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Interaction of the *Hpa*I Endonuclease with Synthetic Oligonucleotides†

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ABSTRACT: To determine which functional groups of bases within the grooves of double-helical DNA interact with the *Hpa*I endonuclease, we have employed chemically synthesized octanucleotides containing base analogues. The 5-methyl group of thymine was probed as a contact between the *Hpa*I endonuclease and its recognition sequence by using the oligonucleotides d(G-G-T-T-A-A-C-C), d(G-G-T-U-A-A-C-C), and d(G-G-T-U(Br)-A-A-C-C). The 2-amino group of guanine was probed as a contact for the *Hpa*I endonuclease by using the octanucleotide d(G-I-T-T-A-A-C-C). The *Hpa*I endonuclease cleaves octanucleotides d(G-G-T-T-A-A-C-C) and d(G-G-T-B-A-A-C-C) according to Michaelis–Menten kinetics. However, both the K_m and turnover number for d(G-G-T-B-A-A-C-C) were severalfold lower than those for cleavage of d(G-G-T-T-A-A-C-C). In addition, d(G-G-T-U-A-A-C-C) was not cleaved by *Hpa*I endonuclease, suggesting that the 5-methyl group of thymine is a contact between the *Hpa*I endonuclease and its recognition sequence. d(G-I-T-T-A-A-C-C) was not cleaved by the *Hpa*I endonuclease which may be due in part to the low thermal stability of the duplex. Nevertheless, our results suggest that the 2-amino group of guanine is a contact for the *Hpa*I endonuclease. A phosphate group 5′ external to the *Hpa*I recognition sequence has been identified as a contact between the *Hpa*I endonuclease and DNA. The *Hpa*I endonuclease cleaved 5′-phosphorylated octanucleotide 30-fold faster than unphosphorylated octanucleotide. In addition, the K_m of the d(G-G-T-T-A-A-C-C) was 8000-fold higher than the K_m of the phage f_1 RFI DNA, suggesting that the octanucleotide is too short to take advantage of the entire DNA binding site of the enzyme.

The simplicity of structure and activity requirements has rendered type II endonucleases and their cognate methylases an attractive system for studying sequence-specific DNA–protein interactions (Modrich, 1979). Since the endonuclease and methylase are normally present as pairs in the cell, they provide an excellent opportunity to study the interactions of two entirely different enzymes with the same DNA sequence. Our laboratory has purified to homogeneity the type II endonuclease and methylase occurring in the bacterium *Haemophilus parainfluenzae*. The *Hpa*I endonuclease, *Hpa*II endonuclease, and their cognate methylases have been characterized with respect to molecular weight, oligomeric state, and kinetic parameters of the DNA cleavage and methylation reactions (Hines, 1979; Hines & Agarwal, 1979; Yoo & Agarwal, 1980; Yoo, 1981; Dwyer-Hallquist, 1981). In this paper, we report studies on the mechanism by which the *Hpa*I

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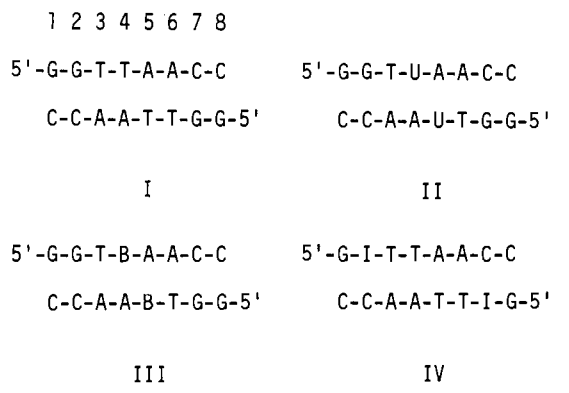

FIGURE 1: Structures of self-complementary octanucleotides. The nucleotides are numbered from the 5′ end. U, B, and I represent deoxyuridine, deoxy-5-bromouridine, and deoxyinosine, respectively.

endonuclease identifies its recognition sequence.

Analysis of double-helical DNA indicates that it is possible for a protein to identify unambiguously a base pair by contacts with functional groups of the bases that are available in the grooves of DNA (Seeman et al., 1976). Interaction of a protein with a DNA sequence does not require disruption of the hydrogen bonds of the base pairs but may depend on the presence or absence of functional groups exposed in the grooves of double-helical DNA. One method of testing functional groups of the bases as contact points between the enzyme and its recognition sequence is to use DNAs containing base analogues as substrates. This approach was followed by Berkner & Folk (1977, 1979), who used phage DNAs containing substituents at position 5 of thymine or cytosine as substrates for several type II restriction endonucleases. They demonstrated that the initial rate of *Hpa*I cleavage of uracil-containing PBS2 DNA was 10 times slower than the rate of *Hpa*I cleavage of thymine-containing phage λ DNA and concluded that the *Hpa*I endonuclease probes the thymine methyl group in its interactions with DNA. However, interpretation of their data is not straightforward, because double-strand cleavage of the DNA was not linear with time and exhibited a lag period. If rates were determined from later times of digestion instead of initial times, then DNA containing uracil appeared to be cleaved 50% more rapidly than thymine-containing DNA. In addition, the preparations of *Hpa*I endonuclease employed were only partially purified, and the identity of the cleaved products was not established. Another disadvantage of their approach of using DNAs highly substituted with base analogues is the uncertainty whether the observed effects on the rate of endonuclease cleavage are due to base substitutions within or outside the recognition sequence. Marchionni & Roufa (1978) found that one of the three *Sma*I sites in bromouracil-substituted DNA is highly resistant to *Sma*I cleavage, even though the *Sma*I recognition sequence does not contain any thymines. Thus, base analogue substitution in sequences external to the recognition site may affect type II endonuclease cleavage of DNA.

