Nucleic Acids Research, Vol. 20, No. 19 5149–5152

Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction

Paul Kong Thoo Lin and Daniel M.Brown
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Received June 2, 1992; Revised and Accepted September 11, 1992

ABSTRACT

Heptadecaoligodeoxyribonucleotides containing one or more of the bases, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P), 2-amino-6-methoxyaminopurine (K), and hypoxanthine (I) and combinations of P with K and I have been synthesised on a DNA synthesiser. The stability of duplexes containing these base-modified oligomers with P/A, P/G, K/C and K/T; P/A, P/G, I/C, I/T and I/A, I/G, I/C, I/T base pairs were compared by measuring their melting transition (T_m) values. Oligomers containing both P and K and P and I were more stable than those with I alone or with mismatches. These oligomers together with one with a P base at the 3'-end were used as primers in polymerase chain reaction (PCR) experiments. They were all effective primers except one with I alone and a triple mismatch. Thus the use of the degenerate bases P and K in primer design is established.

INTRODUCTION

In recent publications we reported separately the synthesis of monomers containing the degenerate bases P, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one and K, 2-amino-6-methoxyamine purine.[1,2] Due to the ability of both bases to exist in their amino and imino tautomers, they can potentially base pair with both A and G, and C and T respectively (see Figure 1). We found that oligomers containing one or more P bases formed DNA duplexes of comparable stability to the parent duplexes and also showed sharp transitions on melting. However, the purine analogue K formed somewhat less stable duplexes. Further evidence from NMR spectroscopy of the octamers d(CGAATPCG)$_2$ and d(CGGATPCG)$_2$ confirmed that the base pairs P/A and P/G were essentially of the Watson-Crick type.[3,4]

We also discussed the potential use of these bases in hybridisation probes and primers when either or both of these bases can be put at positions of degeneracy thus either avoiding the need for multiple-chain primers (or probes) or significantly reducing the chain multiplicity. Indeed oligonucleotides containing several P bases were effective in dot blot hybridisation and DNA sequencing experiments.[5] The most common methods applied in probing genomic or copy DNA based on protein amino acid sequences are the use of mixed probes to cover all possible codon assignments[6,7] or by inserting the 'universal' base hypoxanthine (I)[8,9,10] at sites of degeneracy .The first method is successful when the multiplicity is not too high. However machine synthesis of high multiplicity probes is probably not perfect and in any case it is not possible to check whether the single perfect sequence is present in the mixture. Moreover when hypoxanthine (I) is used the latter can base pair with low discrimination with all the bases; non specific hybridisation can occur and as a consequence result in a high background signal.[11] As for the PCR, mixed oligonucleotide primer amplification (MOPAC) technology is very often used, the rate of success usually depending on the primer complexity (up to 1024 has been used successfully). Increased primer complexity is associated with an increase in non specific priming.[12,13] In this paper we describe the synthesis of oligomers containing both bases P and K, P and I and I alone. The stability of these base modified oligomers are compared with the perfect complementary primers and those with mismatches in PCR reactions . As far as we are aware, it is the first time that degenerate bases such as P and K have been used for PCR amplification. To confirm that recognition of base P in the primer by the *taq* polymerase enzyme leads to chain extention, an oligomer with P at the 3'-end was synthesised. The potential use of bases P and K and P and I in primers, as a way of largely reducing multiplicity without background enhancement, will be discussed.

MATERIALS AND METHODS

Phosphoramidite monomers P and K were synthesised as described previously[1,2] and the deoxyinosine monomer was from Applied Biosystems Inc.

Synthesis of functionalised controlled pore glass (CPG) support carrying the 3'-O-succinate of (5-O-dimethoxytrityl-2-deoxyribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c][1,2]oxazin-7-one

This was carried out by the method of Atkinson and Smith.[14] The nucleoside loading of the functionalised CPG was 57.2μmol./g.

