Effects of Functional Group Changes in the *Eco*RV Recognition Site on the Cleavage Reaction Catalyzed by the Endonuclease†

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ABSTRACT: Oligodeoxynucleotides have been prepared which contain changes in the functional group pattern present in the *Eco*RV recognition site d(GATATC). These modifications involve the deletion of specific functional groups or the reversal of the relative positions of functional groups within the canonical six base pair recognition site. The duplex stability of these modified oligodeoxynucleotides has been assessed by determining the thermodynamic parameters characterizing helix formation. Steady-state kinetic parameters have been used to characterize the interaction of the modified oligodeoxynucleotides with the *Eco*RV endonuclease. The enzyme is very sensitive to the deletion of either of the adenine amino or thymine methyl groups, or the reversal of the relative positions of the adenine amino group and thymine carboxy group which form an interstrand hydrogen bond in the major groove of the B-DNA helix. Conversely, deletion of the guanine amino group had only minimal effects upon the measured kinetic parameters. Deletion of the exocyclic amino group from the "inner" dA-dT base pair resulted in the fragment which interacted with the enzyme on the basis of observed inhibition experiments but was not cleaved. The results suggest that the endonuclease interacts with its recognition sequence via contacts in the major groove of the B-DNA helix and that both hydrogen bonding to the adenine amino groups and also hydrophobic interactions with the thymine methyl groups are involved.

Sequence-specific "recognition" between proteins and nucleic acids can be most easily understood in terms of multidentate hydrogen-bonding contacts and van der Waals interactions between the amino acid side chains of the protein and the nucleobases of the DNA sequence. Multiple ionic interactions between the protein and the phosphate backbone contribute to the overall binding affinity, but it is the direct or indirect readout of the bases that allows the protein to discriminate correct from incorrect sequences. With a direct readout mechanism, it is likely that the protein recognizes or interacts with a unique functional group pattern characteristic of a particular nucleic acid sequence. In the case of indirect readout, the functional group pattern available with the recognition sequence will contribute to the geometry of the site and may be sterically important in allowing the intimate association of the protein and the nucleic acid. With the noteworthy exception of the Trp repressor–operator complex (Otwinowski et al., 1988), the crystal structures reported to date of repressor–operator complexes (Anderson et al., 1987; Jordan & Pabo, 1988; Aggarwal et al., 1988) or the *Eco*RI endonuclease with its recognition site (McClarin et al., 1986) generally suggest a direct readout of the bases. The Trp repressor–operator interaction provides an example of indirect readout of a base sequence; that is, the sequence is recognized as a result of changes in the geometry of the phosphate backbone as a result of a given series of nucleobases (although water-mediated hydrogen bonding may provide some direct readout).

Oligodeoxynucleotides can often substitute for the longer DNA polymers in the study of these phenomena. The incorporation of modified nucleobases into oligodeoxynucleotides can be a useful approach for the study of recognition processes in that the functional group pattern available within a given sequence can be systematically altered. This approach has been employed in studies with repressor proteins (Yansura et al., 1977, 1979; Goeddel et al., 1977, 1978; Fisher & Caruthers, 1979; Caruthers, 1980), restriction endonucleases (Dwyer-Hallquist et al., 1982; Ono et al., 1984; Yolov et al., 1985; Seela & Driller, 1986; Jiricny et al., 1986; Fliess et al., 1986; Brennan et al, 1986a; McLaughlin et al., 1987), and modification enzymes (Brennan et al., 1986b) and the interaction between RNA polymerase with its promoter site (Dubendorff et al., 1987). The modifications which are often most useful in this respect are those which result in the deletion of a functional group from the sequence of interest. The substitution of 2′-deoxyuridine for 2′-thymidine is one of the simplest such functional group deletions in which the thymine methyl group is removed. In a similar fashion, the substitution of 2′-deoxynebularine for 2′-deoxyadenosine replaces the adenine base with the purine base, resulting in the deletion of the adenine exocyclic amino group. However, deletion modifications may perturb nucleic acid structure or stability in that interstrand hydrogen bonds are often lost. It is possible to maintain the "normal" number of hydrogen bonds present in a given sequence but alter its functional group characteristics by reversing the relative positions of functional groups involved in hydrogen bonding. The substitution of an adenine-thymine base pair by a hypoxanthine-5-methylcytosine base pair maintains the two hydrogen bonds normally present but reverses the relative positions of the amino and carboxy groups present in the major groove (McLaughlin et al., 1987). With this functional group change, the positions of other important groups such as the pyrimidine 5-methyl group or the purine N^7 functionality remain largely unchanged.

