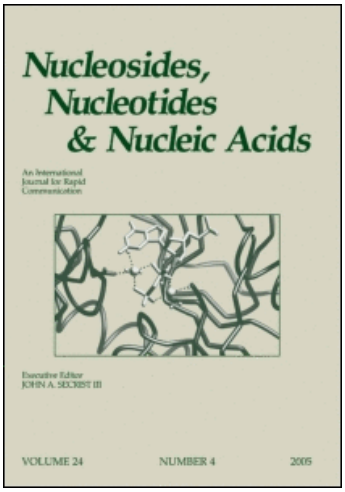

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ENZYMATIC RECOGNITION OF ACYCLIC UNIVERSAL BASE ANALOGUES IN OLIGONUCLEOTIDES

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ABSTRACT. The effects of the acyclic analogues **3** and **4** on recognition by commonly used enzymes in recombinant DNA work have been investigated. This report shows that acyclic sugars can function in place of the normal 2'-deoxyribose moiety with some enzymes, expanding the range of possible manipulations.

INTRODUCTION

Modification of nucleoside bases can alter recognition of DNA by enzymes, but the effects of modifying the deoxyribose sugar on enzymatic recognition have been studied less frequently. We have examined whether enzymes commonly used in recombinant DNA manipulations can recognise universal base nucleosides containing modified sugar residues. There have been many attempts to synthesise nucleosides containing modified bases which behave in a non-discriminatory manner towards the natural bases. Oligonucleotides containing such compounds have been used as hybridisation probes and less frequently for screening libraries and for priming polymerase catalysed chain extensions. In this limited sense the term universal has been applied to such analogues. Among these, hypoxanthine, in the form of its 2'-deoxynucleoside has been the most widely and successfully used,[1] not least because inosine is a natural nucleoside with favourable base-pairing properties.

Recently, two new candidate bases, 3-nitropyrrole,[2] **1**, and 5-nitroindole,[3,4] **2**, (Figure 1) have been compared with hypoxanthine in PCR primers. The acyclic

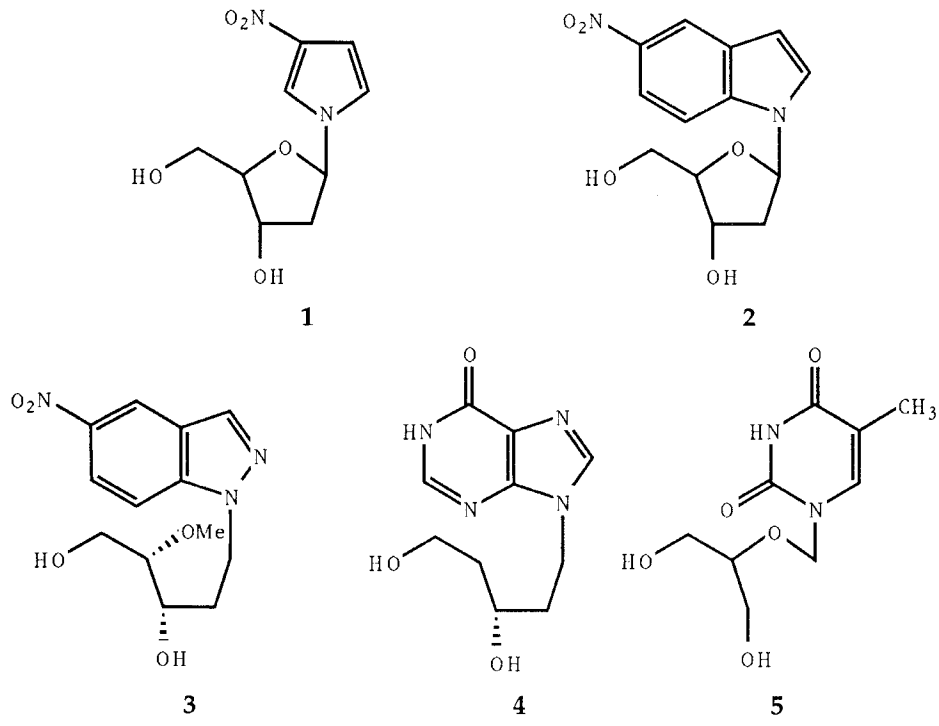

FIG. 1. Structures of nucleoside analogues. The numbers used in the text represent the modified nucleosides 2'-deoxyribosyl-5-nitroindole, **2**, and acyclic 5-nitroindazole, **3**; "I" and "i" indicate 2'-deoxyinosine and acyclic inosine, **4**, respectively.