Oligonucleotide synthesis

Oligonucleotides (Table) were synthesised using an Applied Biosystem 380B instrument with the normal synthesis cycle. The CPG functionalised with the dimethoxytrityl derivative of the

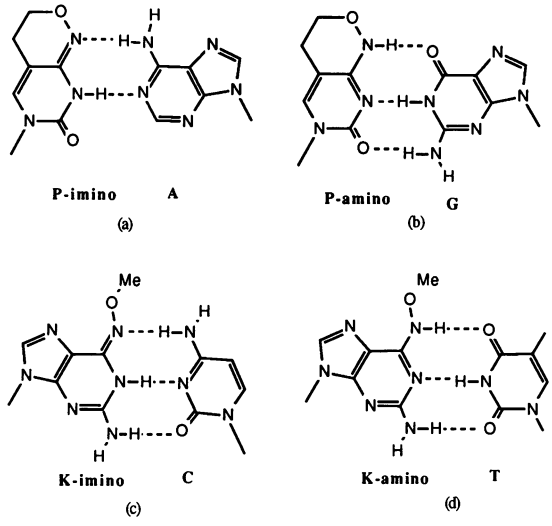

Figure 1. Pyrimidine analogue P in its imino (a) and amino (b) tautomeric form pairing with adenine and guanine. Purine analogue K in its imino (c) and amino (d) tautomeric form pairing with cytosine and thymine. Both analogues form Watson-Crick base pairs.

Table 1. Melting temperatures (T_m) of heptadecamer duplexes in 6×SSC buffer at pH 7.0.

		T_m(°C)			T_m(°C)
1.	ACTTGGCCACCATTTTG TGAACCGGTGGTAAAAC	72	7.	ACTTGICCICIATTTTG ----------C----T-G-----A---	50
2.	ACTTGGCCGCCATTTTG TGAACCGGCGGTAAAAC	75	8.	ACTTGKCCKCPATTPTG ----------C-----T--G------A----	55
3.	ACTTGGCCACCATTTTG ---------T-------C---- C----------	43	9.	ACTTGICCICPATTPTG ----------C----T-G-------A----	59
4.	ACITGICCACCITTITG ----A---C---------- T---A---	57	10.	ACTTGICCICIATTITG ----------C----C-G-----A---	53
5.	ACPTGKCCACCKTTPTG -----A----C----------T----A----	60	11.	ACTTGKCCKCPATTPTG ----------C-----C--G------A----	55
6.	ACPTGICCACCITTPTG ----A----C----------T---A----	60	12.	ACTTGICCICPATTPTG ----------C---C--A-----A----	63

nucleoside P was used to provide an oligonucleotide having the P nucleoside at the 3′-end of the oligomer.

Deprotection was complete after treatment with aqueous NH_3 at 55°C overnight. Purification was carried out by h.p.l.c. on a Waters system using a Whatman Sax partisphere or a Hichrom partisil 10 sax column and a potassium phosphate (pH 6.3) gradient in aqueous 60% formamide.

Melting transitions (T_m) of oligonucleotide duplexes

Melting transitions were measured at 260nm in 6×SSC (0.9M sodium chloride, 0.09M sodium citrate, pH 7.0) buffer at an oligomer strand concentration of ~ 3μM. Absorbance vs. temperature for each duplex was obtained using a Perkin Elmer Lambda 2 spectrometer connected with a Peltier block and temperature programmer. The temperature was increased by 1°C/min. and melting temperatures (T_m) were determined as the maxima of the respective differential curves with an errror of ±1°C.

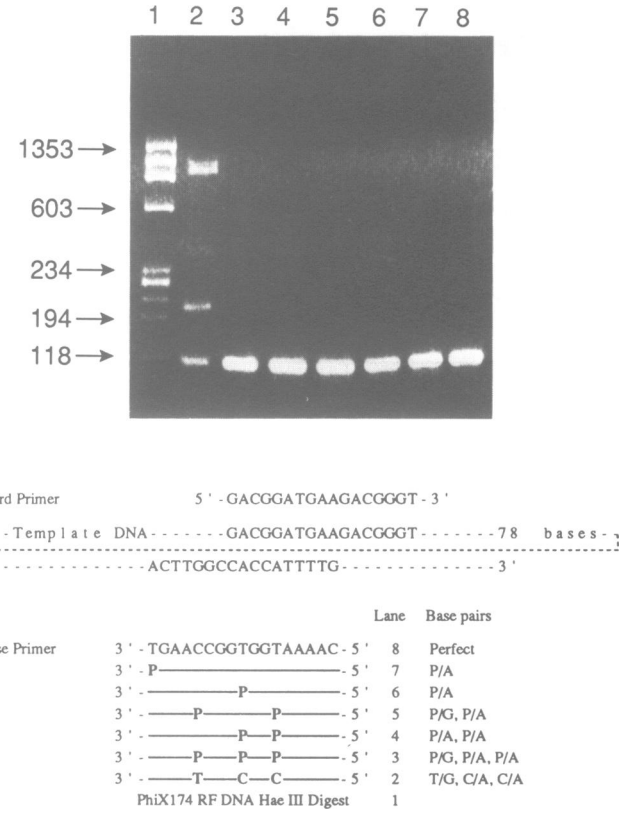

Figure 2. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 44°C for 1 min, chain extension at 70°C for 1 min, final extension at 70°C for 5 min and the number of cycles = 30.

