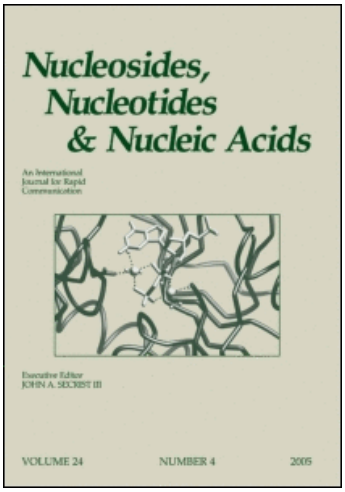

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NUCLEOTIDE INCORPORATION OPPOSITE DEGENERATE BASES BY *Taq* DNA POLYMERASE§

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ABSTRACT: Oligodeoxyribonucleotides containing degenerate bases, 6*H*,8*H*-3,4-dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (P), 2-amino-6-methoxyaminopurine (K), and N^6-methoxyadenine (Z) were made on a DNA synthesizer. The oligonucleotides were used for the preparation of DNA templates by enzymatic ligation of DNA fragments. The templates were annealed with a primer and extended with *Taq* DNA polymerase and dNTPs to reveal the nucleotide(s) incorporated opposite the modified bases. Analysis of the products showed that A was inserted opposite the pyrimidine P and T opposite the purines, K and Z. Incorporation of a small amount of C opposite K was also observed.

INTRODUCTION

A number of approaches have been used in nucleic acids hybridization to overcome the degeneracy of the genetic code.[1-5] The most common method is the use of probes with mixed bases.[1,2] This approach is successful when a target has a relatively low potential multiplicity. Another useful way is to incorporate deoxyinosine at degenerate positions in probes,[3-5] since the "universal base", hypoxanthine (I) forms base pairs with all the normal bases. Its ability to pair with any base, however, causes nonspecific binding to untargetted sites, resulting in a high background signal.[6]

We recently reported that the pyrimidine analogue 6*H*,8*H*-3,4-dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (P)[7] and the purines 2-amino-6-

§ This paper is dedicated to Professor M. Ikehara for his 70th birthday.

methoxyaminopurine (K) and N^6-methoxyadenine (Z)[8] (Figure 1) can pair with A and G, and C and T, respectively. Oligonucleotides with one or more P residues formed duplexes with little decrease in stability; those with K and Z showed the expected degenerate behaviour and T_ms much higher than those with mismatches.[7,8] Indeed,oligonucleotides with P bases were shown to be useful in hybridization and sequencing experiments.[9]

We recently, too, reported that oligonucleotides with P and K or I were effective primers[10] in the polymerase chain reaction (PCR).[11] As a result it became important to know which nucleotides were incorporated opposite the degenerate bases during PCR in order to use such primers more effectively.

In this report, we describe the preparation of DNA templates with P, K and Z and analysis of incorporated nucleotides opposite the bases by the commonly used *Taq* DNA polymerase. In addition, the outcome of using the Klenow polymerase fragment in the reaction is described.

MATERIALS AND METHODS

Enzymes

Taq DNA polymerase and Klenow fragment were purchased from Wako Pure Chemical Industries and Takara, respectively. Other enzymes used were essentially the same as described previously.[12,13]

Oligonucleotide synthesis

Phosphoramidite monomers of P, K and Z were synthesized as described previously.[7,8] Solid-phase synthesis and purification of oligonucleotides containing P, K and Z were carried out as described previously.[7,8] The nucleotide sequences of the oligonucleotides were 5' dGCCGNCGGTGTG 3' (N means P, K, or Z).

Preparation of DNA templates

DNA templates were prepared by enzymatic ligation of the modified oligonucleotides with oligonucleotides, HRU1 and HRU3, as described earlier.[13] Namely, the oligonucleotides with P, K, or Z and HRU3 were phosphorylated with T4 polynucleotide kinase. The phosphorylated DNA fragments and HRU1 were annealed with a splint HRL1 and then treated with T4 DNA ligase (Figure 2). The ligated fragment was purified by 8M urea-20% polyacrylamide gel electrophoresis (PAGE).

