The Paradoxical Influence of Thymine Analogues on Restriction Endonuclease Cleavage of Oligodeoxynucleotides†

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ABSTRACT: Thymine residues in the DNA of eucaryotes may be replaced occasionally by uracil (U) or 5-(hydroxymethyl)uracil (H) as consequences of dUMP misincorporation or thymine oxidation, respectively. In this study, we constructed a series of 44-base oligonucleotides containing site-specific U or H residues and 5′-fluorescein labels in order to probe the influence of such modifications on sequence-specific DNA−protein interactions using several type II restriction endonucleases. We find that substitution within the recognition sites of several restriction endonucleases increases initial cleavage velocity by up to an order of magnitude. These results contrast dramatically with several previous studies which demonstrated that U substitution in short oligonucleotides inhibits or prevents nuclease cleavage. We propose that this apparent paradox results because the rate-limiting step in the cleavage of longer oligonucleotides is product release whereas for shorter oligonucleotides substrate binding is most probably rate-limiting. For longer oligonucleotides and DNA, more rapid release of the cleaved, substituted oligonucleotides results in more rapid turnover and a faster apparent cleavage rate. The sequence length at which the transition in rate-limiting step occurs likely corresponds to the size of the enzyme footprint on its DNA recognition site. We conclude that both U and H do perturb sequence-specific DNA−protein interactions, and the magnitude of this effect is site-dependent.

The DNA of eucaryotes is comprised predominantly of the normal base residues adenine, thymine (T),[1] guanine, and cytosine, whereas in some lower organisms (McClelland et al., 1994), thymine may be replaced completely by either uracil (U) or 5-(hydroxymethyl)uracil (H) (Figure 1). Although these modified pyrimidines may be normal constituents in some procaryotes, their presence in eucaryotic DNA is potentially harmful. Thymine residues of canonical A·T base pairs may be replaced by U because of dUMP misincorporation during DNA synthesis (Richards et al., 1984), and H residues may be introduced into DNA via *in situ* oxidation of the T methyl group (Frenkel, 1991). In eucaryotes, both U (Lindahl, 1974) and H (Hollstein et al., 1984; Cannon-Carlson et al., 1989) residues are removed from both single- and double-stranded DNA by glycosylases specific to each modified base.

The 5-methyl group of T lies in the major groove of a B-form DNA helix. Uracil substitution does not significantly perturb base pair formation or DNA conformation (Delort et al., 1985; Carbonnaux et al., 1990). However, substitution in the 5-position might be expected to have a significant influence on DNA−protein interactions (Ivarie, 1987). A hydrogen atom is considerably smaller than a methyl group and is less hydrophobic. The steric parameter (MR) of the 5-substituent decreases from 5.65 to 1.03, and the hydrophobic parameter (pi) decreases from 0.56 to 0 upon substitution of T by U (Hansch et al., 1973).

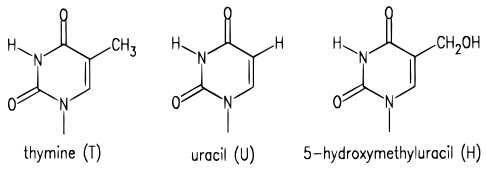

FIGURE 1: Structures of thymine (T), uracil (U), and 5-(hydroxymethyl)uracil (H).

In several previous studies, it has been reported that U substitution in short oligonucleotides inhibits several restriction endonucleases including *Bam*HI (Kang et al., 1994), *Mbo*I (Hayakawa et al., 1988), *Eco*RV (Fliess et al., 1988; Mazzarelli et al., 1989; Newman et al., 1990), and *Eco*RI (Brennan et al., 1986; Fliess et al., 1986; McLaughlin et al., 1987). Inhibition of nuclease cleavage upon substitution of T by U has been interpreted as demonstrating that the T methyl group is an important recognition element. Paradoxically, however, these same nucleases are known to cleave high molecular weight DNA when their recognition sequences are located within U-containing phage DNAs (Berkner & Folk, 1977, 1979; Huang et al., 1982; Hoet et al., 1992). The explanation for why U substitution inhibits cleavage in short oligonucleotides, but not in high molecular weight DNA, has not previously appeared in the literature.

Substitution of T by H, as with U, does not perturb base pair formation or DNA conformation (Mellac et al., 1993). The hydroxymethyl substituent is somewhat larger than the methyl group (MR, 7.19) yet smaller than bromine (MR, 8.88). 5-Bromouracil can substitute for T with most restriction endonucleases (Petruska & Horn, 1983). However, unlike the methyl group and bromine which are hydrophobic, the hydroxymethyl group is hydrophilic (pi, −1.03; Hansch et al., 1973). Substitution of T by H might be expected to perturb DNA−protein interactions to a greater degree than

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[1] Abbreviations: T, thymine; U, uracil; H, 5-(hydroxymethyl)uracil; MR, molar refractivity; GC/MS, gas chromatography/mass spectrometry.