Class II restriction and modification enzymes provide an attractive system for studying sequence-specific interactions [for reviews, see Wells et al. (1981), Modrich (1982), and

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Chirikjian (1987)]. The recognition sites are relatively short, typically four to six base pairs, which provides fewer potentially important functional groups for consideration than corresponding operator sites or other enzyme initiation or recognition sites. Upon sequence-specific interaction, these enzymes also result in an irreversible catalytic event (phosphodiester bond hydrolysis or methylation) which can simplify analysis of the recognition phenomenon. We have reported previously on the effects of functional group changes on the cleavage reaction catalyzed by the *Eco*RI endonuclease (McLaughlin et al., 1987). We have taken a similar approach in studying the *Eco*RV restriction endonuclease which recognizes the hexameric sequence d(GATATC) and catalyzes the hydrolysis of the central phosphodiester bond. The protein is a dimer in solution of approximately 55 kDa and requires Mg^{2+} for catalysis. The endonuclease has been crystallized to 6-Å resolution, and X-ray diffraction studies are in progress (Winkeler et al., 1986). The endonuclease has also been crystallized in co-complexes with self-complementary oligodeoxynucleotides and was found to contain one duplex of the oligodeoxynucleotide per protein dimer (Winkeler et al., 1986). The mechanism of binding and catalysis by the endonuclease has been examined recently (Halford et al., 1988) as well as some preliminary studies utilizing base analogue substitution for the endonuclease (Fliess et al., 1986) and methylase (Nwosu et al., 1988).

We have prepared a series of eight modified *Eco*RV recognition sequences containing either functional group "deletions" or "reversals" to examine the importance of specific functional groups present in the recognition site. The thermodynamic parameters of these modified sequences have been determined to assess the overall helical stability of the functionally modified recognition sites, and we have examined the modified recognition sequences for their ability to function as substrates for the endonuclease and determined steady-state kinetic parameters where appropriate.

Experimental Procedures

Materials

Sep-Pak cartridges were obtained from Millipore (Milford, MA). [γ-^{32}P]ATP was obtained from New England Nuclear (Billerica, MA). DEAE-cellulose thin-layer plates were obtained from J. T. Baker (Phillipsburg, NJ). Homomix was prepared as described (Frank & Blocker, 1982). HPLC was performed on a Beckman (Berkeley, CA) liquid chromatograph with a two-pump solvent delivery system and a 421 controller. The system was fitted with a variable-wavelength detector. ODS–Hypersil was a product of Shandon Southern Products Ltd. (Runcorn, England). Integration of HPLC peaks was performed on a Shimadzu C-R3A chromatopac integrator (Kyoto, Japan). Radioactivity was measured with a LKB Wallac Rack β liquid scintillation counter (Uppsala, Sweden). T4 polynucleotide kinase was a product of New England Biolabs (Beverly, MA). Thermal melting points of the oligodeoxynucleotides were obtained with a Perkin-Elmer Lambda 3 B spectrophotometer equipped with a C570-0701 digital temperature controller and a temperature programmer. Solution temperature was measured directly by using an immersible probe and a Telethermometer (Omega Engineering Inc., Stamford, CT). The oligodeoxynucleotides were synthesized on an Applied Biosystems 381 automated DNA synthesizer.

Methods

Synthesis of Oligodeoxynucleotides. The DNA sequences were synthesized by using phosphite triester methodology on solid-phase CPG-modified supports. The syntheses of the modified β-cyanoethyl phosphoramidites have been described (McLaughlin et al., 1988; Clore et al., 1988) or will be described elsewhere. The oligodeoxynucleotides were synthesized on an Applied Biosystems 381 automated DNA synthesizer and purified by reversed-phase HPLC (McLaughlin & Piel, 1984).

Determination of Thermodynamic Parameters. Thermal melting point (T_m) values were obtained in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM $MgCl_2$ (endonuclease buffer). Absorbance and temperature data were collected and stored after analog to digital conversion (DT-2800, Data Translation, Marlboro, MA) using an IBM-XT personal computer employing the ASYST (Ver.1.53) scientific package (MacMillan Software, New York, NY). From absorbance vs temperature graphs, T_m values could be obtained directly, or first and second derivatives were derived from the collected data.

Isolation of the EcoRV Endonuclease. The *Eco*RV endonuclease overproducing strain of *Escherichia coli* was constructed by M. Zabeau in the same way as the *Eco*RI endonuclease overproducing strain (Bottermann & Zabeau, 1985). It was grown at 28 °C in 3.7 L of L-broth containing 10 g of tryptone/L, 10 g of yeast extract/L and 5 g of sodium chloride/L. After 4 h of growth, overproduction was induced for by a temperature shift to 42 °C. After a 3-h incubation, 8.1 g of wet cell paste was harvested by centrifugation.