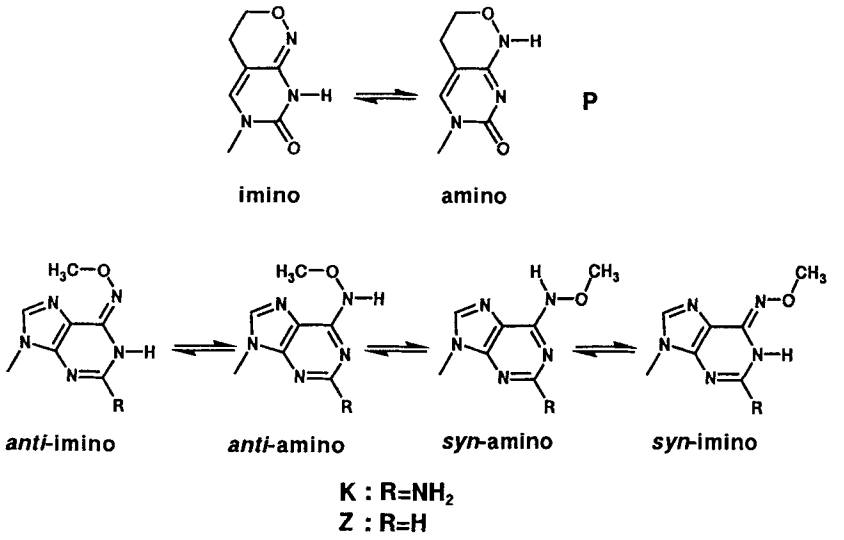

FIG. 1. Structures and tautomerism of P, K, and Z.

In vitro DNA synthesis

The purified DNA template (10 pmol) was annealed with a primer Seq2 (20 pmol, Figure 2). *In vitro* DNA synthesis was carried out at 72°C for 10 min using 4U of *Taq* DNA polymerase in a total volume of 100 μl under the conditions recommended by the supplier {10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 200 μM each dNTP}. Reactions with the Klenow fragment were carried out at 37°C for 30 min using 4U of the enzyme in a buffer solution containing 7 mM Tris-HCl (pH 7.5), 20 mM NaCl, 7 mM $MgCl_2$, 700 μM EDTA, and 50 μM each dNTP.

Analysis of incorporated nucleotides

The product of *Taq* DNA polymerase extension was analyzed with or without purification by 20% PAGE. The product of Klenow fragment was purified by PAGE and single strand DNA without the modified bases was prepared for sequence analysis by *Alu*I digestion and subsequent treatment with λ exonuclease to digest the template strand as described.[13]

Nucleotide(s) incorporated opposite modified bases were identified after polymerase chain reaction amplification followed by restriction analysis (PCR-RE) as described previously.[12,13]

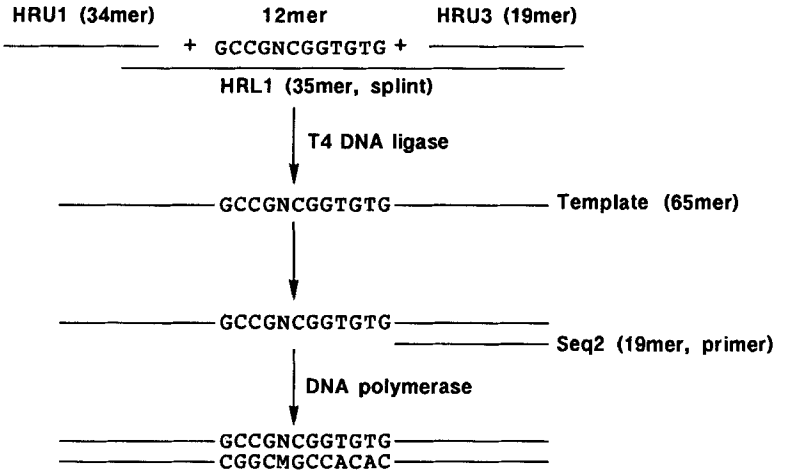

FIG. 2. Preparation of DNA templates with P, K, and Z and primer extension reaction. N means P, K, and Z. M means the base incorporated opposite the degenerate bases.

