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Reinvestigation of the Molecular Influence of Hypoxanthine on the DNA Cleavage Efficiency of Restriction Endonucleases BglII, EcoRI and BamHI

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Hypoxanthine (Hyp), a deaminated base of adenine (Ade), can be employed as a good probe molecule to reveal the significance of the minor groove of guanine (Gua) in biomolecular interactions because Hyp possesses a similar structure to Gua lacking its 2-amino group. In this study, we examined cleavage efficiencies of restriction endonuclease enzymes on DNA substrates with Hyp in their recognition sequences. As a substrate for BglII, EcoRI and BamHI, 24-mer DNA oligomer with Hyp (in place of Gua) was prepared together with its complementary sequences with cytosine (Cyt) or thymine (Thy) as the counter base. At 37°C incubation for 1 h, BglII and EcoRI showed higher DNA cleavage reactivity on Hyp-containing DNA substrates than on normal ones, whereas BamHI showed lower values on Hyp-containing substrates. Such high cleavage performance of BglII and EcoRI on Hyp-containing DNA substrates is in contrast to the results obtained 20 years ago, in which short DNA substrates (8- or 10-mer) and low reaction temperatures (15–20°C) were employed. These new results suggest that the lack of the exocyclic 2-amino group of Gua could contribute to enhanced recognition access of BglII and EcoRI to DNA substrates.

Key words: BglII, EcoRI, hypoxanthine, minor groove, restriction endonuclease.

Abbreviations: Hyp (H), hypoxanthine; Gua (G), guanine; Ade (A), adenine; Cyt (C), cytosine; Thy (T), thymine; Ino (I), inosine; T_m, melting temperature.

Hypoxanthine (Hyp, H) is a major mutagenic DNA lesion in DNA strands produced by oxidative deamination of adenine (Ade, A). Hyp, which possesses a similar structure to guanine (Gua, G) lacking its 2-amino group, induces AT to GC transversions *in vivo* after DNA replication by virtue of its ability to form base pairings with both cytosine (Cyt, C) and thymine (Thy, T), as shown in Fig. 1 (*1–3*). Such Hyp-induced mutations have been confirmed in *Escherichia coli* (*4*) and mammalian cells (*5*). Repair enzymes for Hyp, such as methylpurine glycosylase, AlkA and endonuclease V were found to readily release (deglycosylase) Hyp from DNA strands (*6–8*).

Hyp (alternatively called inosine; Ino, I) is also naturally found in cellular systems and its relevant mechanisms have been well studied (*9*, *10*). In particular, in an anticodon of tRNA, Hyp is known to play an important role in codon degeneracy, *i.e*. Hyp shows a universal binding property (*2*, *11*, *12*). This binding nature of Hyp has been examined in some experimental studies and computational characterizations in terms of its hydrogen bonds and stacking interactions (*12*). Furthermore, the unique property of Hyp has been usefully employed as a practical tool. As a universal nucleobase, Hyp is incorporated in oligonucleotides synthesis for several biotechnological applications such as polymerase chain reaction, hybridization probes and gene therapy (*2*, *12–15*).

On the other hand, Hyp is used as a nucleobase to investigate the mechanisms of DNA-binding proteins or enzymes, and particularly, to examine the importance of the minor groove of Gua in enzymatic recognition, because Hyp has a similar structure to Gua lacking its 2-amino group. One example of the application of Hyp as a useful nucleobase is the report of Ono *et al*. (*16*), which analysed the enzymatic response of Hyp to restriction endonucleases 20 years ago. In their previous studies, a decanucleotide containing Hyp in place of Gua in the restriction site was observed to show low cleavage efficiency with restriction endonuclease BglII compared to normal decanucleotides, thus suggesting that the 2-amino group of the Gua moiety might be important for recognition or binding by BglII. Similarly, another group analysed the enzymatic response of Hyp to EcoRI using octanucleotide (*17*). As in the case of BglII by Ono *et al*., Hyp-containing octanucleotide in the restriction site was more efficiently cleaved by restriction endonuclease EcoRI than normal decanucleotide.

These studies employed a short Hyp-containing DNA oligomer as the DNA substrate so that they could perform the enzyme reaction at relatively low temperature (10–17°C), possibly to allow the low melting temperature (T_m) of Hyp-containing DNA to form a duplex substrate during the enzyme reaction. However, to extend our

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knowledge of the role of the 2-amino group of Gua in recognition by restriction enzymes, performing enzyme reactions under physiological conditions is desirable.