We have approached the problem of nucleic acid–protein interactions by exploring the effects of base analogue substitutions on *Hpa*I endonuclease cleavage of a variety of chemically synthesized oligonucleotides. On the basis of the studies of Greene et al. (1975), who showed that the octanucleotide d(pT-G-A-A-T-T-C-A) is cleaved by the *Eco*RI endonuclease, and our own observations that the octanucleotide d(G-G-T-T-A-A-C-C) (I, Figure 1) is cleaved by the *Hpa*I endonuclease, we synthesized three octanucleotides (II–IV, Figure 1) containing base analogues. Octanucleotides II and III contain the thymine analogues uracil and bromouracil; octanucleotide IV contains the guanine analogue hypoxanthine. These oligonucleotides were used as substrates in steady-state kinetic experiments with homogeneous *Hpa*I endonuclease in order to determine whether the 5-methyl of thymine and the 2-amino of guanine are contacts between the enzyme and its DNA recognition sequence. The major advantage of using chemically synthesized oligonucleotides instead of DNA as substrates for DNA enzymes is that the base analogues can be placed at any position within the sequence at will. In addition, the effect of base analogue substitution on the stability of the duplex form of the oligonucleotide can be determined by measuring the melting curve of the oligonucleotide.

Experimental Procedures

Materials

A Whatman Partisil PXS 10/25 ODS-2 column was used for C_{18} μBondapak silica gel reverse-phase chromatography. The high-speed gel permeation column SW type 2000 was obtained from Toyo-Soda Manufacturing Co. The high-performance anion-exchange column Ultrasil-AX was obtained from Altex Scientific, Inc.

A Perkin-Elmer Series 3 chromatograph was used for high-performance reverse-phase chromatography. An Isco Model 384 Dialagrad pump and a Perkin-Elmer Model LC-55 spectrophotometer were used for high-speed gel permeation and high-performance anion-exchange chromatography.

DEAE-cellulose (1:7.5) glass-backed plates (0.25 mm) were obtained from AnalTech. DEAE-cellulose sheets (0.1 mm) were obtained from Brinkmann. RNA homomix was prepared according to a published procedure (Jay et al., 1974). Agarose type I low EEO was purchased from Sigma. Bovine serum albumin was purchased from Pentax. Snake venom phosphodiesterase was purchased from Worthington. T_4 polynucleotide kinase was isolated by a modified procedure of Panet et al. (1973).

The *Hpa*I endonuclease used in these studies was purified to homogeneity by Hines et al. (Hines & Agarwal, 1979; Hines, 1979; Hines et al., 1980). Since there was no significant loss of *Hpa*I activity in the stock solution, the *Hpa*I endonuclease concentration was calculated from the actual protein concentration and is always given in monomer concentration.

The nucleotides pdT, pdA, and pdG were obtained from Sigma. pdC was obtained from Pharma Waldhof and dG from P-L Biochemicals. [γ-^{32}P]ATP (1400 mCi/μmol) was synthesized as described by Maxam & Gilbert (1977).

f_1 RFI DNA containing [^{3}H]thymidine was prepared according to a published procedure (Model & Zinder, 1974). The specific activity of the various DNA preparations ranged from 1300 to 11 000 cpm/μg of DNA. On the average, about 20% of the DNA was nicked circles.

Methods

Synthesis, Purification, and Characterization of Oligonucleotides. The octanucleotide d(G-G-T-T-A-A-C-C) was synthesized by the diester method (Agarwal et al., 1972) or by the triester method (Agarwal & Riftina, 1978). Octanucleotides II, III, and IV were synthesized by the modified triester method (Agarwal & Riftina, 1978). After DEAE-cellulose chromatography of the completely deprotected octanucleotides, additional purification was achieved by chromatography on a C_{18} μBondapak silica gel reverse-phase column (Fritz et al., 1978). A typical chromatographic run consisted of a 30-min linear gradient of 12–13% acetonitrile in 100 mM triethylammonium acetate, pH 7.0, with a flow rate of 2 mL/min. In certain cases a gradient of 9–11%

acetonitrile in 30 mM triethylammonium acetate was used.

Each octanucleotide was 5′ phosphorylated with T_4 polynucleotide kinase and [γ-^{32}P]ATP (Sgaramella & Khorana, 1972). Generally 200 pmol of the octanucleotide was labeled and separated from unreacted ATP and inorganic phosphate by chromatography on a 10-mL column of Sephadex G-50 superfine in 20 mM triethylammonium bicarbonate, pH 7.8. For confirmation of the sequence of each oligonucleotide, the 5′-labeled octanucleotide was subjected to partial digestion with snake venom phosphodiesterase followed by two-dimensional homochromatography (Jay et al., 1974).

Melting curves of the octanucleotides were determined by measuring absorbances at 260 nm with a Gilford 240 spectrophotometer at temperatures from 6 to 70 °C. Solutions were 18–20 μM total strand concentration of octanucleotide in 0.25 mL of buffer [20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 100 mM NaCl, and 5 mM $MgCl_2$].

Isolation and Identification of Products of HpaI Endonuclease Cleavage of Octanucleotides. d(G-G-T-T-A-A-C-C) (132 μM) in 45 μL of buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, and 89 μg/mL bovine serum albumin) was incubated with 8.3 μM *Hpa*I endonuclease monomer at 20 °C for 4 h. The product peaks were isolated by chromatography on the high-speed gel permeation column equilibrated with 100 mM ammonium acetate, pH 7.6, lyophilized with 1 equiv of triethylamine, dissolved in 400 μL of H_2O, relyophilized, and dissolved in 50 μL of H_2O. Rechromatography of individual peaks revealed cross-contamination of each tetranucleotide with the other, so the purification of each product was repeated. Each of the pure tetranucleotides was then incubated with 2 μg of snake venom phosphodiesterase in 30 μL of buffer (110 mM Tris-HCl, pH 8.9, 110 mM NaCl, and 15 mM $MgCl_2$) at room temperature. A 400-pmol sample of pd(A-A-C-C) and a 900-pmol sample of d(G-G-T-T) were digested for 10 and 60 min, respectively. The reaction mixtures were analyzed by high-pressure anion-exchange column (Ultrasil-AX) chromatography at a flow rate of 90 mL/h. Nucleotides and nucleosides were detected by UV absorbance at 270 nm. The column was eluted with the following gradient: 0–2 min, 0.02 M KH_2PO_4, pH 3.4, to 0.116 M KH_2PO_4, pH 3.4; 2–4 min, 0.116 M KH_2PO_4, pH 3.4; 4–7 min, 0.116 M KH_2PO_4, pH 3.4, to 0.332 M KH_2PO_4, pH 3.4.