nucleoside analogue, 1-(4-O-methyl-2-deoxy-D-ribityl)-5-nitroindazole, **3**, which resembles **2** in its base moiety has also been investigated as well as the related 9-(2,4-dideoxy-D-ribityl)hypoxanthine,[5] **4**. Oligonucleotides containing the latter compounds exhibited remarkably good hybridisation properties with very little discrimination towards the natural DNA bases, producing minimal decreases in duplex stability.[6] This was in contrast to other acyclic nucleosides which had been previously prepared[7-9] which had a strong destabilising effect on oligonucleotides containing them. The 4-O-methyl-2-deoxy-D-ribityl group is an acyclic mimic of the 2-deoxyribose residue in which the O^4-C^1 bond is absent, while the 2,4-dideoxy-D-ribityl residue lacks both O^4-C^1 and O^4-C^4 bonds. Nevertheless, both acyclic residues have very similar effects on the stability of oligonucleotide duplexes.[6]

Our intention has been to investigate the utility and broaden the scope of these acyclic nucleoside analogues, generally, in DNA manipulations. So far as we are aware, oligonucleotides containing acyclic nucleosides have not been shown to prime DNA synthesis in either PCR or sequencing reactions. We have determined the efficacy of primers containing the acyclic nucleosides derived from 5-nitroindazole, **3**, and hypoxanthine, **4**, in comparison with those containing 2'-deoxyinosine and 2'-deoxyribosyl-5-nitroindole, **2**. The experiments have been extended to oligomers containing these residues at their 3'- and 5'-termini, and using them to investigate enzymatic phosphorylation and ligation.

MATERIALS AND METHODS

Preparation of oligonucleotides. The acyclic phosphoramidite monomers of 5-nitroindazole and inosine were synthesised as previously described,[5,6,10] the 5-nitroindole phosphoramidite monomer and the 5'-phosphate-ON reagents were purchased from Glen Research, and 2'-deoxyinosine monomer was supplied by Cruachem. Oligonucleotides were synthesised on an Applied Biosystems 380B synthesiser with the normal synthesis cycle except for the acyclic monomers when a 5 minute coupling time was used. Purification was carried out by electrophoresis using a 20% polyacrylamide gel, extraction (0.5M ammonium acetate, 1mM EDTA) then concentration and desalting using a Sephadex G-25 column (NAP-10, Pharmacia).

Thermal dissociation (Tm) measurements. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer fitted with a Peltier cell. Melting transitions were measured at 260nm in PCR buffer (10mM Tris, 50mM potassium chloride, 1.5mM magnesium chloride, 0.001% w/v gelatin, pH 8.3 at 25°C) or 6xSSC (0.9M sodium chloride, 0.09M sodium citrate, pH 7), at an oligomer strand concentration of approximately 3μM. Absorbance vs temperature for each duplex was obtained at a heating and cooling rate of 0.5°C/minute and melting temperatures (Tm) were determined as the maxima of the differential curves, with an error of ±1°C.

Template preparation. The plasmid used as a template contained a 1288bp Eco47III to HindIII fragment of DNA from the dUTPase gene of the nematode worm *Caenorhabditis elegans*. The Eco47III site was blunt ended with Klenow and dNTPs and cloned in the polylinker of pBluescript II SK$^-$ (Stratagene) between the XhoI (also blunt ended) and HindIII sites. The nucleotide sequence of the

fragment is known (F. Hill and S. Linde, manuscript in preparation). All primers were designed to be complementary to the sequence 5'-GGAGGATTTGGATCCACGGG, and these are listed in Table 1. For sequencing reactions, single-stranded DNA was prepared using the helper phage M13K07[11]. For PCR reactions, double-stranded plasmid DNA was first linearized at the unique SacI site in the polylinker.