In the present study, to verify previous reports on restriction mechanisms under physiological conditions, we performed enzyme reactions with BglII and EcoRI at 37°C, using long sequence oligodeoxynucleotides (24-mer) whose T_m was above 65°C (Tables 1–3). To compare the enzyme reactions of BglII and EcoRI, we employed BamHI as a control because BamHI has a similar recognition sequence to both BglII and EcoRI. Restriction enzyme activities were also examined to investigate the types of Hyp base pairs. In this study, new patterns for DNA cleavage efficiencies of BglII and

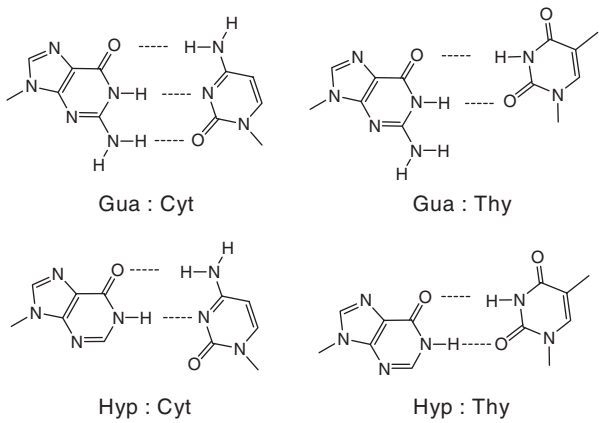

Fig. 1. **Hydrogen bonds of guanine (Gua) and hypoxanthine (Hyp) paired with cytosine (Cyt) or thymine (Thy) (*2*).**

Table 2. **Comparison of melting and reaction temperatures of our study and the previous study on the BglII reaction.**

Sequence	Oligomer length	T_m (°C)	Reaction (°C)	cf
GGAGATCTCC	10	42	15	Previous study[a]
GGAHATCTCC		32		
GAGTG CGGCAGATCT GCGGC TCAG	24	78.8	15-37	This study[b]
GAGTG CGGCAHATCT GCGGC TCAG		77.4		

[a]The sequence and its analysed T_m is from the report by Ono *et al.* (*16*). [b]The sequence was used in this study and its T_m was analysed in this study.

Table 3. **Comparison of melting and reaction temperatures of our study and the previous study on the EcoRI reaction.**

Sequence	Oligomer length	T_m (°C)	Reaction (°C)	cf
GGAATTCC	8	31	20	Previous study[a]
GHAATTCC		24.9[c]		
GAGTG CGGCGAATTC GCGGC TCAG	24	85	15–37	This study[b]
GAGTG CGGCHAATTC GCGGC TCAG		82.0		

[a]The sequence and its analysed T_m is from the report by Brennan *et al.* (*17*). [b]The sequence was used in this study and its T_m was analysed in this study. [c]T_m value for GHAATTCC was calculated on the basis of the T_m calculation program provided by Sigma-Aldrich Japan (Tokyo, Japan).

Table 1. **Oligonucleotide sequences used in this study.**

Enzyme	Substrate sequence	Substrate information
BglII	5′-d(GAGTG CGGC **AXA TCT** GCGG CTCAG)-3′ X = G, H	sense for ds-BglII-**XN** or ds-BglII-**X/Y**
	3′-d(CTCAC GCCG **TNT AGA** CGCC GAGTC)-5′ N = C, T	anti-sense for ds-BglII-**XN**
	3′-d(CTCAC GCCG **TCT AYA** CGCC GAGTC)-5′ Y = G, H	anti-sense for ds-BglII-**X/Y**
EcoRI	5′-d(GAGTG CGGC **XAA TTC** GCGG CTCAG)-3′ X = G, H	sense for ds-EcoRI-**XN** or ds-EcoRI-**X/Y**
	3′-d(CTCAC GCCG **NTT AAG** CGCC GAGTC)-5′ N = C, T	anti-sense for ds-EcoRI-**XN**
	3′-d(CTCAC GCCG **CTT AAY** CGCC GAGTC)-5′ Y = G, H	anti-sense for ds-EcoRI-**X/Y**
BamHI	5′-d(GAGTG CGGC **XGA TCC** GCGG CTCAG)-3′ X = G, H	sense for ds-BamHI1-**XN** or ds-BamHI1-**X/Y**
	3′-d(CTCAC GCCG **NCT AGG** CGCCG AGTC)-5′ N = C, T	anti-sense for ds-BamHI1-**XN**
	3′-d(CTCAC GCCG **CCT AGY** CGCCG AGTC)-5′ Y = G, H	anti-sense for ds-BamHI1-**X/Y**
	5′-d(GAGTG CGGC **GXA TCC** GCGG CTCAG)-3′ X = G, H	sense for ds-BamHI2-**XN** or ds-BamHI2-**X/Y**
	3′-d(CTCAC GCCG **CNT AGG** CGCC GAGTC)-5′ N = C, T	anti-sense for ds-BamHI2-**XN**
	3′-d(CTCAC GCCG **CCT AYG** CGCC GAGTC)-5′ Y = G, H	anti-sense for ds-BamHI2-**X/Y**

Note: **H:** hypoxanthine.

EcoRI on DNA substrates were found. Thse results will provide new guidelines to explain molecular recognition access of EcoRI and BglII to DNA substrates, especially their interaction with the minor groove of Gua on the recognition sequence.