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U. Studies examining restriction endonuclease cleavage of oligonucleotides containing site-specific H substitution, which parallel the studies with U-containing oligonucleotides, have not been previously reported because of synthetic difficulties which have only recently been solved (Sowers & Beardsley, 1993). It is known, however, that uniform H substitution in bacteriophage DNA does inhibit cleavage by several restriction endonucleases (Berkner & Folk, 1979; Huang et al., 1982; Hoet et al., 1992). Whether this inhibition results from site-specific effects or from a more global effect upon DNA because of the T to H substitution in the major groove is as yet unknown.

In this study, we have prepared a series of oligonucleotides containing either U or H at selected sites. The sequence of the oligonucleotide duplex was designed to contain the recognition sites for several restriction endonucleases. Using this system, we have probed the influence of T substitution by U and H on restriction endonuclease cleavage. In addition to specifically probing the influence of site-specific U or H residues on the interaction with endonucleases, we have investigated the utility of fluorescently labeled oligonucleotides and fluorescent detection for monitoring cleavage reactions.

MATERIALS AND METHODS

Materials. Normal phosphoramidites and the fluorescein phosphoramidite were obtained from Glen Research. Restriction endonucleases and buffers were obtained from Promega. Uracil−DNA glycosylase was obtained from United States Biochemical Corp., and (hydroxymethyl)uracil glycosylase was obtained from Dr. George Teebor (Department of Pathology, New York University). Examination of cleavage in initial enzyme assays was performed by transillumination of gels containing fluorescently labeled restriction fragments with UV light. Fluorescent gels were scanned and digitized with a Scanalytics Docugel V CCD camera, and results were quantitated using RFLPscan software (Scanalytics). Kinetic data for DNA cleavage were collected by scanning fluorescent gels with a Molecular Dynamics Fluoroimager SI, and band quantitation was performed with ImageQuantNT software. When very low DNA concentrations were required, kinetic assays were carried out using an ABI 370A DNA sequencer equipped with Genescan 672 software.

Synthesis and Characterization of Synthetic Oligonucleotides. Oligonucleotides were synthesized by standard phosphoramidite methods (Gait, 1984) using a Pharmacia Gene Assembler. The deoxyuridine phosphoramidite was prepared by established methods (Gait, 1984). The 5-(hydroxymethyl)-2′-deoxyuridine phosphoramidite was prepared as previously described (Sowers & Beardsley, 1993). Fluorescein was added to the 5′-end of the oligonucleotides using a fluorescein phosphoramidite. Oligonucleotides were deprotected by treatment in aqueous ammonia overnight at 60 °C. Oligonucleotides still containing a 5′-dimethoxytrityl group were purified by HPLC using a polystyrene column and a gradient of triethylammonium acetate in aqueous acetonitrile. Oligonucleotides were detritylated using NAP columns from Millipore Corp.

The composition of the oligonucleotides was verified by gas chromatography/mass spectrometry (Djuric et al., 1991) and HPLC using photodiode array detection following enzymatic digestion with nuclease P1 and bacterial alkaline phosphatase (Kasai et al., 1986). Both methods confirmed the correct base composition including the presence of U or H in the modified oligonucleotides.

The sequence of each oligonucleotide duplex was verified by cleavage with a spectrum of restriction nucleases as described below. Further, the locations of U and H were verified by cleaving single-stranded oligonucleotides with either uracil glycosylase or (hydroxymethyl)uracil glycosylase. In all cases, substituted oligos were cleaved at the appropriate position.

Restriction Endonuclease Cleavage Conditions and Determination of Initial Cleavage Rates. Complementary oligonucleotides, fluorescently labeled in both strands, were mixed in equimolar amounts, heated to 90 °C for 10 min, and allowed to cool to room temperature. Duplexes were stored frozen for at least a year with no evidence of degradation. Except when otherwise indicated, the buffers and reaction conditions used for each restriction endonuclease were those recommended by the supplier.