Assay of HpaI Endonuclease Cleavage of Octanucleotide. Typical endonuclease reactions contained octanucleotide (5–500 μM) and 3.1 μM *Hpa*I endonuclease monomer in 10–50 μL of buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, and 100 μg/mL bovine serum albumin). The reaction was stopped by boiling an aliquot for 2 min and chilling it in ice. The sample was analyzed by high-speed gel permeation column chromatography, which separated the octanucleotide from each of the tetranucleotide products. The buffer (100 mM NaCl and 10 mM Tris-HCl, pH 7.5) was pumped through the column at a flow rate of 80 mL/h at room temperature. UV absorbance of the oligonucleotides was monitored at 260 nm. Peak areas were measured by a Hewlett-Packard 3388A integrator. The amount of reaction was calculated by

$$\mu\text{M phosphodiester bonds cleaved} = [\text{area of d(G-G-T-T)}][\epsilon_{260}[\text{d(G-G-T-T-A-A-C-C)}]][\mu\text{M d(G-G-T-T-A-A-C-C)}_{orig}]/[(\text{total area}) \times [\epsilon_{260}[d(\text{G-G-T-T})]]]$$

The extinction coefficients are for the single-stranded oligonucleotides and were calculated by addition of the extinction coefficients of the nucleotides in each oligonucleotide. μM d(G-G-T-T-A-A-C-C)$_{orig}$ is the initial total strand concentration of the octanucleotide and was determined from the absorbance at 260 nm. The K_m and V_{max} values were calculated by an iterative least-squares fit of initial velocities and substrate concentrations to a hyperbola.

Assay of HpaI Endonuclease Cleavage of 5′-^{32}P-Labeled Octanucleotide. Cleavage of [5′-^{32}P]d(G-G-T-T-A-A-C-C) was assayed by following the release of [5′-^{32}P]d(G-G-T-T). Aliquots of the reaction were spotted on a DEAE-cellulose thin-layer plate which was developed in the appropriate RNA homomix (Jay et al., 1974). Spots corresponding to the octanucleotide and tetranucleotide were located by autoradiography. The spots corresponding to oligonucleotides were cut out from the plastic-backed TLC plate or scraped from the glass-backed TLC plate, and their ^{32}P content was determined by Cherenkov counting. The amount of reaction was calculated by

$$\mu\text{M phosphodiester bonds cleaved} = [[5'\text{-}^{32}\text{P]d(G-G-T-T)}][\mu\text{M [5'-}^{32}\text{P]d(G-G-T-T-A-A-C-C)}_{orig}]/([[5'\text{-}^{32}\text{P]d(G-G-T-T)}] + [[5'\text{-}^{32}\text{P]d(G-G-T-T-A-A-C-C)}])$$

μM [5′-^{32}P]d(G-G-T-T-A-A-C-C)$_{orig}$ is the initial total strand concentration. This was calculated from the specific activity of the [γ-^{32}P]ATP assuming that almost all of the octanucleotide was phosphorylated by treatment with T_4 polynucleotide kinase and [γ-^{32}P]ATP.

Assay of HpaI Endonuclease Cleavage of f_1 RFI [^{3}H]DNA. For determination of the kinetic parameters for *Hpa*I cleavage of DNA, f_1 RFI [^{3}H]DNA (20–160 nM) and 0.69 nM *Hpa*I monomer were incubated at 25 °C in 10 μL of buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 39 μg/mL bovine serum albumin). Time points were taken up to 15 min. Reactions were stopped by mixing a 5-μL aliquot of the reaction with 20 μL of 25 mM ethylenediaminetetraacetic acid (EDTA), 30% (v/v) glycerol, and 0.02% bromphenol blue. The DNA products were analyzed by electrophoresis on 1% agarose slab gel (15 × 15 × 0.3 cm) as described previously (Sharp et al., 1973). DNA was visualized by staining with 1 μg/mL ethidium bromide. The DNA bands were cut out from the gel and melted in 0.4 mL of H_2O, and their ^{3}H content was determined after addition of 5 mL of Triton X scintillant. The amount of reaction was calculated by

$$\text{nM phosphodiester bonds cleaved} = \frac{2[\text{linear DNA}][\text{nM total DNA}]}{[\text{total DNA}]}$$

nM total DNA is the initial concentration of RF DNA and was determined from the absorbance at 260 nm of the stock solution of DNA.

Results

Synthesis and Characterization of Oligonucleotides. Using a modified triester method, Riftina and Agarwal successfully synthesized the self-complementary octanucleotide d(G-G-T-T-A-A-C-C) that contains the *Hpa*I recognition sequence d(G-T-T-A-A-C) (Agarwal & Riftina, 1978). When this procedure was followed, three additional octanucleotides (II–IV, Figure 1) containing base analogues within the *Hpa*I recognition sequence were synthesized. As shown in Figure 1, the base analogue in each oligonucleotide is located in the 5′ half of the octanucleotide, and therefore the synthesis involved the coupling of the common tetranucleotide d(A-A-C-C) with the three tetranucleotides d(G-G-T-U), d(G-G-T-B),