Sequencing reactions. Sanger dideoxy sequencing reactions were carried out according to the manufacturers recommendations using the commercially available Sequenase™ Version 2.0 DNA sequencing kit (USB). One picomole of the primer being tested was used to prime synthesis on single-stranded DNA. Reaction products were separated by electrophoresis on a 6% buffer gradient polyacrylamide gel. Lanes were loaded in the order TGCA.

Polymerase chain reactions. Each 50µl reaction contained: 100ng of linearized plasmid as template DNA, 200µM of each dNTP, 2.5 units of *Taq* polymerase (AmpliTaq), 5µl of 10X buffer (100mM Tris, 500mM potassium chloride, 15mM magnesium chloride, 0.01% w/v gelatin, pH8.3 at 25°C), 50pmoles of T7 primer (5'GTAATACGACTCACTATAGGGC), and 50pmoles of the primer being tested. The T7 primer sequence flanks the polylinker in pBluescript II. The reactions were overlaid with 35µl of paraffin oil. PCR reactions were carried out on a Techne PHC-3 apparatus and thermal cycling conditions were: denaturation at 96°C for 5 minutes (during which *Taq* polymerase was added), followed by 30 cycles of: denaturation at 96°C for 5 seconds; annealing at 50°C for 5 seconds (Tm range of the primers 51-60°C); extension at 72°C for 30 seconds. A final extension was performed at 72°C for 10 minutes. After cooling to room temperature, the reaction products were analysed by electrophoresis on standard 2% agarose gels. The expected size of the correct PCR product was 430 base pairs (bp).

Ligation reactions. Linkers were phosphorylated with T4 polynucleotide kinase (New England Biolabs): in 25µl reactions containing 250pmoles of each linker, 2.5µl 10xkinase buffer, 1µl 0.1M ATP and 20 units T4 polynucleotide kinase. The reactions were incubated at 37°C for 1 hr. Each reaction was heat inactivated at 65°C for 15-20 minutes and then allowed to cool slowly to room temperature to allow annealing. 50pmoles of the kinased and annealed linkers were ligated in 20µl reactions containing 2µl 10xligase buffer and 400 units T4 DNA ligase (New England Biolabs) at 15°C for 16 hrs: the ligase was heat inactivated by heating to 65°C and again annealing was carried out by slow cooling to room temperature. Restriction endonuclease digestions were carried out using either *Bgl* II (New England Biolabs), recognition site A↓GATCT, or *Bsp*E I (New England Biolabs),

TABLE 1. Primers for PCR and sequencing and their melting temperatures (Tm) in PCR buffer.

3'-	GGG CAC CTA GGT TTA GGA GG	
5'-	CCC GTG GAT CCA AAT CCT CC	68°C
5'-	--- --3 --- --- --- --- --	63°C
	--- --- --- -33 --- --- --	60°C
	--- --- --- 333 --- --- --	56°C
	--- --- --- III --- --- --	52°C
	--- --- --- iii --- --- --	51°C
	--- --- --- 222 --- --- --	56°C

where 3 is the acyclic 5-nitroindazole, i is the acyclic inosine, I is 2'-deoxyinosine and 2 is 5-nitroindole-2'-deoxyriboside.

recognition site T↓CCGGA: 20µl reactions containing 50pmoles of the annealed ligated linker, 2µl NEB 3 buffer and 10 units of the appropriate restriction enzyme were incubated at 37°C for 16 hrs. The products were analysed by electrophoresis on standard 2% agarose gels.