MATERIALS AND METHODS

Oligonucleotides, Enzymes and Chemicals—DNA oligodeoxynucleotides (24-mer) containing hexanucleotide recognition sequences of BglII, EcoRI and BamHI in the middle positions, with or without the modified base Hyp, were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Sequence or substrate names of DNA oligomers used in this study are listed in Table 1. [γ-^{32}P]ATP (6.0 Ci μmol^{-1}) was purchased from GE Healthcare (Piscataway, NJ, USA). Restriction endonucleases BglII, EcoRI and BamHI were obtained from Roche Diagnostics (Mannheim, Germany). T4 polynucleotide kinase (T4 PNK) was obtained from TaKaRa (Shiga, Japan). Other chemical reagents or solvents were purchased from Wako (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Restriction Endonuclease Assays—Ultraviolet (UV) spectra of DNA oligomers were measured at 260 nm on a Shimadzu UV-260 UV-visible light Spectrophotometer for preparation of 20 μM DNA oligomers. The DNA sample (20 μM) was incubated with T4 PNK (40 U) and [γ-^{32}P]ATP (4.5 MBq) in 50 μl of reaction buffer [50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT)] at 37°C for 45 min to prepare radiolabeled DNA oligomers. The reaction was terminated by heat deactivation (70°C, 10 min) and the radiolabeled DNA oligomers were separated using a Centri-Sep purification column (Princeton Separations, Adelphia, NJ, USA). Fragments of 5′-end radiolabeled 24-mer DNA oligomers (100 nM) with 24-mer template DNA strands (100 nM) were incubated with the respective restriction endonucleases (BglII, 10 U; EcoRI and BamHI, 2 U) in 50 μl of reaction buffer [BglII, 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT; EcoRI, 5 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM DTT; BamHI, 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol] at 15 or 37°C for 1 h. The reaction was terminated by heat deactivation (90°C, 10 min). Thereafter, the reaction products were subjected to 20% denaturing PAGE containing 6 M urea at 20 mA, and the amount of product was quantified using a Storm 820 phosphor-imaging scanner (GE-Healthcare, Piscataway, NJ).

Steady-state Kinetic Studies—Kinetic parameters associated with hydrolysis by restriction enzymes were determined at 15°C (BglII and EcoRI) and 37°C (BglII, EcoRI and BamHI), using varying amounts of DNA substrates (50–5000 nM). For the hydrolysis kinetics, reaction mixtures containing DNA substrates and restriction endonucleases BglII (10 U), EcoRI (2 U) or BamHI (2 U) were incubated at 15 or 37°C for 10 min in 40 μl of reaction buffer, as described in 'Restriction endonuclease assays'. The reaction samples were subjected to 20% denaturing PAGE containing 6 M urea at 20 mA, and the amount of product was quantified using a Storm 820 phosphor-imaging scanner. Michaelis constants (K_m) and maximum rates of reaction (V_{max}) values were obtained from Lineweaver–Burk plots.

Melting Temperature Analysis—All DNA solutions (2 μM) were prepared in a phosphate buffer (1 M NaCl, 10 mM Na_2HPO_4 and 1 mM Na_2EDTA adjusted to pH 7.0 with HCl) for melting temperature analysis. Absorbance of solutions containing 1:1 strand ratio of oligodeoxynucleotides at 260 nm was obtained using a Shimadzu TMSPC-8 T_m analysis system at a variable temperature, ranging from 20°C to 90°C at a rate of 0.2°Cmin^{-1}.

The T_m of GHAATTCC in a previous study by Brennan *et al*. (*17*) was calculated on the basis of a T_m calculation program provided by Sigma-Aldrich Japan (Tokyo, Japan) because the previous report did not provide the measured T_m data.

RESULTS AND DISCUSSION

Influence of Hypoxanthine on the Reactivity of BglII—Ono *et al*. (*16*) reported that the minor groove modification of Gua, with no exception of Hyp, inhibited the recognition of BglII and that the 2-amino group of Gua plays an important role in the recognition of BglII. In their study, a short DNA sequence, decamer, with a low T_m was used (Table 2); the temperature of the enzyme reaction was 15°C, which is lower than the optimum temperature for restriction reactions (37°C).

To verify previous studies at physiological conditions with long DNA sequences, we designed a 24-mer Hyp-containing DNA oligomer with a T_m around 78°C (Table 2) and incubated it with BglII at 37°C. As shown in Figs 2 and 3, the Hyp-containing DNA substrate (ds-BglII-HC) was more efficiently cleaved by BglII than the normal DNA substrate (ds-BglII-GC). In fact, the enzymatic cleavage efficiencies on ds-BglII-HC in a 1 h reaction were 2-fold higher than those on ds-BglII-GC (Fig. 3). This is an interesting result because it shows high cleavage efficiencies of BglII on Hyp-containing DNA, which is in contrast to the results reported by Ono *et al*. (*16*).