RESULTS

Preparation of DNA templates with degenerate bases

DNA templates (Figure 2) with P, K, and Z were prepared by enzymatic joining of three DNA fragments. The position where the degenerate bases were introduced corresponds to the second position of codon 12 of a c-Ha-*ras* gene. Any bases at the site can be detected by the PCR-RE method and we can readily analyze nucleotide(s) incorporated opposite the modified bases semi-quantitatively (Table 1).

Analysis of nucleotides incorporated by *Taq* polymerase

First, we analyzed nucleotides inserted opposite P and K. Figure 3a shows the results of the PCR-RE analysis of the P-template. Cleavage was observed when the PCR product was treated with *Aat*II (lane 3). That means dAMP was incorporated opposite P (Table 1). Since none of the other enzymes cut the PCR product, it would appear that insertion of other nucleotides did not occur. In the case of K, cleavage was detected when the PCR product was incubated with *Sal*I (Figure 3b, lane 5), indicating that dTMP was incorporated (Table 1). Furthermore, a faint cleaved band was observed by *Hap*II treatment of the PCR product (lane 1), showing that incorporation of dCMP occurred but was much less frequent (Table 1).

TABLE 1. Detection of incorporated nucleotide.

Enzyme	Recognition Sequence [a]	Incorporated nucleotide, detectable [b]
*Hap*II	5' CCG**G** 3'	C
*Aat*II	5' GACG**T**C 3'	A
Sal	5' GTCG**A**C 3'	T
*Bbe*I	5' GGCG**C**C 3'	G

[a]The positions where the modified bases were introduced are underlined.
[b]Nucleotides which form the recognition sites when incorporated are shown.

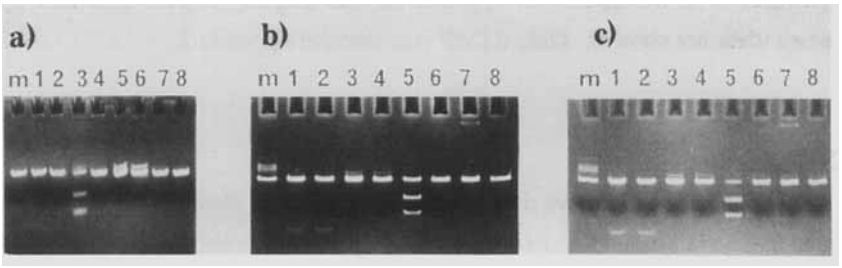

FIG. 3. The PCR-RE analysis of the PCR products originated from templates with **a)** P, **b)** K, and **c)** Z. Incorporation of dCMP, dAMP, dTMP and dGMP were indicated by cleavage of the PCR products by *Hap*II, *Aat*II, *Sal*I and *Bbe*I, respectively (shown as *Hap*II +, for example). To confirm the cleavage, the non-digested controls are also shown (as *Hap*II -, for example). Lane 1, *Hap*II +; lane 2, *Hap*II -; lane 3, *Aat*II +; lane 4, *Aat*II -; lane 5, *Sal*I +; lane 6, *Sal*I -; lane 7, *Bbe*I +; lane 8, *Bbe*I -. m indicates an uncleaved PCR product.

The nucleotide incorporation opposite Z was also investigated for comparison. Figure 3c shows that the PCR products were digested only with *Sal*I (lane 5), indicating that dTMP was specifically incorporated and little, if any, dCMP was inserted.