Initially, nine endonucleases were tested with a T-containing duplex as shown in Figure 2 in order to determine the time required to cleave half of the substrate. Reactions were performed in a total volume of 20 μL containing buffer, 73 pmol of oligonucleotide duplex, and 10−24 units of endonuclease, depending upon the efficiency of the particular enzyme. Reactions were quenched by the addition of 4 μL of 120 mM EDTA solution, dried under reduced pressure, resuspended in urea-saturated formamide, heated for 3 min at 90 °C to denature the duplex, and cooled in a dry ice−acetone bath. Samples were loaded onto a 20% polyacrylamide, 8 M urea denaturing gel and the products resolved by electrophoresis at 500 V for 90 min. Gels were transilluminated with UV light and scanned with a CCD camera (see Figure 3). Percent cleavage was determined by integration of fluorescent gel bands using RFPLscan. These cleavage conditions were then used with each endonuclease to screen a panel of duplex oligonucleotides containing U and H residues at selected sites.

In cases where the cleavage efficiency of a substrate containing either U or H deviated significantly from the control oligo containing all normal bases, time-course experiments were performed to determine initial cleavage velocities. Reaction conditions were identical to those described above for the screening assay except that 3 μL aliquots were removed at selected time intervals. The total amount of fluorescent oligonucleotide in each aliquot in the time-course analysis was below the level of detection by the CCD camera. Therefore, fluorescent gels for the time-course assays were scanned with the Molecular Dynamics Fluoroimager. The resulting measurements of percent cleavage as a function of time were fit to a first-order kinetic reaction curve, and this curve was used to calculate the initial cleavage velocity (v_i) with each substrate.

Steady-State Reaction Assay with EcoRI. Michaelis−Menten kinetic parameters were obtained for *Eco*RI with the normal T-containing and several modified duplex oligonucleotides. These reactions were performed in 200 μL total volume containing between 0.21 and 10.5 pmol of duplex. The DNA concentration ranged from 1.1 to 52 nM with 6.7 units of *Eco*RI and 50 μg/mL BSA. Aliquots containing 10 fmol of DNA were removed at selected times. Reactions were quenched by addition of EDTA and immediately placed

into a dry ice−acetone bath. Samples were dried under vacuum, and oligonucleotide products were separated by electrophoresis in a 10% polyacrylamide, 6 M urea sequencing gel in an ABI 370A sequencer.

In order to measure K_m, it was necessary to use DNA concentrations which were below the limit of detection sensitivity of both the CCD camera and the Molecular Dynamics Fluoroimager. Therefore, for these experiments, DNA was electrophoresed and quantitated directly on the laser-induced fluorescence detector of the ABI sequencer. For each substrate concentration, product versus time data were fit to a first-order reaction curve to determine initial reaction velocity. The resulting v_i was plotted versus substrate concentration. The values of K_m and V_{max} were obtained by fitting experimental data to the Michaelis−Menten equation using nonlinear regression (Enzfitter).

Single-Turnover with EcoRI. The single-turnover velocity of *Eco*RI with the T-containing and modified substrates was determined as described by Zebala et al. (1992a). Briefly, 1.05 pmol of duplex substrate was incubated with a large excess (8400 units) of *Eco*R1 in 400 μL total volume containing the proper enzyme buffer with the exception of Mg^{2+}. After incubation at 25 °C for 5 min, 400 μL of an initiation buffer containing 20 mM Mg^{2+} was added. Aliquots (80 μL) were removed at 2 s intervals and immediately quenched by the addition of 25 mM EDTA at 90 °C followed immediately by immersion in dry ice−acetone. In these experiments, two individuals worked in concert, one pipeting and the other counting the time interval. Samples were analyzed on the ABI sequencer, and initial velocity was determined as indicated above.

Cleavage of Short Oligonucleotides with EcoRI. Shorter, self-complementary, fluorescein-labeled oligonucleotides containing T and U were prepared as described above. The sequences of the oligonucleotides, where X is fluorescein, were as follows:

10-mer: 5′X-CTGAAT(T/U)CAG-3′

12-mer: 5′XGCTGAAT(T/U)CAGC-3′

Cleavage assays were performed as described above using the CCD camera detection system.

RESULTS

A series of 5′-fluorescein-labeled oligonucleotides containing either U or H at selected positions were prepared using standard phosphoramidite methods. The composition of the oligonucleotides was confirmed by both GC/MS and HPLC analysis. The position of each modified base was confirmed by selectively cleaving the single-stranded oligo with either uracil−DNA glycosylase or 5-(hydroxymethyl)uracil−DNA glycosylase. With all modified oligonucleotides, a fragment of the correct length was generated.

The sequence of the oligonucleotide used in this study is shown in Figure 2. When an oligonucleotide of this sequence is cleaved by a series of restriction nucleases, a series of fragments is generated as shown in Figure 3. In these studies, both strands were 5′-fluorescein-labeled to probe for potential strand bias in cleavage reactions.