All procedures pertaining to the isolation were carried out at 4 °C. The 8.1 g of wet cell past was suspended in 230 mL of 30 mM potassium phosphate, 1 mM EDTA, and 0.1 mM DTE at pH 7.2 (buffer A) containing 0.5 M NaCl and sonicated with a MSE MK 2 sonicator at 15 W for 20 min. In 230 mL of crude cell extract, we estimated a total of 2.6×10^6 units of the *Eco*RV endonuclease. Cell debris was removed by centrifugation for 30 min at 100000*g* in a Sorvall high-speed centrifuge. The supernatant was diluted with a 2-fold excess of buffer A, and this solution was applied to a 2.5 × 20 cm column of phosphocellulose (Whatman P-11). The column had been equilibrated with buffer A containing 0.2 M NaCl and after addition of the crude extact was washed with 100 mL of the same buffer. Elution of the column proceeded with 500 mL of buffer A and a linear gradient from 0.2 to 0.8 M NaCl. The *Eco*RV endonuclease activity eluted at approximately 0.45 M NaCl. Fractions containing significant activity were pooled, and ammonium sulfate was added to 80% saturation. After 3 h, the precipitate was isolated by centrifugation and dissolved in 1.5 mL of buffer A containing 0.2 M NaCl. This solution was applied to a 1.6 × 90 cm gel filtration column (LKB Ultogel AcA 44) and eluted with buffer A containing 0.2 M NaCl. Those fractions containing endonuclease activity were analyzed by SDS–polyacrylamide gel electrophoresis. The purest fractions (estimated at 99% pure) were pooled and precipitated with ammonium sulfate (70% saturation). The precipitate was collected (centrifugation) and dissolved in 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 10 mM mercaptoethanol, and 50% glycerol, pH 7.2, and dialyzed overnight against the same buffer. The final enzyme concentration was 0.53 mg of protein/mL. The yield of enzyme with this procedure was 81 μg (1 500 000 units/mg) from each gram of wet cell paste.

EcoRV Endonuclease Assays. The oligodeoxynucleotide concentrations are expressed as duplex concentrations estimated from melting curves obtained by plotting the absorbance at 260 nm vs temperature. The concentration of endonuclease is reported as dimer concentration. The DNA sequences were

initially tested as substrates for the enzyme at 15 °C in 100-μL reaction mixtures containing from 4 to 10 μM oligodeoxynucleotide, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 50 μg/mL BSA. All reaction mixtures were heated to 65 °C and cooled slowly to 15 °C and then initiated with the addition of endonuclease to concentrations varying from 82 to 410 nM. Aliquots were withdrawn and immediately frozen in liquid nitrogen. The aliquots were analyzed by reverse-phase HPLC using a 4.6 × 250 mm column of ODS–Hypersil developed in 20 mM KH_2PO_4, pH 5.5, and a gradient of 0–70% methanol in 60 min. Oligodeoxynucleotides which functioned as effective substrates were more than 98% hydrolyzed within 2 h under these conditions.

Radioisotopic Labeling of Oligodeoxynucleotides. Kinetic parameters were determined by using ^{32}P-end-labeled oligodeoxynucleotides. Isotopic labeling and isolation of the phosphorylated DNA fragments were performed exactly as described previously (McLaughlin et al., 1987).

Determination of Kinetic Parameters. Reaction mixtures (19 μL) contained a mixture of ^{32}P-end-labeled oligodeoxynucleotides (specific activity 15 000–80 000 cpm/pmol) in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 50 μg/mL BSA. The reaction mixture was warmed to 65 °C, cooled slowly, and kept at 15 °C for 10–15 min. The reactions were initiated by the addition of 1 μL of endonuclease to give a concentration of 30–50 nM. One-microliter aliquots were spotted onto DEAE-cellulose thin-layer plates at 0-, 2-, 4-, 6-, 8-, and 10-min intervals. The plates had previously been spotted with 1 μL of 50 mM EDTA, pH 7.6. The plates were developed in Homomix (Frank & Blocker, 1982) at 65 °C, and the radioactivity was located by autoradiography. The areas containing radioactivity were removed and counted using liquid scintillation counting. Cleavage of the decamer results in the formation of the radioisotopically labeled pentamer d(pCTGAT) product. Lineweaver–Burk plots were prepared from initial velocity data, and the kinetic parameters K_m and k_{cat} were obtained by linear regression analysis of these graphs.

The amount of phosphodiester bonds hydrolyzed was calculated from the fraction of radioactivity of the pentamer multiplied by the total picomoles of oligodeoxynucleotide in the assay mixture initially (Brennan et al., 1986a):

$$\text{pmol of d(pCTGAT)} = \frac{\text{cpm d(pCTGAT)}}{\text{cpm d(pCTGAT)} + \text{cpm d(pCTGATATCAG)}} \times \text{pmol of d(pCTGATATCAG)}_i$$

