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Non-hydrogen bonding 'terminator' nucleosides increase the 3′-end homogeneity of enzymatic RNA and DNA synthesis

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ABSTRACT

We report the use of novel non-polar nucleoside analogues as terminators of enzymatic RNA and DNA synthesis. Standard 'runoff' RNA synthesis by T7 RNA polymerase gives RNA products which have ragged ends as a result of transcription which often extends beyond the end of the template DNA strand. Similarly, the Klenow fragment of *Escherichia coli* DNA polymerase I tends to run past the end of the template strand during DNA synthesis. We report here that certain non-hydrogen-bonding nucleoside analogues, when placed at the downstream 5′-end of a template DNA strand, cause the polymerases to stop more abruptly at the last coding nucleotide. This results in a considerably more homogeneous oligonucleotide being produced. Three novel nucleosides are tested as potential terminators: 4-methylindole β-deoxynucleoside (M), 1-naphthyl α-deoxynucleoside (N) and 1-pyrenyl α-deoxynucleoside (P). Comparison is made to an abasic nucleoside (φ) and to unterminated synthesis. Of these, M is found to be the most efficient at terminating transcription, and both P and M are highly effective at terminating DNA synthesis. It is also found that the ability of a nucleoside to stall synthesis when it is internally placed in the template strand is not necessarily a good predictor of terminating ability at the end of a template. Such terminator nucleosides may be useful in the preparative enzymatic synthesis of RNA and DNA, rendering purification simpler and lowering the cost of synthesis by preventing the uptake of potentially costly nucleotides into unwanted products.

INTRODUCTION

In vitro RNA transcription (1–4) is commonly used in the synthesis of milligram amounts of RNA oligonucleotides (5–6) and in the preparation of longer RNA strands as well (7). The standard approach for the synthesis of short RNA oligonucleotides is the use of synthetic DNA template strands which contain an RNA polymerase promoter at the 3′-end (1–4). The 5′-end of the RNA product is well-defined by the transcription start site immediately following the promoter, but the 3′-end is less well-defined. When the polymerase reaches the end of the DNA template strand, it is common for the enzyme to add one or more additional nucleotides to the RNA chain (1–4).

This is undesirable for a number of reasons. First, the separation of an oligonucleotide only one base longer than the desired product can be difficult and time consuming, and thus purification to a homogeneous product can be problematic. Secondly, nucleotides are wasted in the production of a considerable amount of unwanted longer RNA strands; this is more of a concern when especially costly nucleotides (such as isotopically labeled ones for NMR studies) are being used (5,6). Finally, ragged termini on the RNA transcripts can hamper further manipulations, such as ligation reactions, which require a homogeneous 3′-end (8,9).

For the preparation of longer RNA strands using a DNA plasmid-derived template, one approach to generation of homogeneous ends is the use of a ribozyme cassette which can be cloned into the template at or near the desired 3′-end (7). Following transcription, the ribozyme motif self-cleaves to give the transcript a well-defined 3′ terminus. For shorter RNA oligonucleotides it is common to use a single-stranded synthetic DNA template which contains a double-stranded RNA polymerase promoter at the 3′-end (1–4). Ribozyme-mediated post-transcriptional cleavage is less practical to apply to shorter RNA oligonucleotides since the synthetic DNA template would be increased in length by dozens of nucleotides. Moreover, hammerhead-type ribozyme cleavage generates 2′,3′-cyclic phosphate termini, whereas 3′-hydroxyl termini are needed for some applications.

A similar problem of 3′-end heterogeneity also exists for enzymatic synthesis of DNA sequences. Synthetic oligodeoxynucleotide templates are used in the production of DNA oligonucleotides for NMR studies (10). For DNA synthesis of oligodeoxynucleotides using Klenow fragment (KF) of *Escherichia coli* DNA polymerase I, a primer is either added to or is part of the template (10). Elongation of this primer to the end of the single-stranded template then gives the desired product. However, as with RNA synthesis, undesired products one or two nucleotides longer are common (10,11).

Here we describe a new approach to making enzymatic synthesis of RNA and DNA oligonucleotides with more homogeneous 3′-ends than was previously possible. We find that the addition of a single non-coding nucleotide analogue to the 5′ terminus of the template DNA strand can result in much more efficient and specific termination at the desired site (3′-end of the product). The use of

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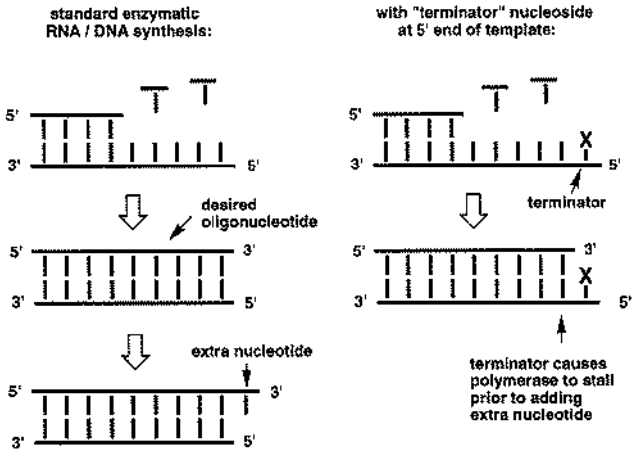

Figure 1. Illustration of poor termination which often occurs during enzymatic RNA and DNA synthesis, yielding oligonucleotides one base longer than desired. On the right is shown how end placement of a 'terminator' nucleoside can cause a polymerase to stop abruptly at the desired site.

such 'terminator' nucleosides results in the production of much cleaner RNA and DNA oligonucleotide products, often in greater yields, and with more efficient use of nucleotides.

RESULTS

Our initial studies of non-polar DNA base analogues and their interactions with DNA and RNA polymerases indicated that some of these unusual nucleosides were efficient at stalling polymerase enzymes. This prompted us to study some of the most efficient terminators for their potential utility in enzymatic DNA and RNA synthesis; placement at the end of a template might stop polymerization at the desired nucleotide (Fig. 1). We therefore constructed several DNA template sequences which could be used to test both DNA and RNA polymerases. We compared the oligonucleotide products of synthesis using normal DNA templates to those with templates containing our non-polar nucleosides at either internal or 5′ terminal positions.