In order to screen for the influence of substitution of each of the thymine residues indicated in the sequence shown in Figure 2, a series of oligonucleotides modified at one position in only one strand were exposed to cleavage conditions with each restriction nuclease. As an example, Figure 4 shows

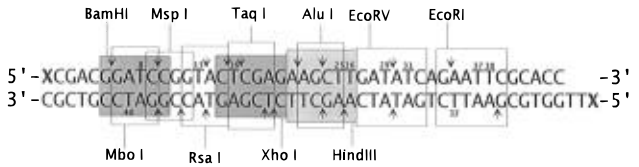

FIGURE 2: Sequence of the oligonucleotide substrate and location of the restriction nuclease recognition cleavage sites. Numbers indicate sites of U or H substitution. The fluorescein label is indicated by the X.

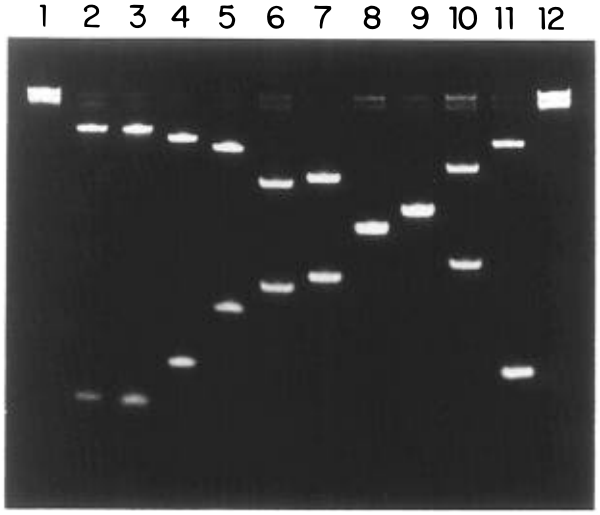

FIGURE 3: Polyacrylamide gel of the oligonucleotide duplex cleaved with a series of restriction endonucleases: lane 1, control; lane 2, *Bam*HI; lane 3, *Mbo*I; lane 4, *Msp*I; lane 5, *Rsa*I; lane 6, *Xho*I; lane 7, *Taq*I; lane 8, *Hin*dIII; lane 9, *Alu*I; lane 10, *Eco*RV; lane 11, *Eco*RI. The duplex was 5′-fluorescein-labeled in both strands. Gel bands were visualized by transillumination on a UV light source.

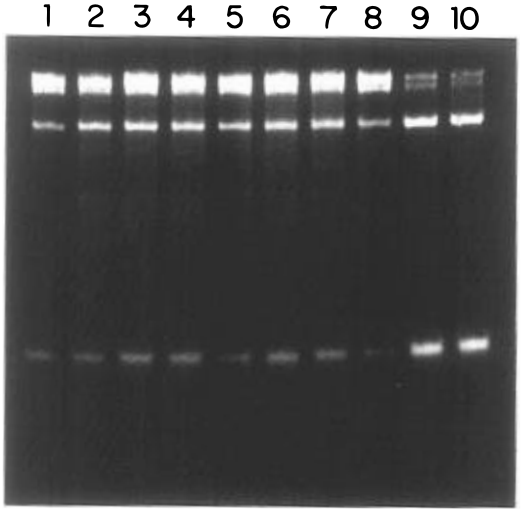

FIGURE 4: Cleavage of a series of oligonucleotides containing H at specific sites cut with *Eco*RI. Lane 1 contains a duplex with only normal bases. Lanes 2−8 contain H at T_8, T_{13}, T_{16}, T_{25}, T_{26}, T_{29}, and T_{31}, respectively. Lanes 9 and 10 contain H at positions T_{37} and T_{38}, respectively. Positions T_{37} and T_{38} are within the *Eco*RI G/A$AT_{37}T_{38}$C-cut site. See Figure 2 for numbering scheme. The amount of oligonucleotide present for this reaction was 73 pmol in 20 μL of reaction buffer containing 10 units of *Eco*RI. The cleavage reaction was conducted at 37 °C for 30 min.

cleavage of the series of H-substituted oligonucleotides with *Eco*RI. As can be seen by inspection of Figure 4, the efficiency of cleavage is unaffected by H when the modification is outside of the *Eco*RI recognition site. However, when