Lambda exonuclease digestion. PCR primers were kinased as described above. PCR reactions were carried out with kinased and unkinased primers, and the products precipitated and resuspended in 20µl water. 10µl of the DNA was digested with 5 units lambda exonuclease (GibcoBRL) and 2µl 10x lambda exonuclease buffer (67mM glycine-KOH, pH 9.4, 2.5mM Magnesium chloride) for 30-45 minutes, then heat inactivated at 65°C for 20 minutes; the reactions were carried out on a Techne PHC-3 apparatus. Products were electrophoretically separated on 2% agarose.

RESULTS

When the acyclic 5-nitroindazole, **3**, was incorporated into the oligonucleotide 5'-CAAAATGG3GGCCAAGT and the melting temperature measured, in 6xSSC buffer, against the complementary strand (5'-ACTTGGCCXCCATTTTG, where **X** is one of the natural DNA bases), there was a melting transition (Tm) range of 2°C. The decrease in the melting temperature (ΔTm 4-6°C) was similar to that for

2'-deoxyribosyl-5-nitroindole[3] and so it is clearly behaving in this respect as a universal nucleoside. A number of oligomers were prepared incorporating the acyclic 5-nitroindazole and it was found that generally they exhibited almost identical melting temperatures as the corresponding oligomers incorporating 2'-deoxyribosyl-5-nitroindole (data not included). Thus in this limited context of hybridisation the acyclic 5-nitroindazole nucleoside causes essentially no destabilisation when incorporated into duplexes. Both the acyclic 5-nitroindazole and 5-nitroindole bases appear to stabilise the duplex by stacking interactions.

A series of oligomers were prepared incorporating one to three consecutive acyclic 5-nitroindazole residues, the latter corresponding to the replacement of an entire codon. These primers were compared with analogous oligomers containing three 2'-deoxyinosine residues[12] and three acyclic inosine residues. Oligomers containing either 2'-deoxyinosine ($T_{3/4}$-$T_{1/4}$ = 9.4°C) or the acyclic inosine, **4**, exhibit similar melting temperatures, though in the case of a duplex containing **4** the melting transition was less cooperative as shown by lower hyperchromicity and a broader transition ($T_{3/4}$-$T_{1/4}$ = 21°C). The sequences of the primers and their melting temperatures (Tm) in PCR buffer are shown in Table 1. Once again their melting temperatures were comparable with corresponding oligonucleotides containing **2**.

Sequenase and *Taq* polymerase

In polymerase chain extension experiments, primers containing one, two or three consecutive 5-nitroindazole substitutions, and three consecutive 2'-deoxyinosine (I) or acyclic inosine (i) substitutions were able to prime DNA synthesis by either Sequenase (Figure 2) or *Taq* polymerase (Figure 3). In sequencing experiments the primers were at least as good as those using the unmodified primer, but in PCR the yield of product decreased as the number of substitutions increased. It is clear therefore that in sequencing experiments the acyclic 5-nitroindazole, **3**, is as effective in primers as **2**,[4] and that acyclic inosine, **4**, is as effective as 2'-deoxyinosine.

However, in PCR, compared to 2'-deoxyribosyl-5-nitroindole the acyclic analogues did not perform quite as well (four contiguous 2'-deoxyribosyl-5-nitroindole substitutions could be used in this system without any significant decrease in yield of product), but what is remarkable is that the acyclic analogue functions at all in the primers. Like 2'-deoxyribosyl-5-nitroindole and -3-nitropyrrole, the acyclic 5-nitroindazole, **3**, does not allow a 20-mer primer to function in either PCR or sequencing when all six substitutions corresponding to codon third positions are made.