We performed restriction enzyme incubation at 15°C, using 24-mer DNA oligomers. At 15°C, ds-BglII-HC showed low enzymatic cleavage efficiencies of BglII compared to ds-BglII-GC, although the difference was quite small (Fig. 3). We also analysed the enzyme reaction when Hyp was paired with Thy, because Hyp is a mutagenic lesion generated from Ade in DNA and the produced Hyp is likely to pair with Thy (*12*, *18*). As shown in Fig. 3, in case of ds-BglII-HT, enzyme activity of BglII was enhanced compared to that in the case of ds-BglII-GT at 15°C, though it showed slightly reduced enzyme activity at 37°C.

Steady-state kinetic values were determined for the oligodeoxynucleotides ds-BglII-GN and ds-BglII-HN that were hydrolyzed by BglII. Michaelis constants (K_m) and maximum rates of reaction (V_{max}) values that were determined for each substrate are summarized in Table 4. At 37°C, V_{max}/K_m for ds-BglII-HC was 2.32 times higher than that for ds-BglII-GC; the K_m value of BglII for ds-BglII-HC was 2.36 times lesser than that for ds-BglII-GC, without a significant difference in V_{max}. These results suggested that substitution of Gua by

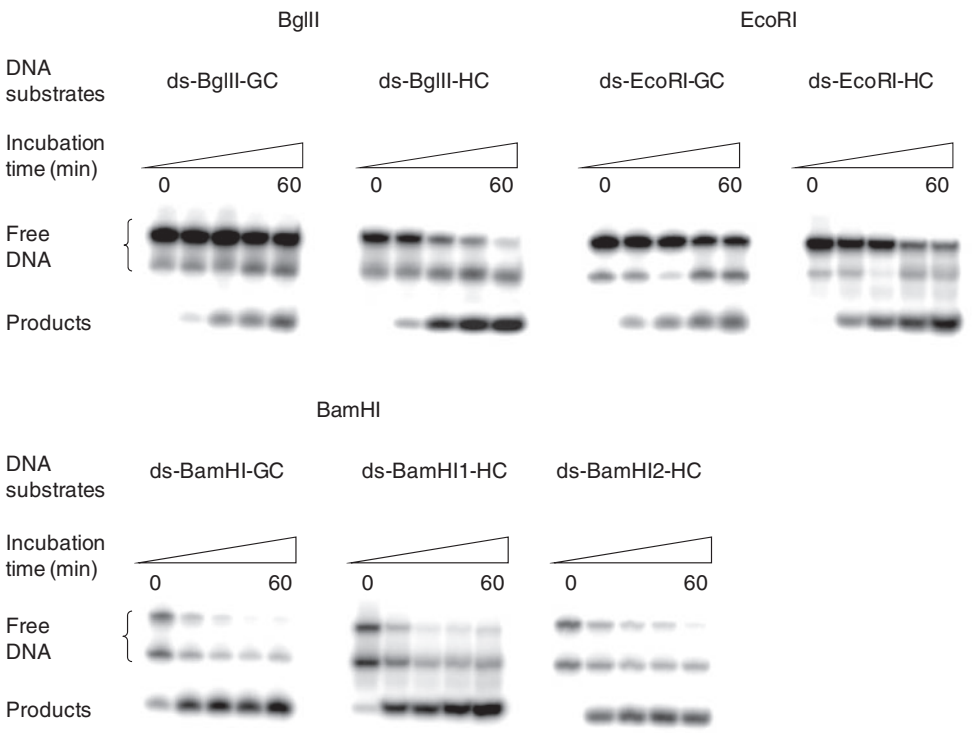

Fig. 2. **Denaturing PAGE separation of nucleotide components in BglII, EcoRI and BamHI reactions.** The bands of free DNA and hydrolysis products are indicated. BglII (10 U), EcoRI (2 U) and BamHI (2 U) endonucleases were incubated with 600 nM 24-mer double strands of ds-BglII-XN, ds-EcoRI-XN, ds-BamHI1-XN and ds-BamHI2-XN (5′-end of sense strand was radiolabeled with ^{32}P) at 37°C for 1 h. Aliquots of the reaction were separated by denaturing PAGE.