Nucleoside design considerations

We have recently undertaken a study of non-polar isosteric analogues of the naturally occurring Watson–Crick bases (12,13). These have been designed to test the importance of hydrogen bonding and shape complementarity in nucleic acid stability (13) and for information transfer via enzymatic polymerization. We observed that when certain of these non-polar nucleoside analogues are included in a single-stranded DNA template, polymerases are very inefficient at inserting normal Watson–Crick nucleoside triphosphates across from them or continuing polymerization past them (see below). In general, nucleoside analogues which are sterically or electronically incompatible with the active sites of polymerases might be especially effective at stopping nucleic acid polymerization at a specific site.

The 4-methylindole β-nucleoside (**M** in Fig. 2A) was designed to mimic the overall shape of deoxyadenosine, but without hydrogen bonding groups. It and the related 4,6-dimethylindole nucleoside have been shown to pair very poorly with natural Watson–Crick bases in duplex DNA (13). The α-naphthalene

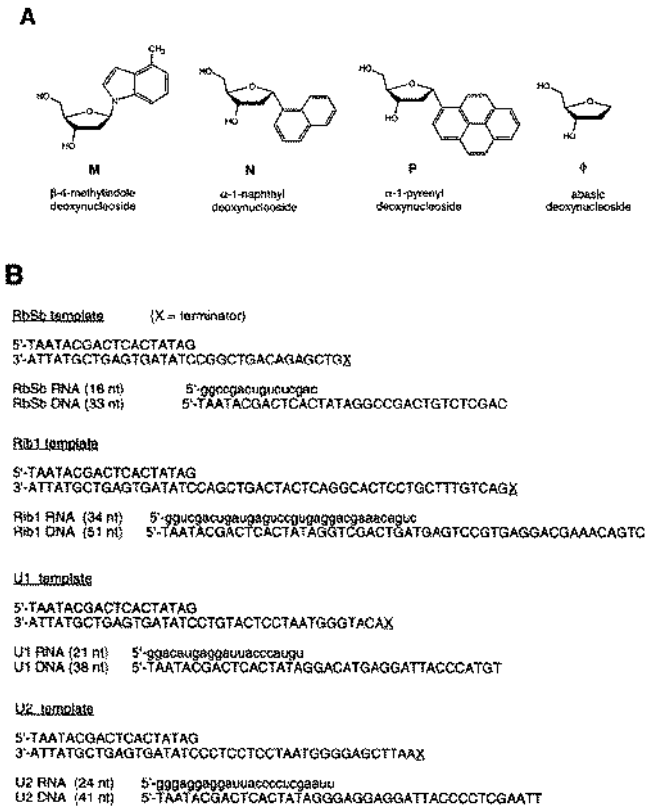

Figure 2. (**A**) Structures of non-polar nucleosides used as potential terminators of RNA transcription and DNA elongation. Note that **N** and **P** nucleosides have alpha configurations about the C1′ carbon. (**B**) Template DNA sequences used for RNA synthesis by transcription with T7 RNA Polymerase and for DNA synthesis with the large fragment of *E.coli* DNA Polymerase I (Klenow fragment, KF). Upper case letters indicate the DNA template and 18 nt T7 promoter for RNA transcription. The sequence of the expected RNA product is shown in lower case letters; its length is indicated in parentheses following the sequence designation. For DNA synthesis, elongation proceeds from the 3′-end of the 18 nt T7 promoter which acts as a primer for DNA polymerization. X indicates the terminator base which is **M**, **N**, **P** or ϕ.

nucleoside (**N**) is slightly larger than a purine base; because of its lack of polar hydrogen bonding groups, its size, and its non-natural α-configuration, it is expected to pair poorly in duplex nucleic acid and hence should be a very poor template for polymerases. The α-pyrene nucleoside (**P**) not only has the unnatural anomeric configuration, but it also possesses an even larger aromatic system whose size approximates that of a full Watson–Crick base pair; these steric demands might be expected to exclude a pairing nucleotide at the enzyme active site. This pyrene nucleoside also is of considerable interest as a biophysical probe because of its fluorescence properties (Paris,P. and Kool,E.T. manuscript in preparation). Finally, an abasic nucleoside (ϕ) (14) is included in these studies to test whether any effects observed are specifically due the 'base' moiety of the nucleoside rather than the sugar–phosphate backbone.

Nucleoside synthesis

The indole nucleoside **M** was prepared in a straightforward manner using the procedure previously described for the 4,6-dimethylindole analogue (12). Naphthalene and pyrene analogues **N** and **P** were synthesized in only two steps from 1-bromonaphthalene and

1-bromopyrene, using a cadmium-mediated aromatic coupling procedure developed in our laboratory (15). All three base-coupled compounds were deprotected, converted in good yield to the 5′-protected DMT ethers and then the 3′ phosphoramidite derivatives using standard methods. The abasic nucleoside and its phosphoramidite derivative were prepared by the published procedure (14). These four phosphoramidites were incorporated into DNA using standard automated coupling cycles.

Template sequences for testing of terminators

Four different templates were constructed to test the ability of these nucleotides to correctly terminate RNA and DNA polymerization at the end of the template (Fig. 2B). In the four sequences, two different bases (A and G) are situated at the last template position to test nearest neighbor effects. The templates are single-stranded and possess T7 promoters at the 3′-end. An 18 nucleotide (nt) top strand acts both as part of the promoter for RNA synthesis and as a primer for DNA synthesis. The expected RNA products of the four templates range from 16 to 34 nt in length. The RNA product of the shortest template (RbSb) is a substrate for a hammerhead ribozyme that is encoded by the longest template (Rib1). The other two templates (U1 and U2) code for RNA hairpins that are recognized by the R17 coat protein; they were used by Uhlenbeck and co-workers to optimize the *in vitro* T7 RNA polymerase system for the synthesis of short RNAs (1,2). The products of RNA synthesis were monitored by gel electrophoresis using 5′-end-labeling by initiating transcription with [γ-^{32}P]GTP or internal labeling with [α-^{32}P]ATP or [α-^{32}P]UTP. For DNA synthesis with Klenow fragment, the 5′-end of the primer strand is radiolabeled, and elongation products are monitored by PAGE. Expected product lengths for DNA synthesis range from 33 to 51 nt.