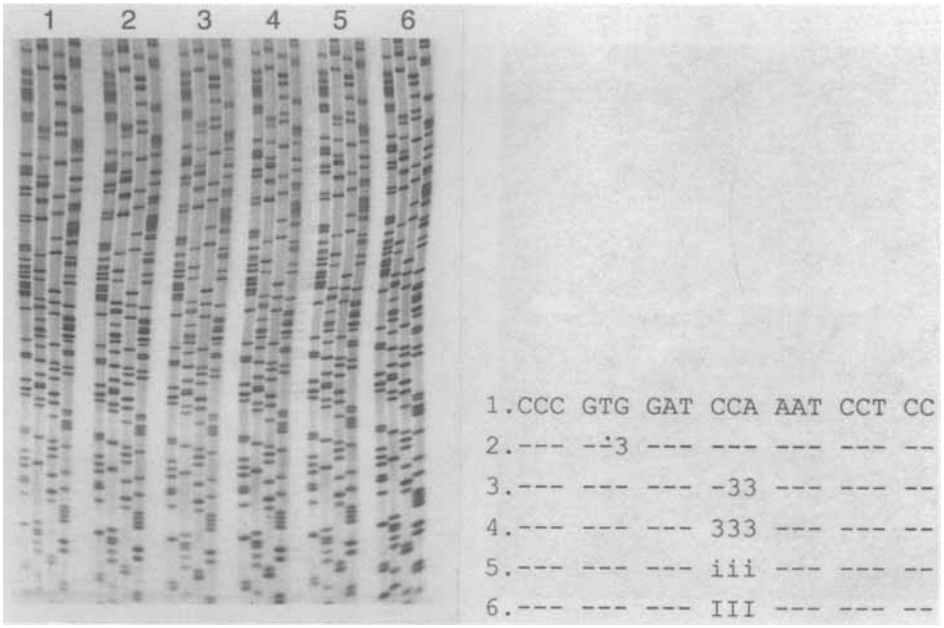

FIG. 2. Dideoxy sequencing reactions using primers containing consecutive acyclic 5-nitroindazole or inosine residues, where 3 is the acyclic 5-nitroindazole, i is the acyclic inosine and I is 2'-deoxyinosine. Lanes are loaded in the order TGCA.

2'-Deoxyinosine has been used to replace an entire codon in a primer for PCR reactions[12] and in the primer/template system used here we show that it can also be used for sequencing. Despite replacing the equivalent of an entire codon by the acyclic analogue of 2'-deoxyinosine a PCR product could still be produced, although the acyclic inosine analogue gave the lowest yield of PCR product, in contrast to 2'-deoxyinosine which gave a yield similar to that of the unmodified primer. This is in contrast to the sequencing reactions where the acyclic inosine was as effective as 2'-deoxyinosine. The Tm values of the two inosine containing primers hybridised to the same complementary 20-mer oligomer show that they have very similar transitions (52°C for 2'-deoxyinosine, 51°C for the acyclic analogue, but note that the acyclic 5-nitroindazole primer has a Tm of 56°C, Table 1). Clearly the presence of the acyclic structure does not destabilise the duplex any more than 2'-deoxyinosine itself does (opposite TGG in these duplexes). So the decrease in yield of PCR product must come as a result of poorer recognition