Inhibition Studies. Cleavage of the unmodified dodecamer d(CGCGATATCGCG) was monitored by HPLC. The cleavage products were resolved from the starting dodecamer, and the extent of cleavage at various time intervals was calculated from the integration of the corresponding peak areas. The concentrations of endonuclease (16 nM) and dodecamer (0.5 μM) were kept constant in 500-μL reaction mixtures (control reaction). Each modified decamer which did not function as a substrate was included in the 500-μL reaction mixture at a concentration of at least 5 μM. Aliquots (200 μL) were removed at 5- and 10-min intervals, heated to 95 °C to inactivate the enzyme, concentrated by vacuum centrifugation, and analyzed by HPLC (buffer A, 20 mM potassium phosphate, pH 5.5; buffer B, same as buffer A but contained 70% methanol; gradient, 0–100% buffer B in 60 min). The dodecamer and cleavage products were resolved from the modified fragments under these conditions such that the extent of cleavage of the dodecamer could be determined in the presence of the modified sequences. These reactions were performed at 15 °C and in some cases also at 37 °C.

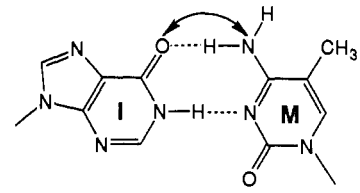

FIGURE 1: Presumed structure of dI-dM base pair.

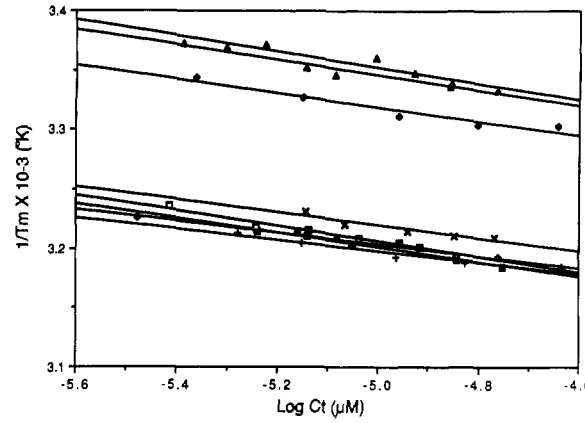

FIGURE 2: Plots of $1/T_m$ vs log C_t for d(CTGATATCAG) (+), d(CTIATATCAG) (♦), d(CTGPTATCAG) (△), d(CTGATAUCAG) (×), d(CTGITAMCAG) (■), d(CTGATPTCAG) (▲), d(CTGAUATCAG) (◇), and d(CTGAMITCAG) (□).

RESULTS

Modified Substrate Design. In order to minimize structural distortion but alter the functional group characteristics of the recognition sequence, two types of functional group modifications were employed. "Deletion modifications" involve the deletion of a single functional group from the recognition site (its substitution by a hydrogen). The second involves reversing the relative positions of functional groups involved in hydrogen bonding in the major groove. In either case, the modification was incorporated by the substitution of the appropriate modified nucleoside phosphoramidite derivative during chemical synthesis of the oligodeoxynucleotide. The desired "deletions" were obtained by the substitution of hypoxanthine (I) or 2-aminopurine (2) for guanine (G), uracil (U) for thymine (T), or purine (P) for adenine (A). The "functional group reversal" results by substitution of 5-methylcytosine (M) for thymine and hypoxanthine for adenine. This creates an dI-dM base pair in place of either of the native dA-dT base pairs (Figure 1) (McLaughlin et al., 1987). Functional group reversals alter the functional group characteristics of the recognition site without changing the number of interstrand hydrogen bonds present within the duplex oligodeoxynucleotide. A total of eight oligodeoxynucleotides were synthesized containing functional group modifications.

Thermodynamic Parameters for Helix–Coil Transitions. Thermodynamic parameters characterizing helix formation by the various modified oligodeoxynucleotides were determined in the concentration range 1.6–25 μM single-stranded concentration from plots of $1/T_m$ vs log C_t (where C_t = total concentration of oligodeoxynucleotide) (Figure 2). Changes in enthalpy (ΔH) and entropy (ΔS) and free energy (ΔG) for helix formation have been derived by assuming a two-state model for the helix to coil transition (Albergo et al., 1981) and are listed in Table I. As a test of the validity of the two-state model in these transitions, we determined ΔH values from the number of individual absorption vs temperature curves (Albergo et al., 1981). These values agreed (within

Table I: Thermodynamic Parameters for Duplex Formation

sequence[a]	$-\Delta H$ (kcal/mol)	$-\Delta S$ (cal mol^{-1} K^{-1})	$-\Delta G$ (kcal/mol) (37 °C)	T_m (10 μM)
(1) d(CTGATATCAG)	84 (62)[b]	246	7.8	40
(2) d(CTIATATCAG)	73	221	4.5	29
(3) d(CT2ATATCAG)	*c*	*c*	*c*	*c*
(4) d(CTGPTATCAG)	67	200	4.6	25
(5) d(CTGATAUCAG)	83	245	7.2	38
(6) d(CTGITAMCAG)	74	215	7.6	39
(7) d(CTGATPTCAG)	73	219	3.9	26
(8) d(CTGAUATCAG)	81	238	7.6	39
(9) d(CTGAMITCAG)	70	202	7.5	39

[a] P = purine, U = uracil, I = hypoxanthine, 2 = 2-aminopurine, and M = 5-methylcytosine. [b] Predicted from Breslauer et al. (1986). [c] No transition in melting curves.