Internal placement of terminators: effects on RNA and DNA synthesis

Since a number of modified DNA bases are known to act within template strands as stops to polymerases, we decided to compare effects of the new nucleosides both internally and at the terminus. We first tested the qualitative effects of nucleosides **M**, **N**, **P** and ϕ on RNA and DNA synthesis when they are internally located in a short template strand (Fig. 3). Templates with natural bases at the same position were examined for comparison. For RNA synthesis with T7 RNA polymerase, all four non-natural nucleosides cause termination with high efficiency (Fig. 3B). There is little or no incorporation of rNTPs across from the non-polar bases or the abasic site, and there are virtually no longer products arising from read-through beyond the sites in question. With the natural nucleotides at this position, synthesis proceeds as expected to the end of the template, and only small amounts of products from normal abortive synthesis are visible at the position where strong stops are seen with the non-natural nucleosides.

For DNA synthesis with KF, the internally-placed non-natural nucleosides also act as strong terminators (Fig. 3C). However, in contrast to the RNA results, all have nucleotides inserted opposite them. The enzyme is apparently slow to elongate past the unusual base pairs, since very small amounts of full length products are seen. Of the four non-natural structures, **M** gives the most full length (and longer) products, although this is minor relative to the amount of truncated product generated after addition of a base across from **M**.

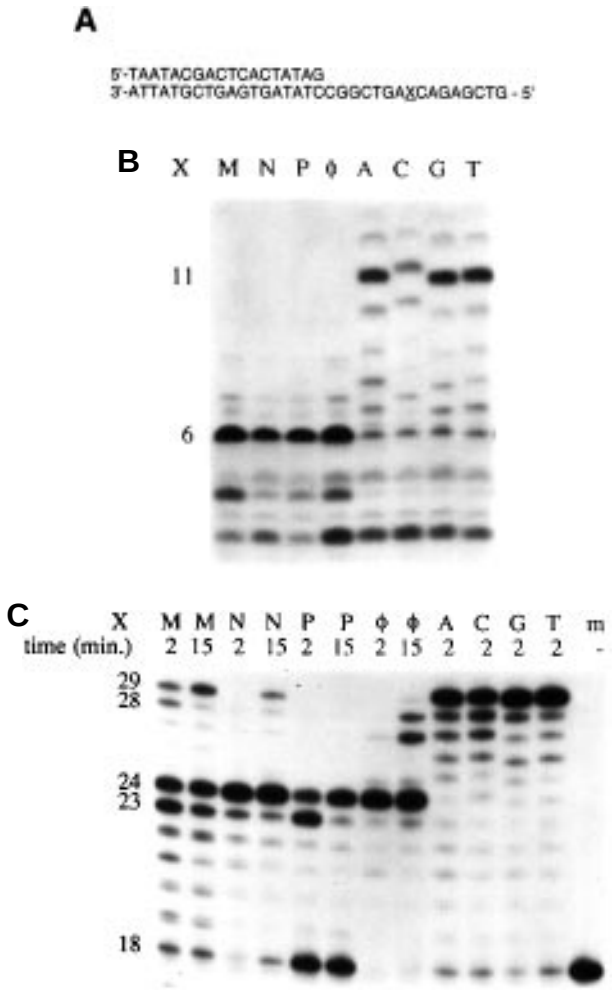

Figure 3. The efficiency of termination at sites within the template sequence. (**A**) Template sequence used to test efficiency of terminating bases at internal position. X indicates the variable natural (**A**, **C**, **G** or **T**) or terminator base (**M**, **N**, **P** or ϕ). (**B**) Autoradiogram of PAGE analysis of RNA synthesis products using T7 RNA polymerase and template with internal non-polar nucleoside analogues. (**C**) Autoradiogram of PAGE analysis of DNA synthesis products using Klenow fragment (exo$^-$) DNA polymerase and template with internal non-polar nucleoside analogues.

Nucleosides **N** and **P** also have nucleotides inserted opposite them, but give very little full length product. Overall, at internal positions, all four compounds act as efficient terminators for both RNA and DNA synthesis, although with DNA synthesis they seem to allow insertion opposite themselves prior to stalling.

Effects of end-placed terminators on RNA synthesis

We then tested the potential terminators at the 5′-end of the templates. T7 RNA polymerase reactions using normal templates and ones with terminator nucleosides at the 5′-end reveal that the non-polar analogues can decrease the appearance of products longer than expected (Fig. 4). For the standard RbSb template (without a terminator) there is a substantial amount of transcription product with one extra base; quantitation of radioactivity in these bands shows the amount of *n*+1 (17mer) product is 45% that of the desired full-length 16 nt oligomer (Table 1). In addition, there are at least four bands of yet greater length present. These bands

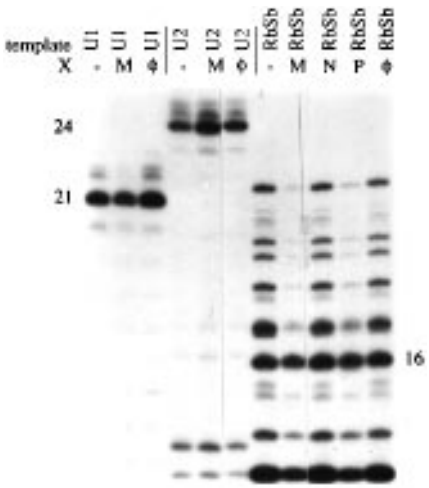

Figure 4. Effect of terminators at the end of the template on RNA synthesis products. Autoradiogram of PAGE analysis of RNA synthesis products using T7 RNA polymerase and templates with non-polar nucleoside analogues (X) at their 5′ termini. Lengths of the expected full-length products are indicated on the side of the gel.

are due to self-priming of the RNA product (results not shown and ref. 16) where nascent transcripts with self-complementary 3′-ends form a duplex which is elongated by the polymerase. The actual full-length product was identified by isolating various bands labeled internally with an [α-^{32}P]rNTP, removing the 5′ triphosphate with calf intestinal phosphatase, 5′-end-labeling with ATP and T4 polynucleotide kinase, and comparing their gel mobility to that of a chemically synthesized, 5′-end-labeled RNA marker with the same sequence as the expected product (results not shown).