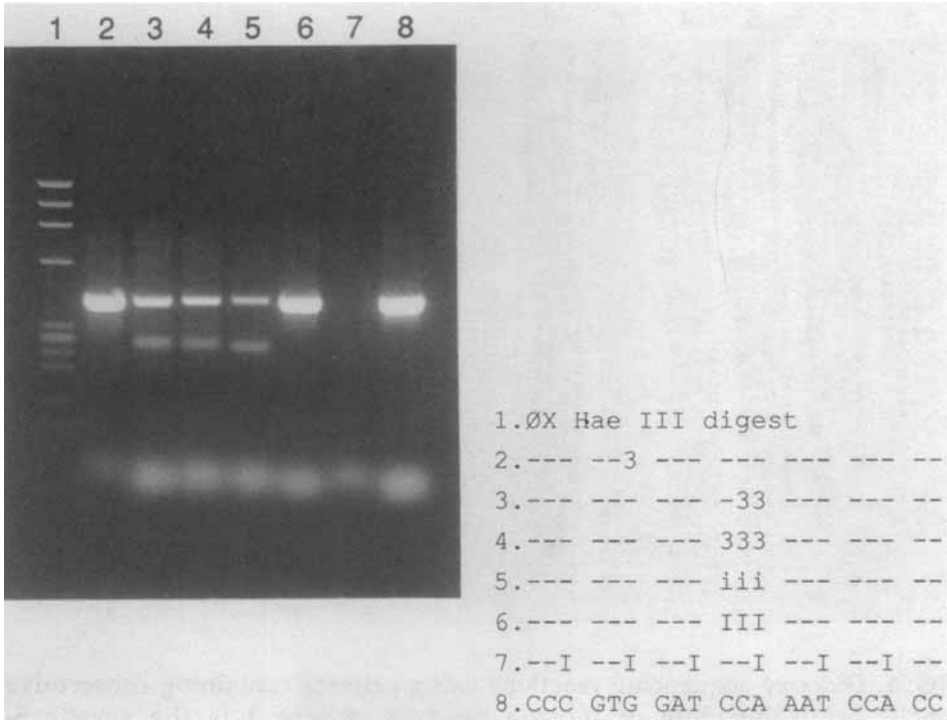

FIG. 3. PCR products using primers containing consecutive acyclic 5-nitroindazole or inosine residues, where 3 is the acyclic 5-nitroindazole, i is the acyclic inosine and I is 2'-deoxyinosine. Lane 7 contains no template, and is therefore a negative control.

by the polymerase rather than by destabilisation of the primer/template complex.

T4 polynucleotide kinase and T4 DNA ligase

A further test for the wider applicability of these bases is whether oligomers containing them are substrates for kinase and ligase enzymes. A series of linkers were prepared corresponding to the *Bgl* II linker d(GGAAGATCTTCC), where **G** is the position at which modifications were made with **1-4**. The corresponding oligomers were also synthesised with a 5'-phosphate group (phosphate 'ON'). These were synthesised to ensure that the ligation experiment did not fail due to poor kinase catalysed phosphorylation, although we have found from other experiments (not shown) that it is possible to phosphorylate both **1** and **2** with T4 polynucleotide kinase. None of the linkers containing these 5'-terminal modified

bases could be ligated with T4-DNA ligase. However, linkers containing either 2'-deoxyinosine or the acyclic inosine, **4**, at the 5'-end of the *Bgl* II linker could both be self ligated. The 2'-deoxyinosine containing linker performed as well as the unmodified one, as expected, giving products up to approximately 1000 bp (as determined from the agarose gel). The linker containing acyclic inosine at the 5'-end was also self-ligated but produced much shorter polymeric products of between 72 and 120 base pairs (Figure 4). This product was characterised by gel electrophoresis on a sequencing gel (data not shown) which gave a ladder of products up to decamers, and there was no detectable monomer present.

The polymerised linkers derived from d(GGAAGATCTTCC), where **G** is replaced by either 2'-deoxyinosine or acyclic inosine, **4**, could be digested with *Bgl* II (recognition site A↓GATCT) as might be expected. Note that the polymerisation of the *Bgl* II linker created a *Bsp*E I site (T↓CCGGA) with the modified base contained within it. When the polymerised linkers were digested using *Bsp*E I the ligation products containing unmodified and the 2'-deoxyinosine linkers were both cleaved, but those containing the acyclic inosine analogue were not.

Lambda exonuclease

The earlier work with these acyclic analogues demonstrated their potential as residues which if placed at the 3'- and 5'-ends of oligonucleotides would largely inhibit digestion by snake venom phosphodiesterase (SV PDE, 3'-exonuclease) and bovine spleen phosphodiesterase (BS PDE, 5'-exonuclease).[6] To further check whether the presence of an acyclic sugar moiety gave protection from nucleases we digested PCR products with lambda exonuclease. Lambda exonuclease is a processive 5'-exonuclease which degrades double-stranded nearly 100 times faster than long single stranded DNA.[13,14] Blunt ended DNA with a 5'-phosphate group is the preferred substrate. PCR was carried out with both kinased and non-kinased primers and the products isolated by precipitation. Treatment of non-kinased ds DNA with lambda exonuclease for 30-45 minutes showed no sign of degradation. Using ds DNA derived from kinased primers, lambda exonuclease digested the phosphorylated strand if the primer contained only natural DNA bases, including hypoxanthine. However, when primers containing acyclic nucleosides were used, the PCR products were protected from digestion, regardless of phosphorylation at the 5'-terminus. The protection afforded by acyclic nucleoside analogues was dependant on the number of residues in the oligonucleotide: with one residue and using a 30 minute digestion time the products were completely protected. However, if the digestion was carried out