Polymerase chain reaction

Single stranded bacteriophage M13 DNA which contained an insert corresponding to the Tyr TS gene of *Bacillus. stearothermophilus* [15] was used as template. PCR reactions were done on a Techne programmable Dri Block PHC-1 apparatus. Each 100μl reaction contained 1μM template DNA, 200pmol of each primers, 50μM of each dNTP, 5 units of *Taq* polmerase in buffer (5μl) provided by Promega and 75μl of paraffin oil. The thermal cycle was: denaturing at 92°C, annealing at 36−44°C, chain extension at 62−70°C each for 1 min, final extension at 62−70°C for 5 min and number of cycles = 30. The regions of the template to be amplified are shown on Figures 2,3 and 4. After amplification the PCR products were electrophoretically separated on standard 2% agrose (BRL Inc.) minigels, containing ethidium bromide and photographed with *uv* illumination.

RESULTS AND DISCUSSION

Examination of the Table, shows the T_m (°C) values of 17-mer DNA duplexes in 6×SSC buffer. Each of them (entries 4−12) contains four modified bases and they should be compared with the respective native (entries 1 and 2) and mismatch (entry 3) duplexes . All the DNA duplexes (entries 4−12) showed higher T_ms than the one with three mismatches(entry 3) (δT_m=10−20°C). In all cases, sharp melting transitions were found indicating cooperativity in their thermal dissociations. Entries 4, 5 and 6 have modified bases at sites of T/A, G/C,

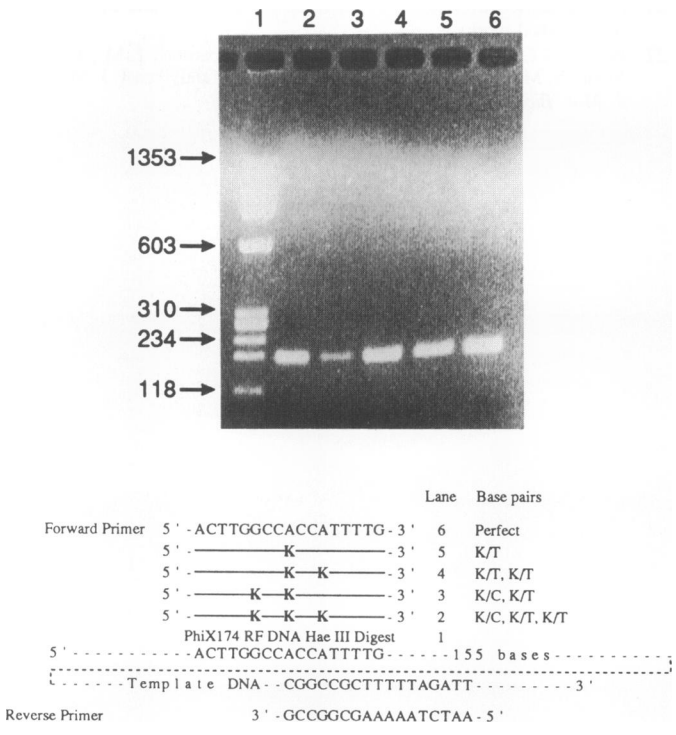

Figure 3. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 36°C for 1 min, chain extension at 62°C for 1 min, final extension at 62°C for 5 min and the number of cycles = 30.