Addition of 4-methylindole β-deoxynucleoside (**M**) to the 5′-end of the RbSb template significantly decreases the amount of product >16 nt. The 17mer and longer bands appear quite significantly lighter on the autoradiogram (Fig. 4); quantitation shows that the n+1 band drops from 45% (when unterminated) to 20% of the full-length product (Table 1). The terminator efficiency, defined as the *n*/*n*+1 ratio for terminated template divided by the ratio for unterminated template, averaged 2.3 for multiple experiments, meaning that the terminator cuts the relative *n*+1mer product amount by 2.3-fold.

The decrease in *n*+1mer was found to be less when the RbSb template is terminated with the naphthalene α-nucleoside (**N**); the terminator efficiency in this case is 1.4, corresponding to a 15% drop in this unwanted product. Results for the pyrene α-nucleoside (**P**) were virtually the same, with a terminator efficiency of 1.7 (Table 1). The nucleoside lacking a base altogether (ϕ) gave no termination at all, with slightly greater amounts of *n*+1mer being observed than with the unterminated template; this establishes that the bases (not the backbones) of **M**, **N** and **P** are responsible for their termination properties.

To test the generality of termination, we then examined three other templates for RNA synthesis; we compared the most successful terminator (**M**) from the previous experiments with results with the abasic nucleoside ϕ and the unterminated template (Fig. 4). For the U1 and U2 templates, termination with **M** also decreases the percentage of *n*+1mer products present. Quantitation of the bands shows that **M** improves the *n*/*n*+1mer ratio by 1.7-fold. The abasic nucleoside slightly increases the *n*/*n*+1 ratio for the U1 template

Table 1. Effects of terminators on RNA synthesis

template	terminator	expected RNA length	n / n+1 [a]	terminator efficiency [b]
RbSb	none	16	2.2 ± 0.8	--
RbSb	M	"	5.1 ± 1.9	2.3
RbSb	N	"	3.3 ± 1.7	1.4
RbSb	P	"	3.4 ± 0.6	1.7
RbSb	ϕ	"	1.4 ± 0.4 [c]	0.7
U1	none	21	2.6 ± 0.7	--
U1	M	"	5.1 ± 1.5 [c]	1.7
U1	ϕ	"	3.5 ± 1.0 [c]	1.1
U2	none	24	1.2 ± 0.2	--
U2	M	"	2.0 ± 0.2	1.7
U2	ϕ	"	2.0 ± 0.6 [c]	1.5
Rib1	none	34	1.9 ± 0.4	--
Rib1	M	"	0.8 ± 0.2 [c]	0.5
Rib1	ϕ	"	1.2 ± 0.4 [c]	0.7

Nucleotides **M**, **N** and **P** are placed at the 5′-end of the template strand, and transcription is carried out with T7 RNA polymerase. For comparison are the results with ϕ (an abasic nucleoside) and with no terminator present. '*n*' is full-length product; *n*+1 is the product resulting from addition of an extra nucleotide.

[a]Quantitation of ^{32}P labeled products by phosphorimaging. Error limits are standard deviations for multiple experiments.

[b]Efficiency is defined as the (*n*/*n*+1) ratio for terminated case divided by the ratio with no terminator.

[c]Error is estimated at ±30% in single experiments.

(10% relative decrease in *n*+1mer), but gives termination roughly equal to that of **M** for the U2 template (Table 1). When the longest template (Rib1) is terminated with the **M** base, an apparent ~2-fold increase in the relative amount of the undesired *n*+1mer product is observed, and results are similar with the abasic nucleoside ϕ. Comparison of the results for templates RbSb and U1, which have different end bases, shows that the terminator **M** gives the same efficiency.

Examination of the overall results shows that four unterminated templates give *n*+1mer amounts which range from 38 to 83% of that for the desired full-length product. The most efficient terminator nucleoside is the 4-methylindole nucleoside **M**, which is effective in three of four cases, giving a ~2-fold reduction in longer products. The abasic nucleoside has little or no effect on product lengths. Transcription reactions both with 500 μM and with 1 mM NTP concentrations gave identical results. In addition, examination of efficiency at different time points showed that relative efficiency of **M** termination stayed approximately constant; however, we did observe slower RNA synthesis overall with the terminated template (data not shown).

Effects of terminators on DNA synthesis

The same templates were then used to study nucleosides **M**, **N** and **P** as potential terminators for DNA synthesis. Comparison was again made with abasic nucleoside ϕ and the unterminated templates. Experiments were carried out with the Klenow fragment of DNA polymerase I, using both a mutant enzyme lacking the 3′–5′ exonuclease activity (designated exo$^-$) and the wild type (exo$^+$).

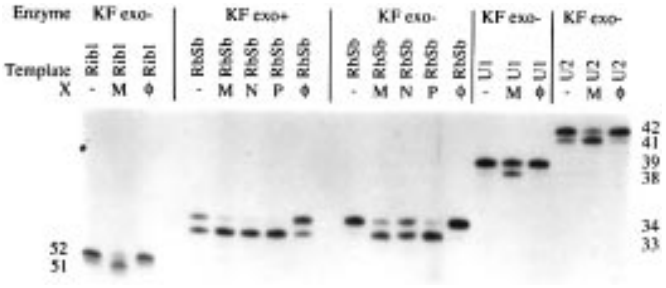

Figure 5. Effect of terminators at the end of the template on DNA synthesis. Autoradiogram of PAGE analysis of DNA synthesis products using the Klenow fragment of DNA polymerase I. Templates have non-polar nucleoside analogues (**X**) at their 5′ termini. Lengths of the expected full-length products (and *n*+1 products) are indicated on the side of the gel. The reactions with the longest template (Rib1) are loaded before the other samples in order to obtain better resolution between the *n* and *n*+1 products of primer elongation.