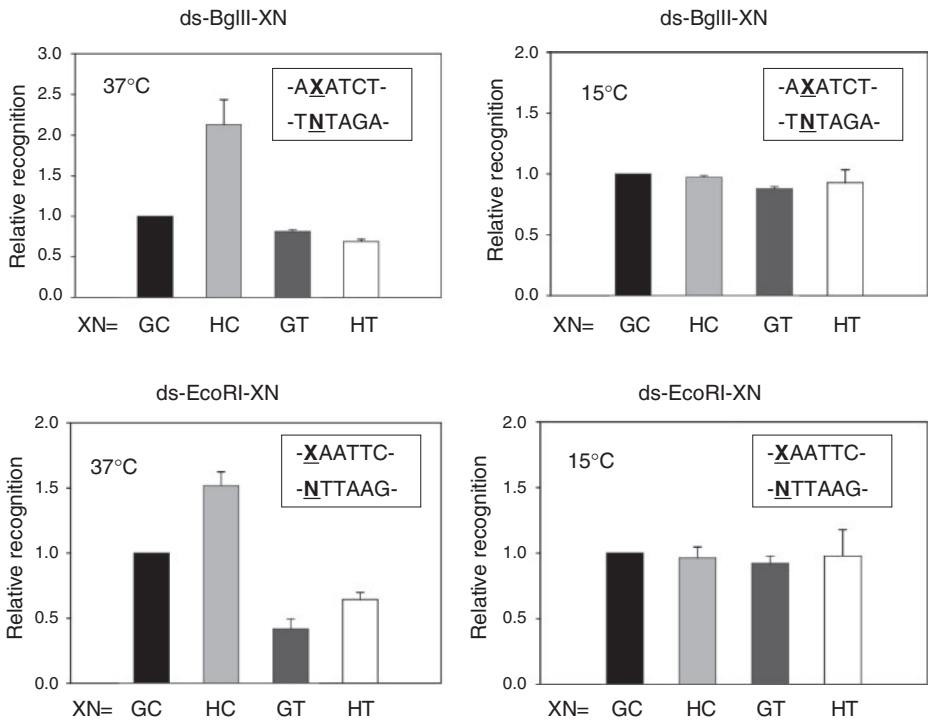

Fig. 3. **Relative recognition of Hyp to Gua (the case of XN = GC was regarded as 1.0) by respective endonucleases BglII and EcoRI.** Hyp or Gua- containing nucleotides, which pair with Cyt and Thy, were incubated with enzymes at 37°C and 15°C for 1 h. The amounts of each product were quantified after separation by denaturing PAGE. Values represent mean ± SD for three experiments.

Table 4. **Steady-state kinetic values for restriction endonucleases BglII and EcoRI.**

Enzyme	DNA substrate	Reaction (°C)	V_{max} (%min^{-1})[a]	K_m (μM)[a]	V_{max}/K_m (%min^{-1}μM^{-1})[a]
BglII	ds-BglII-GC	37	4.57 ± 0.04	0.45 ± 0.02	15.08 ± 0.50
	ds-BglII-HC		4.24 ± 0.11	0.19 ± 0.05	35.01 ± 8.14
	ds-BglII-GT		2.31 ± 0.06	0.36 ± 0.02	6.46 ± 0.20
	ds-BglII-HT		2.64 ± 0.48	0.81 ± 0.35	3.66 ± 1.98
	ds-BglII-GC	15	3.03 ± 0.03	1.04 ± 0.17	2.97 ± 0.59
	ds-BglII-HC		3.30 ± 0.26	1.11 ± 0.45	3.47 ± 1.72
	ds-BglII-GT		2.45 ± 0.26	1.06 ± 0.20	2.40 ± 0.79
	ds-BglII-HT		2.25 ± 0.19	0.52 ± 0.07	4.34 ± 0.23
EcoRI	ds-EcoRI-GC	37	1.76 ± 0.20	0.20 ± 0.03	8.90 ± 1.82
	ds-EcoRI-HC		1.89 ± 0.12	0.16 ± 0.04	12.1 ± 2.3
	ds-EcoRI-GT		1.42 ± 0.29	0.44 ± 0.10	3.30 ± 0.21
	ds-EcoRI-HT		1.63 ± 0.36	0.18 ± 0.04	9.29 ± 1.07
	ds-EcoRI-GC	15	2.29 ± 0.14	0.91 ± 0.38	2.73 ± 0.89
	ds-EcoRI-HC		2.11 ± 0.06	0.78 ± 0.60	3.48 ± 1.54
	ds-EcoRI-GT		2.27 ± 0.17	1.55 ± 0.22	1.43 ± 0.26
	ds-EcoRI-HT		2.29 ± 0.37	1.45 ± 0.35	1.47 ± 0.48

Kinetics of restriction reactions were determined as described in MATERIALS AND METHODS section. [a]Values represent mean ± SD for three experiments.

Hyp may increase the recognition or affinity of BglII on the DNA substrate. However, such increased recognition or affinity induced by Hyp was not observed when tested at 15°C.