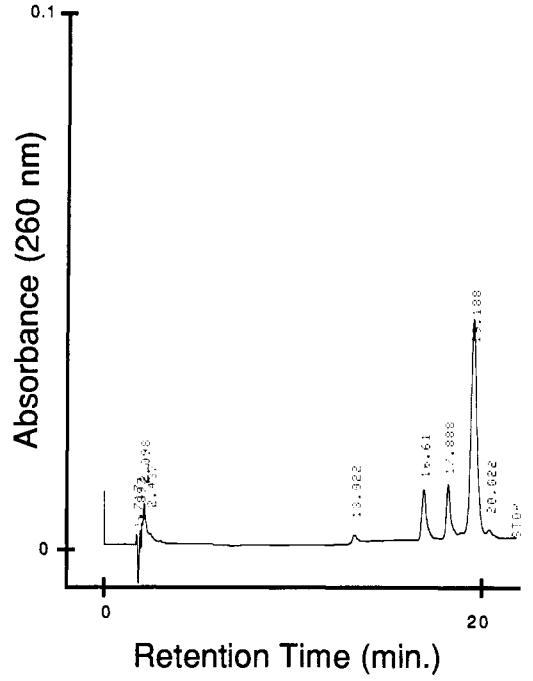

FIGURE 3: HPLC analysis of *Eco*RV-catalyzed hydrolysis of the native recognition sequence. Chromatography conditions as described under Experimental Procedures. The peaks were identified as follows: 13.0 min, BSA; 16.6 min, d(pATCAG); 17.9 min, d(CTGAT); 19.2 min, d(CTGATATCAG).

15%) with those obtained from $1/T_m$ vs log C_t plots.

Reactivity of the Native EcoRV Recognition Sequence. At a single-strand concentration of 10 μM, the decamer containing the native recognition site, d(CTGATATCAG), exhibited a T_m of 40 °C (Table I), when assayed in endonuclease buffer (see Methods). On the basis of the melting curve, the decamer was almost exclusively in the double-stranded form at 15 °C, the temperature used for the enzyme assays. HPLC analysis confirmed that the decamer could be hydrolyzed to form the two pentamer cleavage products in the absence of nonspecific nuclease activity (Figure 3).

The enzyme exhibited Michaelis–Menten kinetic parameters. It could be saturated with substrate and was first order in enzyme concentration over the range 16–66 nM (data not shown). Kinetic parameters were obtained in a substrate concentration range of 0.2–1.5 μM and were derived from Lineweaver–Burk plots of initial velocity data. The decamer exhibited a K_m of 0.46 ± 0.08 μM and a k_{cat} of 0.70 ± 0.10 phosphodiester bond hydrolyzed min^{-1} (enzyme dimer)$^{-1}$.

Reactivity of Modified Oligonucleotides. The modified oligodeoxynucleotides were initially assayed for nonspecific hydrolytic reactions and for their ability to function as substrates for the *Eco*RV endonuclease using HPLC. Hydrolytic reactions, specific or nonspecific, which resulted in hydrolysis of 1% of the oligodeoxynucleotide could be detected in this manner. Oligodeoxynucleotides which appeared to function as enzyme substrates could be completely hydrolyzed within 2 h at 15 °C. Some DNA fragments produced small amounts of the specific cleavage products after a 20-h incubation but not enough to suggest that the fragments were effective substrates. In these cases, a "trace" of hydrolysis was observed, but this never represented more than 5% of the initial concentration after 20 h at 15 °C.

Table II: Kinetic Parameters at 15 °C for the Cleavage of the Native and Modified *Eco*RV Recognition Site

sequence[a]	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m(rel)
(1) d(CTGATATCAG)	0.46 ± 0.08	0.70 ± 0.10	1.0
(2) d(CTIATATCAG)	0.20 ± 0.04	0.32 ± 0.04	1.05
(3) d(CT2ATATCAG)	*b*	*b*	*b*
(4) d(CTGPTATCAG)	t[c]	t	t
(5) d(CTGATAUCAG)	t	t	t
(6) d(CTGITAMCAG)	nc[d]	nc	nc
(7) d(CTGATPTCAG)	t	t	t
(8) d(CTGAUATCAG)	t	t	t
(9) d(CTGAMITCAG)	nc	nc	nc

[a] P = purine, U = uracil, I = hypoxanthine, 2 = 2-aminopurine, and M = 5-methylcytosine. [b] The T_m value could not be determined, and this fragment must be assumed to be single stranded at the assay temperature. [c] A trace (t) of cleavage could be observed after a 20-h incubation (less than 5%). [d] No cleavage (nc) observed after 20 h.