Table 2. Effects of terminators on DNA synthesis

template	terminator	expected DNA length	exo -		exo +	
			n / n+1 [a]	terminator efficiency [b]	n / n+1 [a]	terminator efficiency [b]
RbSb	none	33	0.2 ± 0.2	--	1.3 ± 0.4 [c]	--
RbSb	M	"	2.3 ± 0.4	14.9	5.1 ± 1.5 [c]	4.0
RbSb	N	"	1.7 ± 0.7	9.9	7.4 ± 2.2 [c]	5.9
RbSb	P	"	4.4 ± 0.3	32.2	11.0 ± 3.3 [c]	8.7
RbSb	ϕ	"	0.2 ± 0.1	1.0	0.6 ± 0.2 [c]	0.5
U1	none	38	0.10 ± 0.01	--		
U1	M	"	0.7 ± 0.2	6.4		
U1	ϕ	"	0.10 ± 0.01	0.8		
U2	none	41	0.5 ± 0.1	--		
U2	M	"	2.00 ± 0.02	4.1		
U2	ϕ	"	0.3 ± 0.1	0.6		
Rib1	none	51	0.8 ± 0.2 [c]	--		
Rib1	M	"	2.1 ± 0.1	2.7		
Rib1	ϕ	"	0.7 ± 0.2	1.0		

Nucleosides **M**, **N** and **P** are placed at the 5′-end of template strands, and synthesis is carried out with KF DNA polymerase (exo$^-$ or exo$^+$). For comparison are the results with ϕ (an abasic nucleoside) and with no terminator present. '*n*' is full-length product; '*n*+1' is the product resulting from addition of an extra nucleotide.

[a]Quantitation of ^{32}P-labeled products by phosphorimaging. Error limits are standard deviations for multiple experiments.

[b]Efficiency is defined as the (*n*/*n*+1) ratio for terminated case divided by the ratio with no terminator.

[c]Error is estimated at ±30% in single experiments.

The results show that the non-natural nucleosides act as very efficient terminators of DNA synthesis. Experiments with KF (exo$^-$) using the RbSb template show (Fig. 5; Table 2) that the unterminated template gives fully five times as much *n*+1 product as the desired full-length product. The addition of the terminators reverses this unfavorable ratio, giving the desired product as the major band. The terminator **M** gives 2.3 times as much full-length product as *n*+1mer, improving the relative full-length amount by almost 10-fold (its terminator efficiency). The naphthalene terminator **N** also worked very well (with a terminator efficiency of ~10) but less so than 4-methylindole **M**. Finally, the pyrene terminator **P** was quite effective, giving 4.4-fold more full-length product than *n*+1mer (an efficiency of >30). By comparison, the template ending with the abasic nucleoside ϕ gives results identical to those with no terminator at all (Table 2).

Experiments with same RbSb templates but with the wild-type KF (exo$^+$) enzyme also show substantial increases in the desired product when the three non-natural nucleosides are used as terminators (Fig. 5; Table 2). With the exonuclease activity, the undesired *n*+1mer product in the unterminated case is lowered significantly, although the band is still 79% the intensity of the full-length product. With the terminators **M**, **N** and **P** the amount of undesired product becomes very small; the percentages of *n*+1 product relative to the desired product range from 19% for **M** to 9% for **P**. With this enzyme, the abasic nucleoside is seen to increase the amount of undesired product, with an efficiency less than one.

Using the (exo$^-$) KF mutant, we then tested the generality of termination with the three other templates using nucleoside **M**, compared to the abasic nucleoside and the unterminated case. Results show that DNA synthesis on the unterminated templates results in greater amounts of *n*+1mer than the actual desired product, with the latter making up only 10–50% of the two bands (Fig. 5). In all three cases, addition of the terminator greatly increases both the absolute and relative amounts of desired full-length product, with terminator efficiencies ranging from 2.7 to 6.4 (Table 2). The abasic nucleoside again has either no effect or a negative effect on desired product.

DISCUSSION

The present studies show clearly that a single non-hydrogen-bonding nucleoside added to the end of a template strand can significantly improve the ratios of desired oligonucleotide products to longer undesired ones. Interestingly, it is also clear that termination ability of nucleosides at positions internally situated in a template is not necessarily a good predictor of their properties when placed at the end of a template. For example, the three analogues **M**, **N** and **P** are only moderate terminators of RNA synthesis at the template end, but are strong terminators internally. It is possible that the strong tendency for termination in this case arises in part from the proximity of the site in question to the promoter, since RNA synthesis is aborted more easily in the first few nucleotides (17). However, our control sequences (with natural nucleotides at the varied position) show very little tendency for abortive synthesis, indicating that by far the majority of the termination is caused by the non-natural nucleoside structures.

Another example of differential behavior internally and at the terminus is the abasic nucleoside, which acts as a strong terminator internally but causes no termination at all at the end of the template. It appears that in general it is not safe to extrapolate results within a template to expectations at the terminus. Many nucleoside analogues have been studied for their mutagenic potential, and a large number of these cause pauses at internal sites (18–21); however, our results indicate that until they are studied at the terminus it is unclear whether they can act as efficient end terminators.

Also interesting is the finding that in DNA synthesis, all three nucleoside analogues in this study allow a natural nucleotide to be inserted opposite themselves when they are internally located. However, when the analogues are at the end of the template, they strongly inhibit this insertion. It is unclear at present what is the origin of this difference in behavior with context. It seems likely that end termination simply causes the enzyme to pause at the unusual

template base after incorporating the last normal nucleotide, and that this pause is long enough to allow enzyme dissociation from the template. In the terminated RNA synthesis we observe substantial lowering of longer products and a slight lowering of full-length product at early reaction times; this finding is consistent with the terminator causing a pause which eventually leads to dissociation. In the terminated DNA synthesis we observe a large decrease in *n*+1 products and a large increase in full-length products; we attribute this to the very strong tendency of the KF enzyme to add an extra nucleotide. Causing dissociation prior to this event allows the enzyme to make the desired product a greater fraction of the time.

In RNA synthesis, the 4-methylindole nucleoside **M** shows repeatable and significant (~2-fold) efficiency in two different nearest neighbor contexts. It gives stronger end termination than do the naphthalene and pyrene analogues **N** and **P**. It is possible that the difference arises because **M** has the natural β-anomeric configuration. It may be that yet larger non-hydrogen bonding base analogues which possess β-configuration might further improve termination efficiency. One such possibility might be a beta-anomeric analogue of pyrene nucleoside **P**; future studies will test the terminating ability of this non-natural nucleoside in comparison to the present results.