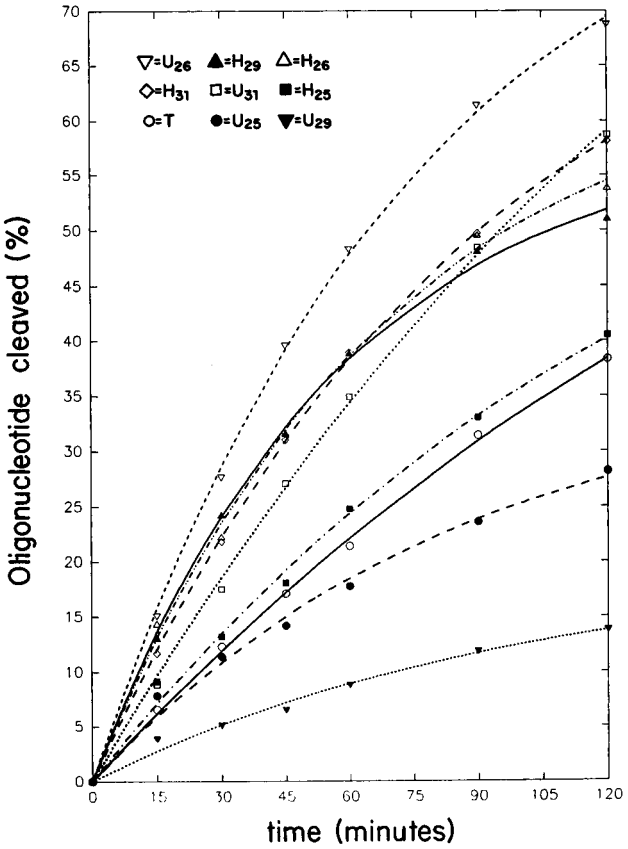

FIGURE 5: Determination of initial cleavage velocity with *Eco*RV. Normal and substituted oligonucleotides were cleaved with *Eco*RV. Fractions were obtained at selected time intervals and analyzed by gel electrophoresis. Percent cleavage was determined by quantitation of fluorescent gel bands. Location and identification of the modifications are shown in the inset.

H is at either T position within the G/AATTC recognition site (lanes 9 and 10, Figure 4), the corresponding oligonucleotides are cleaved to a greater extent.

When the initial screening assay indicated that substitution did influence the cutting efficiency, a second series of experiments was conducted to measure the initial cleavage velocity, relative to the unsubstituted control. For example, the percent cleavage as a function of time is shown in Figure 5 for a series of modified duplexes and nuclease *Eco*RV. Table 1 contains relative initial velocity measurements for each enzyme and each substitution. In some cases, the substitution decreases the initial velocity; in others, the relative velocity is increased. Deviations in cleavage velocity are generally only observed when the modification is located in or near the recognition site of each nuclease.

The largest influence of the modifications was seen with *Eco*RI. We therefore investigated further the basis of this effect by determining the Michaelis−Menten kinetic parameters. We observed that both V_{max} and K_m were increased for the duplexes containing modifications within the *Eco*RI recognition site. In order to determine the source of the elevated V_{max} for the substituted oligos, a series of single-turnover experiments were conducted. Values obtained for K_m, V_{max}, and single turnover velocity are given in Table 2.

In order to examine the possible influence of oligonucleotide length on substituent effects, self-complementary oligos containing either T or U of 10 or 12 bases in length were synthesized. These were digested with *Eco*RI for 45 min. The reaction products are shown in Figure 6. We observe that, for a 10-base oligonucleotide duplex, substitution of T by U decreases the cleavage efficiency by a factor of 2. In contrast, however, when an additional base is added to each end, increasing the oligonucleotide length to 12 and leaving the central sequence unaltered, U substitution doubles cleavage efficiency.

DISCUSSION

The importance of the thymine methyl group for the interaction of sequence-specific DNA binding proteins with DNA has been inferred from several studies using synthetic oligonucleotides containing site-specific U substitution. Restriction endonucleases such as *Bam*HI (Kang et al., 1995), *Mbo*I (Hayakawa et al., 1988), *Eco*RV (Fliess et al., 1988; Mazzarelli et al., 1989), and *Eco*RI (Brennan et al., 1986; Fliess et al., 1986; McLaughlin et al., 1987) were either unable to cleave oligonucleotides with U in the recognition site or cleaved with significantly diminished efficiency. In contrast, however, it has been known for some time these same endonucleases recognize and cleave phage DNA in which T is uniformly substituted by U (Berkner & Folk, 1977, 1979; Huang et al., 1982). To date, this apparent paradox has not been explained.