(A) Modifications to the Guanine-Cytosine Base Pairs. Two modifications of the dG-dC base pairs in the recognition sequence were performed. The DNA fragment containing the substitution of hypoxanthine for guanine (deletion of the guanine exocyclic amino group) functioned as a substrate for the enzyme, and kinetic parameters were determined (Table II). The hydrolysis could be characterized by a K_m of 0.20 ± 0.04 μM and a k_{cat} of 0.32 ± 0.04 phosphodiester bond hydrolyzed min^{-1} (enzyme dimer)$^{-1}$. The second modification of the dG-dC base pair was the substitution of 2-aminopurine for guanine (deletion of the carbonyl at the 6-position). This deletion resulted in an oligodeoxynucleotide which did not exhibit a helix to coil transition in the temperature range 10–15 °C and must be assumed to be largely a random coil at 15 °C. This fragment was inactive with the endonuclease.

(B) Modifications to the "Outer" Adenine-Thymine Base Pairs. The deletion of the methyl group from the thymine residue was accomplished with the substitution of uracil in this position. This substrate analogue did not function as an efficient endonuclease substrate although some cleavage was observed after a 20-h incubation at 15 °C (less than 5%). Substitution of purine for adenine (deletion of the exocyclic amino group) also produced a fragment for which a trace of reaction was present, but never more than 5% of the fragment was hydrolyzed after a 20-h incubation. The substitution of the dI-dM base pair at this position (reversing the positions of the exocyclic functional groups in the major groove) was not cleaved by the enzyme.

(C) Modifications to the "Inner" Adenine-Thymine Base Pairs. Neither the substitution of uracil for thymine nor the substitution of purine for adenine at the inner dA-dT base pair resulted in an efficient substrate for the endonuclease (less than 5% cleavage in 20 h). Reversing the relative positions of the functional groups in the major groove by substitution of the dI-dM base pair also did not produce an endonuclease substrate.

(D) Inhibition Studies. We examined the ability of all modified sequences to inhibit the cleavage of the dodecamer

d(CGCGATATCGCG) (T_m = 57 °C) containing the native recognition site for two reasons: (i) It was necessary to confirm that the inactivity or extreme low activity of some of the sequences was due to the presence of the modification in the recognition site and not the result of foreign contaminants in the oligodeoxynucleotide preparation. (ii) By examining any inhibition present as a function of temperature, it is possible to assess whether, in some cases, there is enzyme substrate binding in the absence of catalysis as we have described for the *Eco*RI endonuclease (McLaughlin et al., 1987). In the present case, the only modified DNA fragment which exhibited inhibition of the hydrolysis of the native dodecamer sequence (at 15 °C) was the purine-containing fragment d(CTGATPTCAG). However, the inhibition by this fragment was temperature sensitive, and at 37 °C above the T_m of this fragment (26 °C), the inhibition largely disappeared.

Discussion

Thermodynamic Stability of the Duplex DNA Sequences. Since the endonuclease requires a double-stranded nucleic acid substrate, the determination of helix stability, especially for functionally modified sequences, is fundamental for the interpretation of experiments related to enzyme recognition. We have determined helix stability and calculated the thermodynamic parameters characterizing helix formation under the conditions of the endonuclease assay. The predicted thermodynamic parameters for the unmodified decamer have also been calculated (Breslauer et al., 1986) but for a higher salt concentration (1 M NaCl) than employed in the present study (50 mM NaCl, 10 mM $MgCl_2$). This may in part account for the differences observed between the experimental and predicted values (Table I). The deletion of functional groups not directly involved in hydrogen-bonding schemes such as the thymine methyl group (substitution of U; entries 5 and 8, Table I) has little or no effect upon the T_m or the thermodynamic parameters for helix formation as would be expected. The deletion of functional groups which are responsible for the formation of an interstrand hydrogen bond such as the exocyclic amino groups of guanine (substitution of I; entry 2, Table I) or adenine (substitution of P; entries 4 and 7, Table I) results in decreased duplex stability, and the T_m values in these three cases are reduced by 11–15 °C. These deletion modifications occur in each half-site of the self-complementary oligodeoxynucleotide such that in comparison with the native sequence, two hydrogen bonds have been deleted with the substitution of hypoxanthine for guanine or purine for adenine. The missing hydrogen bonds are largely responsible for the greater than 10 kcal/mol decrease in enthalpy and a 3.2–3.9 kcal/mol change in free energy for these three sequences when compared with the native sequence (Table I). We cannot, however, rule out the contribution made to the calculated thermodynamic values arising from differences in base stacking for the "normal" and modified base. The substituion of 2-aminopurine for guanine deletes the carbonyl involved in a single interstrand hydrogen bond but also results in a tautomeric change in the character of the guanine N^1 such that two of the three hydrogen bonds normally present in the dG-dC base pair are lost. This modification results in the deletion of four hydrogen bonds from the modified DNA duplex which in turn decreases the helical stability such that in the range of 10–50 °C we were unable to observe a well-characterized helix to coil transition. Modifications which we have termed "functional group reversals" (substitution of dI-dM for dA-dT) maintain the number of hydrogen bonds equal to that found in the native sequence. The T_m values for both dI-dM-containing sequences (entries 6 and 9, Table I) are within 1 °C of the value obtained for the unmodified sequence. The free energy change for helix formation is also essentially the same for the native dA-dT or modified dI-dM sequences while the change in enthalpy for both sequences with functional group reversals decreased by approximately 10 kcal/mol. Although enthalpy changes are often related to the number of hydrogen bonds present, base stacking also plays a crucial role in helix stability (Petruska et al., 1986), and differences in base stacking for the native and modified sequences could account for the observed enthalpy values in this case.