In DNA synthesis, all three terminators dramatically improved the amounts of full-length products with the KF enzyme. It appears that both β- and α-oriented bases can be effective. The fact that the pyrene analogue is somewhat better at termination than the other two may be because its size is larger, allowing it to occupy a greater volume in the active site and excluding more effectively the incoming nucleoside triphosphates.

The terminator nucleosides studied here are more successful in application to DNA synthesis than to RNA synthesis, at least for the enzymes studied. It is also true that the magnitude of the problem is greater for DNA synthesis, in that the tendency for T7 RNA polymerase to add extra nucleotides is much less than that for KF DNA polymerase. Nonetheless, a 2-fold reduction in *n*+1mer product during RNA synthesis, as seen for terminator **M**, would seem to make its use worthwhile. The application of such a terminator adds very little effort to enzymatic oligonucleotide synthesis; indeed, it adds only a single step to the synthesis (carried out automatically on the synthesizer) and requires no other changes from standard methods. Since we observed apparently poor termination in one of the four sequences, it would be worthwhile to test this compound in additional sequences and in larger preparative reactions to evaluate the general effects in RNA synthesis. At present, since **M** is the best RNA terminator and is also a good DNA terminator, it would appear to be the single analogue with the most general terminator utility. Future studies may find new nucleosides with yet better termination abilities in RNA synthesis.

We observe here that the KF DNA polymerase has a greater tendency to add an extra nucleotide beyond the template than does T7 RNA polymerase. Enzymatic synthesis of DNA oligonucleotides is often carried out with the KF (exo^-) mutant, to limit degradation of the full-length products by the exonuclease activity under the reaction conditions. However, as seen in our results, the enzyme lacking this activity has a strong tendency to add an extra nucleotide at the end. In enzymatic DNA synthesis for structural studies, this fact has caused other workers to choose the (exo^+) variant of KF, which has a less strong tendency to add an extra nucleotide (10). In our experiments, however, even this latter enzyme gives a large amount of the undesired longer product, and so terminators such as **M**, **N** and **P** should be of general value in DNA synthesis.

The present study has focused on short synthetic oligonucleotides as templates. In the case of enzymatic synthesis of longer RNAs, it is common to generate template duplexes from restriction cleavage of biologically derived DNAs. For some applications it is necessary that the 3′ terminus of the RNA be well-defined and homogeneous, such as when a subsequent ligation of the RNA is to be performed. One approach to achieving this has been the construction of cassettes in which a *cis*-cleaving ribozyme is included in order to generate a clean 3′-end. It seems possible that the present method might be a useful alternative, in that a short sticky-ended segment of DNA carrying a terminator could be ligated onto the end of any restriction fragment. This would also have the advantage of giving free hydroxyl groups at the RNA terminus rather than phosphorylated hydroxyls which result from ribozyme cleavage. This possibility awaits future experiments.

CONCLUSIONS

We conclude that simple placement of a non-hydrogen-bonding 'terminator' nucleoside at the 5′-end of a given DNA template strand can be used to significantly improve the 3′-end homogeneity of products of RNA and DNA synthesis. Of the structures studied to date, 4-methylindole nucleoside **M** is the most efficient terminator for T7 RNA polymerase. For DNA synthesis with the Klenow fragment, the α-pyrene nucleoside **P** is the most effective terminator, with **M** also showing good efficiency. The application of such terminators may be useful in simplifying purification and lowering the cost of enzymatic RNA and DNA synthesis *in vitro*.

MATERIALS AND METHODS

^1H and ^{13}C NMR spectra were recorded with a 300 MHz spectrometer unless otherwise noted, and chemical shifts are reported in p.p.m. on the δ scale with the solvent as an internal reference, and the coupling constants are in Hertz (Hz). The mass spectra were performed using fast atom bombardment or electron impact.

All reactions were monitored by thin-layer chromatography (TLC) using EM Reagents plates with fluorescence indicator (SiO_2-60, F-254). Flash column chromatography was conducted using EM Science Silica Gel 60 (230–400 mesh). Mass spectral analyses were performed by the Riverside Mass Spectrometry Facility (University of California, Riverside, CA).

All reactions were carried out under a nitrogen atmosphere in dry, freshly distilled solvents under anhydrous conditions unless otherwise specified. THF was distilled from sodium metal/benzophenone, methylene chloride was distilled from NaH, and pyridine was distilled from BaO prior to use.

The abasic nucleoside (φ) was synthesized according to the published procedure (14).

1′,2′-Dideoxy-1′-(4-methylindol-1-yl)-3′,5′-di-*O*-toluoyl-β-D-ribofuranose (M)

4-Methylindole (0.92 g, 7 mmol) was added to a flame-dried, nitrogen flushed flask and dissolved in 35 ml acetonitrile. The solution was cooled to 0°C when 60% sodium hydride dispersion (0.393 g, 9.8 mmol) was added and the blue–green solution stirred for 30 min. Solid 1-α-chloro-3,5-di-*O*-toluoyl-2-deoxyribose

(22) (2.781 g, 7.2 mmol) was added in several portions over 90 min. The reaction was cooled for ≥2 h then stirred overnight while warming to room temperature. The acetonitrile was evaporated and the brown residue redissolved in diethyl ether. The ether solution was washed with saturated sodium bicarbonate solution and brine before drying over anhydrous magnesium sulfate. Concentration and chromatography on silica eluting with methylene chloride gave 54% yield as a beige foam, R_f = 0.47 (methylene chloride). ^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.03 (2H, d, J = 8), 8.00 (2H, d, J = 8), 7.42 (1H, d, J = 8.4), 7.33 (3H, m), 7.28 (2H, d, J = 8), 7.12 (1H, t, J = 8), 6.97 (1H, d, J = 7.2), 6.60 (1H, d, J = 3.2), 6.51 (1H, dd, J = 5.6, 8.4′), 5.75 (1H, m), 4.69 (2H, d, J = 6), 4.61 (1H, br s), 2.89 (1H, m), 2.69 (1H, m), 2.56 (3H), 2.48 (3H, s), 2.46 (3H, s); ^{13}C NMR ($CDCl_3$, p.p.m.) 18.6, 21.7, 37.8, 64.3, 75.1, 81.5, 85.6, 101.9, 102.1, 107.5, 120.5, 122.3, 123.2, 126.6, 126.9, 129.0, 129.2 (d),129.7 (d), 130.6, 135.6, 143.9, 144.4; HRMS (EI+) calcd for $C_{30}H_{29}NO_5$ 483.2045, found 483.2028.

