an S100 fraction of lysed isolated macronuclei and an S100 extract of whole cells. When a *Hha*I–*Hin*dIII fragment of the promoter containing plasmid pEN 19-1 is employed as a template, transcription yields two major products of about 560 (A) and 510 (B) bases in length. The analysis of the transcription products of truncated templates showed that RNA A is a runoff transcript and RNA B is produced by nucleolytic cleavage of RNA A at a site about 50 nucleotides to the left of the *Hin*dIII cleavage site. S1 nuclease mapping was used to demonstrate that the 5′ end of RNA A is identical with that predicted for a transcript which was initiated at the same site on the gene as the in vivo 35S rRNA precursor. Transcription is dependent upon the addition of promoter containing DNA, is inhibited by 1 μg/mL actinomycin D, and is insensitive to 200 μg/mL α-amanitin. Transcription is dependent upon the salt levels in the assay exhibiting activity peaks at 58 mM KCl, 28 mM $(NH_4)_2SO_4$, and 3 mM $MgCl_2$. Several minor transcription start sites to the left of the major initiation site become active at high salt, yielding several minor longer transcripts. High salt also inhibits the RNA cleavage activity, reducing the levels of RNA B produced.

In eukaryotes there are three forms of RNA polymerase, I, II, and III, which catalyze the synthesis of precursors to rRNA, mRNA, and tRNA and 5S rRNA, respectively. Since transcription initiation is a likely site for the regulation of gene expression, a major effort has been expended in order to identify the functional regions of eukaryotic promoters. DNA sequence analysis and in vitro transcription studies of several genes transcribed by form II RNA polymerases have revealed that high levels of specific initiation require an A-T-rich region (TATA box) centered at about −25 from the 5′ end of the mRNA plus, in some cases, additional upstream sequences (Breathnack & Chambon, 1981). The form III RNA polymerase requires an intragenic region which binds an activator protein plus additional sequences to the left of the transcription start site (Hall et al., 1982).

Less is known about transcription initiation mediated by the form I RNA polymerase. Transcription initiation sites have been mapped and their base sequences determined for rRNA genes cloned from *X. laevis* (Sollner-Webb & Reeder, 1979; Bakken et al., 1982), yeast (Bayev et al., 1980; Swanson & Holland, 1983), mouse (Urano et al., 1980; Bach et al., 1981; Miller & Sollner-Webb, 1981), *D. melanogaster* (Long et al., 1981), human (Finanscsek et al., 1982), *Tetrahymena pyriformis* (Niles & Jain, 1981; Niles et al., 1981a,b; Higashinakagawa et al., 1981; Saiga et al., 1982), *Dictyostelium discoideum* (Hoshikawa et al., 1983), and *Physarum polycephalum* (Blum et al., 1983). Contrary to the genes transcribed by RNA polymerase *Pol*II and *Pol*III, only limited sequence conservation is noted in the rRNA gene transcription

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