Interaction of the Endonuclease with the Native Recognition Site. The decamer d(CTGATATCAG) containing the canonical hexameric recognition site d(GATATC) was a substrate for the endonuclease and was hydrolyzed to form the two pentamer products. This is in agreement with previously reported results with other oligodeoxynucleotides (D'Arcy et al., 1985; Fliess et al., 1986). The enzyme could be saturated with substrate and exhibited Michaelis–Menten kinetics as judged from the initial velocity data at various substrate concentrations. The K_m obtained for the decamer is higher than previously reported for DNA polymers (Halford et al., 1988), but that is not unusual for oligodeoxynucleotide substrates (Greene et al., 1981; Dwyer-Halquist et al., 1982; Brennan et al., 1986a; McLaughlin et al., 1987). The differences appear to be related to the length of the substrate and/or flanking sequences outside the recognition sequence. We have not varied the flanking sequences in this study but limited the modifications to the canonical six base pair recognition site. Variations in assay conditions may also in part account for differences in the observed Michaelis constant by effecting product release from the endonuclease, an apparent rate-limiting step for the enzyme (Halford et al., 1988). There are small differences for the catalytic activity of the endonuclease as characterized by k_{cat} for the present native oligodeoxynucleotide with respect to that reported for DNA substrates (Halford et al., 1988). This reflects in part differences in assay conditions including the low temperature (15 °C) necessary in the present study in order to maintain the fragments in the double-stranded form.

Modified Recognition Sequences. (A) Modifications to the Guanine-Cytosine Base Pairs. Two DNA fragments were synthesized containing modifications of the dG-dC base pair within the recognition site. Substitution of 2-aminopurine for guanine did not appear to result in a double-stranded structure at the assay temperature (15 °C), and the lack of substrate activity in this case is directly a result of poor helix stability. The substitution of hypoxanthine or guanine (deletion of the guanine exocyclic amino group) results in a fragment which functions as an endonuclease substrate with only a slight decrease in K_m and k_{cat}. Enzyme efficiency as measured by the relative specificity constant (k_{cat}/K_m) is essentially unchanged (Table II). Although the deletion of this amino group might result in a small perturbation of DNA structure which marginally affects the interaction of the nucleic acid with the protein, the results suggest that the guanine amino group, located in the minor groove the DNA helix, is not an important contact for the enzyme.

(B) Modifications to the Adenine-Thymine Base Pairs. Modifications to either the "outer" or the "inner" adenine-thymine base pairs were very similar in terms of their affects on hydrolysis by the endonuclease. Deletion of either methyl group from the thymine residues or either exocyclic amino group from the adenine residues produced fragments which were inactive as substrates or exhibited only small amount of hydrolysis after an extended incubation. This is in agreement

with the reported preliminary studies involving similar modifications (Fliess et al., 1986; Nwosu et al., 1988). The high-resolution NMR-derived structure of an oligodeoxynucleotide containing a purine-thymine base pair indicates that, although an interstrand hydrogen bond is missing, the purine occupies the same position in the helix and its orientation with respect to thymine is the same as for a native adenine-thymine base pair (Clore et al., 1988). On the basis of these observations, it is unlikely that the lack of substrate activity observed with the deletion of either amino group is a function of a significant structural aberration induced by the presence of the purine base. Reversing the relative positions of the carbonyl and amino groups involved in the hydrogen bonding at either base pair also produced sequences which were not hydrolyzed by the endonuclease. These results suggest that both amino groups and both methyl groups present in the major groove represent significant contacts to the enzyme and affect either binding (K_m) or catalysis (k_{cat}).

In general, the sensitivity of the endonuclease to modifications in the major groove of the B-DNA helix and the insensitivity to the modification made in the minor groove (at the dG-dC base pair) suggest that the enzyme recognizes the correct sequence largely via interactions in the major groove.