Recent structural data of BglII suggested that the 'bent' conformation of the DNA substrate is critical for efficient recognition of BglII on the DNA substrate (*19*), *i.e*. Hyp in the restriction site might help BglII to bind its recognition sequence because the lack of the 2-amino group in the minor groove of Gua may increase the local flexibility of the DNA (*20*). To further understand this mechanism, we performed enzymatic reactions with Hyp in place of Gua in both sense and anti-sense DNA (ds-BglII-X/Y). As shown in Fig. 5, when 2 Gua were replaced by Hyp in both sense and anti-sense DNA (ds-BglII-H/H), the highest cleavage efficiency of BglII was observed compared to normal DNA (ds-BglII-G/G) or single Hyp substitution (ds-BglII-H/G, ds-BglII-G/H), thus supporting the restriction mechanism of BglII. These tendencies were also observed at 15°C, though cleavage efficiencies of BglII were decreased compared to the enzyme reaction at 37°C (data not shown). Furthermore, it was expected that the previous results by Ono *et al*. (*16*) could be affected by the instabilities of short DNA substrates, in which 2 Gua were replaced by Hyp. The biophysical stability of DNA substrate is critically important for examining the restriction reaction, especially when DNA bending is involved in the restriction mechanism.

Influence of Hypoxanthine on the Reactivity of EcoRI—Brennan *et al*. reported that Hyp substituted for Gua decreased the recognition efficiency of EcoRI (*17*). They used a short sequence of octamer as the DNA substrate and performed enzymatic reactions at a low temperature of 20°C (Table 3), as in the experiments with BglII by Ono *et al*. (*16*). In our study, we prepared a 24-mer DNA sequence and incubated it with EcoRI at 37°C for 1 h (Fig. 2) to verify previously reported results of EcoRI. As shown in Fig. 3, the enzymatic cleavage efficiency of EcoRI on ds-EcoRI-HC was 1.5-fold higher than that on ds-EcoRI-GC, whereas the enzymatic cleavage efficiency of EcoRI on ds-EcoRI-HT was 2-fold higher than that on ds-EcoRI-GT. However, at a low temperature of 15°C, there was no significant difference among ds-EcoRI-GC, ds-EcoRI-HC, ds-EcoRI-GT and ds-EcoRI-HT. Although the enzymatic cleavage efficiency of EcoRI on ds-EcoRI-HC was lower than that on ds-EcoRI-GC at 15°C, the difference was quite small.

Steady-state kinetic values were determined for the oligodeoxynucleotides ds-EcoRI-GN and ds-BamHI2-HN that were hydrolyzed by EcoRI. Michaelis constants (K_m) and maximum rates of reaction (V_{max}) values that were determined for each substrate are shown in Table 4. At 37°C, V_{max}/K_m for ds-EcoRI-HC was 1.36 times higher than that for ds-EcoRI-GC; the K_m value of EcoRI for ds-EcoRI-HC was 1.25 times lesser than that for ds-EcoRI-GC, without a significant difference in V_{max}. These results suggested that substitution by Hyp (the lack of the 2-amino group of Gua) may increase the recognition or affinity of EcoRI on the DNA substrate. As in the case of BglII, 'bending' of the DNA substrate is required for EcoRI to recognize the DNA substrate more efficiently (*21*), *i.e*. the substitution by Hyp in the restriction site and lack of the 2-amino group in the minor groove of Gua might help EcoRI to bind its specific sequence, thus resulting in high cleavage efficiency.

In addition to the BglII reaction, we also analysed the enzymatic reaction when Gua was replaced by Hyp in both sense and anti-sense DNA (ds-EcoRI-X/Y), which is similar to the investigation reported by Brennan *et al*. (*17*). As shown in Fig. 5, when 2 Gua were replaced by Hyp (ds-EcoRI-H/H), a higher cleavage efficiency of EcoRI was observed compared to normal DNA (ds-EcoRI-G/G) or a single Hyp in place of Gua (ds-EcoRI-H/G, ds-EcoRI-G/H). These tendencies were also observed at 15°C, though cleavage efficiencies of EcoRI were decreased compared to the enzyme reaction at 37°C (data not shown). As discussed in the case of BglII, it was expected that the previous results by Brennan *et al*. (*17*) could be affected by the instabilities

