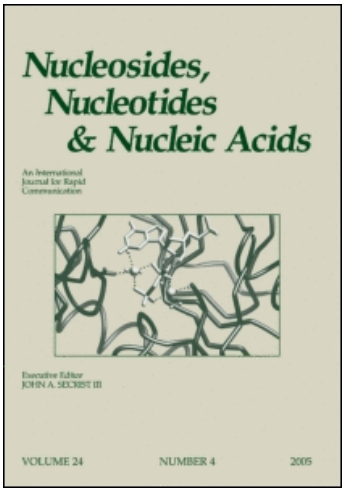

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Properties of Oligonucleotides Containing the Transition Bases P and K

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PROPERTIES OF OLIGONUCLEOTIDES CONTAINING THE TRANSITION BASES P AND K.

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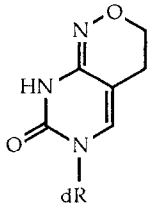
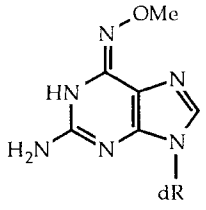
ABSTRACT. The properties of the degenerate nucleosides d**P** and d**K**, in templates and primers were determined. d**P** was copied as either pyrimidine, d**K** as either purine. In primers, an equimolar mixture of the two nucleosides functioned as a universal base equivalent in both sequencing and the polymerase chain reactions. Cloning of DNA containing d**P** or d**K** produced transition mutations *in vivo*.

The degenerate pyrimidine (d**P**) and the degenerate purine (d**K**) have been prepared (1), and incorporated into oligonucleotides. They may replace the natural DNA bases with little destabilisation, and it has been demonstrated that they could be substituted independently into oligonucleotide primers for the polymerase chain reaction (2). We have examined the templating properties of these nucleosides and demonstrate that they may be used as a mixture for primers in PCR and sequencing.



d**P**



d**K**

Template properties: To examine the behaviour of the degenerate bases when copied by *Thermus aquaticus* (*Taq*) DNA polymerase, two oligonucleotide templates were synthesised (Figure 1). One template contained six d**P** residues, and the other six d**K** residues. Each template was amplified in forty-eight separate PCR reactions using two flanking primers:

```
5'GAATTTTGACCTTCTTAAGCTT
5'GAATTTTGACCTTCTTAAGCTTGCPGGPGAPPTCPAGTPCAACCCTGGGCCCATGTTACGT 3'
                                                GGACCCGGGTACAATGCA 5'
```

Figure 1: Template and primers used for determining the template properties of dP. dK was exchanged for dP in the same sequence for determining the template behaviour of dK.

The PCR products from each template were pooled and cloned. Sixteen clones from each pool were sequenced, ensuring that the vast majority of clones derived from independent amplifications. The dP nucleotide was copied as if it were either T or dC, with T predominating by a small margin (ratio T:C was 1.5:1). The dK nucleotide was copied as if it were dG or dA, but showed a more marked preference for directing the incorporation thymidine (ratio of A:G was 7:1).

P & K in Sequencing primers: Oligomers containing either a mixture of **P** and **K** or 2'-deoxyinosine, which is often used as a "universal" nucleoside (3), were compared as sequencing primers on three plasmids containing homologous but not identical sequences. The **P/K** primer proved to be more effective, priming T7 DNA polymerase specifically from each of three related sites; the primer containing six dI residues primed well on only two of the templates and not at all on the third (manuscript in preparation).

P & K in PCR primers: To test primers containing **P/K** mixtures in PCR, a second pair of primers, again containing six modified bases was synthesised. The primers and their priming sites are shown:-

```
dI  primer: 5'atIgaIagIgaItaIcgIgg 3'
P/K primer: 5'atMgaMagMgaMtaMcgMgg 3'  M = P/K

 1st template  atcgacagtgattatcgcgg
 2nd template  attgatagtgattaccgtgg
 3rd template  atcgatagcgactatcgtgg
```

These primers direct synthesis towards the priming site used for sequencing; the pair of modified primers should amplify a 185 base-pair product from each of the three plasmids. The **P/K** pair of primers amplified the correct product from each of the templates using 50 picomoles of each primer per reaction. The products obtained using 200 picomoles of each **P/K** primer are shown in Figure 2, lanes 2-4. Even at 200 picomoles of each primer, the dI pair of primers only amplified from two of the templates; no product was obtained using 50 picomoles (results not shown). As a more stringent test of the **P/K** pair of primers, we used genomic DNA from the nematode *Caenorhabditis elegans* as a template. Each plasmid template contains a distinct cDNA