The results of our study utilizing a 44-base synthetic oligonucleotide containing site-specific U and H modifications are summarized in Table 1. U substitution decreased cleavage rates for two endonucleases which have four base recognition sites, *Alu*I and *Rsa*I. A similar result has been noted previously for *Rsa*I cleavage of U-containing bacteriophage DNA (Bodnar et al., 1983). Substitution with U immediately adjacent to the *Taq*I site had a slight inhibitory effect; however, substitution within the *Taq*I site has no apparent effect upon cleavage rates, consistent with previous findings (Zebala et al., 1992b; Bodnar et al., 1983).

The majority of our results, however, are somewhat striking when viewed within the context of the prior literature. In most cases, U substitution within the recognition site of a particular endonuclease increased the initial cleavage velocity. Our results are therefore more consistent with previous studies with U-substituted phage DNA and contrast sharply with previous studies with synthetic oligonucleotides. Within this context, Berkner and Folk reported that U-substituted PBS2 DNA was cleaved by *Eco*RI at approximately twice the rate observed for cleavage of T-containing lambda DNA (Berkner & Folk, 1977). We note that the composition of our oligonucleotides was rigorously confirmed using both HPLC and GC/MS methods, the location of U or H in each substituted oligonucleotides was verified by cleavage with a base-specific glycosylase, and differences in relative cleavage velocity are noted only when the modification is in or near the recognition site of the respective endonuclease.

The significant question generated by our data is why our U-substituted oligonucleotides are cleaved at higher relative rates whereas U substitution in several previously reported studies with synthetic oligonucleotides resulted in significant inhibition. One possible explanation considered relates to the symmetry of the endonuclease recognition site. In our system, the U modification was only in one strand. In previous studies, self-complementary oligonucleotides containing a single U substitution generated a duplex containing two U substitutions, one in each of the symmetric half-sites.

Table 1: Relative Initial Velocity Grid[a]

	*Bam*HI G/GAT$_8$CC	*Mbo*I /GAT$_9$C	*Msp*I C/CGG	*Rsa*I GT$_{13}$/AC	*Xho*I C/T$_{16}$CGAG	*Taq*I T$_{16}$/CGA	*Hin*dIII A/AGCT$_{25}$T$_{26}$	*Alu*I AG/CT$_{26}$	*Eco*RV GAT$_{29}$/AT$_{31}$C	*Eco*RI G/AAT$_{37}$T$_{38}$C
U08	**6.7**	**3.2**	1	1	1	1	1	1	1	1
H08	**1.5**	**1.9**	1	1	1	1	1	1	1	1
U13	1	1	1	**0.31**	1	**0.88**	1	1	1	1
H13	1	1	1	**0.92**	1	**1.1**	1	1	1	1
U16	1	1	1	1	**1.4**	**1**	1	1	1	1
H16	1	1	1	1	**0.68**	**1.1**	1	1	1	1
U25	1	1	1	1	1	1	**1.9**	**0.36**	**1**	1
H25	1	1	1	1	1	1	**2.2**	**1.1**	**1.2**	1
U26	1	1	1	1	1	1	**1.5**	1	**2.7**	1
H26	1	1	1	1	1	1	**1.2**	1	**2.3**	1
U29	1	1	1	1	1	1	1	1	**0.47**	1
H29	1	1	1	1	1	1	1	1	**2.4**	1
U31	1	1	1	1	1	1	1	1	**1.6**	1
H31	1	1	1	1	1	1	1	1	**2.0**	1
U37	1	1	1	1	1	1	1	1	1	**2.1**
H37	1	1	1	1	1	1	1	1	1	**7.9**
U38	1	1	1	1	1	1	1	1	1	**6.6**
H38	1	1	1	1	1	1	1	1	1	**6.7**
T/U12										**7.3**
U37/U12										**10.1**
U38/U12										**11.1**
T/U40	**4.6**									
U08/U40	**4.6**									
T/H40	**1.6**									
H08/H40	**3.4**									

[a] The values presented represent the initial cleavage velocity of the substituted oligonucleotide, relative to the thymine-containing control.

Table 2: K_m, V_{max}, and Single-Turnover Data for *Eco*R1

	K_m (nM)	V_{max} (pmol/min)	V_{max}/K_m	single-turnover number (min^{-1})
-GAATTC- -CTTCCG-	4.35 ± 1.5	0.12 ± 0.01	0.0269	19 ± 3
-GAATUC- -CTTATG-	11.5 ± 0.72	0.68 ± 0.01	0.0591	8 ± 1
-GAATHC- -CTTAAG-	25.4 ± 3.38	0.80 ± 0.04	0.0316	14 ± 3
-GAATUC- -CUTAAG-	34.4 ± 6.82	0.86 ± 0.07	0.0250	1 ± 0.3