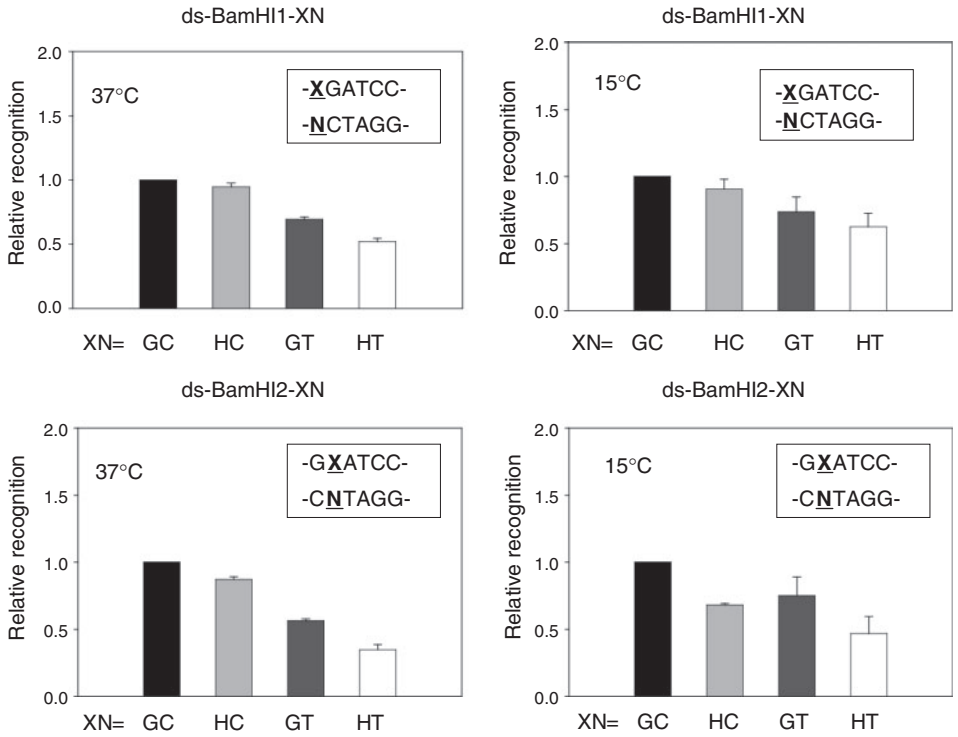

Fig. 4. **Relative recognition of Hyp to Gua (the case of XN = GC was regarded as 1.0) by endonuclease BamHI.** Hyp or Gua-containing nucleotides, which pair with Cyt and Thy, were incubated with enzymes at 37°C and 15°C for 1 h. The amounts of each product were quantified after separation by denaturing PAGE. Values represent mean ± SD for three experiments.

of short DNA substrates, in which 2 Gua were replaced by Hyp.

Influence of Hypoxanthine on the Reactivity of BamHI—We analysed the influence of Hyp on the enzymatic reaction of BamHI, which recognizes d(GGATCC) sequence. Two types of Hyp-containing DNA substrates were prepared by substituting each Gua in the recognition sequence of BamHI with Hyp, *i.e.* ds-BamHI1-HN containing d(HGATCC) and ds-BamHI2-HN containing d(GHATCC).

Contrary to the results of BglII and EcoRI, BamHI showed lower cleavage efficiency for Hyp-containing DNA substrates such as ds-BamHI1-HN and ds-BamHI2-HN than for normal DNA substrates (Figs 2 and 4). The cleavage efficiency of BamHI on ds-BamHI1-HN was not much lower than that on ds-BamHI-GN. Furthermore, this behavior of BamHI on Hyp-containing DNA substrates did not change, regardless of the reaction temperature or the types of base pairs (Fig. 4).

We determined steady-state kinetic values for BamHI according to their DNA substrates. It was also shown that BamHI had slightly lower V_{max}/K_m values for Hyp-containing DNA substrates (ds-BamHI1-HC and ds-BamHI2-HC) than for normal DNA substrate (data not shown). In particular, V_{max}/K_m for ds-BamHI2-GC decreased more than that for ds-BamHI1-GC when they were compared with the normal DNA substrate.

In fact, BamHI does not require the 'bending' of the DNA substrate (*22*) and thus, the substitution by Hyp in DNA substrates may not affect the cleavage efficiency of BamHI. Instead, BamHI recognizes the minor groove of the second Gua in the recognition site (G**G**ATCC) of sense strand DNA through its Asp residue (Asp196 of BamHI), which is found to interact with the 2-amino group of Gua by direct contact of BamHI. Therefore, the substitution of Hyp for the second Gua in the restriction site of the sense strand DNA d(GHATCC) may decrease the recognition and affinity efficiency of BamHI on the DNA substrate because the lack of the 2-amino group of Gua induces the loss of important minor groove contact with BamHI. As observed in this study, the cleavage efficiency of BamHI on ds-BamHI2-HC decreased more than that on ds-BamHI1-HC.

We also analysed the enzymatic reaction when Gua was substituted by Hyp in both sense and antisense DNA (ds-BamHI1-X/Y and ds-BamHI2-X/Y). As shown in Fig. 5, when 2 Gua were replaced by Hyp (ds-BamHI1-H/H and ds-BamHI2-H/H), the lowest cleavage efficiency of BamHI was observed compared to that on normal DNA (ds-BamHI-G/G) or single Hyp in place of Gua (ds-BamHI1-H/G or ds-BamHI1-G/H and ds-BamHI2-H/G or ds-BamHI2-G/H). The substitution of Hyp for Gua on ds-BamHI1-X/Y did not affect the enzyme reaction with BamHI compared to that on ds-BamHI2-X/Y. These results also support the recognition mechanism of BamHI.