Analysis of nucleotides incorporated by Klenow fragment

To compare nucleotide incorporation opposite the purine base analogues, Klenow fragment of *E. coli* DNA polymerase I was also used. Single strand DNA without the modified base was prepared by selective digestion of the template strand with combination of *Alu*I and λ exonuclease as described.[13] The DNA was analyzed by the PCR-RE method and showed that dTMP and dCMP were inserted opposite K (Table 2)

TABLE 2. Nucleotide incorporated opposite P, K, Z and I.

Enzyme	P	K	Z	I [a]
Taq polymerase	A	T>>C	T	C
Klenow fragment	ND [b]	T>>C	T	ND [b]

[a]From reference 13.
[b]Not determined.

although ratio of C-incorporation opposite K was slightly higher than that by *Taq* polymerase (data not shown). Only dTMP was inserted opposite Z.

DISCUSSION

In recent publications we described the synthesis of purine and pyrimidine base analogues that have ambivalent hydrogen-bonding modes as shown in Figure 1.[7,8] We also reported thermal stabilities of oligomer duplexes containing the modified bases.[7,8] In these studies we showed that the pyrimidine (P) and purine (K) base analogues could be useful in probing DNA and that primers containing P and K, and P and I were effective in PCR.[9,10] In order to use primers with the modified bases more effectively, it is very important to know the deoxynucleotide inserted opposite each base analogue when it is present in PCR primers.

To reveal inserted nucleotide(s) we used the PCR-RE method that was originally designed for detection of point mutations at a specific position.[12] The analysis indicated that dAMP was inserted opposite P and that dTMP and a small amount of dCMP were inserted in the case of K (Figure 3a and b, and Table 2). We then analyzed nucleotide(s) inserted opposite Z for comparison. Exclusive incorporation of dTMP was detected (Figure 3c). Similar results were obtained when the Klenow fragment was used in an *in vitro* DNA synthesis (Table 2). The results which were not in accordance with thermal stability appeared to be related to the base-pairing pattern, as discussed in the case of O^6-methylguanine,[13-19] xanthine,[13,20] and Z.[21]

NMR studies of octanucleotide duplexes with P:A and P:G pairs indicated that P:A is isostructural with T:A with the same stability. P:G adopts Watson-Crick and wobble pairing which are slow exchange in the duplex on the NMR time scale (Figure

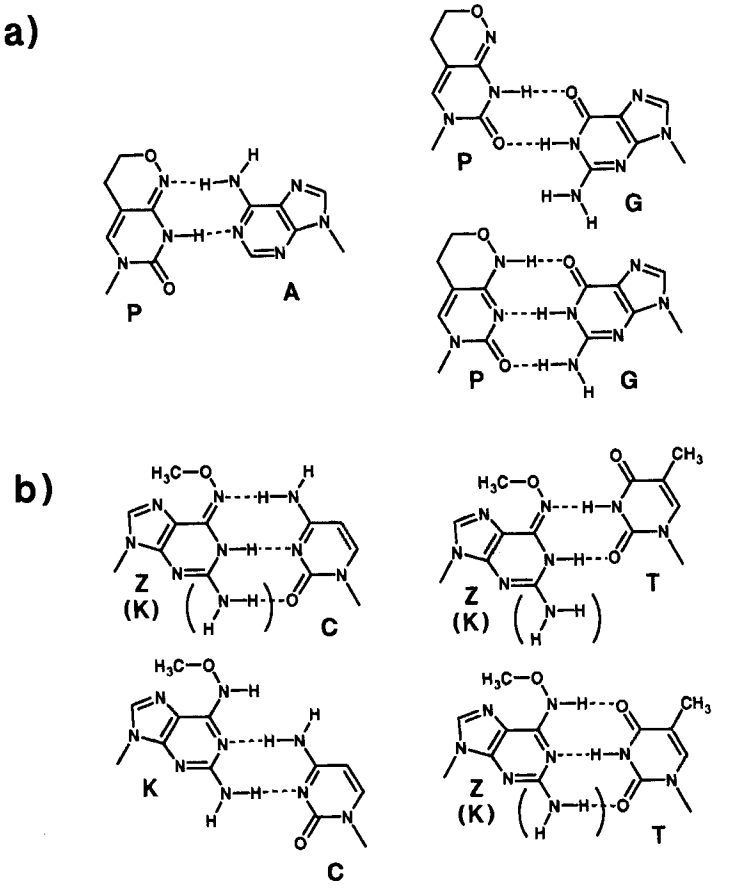

FIG. 4. Base pairs involving **a)** P and **b)** K and Z.