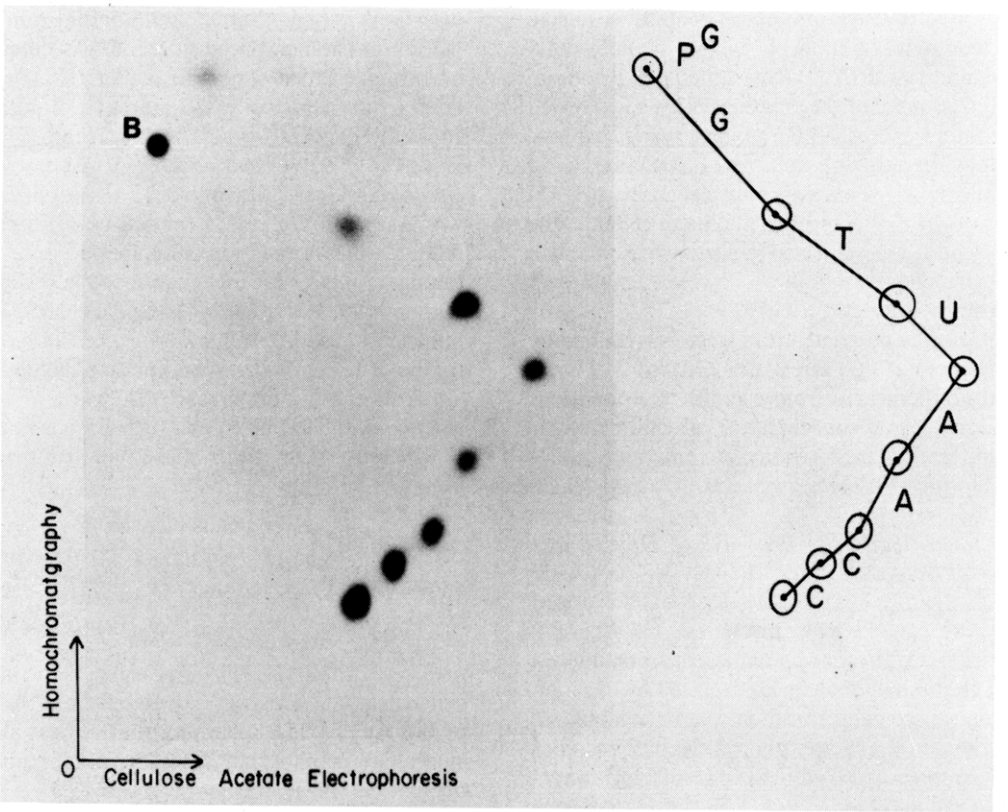

FIGURE 2: Autoradiogram of the fingerprint obtained on partial venom phosphodiesterase digestion of the 5′-^{32}P-labeled octanucleotide II. Oligonucleotide (10 pmol) containing 10 μg of yeast RNA was incubated with 2 μL of snake venom phosphodiesterase at 500 μg/mL in 5 mM Tris-HCl, pH 8.0. After 30 min at 37 °C, the digest was subjected to two-dimensional fingerprint analysis using homomix IV of Jay et al. (1974). The sequence of the octanucleotide is derived from the pattern as shown in the reproduction to the right of the fingerprint. B is the blue dye marker.

Table I: Kinetic Parameters for *Hpa*I Endonuclease Cleavage of Octanucleotides and DNA

octanucleotide	K_m^{duplex} (μM)[a]	turnover no. (min^{-1})[b]	turnover no./K_m^{duplex} ($\mu M\ min^{-1}$)	T_m (°C)	K ($M^{-1} \times 10^{-5}$)[c]
d(G-G-T-T-A-A-C-C)	170 ± 30	0.177 ± 0.019	(1.04 ± 0.21) × 10^{-3}	29	3.28
d(G-G-T-U-A-A-C-C)[d]				28	1.69
d(G-G-T-B-A-A-C-C)	36 ± 10	0.030 ± 0.003	(0.83 ± 0.24) × 10^{-3}	33	8.24
d(G-I-T-T-A-A-C-C)[d]				17	0.067
f_1 RFI DNA[e]	0.020 ± 0.007	0.46 ± 0.07	23 ± 9		

[a] K_m^{duplex} calculated from K_m^{CT} using the equilibrium constant K for the association of octanucleotides. [b] Turnover no. = phosphodiester bonds cleaved per minute per *Hpa*I endonuclease monomer. [c] K = [helix]/[coil]^2 at 25 °C. [d] Not cleaved by *Hpa*I. [e] Concentration of DNA is expressed in terms of double-stranded molecules.

and d(G-I-T-T) to yield the respective octanucleotides. The synthesis of the four tetranucleotides was made by coupling the respective dinucleotides in high yield. In a typical synthesis of the octanucleotide d(G-G-T-U-A-A-C-C), the appropriately protected tetranucleotides d(G-G-T-U) and d(A-A-C-C) were isolated in 64% and 81% yield, respectively (data not shown). Coupling of the two tetranucleotides gave the octanucleotide that was isolated in 62% yield by thin-layer chromatography. The fully deprotected octanucleotides were purified by DEAE-cellulose column chromatography, and their purity was assessed by C_{18} μBondapak reverse-phase and high-pressure gel permeation chromatography. Octanucleotides II and III appeared homogeneous by gel permeation chromatography and 95% pure by reverse-phase chromatography. Octanucleotide IV appeared as one major peak with a small shoulder on gel permeation chromatography and 85% pure by reverse-phase chromatography. Octanucleotide IV was further purified by reverse-phase chromatography before use. The nucleotide sequence of each of the octanucleotides, as determined by two-dimensional homochromatography (Jay et al., 1974), is in complete agreement with the assigned structures. As an example, an autoradiogram displaying the sequence of the octanucleotide d(G-G-T-U-A-A-C-C) is shown in Figure 2.

For determination of the thermal stabilities of the octanucleotides, the melting profile of each octanucleotide was determined in the reaction buffer. Comparison of the T_m values, the midpoints of the melting curves, indicates that octanucleotide III forms the most stable duplex, octanucleotides I and II form slightly less stable duplexes, and octanucleotide IV forms the least stable duplex; the data are summarized in Table I. Association constants [K (M^{-1})] for the single strand to duplex transition of each of the octanucleotides were calculated from the melting curve data and were used to calculate the concentration of the duplex form of each octanucleotide (Table I). From the ΔG obtained by Patel (1979) for the melting transition d(G-G-A-A-T-T-C-C) in 100 mM NaCl buffer, an association constant of 9.4 × 10^4 M^{-1} for this oligonucleotide is calculated and is similar to the association constants obtained by us for octanucleotides I and II.

Cleavage of Oligonucleotides by the HpaI Endonuclease. *Hpa*I endonuclease cleavage of the octanucleotides was monitored by high-speed gel permeation column chromatog-

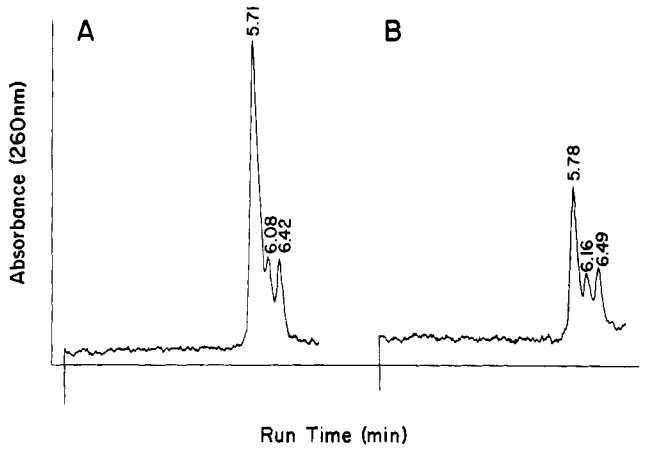

FIGURE 3: Assay of *Hpa*I endonuclease cleavage of the octanucleotides I and II by high-speed gel permeation column chromatography. The octanucleotides were cleaved by *Hpa*I endonuclease under the conditions described under Experimental Procedures, and the reaction products were analyzed by high-speed gel permeation column chromatography. (A) Octanucleotide I (5.71 min) and the two tetranucleotides (6.08 and 6.42 min). (B) Octanucleotide III (5.78 min) and the two tetranucleotides (6.16 and 6.49 min). The details of chromatography are given under Experimental Procedures.