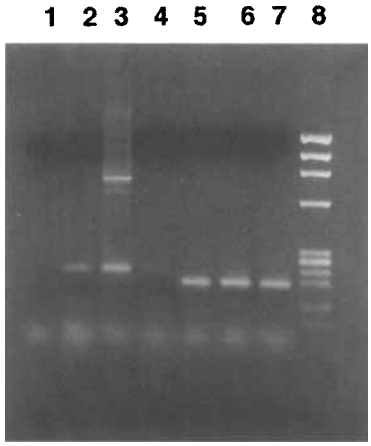


Figure 2. PCR reactions using each **P/K** primer. Lane 8 contains ØX 174 markers, lanes 7-5 shows the reaction products from each of the three plasmid templates; lanes 4 and 3 the amplification products from a genomic clone containing all three targets and lane 2 the products amplified from genomic DNA of C. elegans. Lane 1 was a negative control, lacking only template DNA.

fragment from this organism. Thus genomic DNA contains all three targets, which are larger by 45 bp, because of the presence of an intron of this length. Figure 2 shows that only a PCR product of the appropriate size was amplified from genomic DNA (lane 7); this was identical to that amplified from a genomic clone containing all three amplification targets (lanes 5&6), and 45 base-pairs larger than the products from the cDNA clones (lanes 2-4). As the three target products could not be separated electrophoretically, the PCR products were Southern blotted and hybridised sequentially with ^{32}P-labelled probes specific for each product. Each product was detected, in approximately equal amounts (not shown).

Cloning DNA containing P and K bases: To determine whether DNA containing **P** or **K** bases could be cloned in *E. coli*, the vector pUC18 was digested with a restriction enzyme and the overhangs repaired using only three dNTPs: d**P**TP or d**K**TP and two normal triphosphates. The fate of the inserted modified bases was determined by sequencing clones of the re-ligated vector.

First, pUC18 was digested with Xba I (T↓CTAGA), and the ends were repaired using only d**P**TP, dATP and dGTP. This forces d**P**TP incorporation in place of dCTP and TTP. The Klenow fragment of DNA polymerase I was used. The repaired vector was re-circularised by ligation and transformed into *E. coli*. White colonies were picked randomly and sequenced. The results are shown in the table. Of note, in all cases where

GTCGACT**CT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA**TC**CCCGGG
GTCGACT**TT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA$^{T}_{C}$$^{T}_{C}$CCCGGG
GTCGACT**TT**AGCT**AG**AGGATCC	TCTAGAG**GG**TCGA**TC**CCCGGG
GTCGACT**TT**AGCT**AG**AGGATCC	TCTAGAG**AG**TCGA**TC**CCCGGG
GTCGACT$^{T}_{C}$**T**AGCT**A**$^{G}_{A}$**A**GGATCC	TCTAGAG**GA**TCGA**TT**CCCGGG
GTCGACT**CT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA**TC**CCCGGG
GTCGACT**CT**AGCT**AG**AGGATCC	TCTAGAG**AG**TCGA**TC**CCCGGG
GTCGACT**CT**AGCT**AA**AGGATCC	TCTAGAG**G**$^{G}_{A}$TCGA**TC**CCCGGG
GTCGACT**CT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA**CT**CCCGGG
GTCGACT**TT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA**CC**CCCGGG
GTCGACT**TT**AGCT**AG**AGGATCC	TCTAGAG**GA**TCGA**TC**CCCGGG

Figure 3: Sequences of filled in restriction sites; on the left, Xba I repaired with d**P**TP, dATP, dGTP and Klenow Fragment. On the right, the BamH I sites, repaired with d**K**TP, TTP, dCTP and exo-Klenow Fragment. Above each set of sequences is the sequence obtained following repair with four normal dNTPs.

d**P**TP was incorporated in place of TTP, this was copied as T. However, following six out of twenty incorporations of dPTP as dCTP, dP was copied as T; furthermore, in clone #4, a mixed sequence was found suggesting that the **P** base in these two positions was copied more than once, sometimes as dC, sometimes as T.

A second experiment was performed using BamH I digested pUC18 (G↓GATCC). The overhangs were filled in with d**K**TP, TTP and dCTP; the enzyme used was the 3'-5' exonuclease-minus version of Klenow. Again, randomly picked clones were sequenced; the results are shown in the table. Transition mutations in both directions were obtained, in contrast to the results with d**P**TP. Incorporation of d**K**TP as dATP twenty times produced six A to G transitions, whereas incorporation as dGTP the same number of times resulted in four G to A transitions. Once again, some mixed sequences were found. Again this might be attributed to the modified base being copied more than once, and in two different ways.

Conclusion: The degenerate bases **P** and **K** are copied as either pyrimidine or either purine respectively. They can be used together in oligomers to prime DNA synthesis by *Taq* polymerase in PCR and T7 DNA polymerase in sequencing reactions. In a direct comparison, they were more effective in primers than 2'-deoxyinosine, and have been successfully used in PCR on genomic DNA. DNA containing d**P** or d**K** can be cloned in *E. coli*, replication of this DNA produces transition mutations.

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