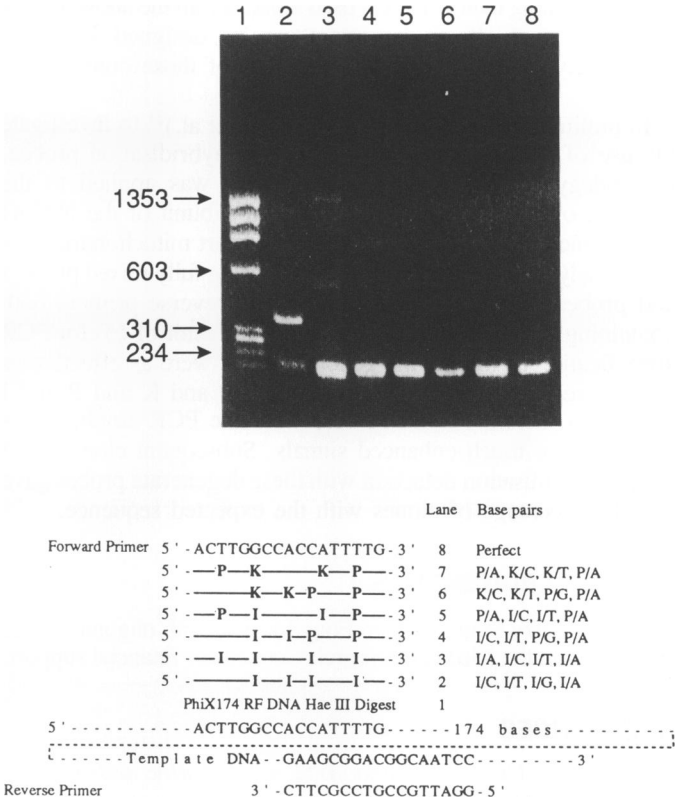

Figure 4. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 36°C for 1 min, chain extension at 62°C for 1 min, final extension at 62°C for 5 min and the number of cycles = 30.

A/T and T/A base pairs and showed the following trend: P/A, I/C, I/T and P/A = P/A, K/C, K/T and P/A > I/A, I/C, I/T and I/A. The difference between entries 5, 6 and 4 is small (~3°C) probably related to the stability of I/A and I/C base pairs.[16,17] Duplexes 8 (K/C, K/T, P/G and P/A) and 9 (I/C, I/T, P/G, P/A) showed more stable duplexes (T_m= 55 and 59°C respectively) when compared with entry 7 (T_m= 50°C) which has I/T and I/A base pairs. However the duplex in entry 12 with I/C, I/C, P/G and P/A pairs showed higher stability by 10°C than the corresponding one (entry 10) which contained solely I residues at these positions. The number of examples in the Table, while allowing relevent comparisons, is not sufficient to let firm conclusions to be drawn, particularly when relatively large sequence-related effects occur.[18] Nevertheless, the use of P as a pyrimidine analogue together with K or I as a purine may provide a more satisfactory solution to reduction in primer multiplicity. The PCR experiments provide clear evidence that this is so.

Figures 2, 3 and 4 show the results of a number of PCR experiments using primers containing P, K and I alone, P and K and P and I in combination. The template employed in these experiments was a single stranded M13 DNA which contained an insert corresponding to the Tyr TS gene of *Bacillus stearothermophilus*. In Figure 2, the forward primer was the perfect complement while all the reverse primers contained modified bases except for the positive control. It shows that all the primers with one or more P residues including the one with the P base at the 3′ end (lane 7) give the PCR product of the correct length (112 base pairs). This confirms that the enzyme *taq* polymerase recognises the P base and chain extends from it. For this to happen the P base must form a Watson–Crick base pair with A (as indeed it does[3]) since a mismatch at the 3′-end is known to result in failed amplification.[19,20] It is worth mentioning that the primer with three mismatches (lane 2) resulted in non specific priming, more than one PCR product being observed. Primers with one, two and three K bases gave a single product (189 base pairs) (Figure 3) in this case the annealing temperature was 36°C in order to achieve amplification. The lower annealing temperature is in accord with the observed T_μs of these K containing duplexes.[2] Figure 4 shows that all the primers containing P and I, P and K and I only give the expected PCR product (208 base pairs) except that in lane 2; the latter primer with four I residues exhibits non specific priming and hence is not effective compared with corresponding primers containing P and K (lane 6) and P and I (lane 4).

CONCLUSIONS

We have shown in this work that duplexes containing P and K, and P and I have better stability than the corresponding duplexes with only I. In duplexes with P and K, these bases have degenerate properties but their lower T_m values compared with the parent duplexes is probably dependent on the *syn* oriented methoxyl group of the K base, interfering with the normal Watson–Crick base pairing. In the case of duplexes containing both P and I, the latter base can form pairs with C and weakly with T but its base stacking properties contribute to the duplex stability.[16] In the PCR experiments, it was shown that P and K and P and I combinations can be used successfully with less non

specific priming than when I is used alone. From the above results it is clear that effective primers can be designed with low multiplicity, avoiding the disadvantages of those containing I alone.