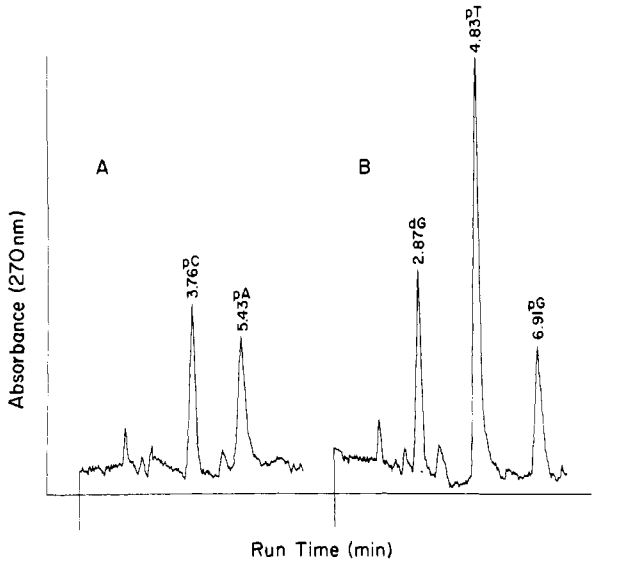

FIGURE 4: Nucleotide analysis of reaction products of *Hpa*I endonuclease cleavage of octanucleotide I. The product peaks with retention times 6.08 and 6.42 min were isolated as shown in Figure 3A and hydrolyzed to mononucleotides by snake venom phosphodiesterase treatment. The mixture of nucleoside and nucleotides was analyzed by high-pressure anion-exchange column (Ultrasil-AX). (A) Nucleotide coomposition of tetranucleotide with retention time of 6.08 min. (B) Nucleoside and nucleotide composition of tetranucleotide with retention time of 6.42 min.

raphy which separates the two tetranucleotide cleavage products from the uncleaved octanucleotide as shown in Figure 3. Octanucleotides I and III had retention times of 5.71 and 5.78 min, respectively, and the tetranucleotide products appeared as peaks that are not completely resolved from each other and the octanucleotide. For identification of the cleavage products, the peaks were isolated, purified further by rechromatography of each peak by high-speed gel permeation chromatography, and subjected to nucleotide analysis. The nucleoside and nucleotide products generated by snake venom phosphodiesterase treatment were separated and quantitated by high-pressure anion-exchange chromatography. As shown in Figure 4, the tetranucleotide with the retention time 6.08 min (Figure 3A) contains dpA and dpC in a 1:1 ratio and

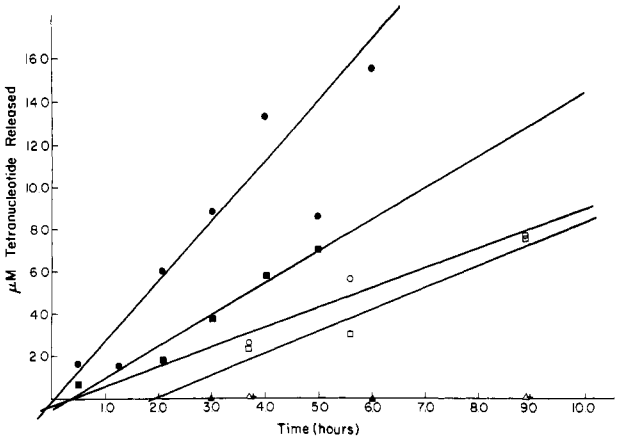

FIGURE 5: Rate of cleavage of the four octanucleotides by *Hpa*I endonuclease. Each of the four octanucleotides was incubated at 25 °C in the reaction buffer (30–50 μL; 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, and 100 μg/mL bovine serum albumin) with 3.1 μM *Hpa*I endonuclease monomer. Aliquots were withdrawn at the indicated times, and the products were separated by high-speed gel permeation column chromatography. The amount of tetranucleotide released was calculated by the equation described under Experimental Procedures. The octanucleotides and total strand concentrations shown are (○) 20 μM octanucleotide I, (△) 20 μM octanucleotide II, (□) 20 μM octanucleotide III, (+) 20 μM octanucleotide IV, (●) 46 μM octanucleotide I, (▲) 46 μM octanucleotide II, and (■) 46 μM octanucleotide III.

therefore corresponds to the tetranucleotide d(pA-A-C-C), and the tetranucleotide with the retention time 6.42 min (Figure 3A) contains dG, pdG, and pdT in a ratio of 1:1:2 and corresponds to tetranucleotide d(G -G-T-T). Cleavage of octanucleotide III resulted in two tetranucleotides which were identified by their retention times as d(G-G-T-B) and d(pA-A-C-C). These results clearly show that the *Hpa*I endonuclease cleaves octanucleotides I and III at the T–A and B–A phosphodiester bonds. This mode of cleavage is identical with that previously established for the natural DNA (Garfin & Goodman, 1974).

The rate at which the *Hpa*I endonuclease cleaved each octanucleotide was measured at low concentrations of substrate. As shown in Figure 5, octanucleotide I is cleaved slightly faster than octanucleotide III, whereas octanucleotides II and IV are not cleaved. The reason the *Hpa*I endonuclease does not cleave octanucleotide IV may be the low thermal stability of the duplex form. At a total strand concentration of 20 μM, the duplex concentration of octanucleotide IV is only 1.7 μM and 4 times less than the duplex concentration of octanucleotide I which is 7.7 μM. In contrast, the duplex concentration of octanucleotide II is 6.6 μM when the total strand concentration is 20 μM.