4a).[22,23] NMR T_m measurements show that the P:G base pair confers lower stability than does P:A on the octamers.[23] It is known that the imino-form of P is strongly favoured as it is in N^4-hydroxycytosine.[24] The imino form of P can pair with A in a Watson-Crick manner while this major tautomer can form a wobble pair with G and the less stable amino-tautomer can adopt Watson-Crick pairing (Figure 4a). Thus in a template, that is, in a single-stranded DNA, P existing as the preferred imino-tautomer leads to the incorporation of dAMP because of the favoured Watson-Crick base-pairing (Figure 4a). On the other hand, dGMP is not inserted at an observable level because of the preferred imino-P:G wobble pairing. Indeed, dGTP may be "rejected" by DNA polymerases before the wobble to Watson-Crick base-pair exchange can be reached (Figure 4a).

Turning to N^6-methoxyadenine (Z), the evidence is clear that both tautomers are present in solution in equilibrium and can be observed by NMR, the amino tautomer predominating in low dielectric solvents[21,24-26], a situation that may pertain at the polymerase active site. Moreover the methoxy group is preferentially in the *syn*-orientation.[27,28]. Since, on steric grounds, base-pair formation in the polymerase extension step requires an *anti*-configuration for the methoxy group and that *syn-anti* equilibrium presumably occurs through the amino-tautomer (Figure 1), it is evident that the favoured pairing is *anti*-amino Z:T (Figure 4b). This falls into place with our observations. Nevertheless Nishio *et al.*[21] observe that with an oligomer containing Z and the Klenow fragment both dTMP and dCMP incorporation occurs in approximately a 2:1 ratio. This is not unexpected since in their experiments there was no competition between triphosphates for the active site, while there was in the present work. This shows that the rate difference for T and C incorporation must be relatively small and can be related to those factors that lead to the stability differences between oligomers containing Z:T and Z:C pairs.[8,21] Furthermore, the contrast between the results of Nishio *et al.* [21] and our own could be ascribed, at least in part, to difference in sequence contexts which affects nucleotide incorporation.[15,29]

T is incorporated preferentially opposite K, together with some C incorporation. There is no information as to which tautomer of K is the preferred one, however several oligomers with K:T and K:C pairs are very similar in stability. It seems reasonable to assume in this case, too, that pairing at the polymerase active site of the incoming triphosphate requires an *anti*-configuration of the methoxy group and that the 2-amino-function in K confers some further stabilization, as it does in a G:C pair (Figure 4b).

The overall conclusion is that with oligomers containing K and Z the polymerase-catalyzed primer extension requires the less favoured *anti*-configuration of the methoxy group, allowing in each case the required Watson-Crick pairing. We hope to clarify this point by extending the experiments to purine analogues in which the aminooxy function is constrained in the *anti*-configuration, as it is in the P-nucleoside.

In this report we find that dAMP and dTMP were inserted opposite P and K, respectively, by *Taq* DNA polymerase (Figure 3a and b). In addition, the enzyme incorporated dCMP opposite hypoxanthine (I)[13]. These findings are helpful when primers containing P and K or P and I, are used in the polymerase chain extension reactions. Thus, such degenerate bases can be employed to decrease chain multiplicity in the first few low temperature cycles of PCR targeted to DNA, involving unknown sites. Corresponding unique primers with T, A and G instead of P, K and I, respectively, can subsequently be used for further amplification at high stringency conditions.[30]

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