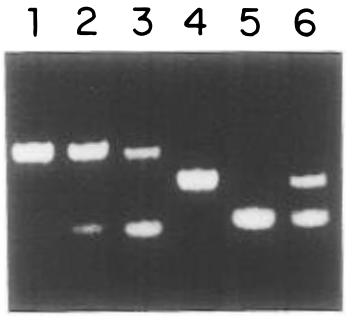

FIGURE 6: Cleavage of 10- and 12-base oligonucleotide duplexes containing either U or T in the recognition site of *Eco*RI. Cleavage of all oligonucleotides was allowed to proceed for 45 min. Lane 1, uncut 12-base T control; lane 2, cleaved T control; lane 3, 12-base duplex with U in the outer T position; lane 4, uncut 10-base T control; lane 5, 10-base cleaved T control; lane 6, cleaved 10-base duplex containing U in the outer T position.

We therefore prepared a series of oligonucleotides containing U residues in the complementary strand in order to generate symmetrically modified sites parallelling those used in previous studies. As indicated in Table 1, the oligonucleotides containing symmetric U substitution were also cleaved at faster rates than the unsubstituted controls. Indeed, with *Eco*RI, symmetric substitution increased the relative cleavage velocity even more than single substitution. Clearly, symmetric versus asymmetric substitution does not reconcile our data with previous studies, but rather increases the dichotomy between our respective findings.

The outer T of the *Eco*RI site has been shown to be more sensitive to modification. In the study by Brennan et al. (1986), it was observed that U substitution at this site in a symmetric 8-base oligonucleotide prevented cleavage. We also find that the outer T position is more sensitive to modification; however, the direction of the effect is opposite. Symmetric U substitution at this site, in our system, increases the relative cleavage velocity by an order of magnitude and represents the largest deviation from the T-containing control observed in our study.

Because the apparent paradox of U substitution was greatest for the *Eco*RI site, we conducted further studies with this nuclease in an attempt to explain the phenomenon. We therefore measured cleavage velocity as a function of substrate concentration in order to determine K_m and V_{max} values. Both the normal and substituted systems obeyed Michaelis−Menten kinetics. As shown in Table 2, substitution within the *Eco*RI site resulted in an increase in both K_m and V_{max}. An increase in K_m can be explained by reduced binding affinity for the substituted site. Recently, Lesser et al. (1993) demonstrated that U substitution within the *Eco*RI site had an unfavorable energetic influence upon the interaction of *Eco*RI and its substrate. An increase in the apparent V_{max}, however, is more difficult to explain.

In 1976, Modrich and Zabel reported that the rate-limiting step for the cleavage of ColE1 DNA was release of endonuclease from the cleaved DNA product (Modrich & Zabel, 1976). This same group subsequently reported that the intrinsic affinity of the endonuclease for its recognition site was independent of chain length between 34 and several thousand base pairs (Jack et al., 1982; Terry & Modrich, 1983). It is likely, therefore, that the rate-limiting step with the 44-base duplex reported here is also product release. The

substitution of T with U could conceivably weaken the complex between cleaved oligonucleotide and endonuclease, resulting in faster product release and a greater apparent rate of cleavage.

In order to eliminate relative binding affinity and product release from the observed cleavage rates, we measured relative cleavage rates after preincubation of duplex with a saturating enzyme concentration. The observed rate constants from these single-turnover experiments are given in Table 2. Under saturating conditions, the substituted oligonucleotides were cleaved more slowly than the T-containing control, and the symmetrically U-substituted duplex was cleaved significantly slower. These data are consistent with the proposal that, under nonsaturating conditions, faster product release explains the faster cleavage of the substituted oligonucleotides. It also indicates that U substitution decreases the rate of the actual cleavage reaction.

Product release appears to be the rate-limiting step for cleavage of DNA and oligonucleotides greater than 34 bases in length by *Eco*RI, including the 44-base sequence reported in this study. Jen-Jacobsen et al. (1983) reported that the association constant between *Eco*RI and its substrate changed little between plasmid DNA and a 12-base oligonucleotide. The association constant for an 8-base oligonucleotide, however, decreased dramatically. Consistent with these findings, Brennan et al. (1986) reported that the dissociation rate of the *Eco*RI−octanucleotide complex in the absence of Mg^{2+} is several orders of magnitude more rapid than release of polymeric DNA. With shorter oligonucleotides, product release may no longer be rate-limiting (Alves et al., 1982), and either substrate binding or cleavage could be the rate-determining step.