Steady-State Kinetic Studies with Octanucleotides and f_1 RFI DNA. The steady-state kinetic parameters of *Hpa*I endonuclease cleavage of octanucleotides I and III and f_1 RFI DNA (one *Hpa*I recognition site) were determined in order to explore the kinetic properties of the enzyme. The assay employed for the cleavage of octanucleotides measures the release of the tetranucleotide d(G-G-T-N) by high-speed gel permeation chromatography as described above. In the case of f_1 RFI DNA, tritium-labeled DNA was used as substrate. The assay measured the amount of unit length DNA after digestion with the *Hpa*I endonuclease as described by Greene et al. (1975). The reaction products were separated by 1% agarose gel electrophoresis, and the radioactivity in unit length DNA was quantitated.

The *Hpa*I endonuclease exhibited Michaelis–Menten kinetics with respect to the total strand concentration (C_T) of

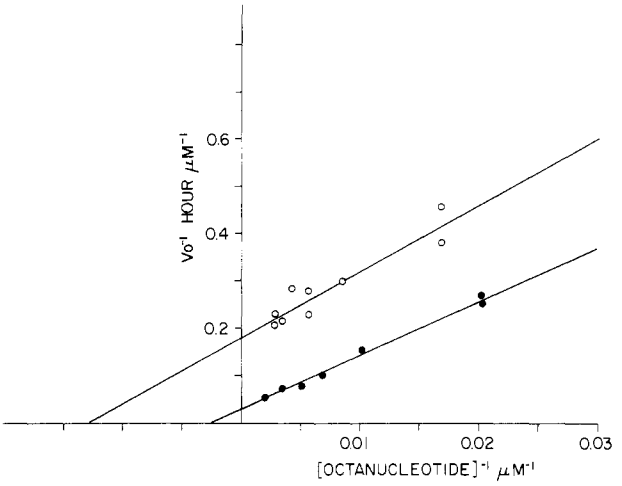

FIGURE 6: Steady-state kinetics of *Hpa*I endonuclease cleavage of octanucleotide I and octanucleotide III. The assays were performed by high-speed gel permeation column chromatography as described under Experimental Procedures. Indicated concentrations of octanucleotide I (●) and 3.1 μM *Hpa*I endonuclease I (monomer) were incubated at 25 °C in 20 μL of buffer, and 5 μL of reaction mixture was withdrawn at 1.5, 3, and 5 h and assayed. Similarly, appropriate amounts of octanucleotide III (○) and 3.1 μM *Hpa*I endonuclease I (monomer) were incubated at 25 °C in 30 μL of buffer, and 3 μL of reaction mixture was withdrawn at 2.2, 4.3, and 7.8 h and assayed. The octanucleotide concentration is expressed in terms of strand concentration.

d(G-G-T-T-A-A-C-C) and d(G-G-T-B-A-A-C-C) as shown in Figure 6. Since 84–95% of d(G-G-T-T-A-A-C-C) and 90–96% of d(G-G-T-B-A-A-C-C) are in the duplex form under the reaction conditions, K_m (duplex) was calculated from K_m (C_T) and the association constant K for each octanucleotide (Table I). The *Hpa*I endonuclease does turn over in vitro, although the turnover numbers are less than 1 min^{-1} (Figure 5 and Table I). Fitting initial velocities to the duplex concentrations of the octanucleotides did not give kinetic parameters significantly different from those listed in Table I.

The kinetic parameters for *Hpa*I endonuclease cleavage of octanucleotides I and III are consistent with the data shown in Figure 5. At low concentrations of substrate, the *Hpa*I endonuclease cleaves octanucleotides I and III at equal rates, because the turnover number/K_m values for octanucleotides I and III are equal. On the other hand, this ratio for DNA is 20 000-fold higher than the ratio for octanucleotides I and III. Therefore, DNA is a more preferred substrate than either octanucleotide for the *Hpa*I endonuclease.

Comparison of the Rate of Cleavage of 5′-Phosphorylated Octanucleotide I with Octanucleotide I. Since DNA is a more specific substrate for the *Hpa*I endonuclease than octanucleotide I by 4 orders of magnitude, we reasoned that the phosphate anions outside of the recognition sequence may be required for the effective interaction of the enzymes with the substrate. To study this problem, we compared the rate of cleavage of octanucleotide I with the rate of cleavage of 5′-phosphorylated octanucleotide I. As shown in Table II, the 5′-phosphorylated octanucleotide was cleaved 30-fold faster than octanucleotide I. Furthermore, the rate of cleavage of the 5′-phosphorylated octanucleotide did not change significantly in the presence of equimolar amounts of octanucleotide I. In addition, consistent with *Hpa*I cleavage of unphosphorylated octanucleotides, *Hpa*I cleaved 5′-phosphorylated octanucleotide I faster than 5′-phosphorylated octanucleotide III, while 5′-phosphorylated octanucleotide II and 5′-phosphorylated octanucleotide IV were cleaved only very slowly as shown in Figure 7.

Table II: *Hpa*I Endonuclease Cleavage of 5′-^{32}P-Labeled Octanucleotide I and Octanucleotide I

reaction[a]	octanucleotides	V_0 (μM h^{-1})
A	7.0 μM [5′-^{32}P]d(G-G-T-T-A-A-C-C)	6.9
B	7.4 μM d(G-G-T-T-A-A-C-C)	0.2
C	7.0 μM [5′-^{32}P]d(G-G-T-T-A-A-C-C) plus 7.4 μM d(G-G-T-T-A-A-C-C)	6.2

[a] Octanucleotide(s) and 3.1 μM *Hpa*I endonuclease were incubated at 25 °C in 20 μL of buffer. 5 μL of reaction mixture was withdrawn up to 6 h and the reaction products were assayed. Cleavage of 5′-labeled octanucleotide I was assayed by one-dimensional homochromatography and the cleavage of octanucleotide I by high-speed gel permeation chromatography as described under Experimental Procedures. In reaction C, only cleavage of 5′-labeled octanucleotide was assayed.