CONCLUSION

In this report, we investigated the cleavage efficiencies of restriction endonucleases (BglII, EcoRI and BamHI) on DNA substrates with Hyp in the DNA duplexes. It was

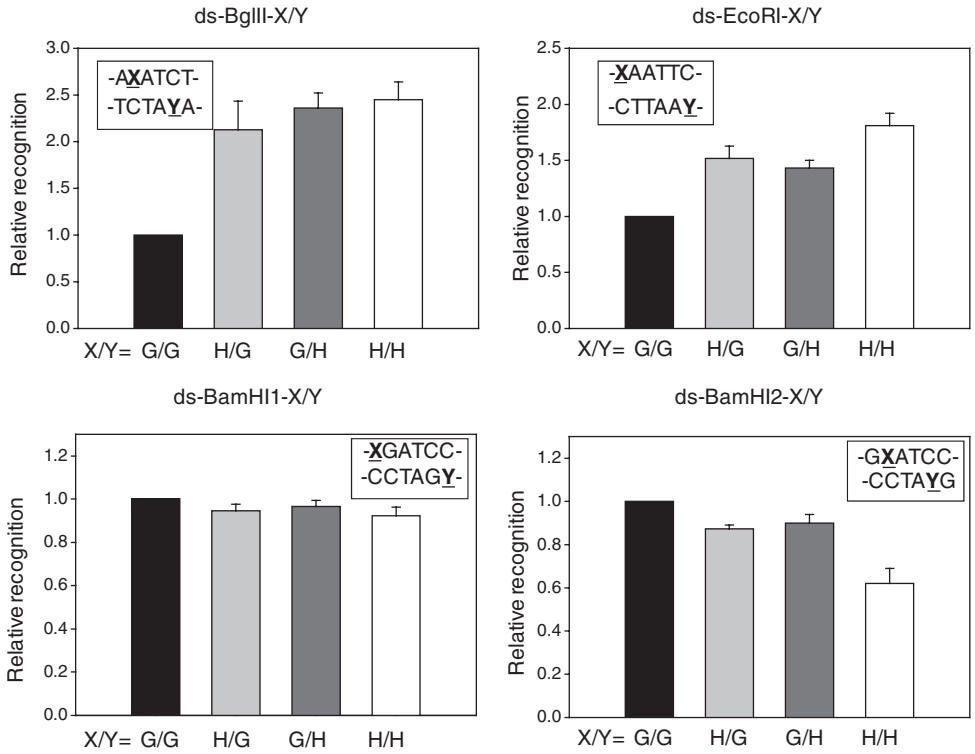

Fig. 5. **Relative recognition of Hyp to Gua (the case of X/Y = G/G was regarded as 1.0) by endonucleases BglII, EcoRI and BamHI.** Normal Gua (G/G) and 1 (H/G, G/H) or 2 Hyp (H/H)-containing DNA duplexes were incubated with enzymes at 37°C for 1 h. The amounts of each product were quantified after separation by denaturing PAGE. Values represent mean ± SD for three experiments.

newly found that the substitution of Hyp for Gua in the recognition sequence did not decrease, but, in fact, increased the cleavage efficiencies of BglII and EcoRI. These results differ from the previous findings that Hyp in DNA substrates could decrease the enzymatic activity of BglII and EcoRI (*16*, *17*). In previous studies, short DNA sequences were used as DNA substrates (8- or 10-mer), and hence, the T_m of the Hyp-containing DNA duplexes was lower than that of normal DNA duplexes (Table 2). In this study, we designed a 24-mer Hyp-containing DNA oligomer with a T_m around 78°C (Tables 2 and 3) and incubated it with BglII, EcoRI and BamHI at 37°C.

While BglII and EcoRI showed higher cleavage efficiencies on Hyp-containing DNA substrates than on normal DNA substrates, BamHI showed lower cleavage efficiencies, especially in the case where Hyp replaced Gua both in sense and anti-sense DNA. These differences are probably related to the recognition mechanisms of the restriction enzymes. For BglII and EcoRI to recognize their recognition sequences, 'bending' of the DNA substrate is required. From this perspective, the substitution of Hyp for Gua in the recognition sequence could provide positive effect on 'bending' of the DNA substrate, because the lack of the 2-amino group of Gua in the minor groove will help to increase the local flexibility of the DNA substrate. In the case of BamHI, such 'bending' of the DNA substrate is not required (the 'non-flexible' structure of the DNA substrate is favourable for BamHI recognition). Furthermore, the second Gua in the recognition sequence of the sense strand is known to provide minor groove contact with the Asp residue of BamHI, which is a direct interaction between the 2-amino group of Gua and Asp when BamHI recognizes the DNA substrate. As observed in this study, the substitution of Gua by Hyp resulted in lower cleavage efficiencies of BamHI. The difference between our results and previous studies by Ono *et al*. (*16*) and Brennan *et al*. (*17*) result from the biophysical instabilities of short DNA duplexes, which are critical for efficient DNA bending.

The complex structure should be determined to confirm the mechanism of action of restriction enzymes on Hyp-containing DNA substrates. The results obtained may be useful in explaining the molecular recognition mechanism relating to molecular interaction in the minor groove of Gua in the DNA substrate.

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CONFLICT OF INTEREST

None declared.

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