In preliminary experiments (Arizmendi et al.)[21] to investigate the use of the analogues in primers and hybridisation probes, the stategy of Walker and co-workers[22] was applied to the isolation of a genomic DNA clone of a subunit of the NADH ubiquinone oxidoreductase from bovine heart mitochondria; this had already been isolated with difficulty using fully mixed primers and probes. We found that forward and reverse primers both containing P and K (multiplicity reduction factor of 15) for PCR amplification of the bovine genomic DNA were as effective as fully mixed primers. Probes containing P and K and P and I (reduction factors 11 and 21 resp.) for the PCR amplification product gave much enhanced signals. Subsequent cloning and plaque hybridisation detection with these degenerate probes gave a high percentage of clones with the expected sequence.

ACKNOWLEDGEMENTS

We thank Terry Smith and Jan Fogg for help with oligonucleotide synthesis and the Medical Research Council for financial support.

REFERENCES

1. Kong Thoo Lin, P. and Brown, D.M. (1989) *Nucleic Acids Res.*, *17*, 10373–10383.
2. Brown, D.M., and Kong Thoo Lin, P. (1991) *Carbohydrate Research*, *216*, 129–139.
3. Stone, M.J., Nedderman, A.N.R., Kong Thoo Lin, P., Brown, D.M., and Williams, D.H. (1991) *J. Mol. Biol.*, *222*, 711–723.
4. Nedderman, A.N.R., Stone, M.J., Kong Thoo Lin, P., Brown, D.M., and Williams, D.H. (1991) *J. Chem. Soc., Chem. Commun.*, *19*, 1357–1359.
5. Brown, D.M. and Kong Thoo Lin, P. (1990) *Coll. Czech. Chem. Commun.*, *55*, 213–215.
6. Ohkawa, J., Okada, N., Shinmyo, A. and Takano, M. (1989) *Proc. Natl. Acad. Sci.*, USA, *86*, 1239–1243.
7. Kennedy, J.E., Wager-Smith, K., Barzilai, A., Kandel, E.R. and Sweatt, J.D. (1988) *Nature*, *336*, 499–500.
8. Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y. and Matsubara, K. (1985) *J. Biol. Chem.* , *260*, 2605–2608.
9. Okada, N., Koizumi, N., Tanaka, T., Ohkubo, H., Nakanishi, S. and Yamada, Y. (1989) *Proc. Natl. Acad. Sci.*, USA, *86*, 534–538.
10. D'onofrio, M., Starr, C.M., Park, M.K., Holt, G.D., Haltiwanger, R.S., Hart, G.W. and Hanover, J.A. (1988) *Proc. Natl. Acad. Sci.*, USA, *85*, 9595–9599.
11. Knight, P. (1991) *The Biochemist*, *13*, 28.
12. Compton, T. (1990) in PCR protocols, A guide to Methods and Applications (Eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J.), Academic. Press Inc., New York, Chapter 5, 39–45.
13. Lee, C.C. and Caskey, C.T., (1990) in PCR protocols, A guide to Methods and Applications (Eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J.), Academic. Press Inc., New York, Chapter 6, 46–53.
14. Atkinson, T. and Simth, M., (1984) in Oligonucleotide synthesis, a practical approach (Ed. Gait, M. J.), IRL Press Limited, Chapter 3, 35–81.
15. Winter, G., Koch, G.L.E., Hartley, B.S. and Barker, D.G. (1983) *Eur. J. Biochem.*, *132*, 383–387.
16. Corfield, P.W.R., Hunter, W.N., Brown, T., Robinson, P. and Kennard, O. (1989) *Nucleic Acids Res.*, *15*, 7935–7949.
17. Kawase, Y., Iwai, S., Inoue, H., Miura, K. and Ohtsuka, E. (1986) *Nucleic Acids Res.*, *14*, 7727–7736.
18. Martin, F.H., Castro, M.M., Abou-Ela, F. and Tinoco, I. (1985) *Nucleic Acids Res.*, *13*, 8927–8938.
19. Ehlen, T. and Dubeau, L. (1989) *Biochem. and Biophys. Res. Commun.*, *160*, 441–447.
20. Newton, C.R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C. and Markham, A. F. (1989) *Nucleic Acids Res.*, *17*, 2503–2516.
21. Arizmendi, J. M., Walker, J. E., Kong Thoo Lin, P. and Brown, D. M. (unpublished work).
22. Walker, J.E., Arizmendi, J. M., Dupuis, A., Fearnley, I. M., Finel, M., Medd, S. M., Pilkington, S. J., Runswick, M. J. and Skehel, J. M. (1992). *J. Mol. Biol.* , *226*, 1051–1072.