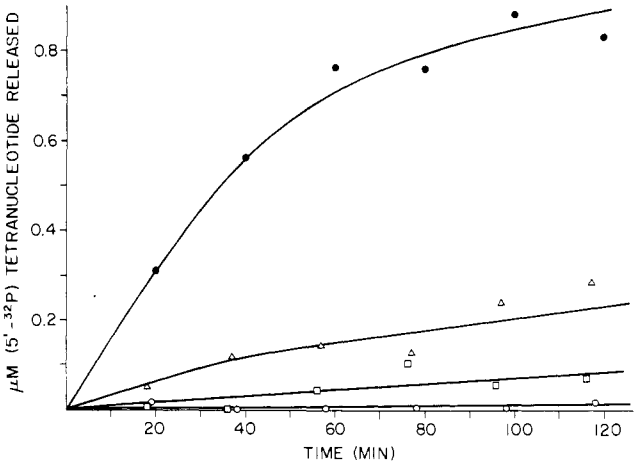

FIGURE 7: Rate of cleavage of 5′-^{32}P-labeled octanucleotides. 3 μM 5′-^{32}P-labeled octanucleotides, (●) octanucleotide I, (○) octanucleotide II, (△) octanucleotide III, and (□) octanucleotide IV, and 0.31 μM *Hpa*I endonuclease monomer were incubated at 20 °C in 10 μL of buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, and 50 μg/mL bovine serum albumin). 0.5 μL of each reaction was withdrawn at indicated time intervals and spotted on a DEAE-cellulose thin-layer plastic plate. The plate was developed in 2% RNA homomix and autoradiographed. The spots corresponding to the 5′-labeled tetranucleotide and the octanucleotide were cut out, and radioactivity was determined by Cherenkov counting. The oligonucleotide concentrations are expressed in terms of total strand concentrations.

Discussion

The goal of these studies was to determine if functional groups of the bases available in the grooves of double-helical DNA are contacts between the *Hpa*I endonuclease and its recognition sequence. We have approached this problem by examining the interactions of the *Hpa*I endonuclease with a variety of chemically synthesized oligonucleotides. On the basis of the observation that the octanucleotide d(pT-G-A-A-T-T-C-A) is an effective substrate for the *Eco*RI enzyme (Greene et al., 1975), we synthesized octanucleotide I (Figure 1) which contains the *Hpa*I recognition sequence d(G-T-T-A-A-C). The four dG·dC pairs in octanucleotide I ensure a reasonable thermal stability for its duplex form.

Our studies of *Hpa*I endonuclease cleavage of octanucleotide I revealed that this oligonucleotide is an effective substrate for the *Hpa*I endonuclease and that the *Hpa*I endonuclease cleaves the same phosphodiester bond in octanucleotide I as in natural DNA (see Results). Three additional octanucleotides (II–IV, Figure 1) containing base analogue substitutions within the *Hpa*I recognition site were chemically synthesized. The oligonucleotides were designed to test functional groups available in the grooves of DNA as contact points for the *Hpa*I

endonuclease. Octanucleotides II and III contain deoxyuridine and deoxybromouridine, respectively, in place of thymidine (position 4 of octanucleotide I, Figure 1) and tested a potential major groove contact, the 5-methyl group of thymidine. Octanucleotide IV contains deoxyinosine in place of deoxyguanosine (position 2 of octanucleotide I, Figure 1) and tested a potential minor groove contact, the 2-amino group of deoxyguanosine.

Our studies of *Hpa*I endonuclease cleavage of octanucleotides I–III indicate that the 5-methyl group of thymidine is a contact point between the *Hpa*I endonuclease and its recognition sequence. While octanucleotide I is cleaved by the *Hpa*I endonuclease, octanucleotide II is resistant to cleavage (Figure 5). In addition, both the K_m and turnover number values for *Hpa*I endonuclease cleavage of octanucleotide III were reduced 5-fold relative to the values for octanucleotide I. The differences in kinetic parameters for *Hpa*I endonuclease cleavage of octanucleotides I–III cannot be explained by the small differences in thermal stabilities of these three octanucleotides. All three octanucleotides base pair to form duplex structures and have similar T_m values (see Results); these results do show that the *Hpa*I endonuclease distinguishes among groups at the 5 position of thymidine analogues (position 4 of octanucleotides I–III, Figure 1).

A possible explanation for the different K_m and turnover number values for *Hpa*I endonuclease cleavage of octanucleotides I and III is that the *Hpa*I endonuclease binds octanucleotide III in a nonproductive mode. As shown by Cornish-Bowden (1979), when nonproductive enzyme–substrate complexes form in addition to productive complexes, the measured values of K_m and turnover number are less than what the values of K_m and turnover number would be if nonproductive complexes did not form. However, the value of the turnover number/K_m ratio is not changed by the occurrence of nonproductive binding. The K_m and turnover number values for *Hpa*I cleavage of octanucleotide III are lower than those values for octanucleotide I, but the ratio turnover number/K_m is equal for both octanucleotides I and III; this result suggests that *Hpa*I endonuclease binds octanucleotide III nonproductively.

Octanucleotides I–III contain substituents at position 5 of thymidine that differ both in size and in electronegativity. Since a bromine atom (octanucleotide III) is about the same size as a methyl group (octanucleotide I), its electronegativity is probably responsible for the decrease in kinetic parameters for *Hpa*I endonuclease cleavage of octanucleotide III relative to octanucleotide I. The small size of the hydrogen atom (position 4, octanucleotide II) may be responsible for the differences in kinetic parameters for *Hpa*I endonuclease cleavage of octanucleotides I and II. The bulky methyl group may force the *Hpa*I endonuclease into a conformation required for cleavage. In the absence of the 5-methyl group of thymidine, perhaps a nonproductive complex forms between the *Hpa*I endonuclease and octanucleotide. Alternatively, there may be a hydrophobic interaction between the 5-methyl groups of thymidine and the *Hpa*I endonuclease that increases the binding affinity of the enzyme for the DNA.

Our conclusion that the 5-methyl group of thymidine is a contact point for the *Hpa*I endonuclease is in agreement with the conclusions of Berkner & Folk (1979), who studied *Hpa*I cleavage of uracil-containing phage DNA. However, we cannot explain their observation that uracil-containing DNA was cleaved faster than the thymine-containing DNA by the *Hpa*I endonuclease after long periods of incubation.