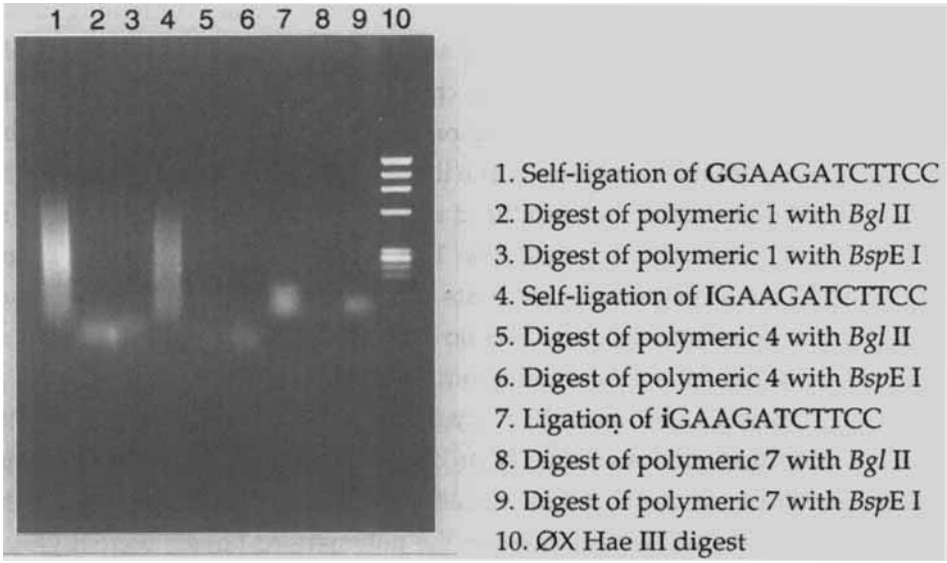

FIG. 4. Ligation and digestion of modified *Bgl* II linkers; I is 2'-deoxyinosine and i is the acyclic analogue.

for 45 minutes then oligonucleotides containing only one residue showed signs of digestion. If three residues were present, containing either modified sugar, then there was no evidence of digestion after 45 minutes. The protection appears to be attributable to the acyclic sugar residue, and not to the unnatural base. When a primer containing 2'-deoxyribosyl-5-nitroindole, **2**, was used the PCR product was digested. The preparation of ss DNA from ds DNA is often a tedious process. By using a primer containing a single 5'-terminal acyclic nucleoside, ds DNA from PCR reactions can be digested with lambda exonuclease to give the single stranded DNA provided the second primer has a 5'-phosphate group. The strand obtained is that primed by the oligonucleotide containing the acyclic nucleoside. To confirm that we were able to prepare ss DNA by this method, digested products containing acyclic residues were sequenced and normal sequencing ladders were obtained.

DISCUSSION

Numerous attempts to design a universal base have been made in the past decade for incorporation into oligonucleotides.[2,3] The oligonucleotides

incorporating the modified bases were used principally for hybridisation studies. More recently, they have been used in primers for PCR and sequencing and in probing cDNA or genomic libraries. 2'-deoxyribosyl-5-nitroindole and 2'-deoxyinosine are among the most useful analogues studied so far. We have demonstrated in this paper that the acyclic analogues of hypoxanthine and 5-nitroindazole can also prime DNA synthesis when incorporated into oligonucleotides. In addition, we have shown that those containing the acyclic sugar residue can be recognised and accepted by some enzymes (for example, in the case of T4 DNA ligase the structure of the base also appears to be important for recognition) and this should give these nucleosides a wide range of uses in recombinant DNA experiments. The results are summarised in Table 2.