General procedure for glycosidic coupling reaction and isolation of major α-epimers as bis-p-toluoyl esters of N and P

Dry THF (5 ml) was placed in a round-bottomed flask equipped with a condenser, drying tube and addition funnel. Magnesium turnings (0.3 g, 1.2 mmol) and a few crystals of iodine were added. 1-Bromopyrene (0.35 g, 1.2 mmol) was added to the mixture. Slight heating was needed (40°C) to drive the reaction to completion. After formation of the Grignard reagent was complete (~1 h), dry $CdCl_2$ (110 mg, 0.6 mmol) was added and the reaction mixture was continuously heated under reflux for 1 h. 1-α-chloro-3,5-di-*O*-toluoyl-2-deoxyribose (22) (0.51 g, 1.3 mmol) was then added to the above mixture in one portion. The solution was stirred at room temperature for 4 h under an atmosphere of N_2. The solution was poured into 10% ammonium chloride (2 × 50 ml) and extracted with methylene chloride. The organic layers were washed with saturated sodium bicarbonate and brine and dried over anhydrous magnesium sulfate. The solution was filtered, concentrated and purified by flash silica gel chromatography eluting with hexanes-ethyl acetate (9:1). The major product 1′,2′-dideoxy-1′-(1-pyrenyl)-3′,5′-di-*O*-toluoyl-α-D-ribofuranose (**P**) was obtained as a pale yellow oil (α-epimer, 48% isolated yield) R_f = 0.29 (ethyl acetate:hexanes 20:80): ^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.80 (2H, d, J = 8.0), 8.72 (2H, d, J = 8.0), 8.05 (1H, s), 7.92–8.00 (2H, m), 7.72–7.60 (4H, m), 7.58 (2H, d, J = 8.0), 7.32 (2H, d, J = 8.0), 6.96 (2H, d, J = 8.0), 6.15 (1H, dd, J = 8.2, 6.0), 5.76 (1H, m), 4.98 (1H, m), 4.75–4.65 (2H, m), 3.30–3.22 (1H, m), 3.50–3.45 (1H, m), 3.44 (3H. s), 3.38 (3H, s); ^{13}C NMR ($CDCl_3$, p.p.m.) δ 21.3, 21.4, 39.2, 64.5, 76.2, 78.0, 82.5, 122.3, 122.9, 123.2, 123.6, 126.0, 126.3, 126.4, 126.6, 126.8, 127.0, 128.7, 128.8, 128.9, 129.0, 129.2, 129.4, 129.6, 129.8, 130.6, 131.4, 136.2, 143.5, 143.6, 165.8, 166.2; HRMS (FAB, 3-NBA matrix) calcd for $C_{37}H_{31}O_5$ (M+1) 554.2093, found 554.2069.

1′,2′-Dideoxy-1′-(1-naphthyl)-3′,5′-di-*O*-toluoyl-α-D-ribofuranose (N)

(α-epimer, 52% isolated yield) R_f = 0.36 (ethyl acetate:hexanes 20:80) ^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.05 (2H, d, J = 8.0), 7.95 (2H, m), 7.83 (2H, overlapped d), 7.71 (2H, d, J = 8.0), 7.55 (3H, m), 7.32 (2H, d, J = 8.0), 7. 19 (2H, d, J = 8.0), 6.10 (1H, dd, J = 8.0, 6.0), 5.69 (1H, m), 4.90 (1H,m), 4.76–4.65 (2H, m), 3.28–3.18 (1H, m), 2,52–2.45 (1H, m), 2.48 (3H, s), 2.42 (3H, s); ^{13}C NMR ($CDCl_3$, p.p.m.) δ 21.4, 21.5, 39.5, 64.5, 76.2, 77.8, 82.2, 122.1, 122.9, 125.1, 125.3, 125.8, 126.6, 127.0, 128.7, 128.8, 128.9, 129.2, 129.4, 129.5, 129.9, 133.6, 137.9, 143.6, 165.8, 166.2; HRMS ((FAB, 3-NBA matrix) calcd for $C_{31}H_{29}O_5$ (M+1) 481.2015, found 481.2025.

General procedure for the deprotection of 1′,2′-dideoxy-1′-(aryl)-3′,5′-di-*O*-toluoyl-D-ribofuranoses

1′,2′-Dideoxy-1′-(4-methylindol-1-yl)-3′,5′-di-*O*-toluoyl-β-D-ribofuranose (**1**) (1.766g, 3.8 mmol) was dissolved in 25 ml MeOH and treated with 2 ml 0.75 M NaOMe solution. The reaction was stirred for 3 h at room temperature under a nitrogen atmosphere and quenched by adding solid ammonium chloride. The mixture was filtered and concentrated before chromatographing on silica eluting with ethanol:diethyl ether (5:95). The product 1′,2′-Dideoxy-1′-(4-methylindol-1-yl)-β-D-ribofuranose (**M**) was obtained in 88% yield as a light pink oil, R_f = 0.30 [ethanol:diethyl ether (5:95)]. ^{1}H NMR ($CDCl_3$, p.p.m.) δ 7.32 (2H, m), 7.18 (1H, t, J = 9), 6.97 (1H, d, J = 6), 6.60 (1H, br s), 6.33 (1H, t, J = 6), 4.40 (1H, m), 3.88 (1H, m), 3.64 (2H, m), 3.30 (2H, br s), 2.56 (3H, s), 2.51 (1H, m), 2.31 (1H, m); ^{13}C NMR ($CDCl_3$, p.p.m.) δ 18.6, 39.5, 62.3, 71.3, 84.5, 85.8, 101.9, 107.4, 120.6, 122.4, 123.2, 128.8, 130.6, 135.8; HRMS (EI+) calcd for $C_{14}H_{17}NO_3$ 247.1208, found 247.1196.