Our studies of *Hpa*I cleavage of octanucleotide IV suggest that the 2-amino group of guanine is also a contact point between the *Hpa*I endonuclease and its recognition sequence. Octanucleotide IV is resistant to *Hpa*I cleavage (Figure 5). Octanucleotide IV does form a duplex structure, although this duplex is less stable than the duplex formed by octanucleotide I. In the experiment shown in Figure 5, 17% of octanucleotide IV is in the duplex form compared to 77% of octanucleotide I. If the duplex form of octanucleotide IV is cleaved by the *Hpa*I endonuclease, the *Hpa*I endonuclease should have cleaved octanucleotide IV under the reaction conditions in Figure 5, although at a slow rate. Instead, octanucleotide IV was not cleaved, suggesting that the 2-amino group of guanine is a contact point for the *Hpa*I endonuclease. However, we cannot rule out the possibility that the single-stranded form of octanucleotide IV inhibits *Hpa*I cleavage of the duplex form of octanucleotide IV, which would also account for the inability of the *Hpa*I endonuclease to cleave octanucleotide IV.

In addition to proving functional groups within the *Hpa*I DNA recognition sequence as contact points for the *Hpa*I endonuclease, we determined that a phosphate group 5′ external to the *Hpa*I recognition site is important in binding of the enzyme to DNA; the *Hpa*I endonuclease cleavage of 5′-phosphorylated octanucleotide I is faster than cleavage of the unphosphorylated octanucleotide. Interaction of the *Hpa*I endonuclease with phosphates external to the recognition sequences explains why the K_m of octanucleotide is 8000-fold higher than the K_m of DNA. The octanucleotide is probably not long enough to take advantage of the entire DNA binding site of the *Hpa*I endonuclease.

The *Eco*RI endonuclease also interacts with nucleotides external to its recognition site when binding and cleaving DNA. Greene et al. (1975) reported a 200-fold difference in the K_ms of d(pT-G-A-A-T-T-C-A) and DNA in the *Eco*RI endonuclease reaction. In addition, Modrich (1980) showed by ethylation protection experiments that phosphates 5′ external to the *Eco*RI sequence are important in formation of specific DNA–*Eco*RI endonuclease complexes. Thus, both the *Eco*RI endonuclease and the *Hpa*I endonuclease interact with phosphate residues 5′ external to their DNA recognition sequence.

Acknowledgments

We thank Drs. Joon Yoo and John Westley for helpful discussions, Dr. K. S. Chiang for the use of the Gilford spectrophotometer, thermosensor, and thermoprogrammer, and Faina Riftina for advice during the synthesis of oligonucleotides.

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Comparison of Transfer Ribonucleic Acid Structures Using Cobra Venom and S_1 Endonucleases†

Philip E. Auron, Lawrence D. Weber, and Alexander Rich*

ABSTRACT: Cobra venom nuclease V_1, which cleaves double-stranded RNA, has been used to study the structure of four *Escherichia coli* tRNAs: Phe, Glu_2, Leu_2, and Ile_1. The cleavage patterns are compared to those found for yeast $tRNA^{Phe}$, the three-dimensional structure of which is known. The cleavage patterns of all the tRNA molecules are similar, and the most sensitive cleavage is found at the central base pair of the anticodon stem. Studies of *E. coli* $tRNA_2^{Leu}$, which has a large variable loop, are consistent with the formation of a base-paired stem and loop structure that is not closely bound to the remainder of the molecule. A survey of the results suggests that the V_1 molecule may interact with the minor groove of the double helix with an affinity for stacked bases and that it may require two or three stacked bases for optimal binding and cleavage.

Enzymatic digestion has proved to be a valuable means of studying nucleic acid structure in solution, especially among transfer RNA (tRNA) molecules. Only a small number of tRNA species have been crystallized to date, and it has been difficult to obtain crystals of high quality. It is thus difficult to get direct structural information for many tRNA species. Although there is convincing evidence supporting the similarity between macromolecules in the crystalline state and their counterparts in solution (Chen et al., 1978; Matthews, 1974), such similarlity cannot be assumed to exist for all crystals or at all levels of detail. In this context, the enzymatic approach to structural study can both serve as a useful complement to high resolution crystallographic studies and provide significant structural information in solution for tRNA and other macromolecular species for which crystallographic information is unavailable.

Previous approaches to the enzymatic analysis of RNA structure have generally depended upon the use of single-strand-specific nucleases such as nuclease S_1 and ribonuclease T_1. The S_1 nuclease by itself is incapable of discerning all single-stranded regions within a nucleic acid molecule like tRNA. This is the case because some regions, such as the D and T loops, are held tightly together by tertiary interactions (Rich & Rajbhandary, 1976). Therefore, S_1 susceptibility testifies to a region's single-strandedness, but a lack of digestion is meaningless in deciding between secondary structure and tertiary structure. The use of other nucleases such as ribonucleases T_1 and A has proven successful in digesting S_1-resistant regions of some tRNA molecules; however, this is dependent upon the presence of specific residues within the region in question and is therefore not generally applicable.

The analysis of secondary structure in RNA molecules requires a method for determining the presence of double-stranded regions. Such information complements the S_1 and T_1 digestion data and can result in a more complete structural picture. An enzyme has been isolated from cobra venom that has the ability to cleave ribonucleic acid specifically within double-stranded regions (Vassilenko & Ryte, 1975). This enzyme, which has been called ribonuclease V_1 (Lockard & Kumar, 1981), can be used in concert with other digestion data for structure mapping tRNA molecules. Such an approach has been used in our laboratory and elsewhere to analyze the solution conformation of yeast $tRNA^{Phe}$, which has a known crystallographic structure. The results have indicated strong agreement between the X-ray crystal structure data and the enzymatic digestion pattern of this tRNA in solution (Lockard & Kumar, 1981). We have extended this enzymatic analysis

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received March 12, 1982.* This work was supported by grants from the National Institutes of Health, the American Cancer Society, the National Science Foundation, and the National Aeronautics and Space Administration. P.A. was supported by Fellowship DRG-425F of the Damon Runyon-Walter Winchell Cancer Fund. L.W. was supported by National Research Service Award 3 F32 GM07078-01S1 BBCA and by Fellowship 61-525 of the Jane Coffin Childs Memorial Fund for Medical Research.

0006-2960/82/0421-4700$01.25/0