(*C*) *Inhibition Studies.* The inhibition studies largely confirmed that the lack of substrate activity observed with many of the modified sequences was the result of the modified recognition site and not a function of any foreign entity present as a result of synthesis or purification procedures. An exception is the sequence containing purine substituted for the "inner" adenine residue. At 15 °C, this fragment, d-(CTGATPTCAG), strongly inhibited the cleavage of the dodecamer d(CGCGATATCGCG), but at increased temperatures (37 °C), above the T_m of the modified fragment, the inhibition largely disappeared. The ability to melt out the inhibition at higher temperature indicates that the observed inhibition was due to the presence of the modified *double-stranded* fragment and unlikely to result from contamination present in the oligodeoxynucleotide preparation. As we have reported before (McLaughlin et al., 1987), such temperature-sensitive inhibition of this type suggests that the enzyme binds the modified sequence but is unable to initiate catalysis. In fact, catalysis appeared to be present after an extended incubation (~5% hydrolysis after 20 h), suggesting that the modification results in severely reduced turnover by the enzyme. The results indicate that most of the modifications in the present study affect the ability of the protein to effectively bind the sequence. Only the decamer, d(CTGATPTCAG), appears to bind to the enzyme effectively, but catalysis is extremely inefficient. This suggests that the amino group of the inner dA-dT base pair is relatively unimportant in the formation of the sequence-specific protein–DNA complex but is a necessary contact for effective catalysis to occur.

(*D*) *Comparison with Results from the EcoRI Restriction Endonuclease.* It is of value to compare the *Eco*RI and the *Eco*RV restriction endonucleases studied with the same series of modified bases. The base compositions of the two recognition sites are the same but differ in the orientation of the central two adenine-thymine base pairs (GAATTC vs GATATC). Deletion of either amino group or either methyl group from the hexameric sequence d(GAATTC) recognized by the *Eco*RI endonuclease produces fragments which can be cleaved by the enzyme although sometimes with poor efficiency (McLaughlin et al., 1987). The *Eco*RI endonuclease also discriminates between modifications made at the inner and outer dA-dT base pairs and is more sensitive to functional group alterations at the outer base pair. By comparison, the present results for the *Eco*RV endonuclease indicate that this enzyme is sensitive to similar modifications made at either the inner or the outer dA-dT base pairs and is unable to effectively hydrolyze sequences from which a single methyl or adenine amino group has been deleted. This suggests that important contacts between the protein and the nucleic acid occur at both adenine amino groups and both thymine methyl groups present in the canonical six base pair recognition sequence. The sensitivity of both enzymes to the deletion of methyl groups from the thymine residues (the *Eco*RI is only sensitive to deletion of the methyl group from the outer base pair) indicates the importance of these functional groups in van der Waals contacts or hydrophobic interactions. The significance of thymine methyl groups in sequence-specific recognition has been noted previously with the lac repressor (Caruthers, 1980) and RNA polymerase (Dubendorff et al., 1987) and appears to be equally important in both of these restriction endonucleases.

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A Novel Primase-Free Form of Murine DNA Polymerase α Induced by Infection with Minute Virus of Mice†

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ABSTRACT: Two species of DNA polymerase α free of primase activity were identified in extracts of Ehrlich mouse cells that had been infected with minute virus of mice. Primase-free forms of DNA polymerase α eluted with 150 and 180 mM NaCl during ion-exchange chromatography on DEAE-cellulose columns, exhibited sedimentation coefficients of 11 S and 8.2 S, respectively, and were inhibited by aphidicolin, N^2-(*p*-*n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate, and 2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate. The ratio of primase-free DNA polymerase α to the DNA polymerase α–primase complex increased from 1.5 to >100 during the course of infection, and free primase was produced during the MVM replicative cycle.

The eucaryotic replicative enzyme DNA polymerase α is characterized by its relatively high molecular weight, complex subunit composition, specific induction during the S-phase of the cell cycle, ability to use RNA primers, and sensitivity to aphidicolin, BuPdGTP,[1] and BuAdATP (Fry & Loeb, 1986). Highly purified preparations of DNA polymerase α from mammalian cells and insects as well as the analogous DNA polymerase I in yeast consist of a DNA polymerase catalytic subunit of M_r = 145 000–185 000 in tight association with two to three additional subunits of M_rs = 47 000–86 000 (Kaguni et al., 1983; Faust et al., 1984; Chang et al., 1984; Karawya et al., 1984; Wahl et al., 1984; Denhardt & Faust, 1985; Plevani et al., 1985; Vishwanatha et al., 1986; Holmes et al., 1986; Wong et al., 1986; Nasheuer & Grosse, 1987; Cotterill

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[1] Abbreviations: BuPdGTP, N^2-(*p*-*n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate; BuAdATP, 2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; DTT, dithiothreitol.

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