Altered enzymatic recognition of DNA containing modified bases or sugars can be advantageous both *in vitro*, for recombinant DNA manipulations, and *in vivo*, for antisense DNA therapeutic strategies. *In vitro*, for example, the characterisation of PCR products, by sequencing or hybridisation, is generally facilitated by selectively removing one of the strands to prevent renaturation. A strategy using lambda exonuclease to selectively degrade strands containing 5'-phosphate groups has been described;[15] however, lambda exonuclease will attack double-stranded DNA lacking 5'-phosphate groups, albeit at a reduced rate. Incorporation of a single nucleoside containing an acyclic sugar into a primer lacking a 5'-phosphate significantly enhances the selectivity of the enzyme. In contrast, a recently described approach, using phosphorothioate primers to prevent, selectively, degradation by the T7 gene 6 exonuclease required a minimum of four modified residues[16] despite the fact that both exonucleases remove single nucleotides per step. Resistance to nucleases is an important requirement for oligonucleotides to be used as antisense DNA therapeutics, as oligonucleotides containing only natural DNA bases are readily hydrolysed by nucleases in serum. Modification of the phosphate ester backbone[17,18] and sugar residues[19] have already been shown to confer protection against digestion. The acyclic sugars used here represent promising candidates for investigation as they have been shown to prevent digestion by the nucleases SV PDE, BS PDE and lambda exonuclease.

The chemical similarity between the acyclic 5-nitroindazole and the related 2'-deoxyribosyl-5-nitroindole is obvious, and is reflected in the similarity of the hybridisation properties of oligonucleotides containing them. Likewise, oligomers containing either 2'-deoxyinosine or the acyclic inosine, **4**, exhibit similar melting temperatures, though in the case of a duplex containing **4** the

TABLE 2. Summary of enzyme recognition of modified nucleosides in oligomers described in the text.

Enzyme	Modified base	Acyclic sugar
Taq polymerase	+	+
Sequenase (T7)	+	+
T4 polynucleotide kinase	+	+
T4 polynucleotide ligase	-*	-*
Restriction enzymes‡	digested	protected
Lambda exonuclease	digested	protected
SV PDE[6]	digested	protected

*with the exception of hypoxanthine which was a substrate.
‡only *Bgl* II and *Bsp*E I have been used

melting transition was less cooperative as shown by lower hyperchromicity and a broader transition. There is an increase in entropy upon duplex formation by nucleotides with a flexible sugar chain in oligonucleotides compared to those with the 2'-deoxyribose moiety.[6] Therefore a normal oligomer has an entropic advantage over flexible oligomers. Additional stability for oligomers containing acyclic sugar residues may arise out of the extra flexibility allowing for the most favourable stacking interactions, and in the case of the acyclic 5-nitroindazole the additional nitrogen atom in the pyrazole ring will alter the electronic charge density distribution of the base compared to **2**. An extended study would need to be carried out to establish the relative contributions. It is of considerable interest that the acyclic nucleoside, **5**, resulting from the removal of the C^1-C^2 and C^2-C^3 bonds, but which otherwise provides the requisite base-phosphate separations in oligonucleotides behaves quite differently.[7,8] The stabilities of oligonucleotide duplexes containing this analogue decrease rapidly with increasing substitutions. This may be accounted for in those cases by poor stacking interactions by the thymine residues of **5** to counteract stability loss due to the acyclic sugar residues.

One obvious advantage of incorporating acyclic nucleoside analogues in oligonucleotides is that they are frequently resistant to digestion by nucleases,[6] and may therefore have potential in antisense DNA therapeutic strategies. In this connection one of the major drawbacks to the use of oligonucleotides containing only natural DNA bases is that they are readily hydrolysed by nucleases within eukaryotic cells. Oligonucleotides containing acyclic nucleosides are known to be

poor substrates for nucleases, and they may therefore be of use in antisense agents.

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