If the rate-limiting step for the *Eco*RI cleavage reaction varies as a function of the oligonucleotide length, as suggested above, the relative influence of substitutions may also change as a function of oligonucleotide length. Previous literature data already suggested that the effect of U substitution might be a function of length. Brennan et al. (1986) showed that U substitution within an 8-base oligonucleotide prevented *Eco*RI cleavage. McLauglin et al. (1987) showed that the same substitution in a 10-base sequence decreased the relative cleavage velocity by a factor of 2, and Fliess et al. (1988) reported that 11-base oligonucleotides with the same U substitution were cleaved with 70% efficiency, relative to the unsubstituted control. We sought to directly examine the potential for oligonucleotide length dependence of the U substitution effect as well as to determine the oligonucleotide length at which the transition would occur. We prepared self-complementary oligonucleotides 10 and 12 bases in length with and without U at the outer position. As shown in Figure 6, the 10 base pair duplex containing the U is cleaved at half the rate of the T control, consistent with previous studies on U substitution in the *Eco*RI site. However, for the 12-base oligonucleotide containing an identical central sequence, the U-containing duplex is cleaved at twice the rate.

The transition we observe for the relative influence of U substitution on the cleavage rate between 10 and 12 bases is consistent with the change in binding affinity observed for sequences of 8 and 12 bases containing the *Eco*RI site (Jen-Jacobson et al., 1983). Lu and Modrich (1981) previously established, using ethylation interference, that the endonuclease interacts with a minimum of 10 base pairs, and that sequences of smaller length will not occupy the entire binding site. Ethylation interference results are in complete accord with results of crystallographic studies (McClarin et al., 1986). Although the sequence-specific binding domain for *Eco*RI is only six bases, nonspecific phosphate contacts beyond the specific recognition site clearly facilitate enzyme−substrate binding.

CONCLUSIONS

We conclude that, for oligonucleotides which are smaller than the footprint of the endonuclease, 10−12 bases in the case of *Eco*RI, substrate binding or cleavage is rate-limiting. Substitution of T by U inhibits both binding and cleavage, consistent with the results reported here as well as in several previous studies. As the length of the oligonucleotide increases, the binding affinity between endonuclease and substrate increases dramatically because of the introduction of nonspecific phosphate contacts, and a change in the rate-limiting step occurs. For the longer oligonucleotides and DNA, product release becomes rate-limiting. Substitution of T by U weakens the affinity between substrate and enzyme, facilitating product release, resulting in faster turnover and faster apparent cleavage. This explanation allows reconciliation of previous studies using synthetic oligonucleotides with our studies and with studies which demonstrated enhanced cleavage of U-substituted phage DNA. We propose that this explanation may be generally applicable to other restriction endonucleases as well.

For oligonucleotides of all lengths, U substitution decreases the affinity of the endonuclease for its substrate. Our study indicates that the effects of H substitution parallel those of U substitution, and although these substitutents decrease affinity, neither prevents endonuclease binding and cleavage. The increase in relative cleavage velocity, a reflection of diminished affinity in the system reported here, is observed when the substitution is in or near the recognition site for a given endonuclease. In some cases, U has the greater effect; in others, H has the greater effect.

We observed that site-specific H substitution does not prevent cleavage by several endonucleases including *Eco*RI, *Bam*HI, and *Hin*dIII whereas these endonucleases fail to cleave H-containing phage DNA. The H-substituted phage DNA, however, is uniformly substituted with H. The significant difference between H and T is not size, as replacement by the larger bromine of 5-bromouracil does not prevent endonuclease cleavage (Petruska & Horn, 1983). In contrast to the hydrophobic methyl group of T, the hydroxymethyl group of H would be expected to be well hydrated. Uniform H substitution may therefore significantly alter hydration in the major groove of a B-form helix, substantially disrupting DNA−protein interactions.

Both U and H may occur randomly in DNA due to misincorporation of dUMP and T oxidation, respectively. The data presented here suggest that such substitution, if it occurs within the binding domain of a sequence-specific DNA binding protein, may diminish the affinity of the interaction. The inhibitory effect of uracil substitution has been shown directly for the interaction of the *lac* repressor and *lac* operator (Goeddel et al., 1977) as well as the binding of Oct2 (Jansco et al., 1994) and GCN4 (Pu & Struhl, 1992) transcription factors to their recognition sequences. Sequence-specific DNA binding proteins which bind to the major

groove of the DNA duplex must place apolar side chains in the major groove to accommodate the thymine methyl group. Replacement of the thymine methyl group with less hydrophobic groups such as hydrogen or hydroxymethyl would then be expected to perturb such interactions. Indeed, modification at the thymine methyl group could be functionally equivalent to a base substitution mutation in the binding domain. Interference with sequence-specific DNA−protein interactions may be an additional explanation for why H and U are excised from DNA in vivo.

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