1′,2′-Dideoxy-1′-(1-pyrenyl)-α-D-ribofuranose (**P**) was obtained by chromatography on silica eluting with ethyl acetate as an off-white solid in 69% yield, R_f = 0.26 (ethyl acetate). ^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.35 (1H, d, J = 8), 8.29–8.00 (8H, m), 6.19 (1H, dd, J = 7.2, 8), 4.63 (1H, m), 4.47 (1H, m), 4.18 (1H, m), 3.85 (1H, m), 3.10 (1H, m), 2.23 (1H, m), 1.87 (2H, bs); ^{13}C NMR 400 MHz (d_6 DMSO, p.p.m.) δ 48.7, 67.0, 77.1, 81.3, 91.8, 128.2 (d), 129.1, 129.2, 130.1 (d), 130.3, 131.3, 131.8, 131.9, 132.4, 132.6, 134.9, 135.4, 136.0, 143.1; HRMS (FAB, MEOH/3-NBA matrix) calcd for $C_{21}H_{18}O_3$ (M^+) 318.1257, found 318.1256.

1′,2′-Dideoxy-1′-(1-naphthyl)-α-D-ribofuranose (N)

1′,2′-Dideoxy-1′-(1-naphthyl)-α-D-ribofuranose (**N**) was obtained by chromatography on silica eluting with ethyl acetate as an off-white solid in 78% yield, R_f = 0.22 (ethyl acetate). ^{1}H NMR ($CDCl_3$, p.p.m.) δ 7.86 (1H, d, J = 8), 7.77 (3H, m), 7.45 (3H, m), 5.62 (1H, dd, J = 7.3, 8), 4.32 (1H, m), 4.13 (1H, m), 3.94–3.62 (4H, m), 2.65 (1H, m), 1.97 (1H, m) ^{13}C NMR 400 MHz ($CDCl_3$, p.p.m.) δ 42.5, 58.2, 72.9, 77.0 (obscured by solvent), 85.3, 121.5, 122.9, 125.4 (d), 126.0, 127.8, 128.8, 129.8, 133.6, 138.0; HRMS (FAB, MEOH/3-NBA matrix) calcd for $C_{15}H_{16}O_3$ (M^+) 244.1102, found 244.1099.

1′,2′-Deoxy-5′-(4,4′-dimethoxytrityl)-1′-(4-methylindol-1-yl)-β-D-ribofuranose (M)

1′,2′-Dideoxy-1′-(4-methylindolyl)-β-D-ribofuranose (0.336 g, 1.4 mmol) was dissolved in 20 ml methylene chloride in a flame dried, nitrogen flushed flask. *N*,*N*-Diisopropylethylamine (0.34 ml, 4.2 mmol) was added, followed by 4,4′-dimethoxytrityl chloride (0.745 g, 2.2 mmol). The reaction was stirred for 3 h at reflux, then quenched with 1 ml methanol. After 30 min methylene chloride (30 ml) was added and the mixture was washed with saturated sodium bicarbonate solution and brine. Drying over anhydrous

sodium sulfate and silica gel chromatography eluting with ethyl acetate:hexanes (40:60 plus 5% triethylamine), R_f = 0.23, gave the product in 72% yield. ^{1}H NMR ($CDCl_3$, p.p.m.) δ 7.42 (2H, m), 7.31 (4H, m), 7.22 (5H, m), 7.06 (1H, t, J = 6), 6.91 (1H, d, J = 6), 6.82 (4H, d, J = 9), 6.50 (1H, d, J = 3), 6.35 (1H, t, J = 6), 4.51 (1H, m), 4.03 (2H, m), 3.74 (6H, s), 3.31 (2H, m), 2.63 (1H, m), 2.54 (3H, s), 2.43 (1H, m), 1.63 (1H, br s); ^{13}C NMR ($CDCl_3$, p.p.m.) δ 11.1, 18.6, 40.1, 45.9, 55.1, 64.0, 72.4, 84.9, 86.4, 101.4, 107.6, 113.1, 120.3, 122.1, 123.5, 126.8, 127.8, 128.1, 129.0, 130.0, 130.3, 135.7, 144.6, 158.4; HRMS (EI+) calcd for $C_{35}H_{35}NO_5$ 549.2515, found 549.2514.

General procedure for preparation of tritylated α-C-nucleosides

1′,2′-Dideoxy-1′-(1-naphthyl)-β-D-ribofuranose (461 mg, 1.88 mmol) was dissolved in dry pyridine (20 ml) and a catalytic amount of DMAP (11 mg) was added. 4,4′-Dimethoxytrityl chloride was added to the above mixture and the reaction was stirred under a nitrogen atmosphere for 3 h and then quenched with 2 ml ethanol. The reaction was diluted with methylene chloride (75 ml) and washed once with 5% sodium bicarbonate solution. The organic mixture was dried over anhydrous sodium sulfate, filtered and concentrated. The product was purified by silica gel chromatography (pre-equilibrated with 5% triethylamine in hexanes) eluting with ethyl acetate:hexanes (25:75). The product 1′,2′-Deoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-naphthyl)-α-D-ribofuranose (**N**) was obtained as a yellowish foam in 83% yield (859 mg, 1.57 mmol), R_f = 0.25 (ethyl acetate:hexanes 1:2): ^{1}H NMR ($CDCl_3$, p.p.m.) δ 7.90 (2H, m), 7.83 (2H, m), 7.62–7.25 (12H, m), 6.92 (4H, d, J = 8.8), 5.95 (1H, unresolved m) 4.55 (1H, m), 4.02 (1H, m) 3.83 (6H, s), 3.45 (2H, m), 3.00 (1H, m), 2.17 (2H, m); ^{13}C NMR 400 MHz ($CDCl_3$, p.p.m.) δ 42.6, 55.2, 64.8, 74.7, 77.0 (obscured by solvent), 85.1, 86.4, 113.3, 122.0, 123.4, 125.6, 126.0, 126.9, 127.8, 128.0, 128.3, 128.9, 130.2, 130.3, 133.8, 136.1, 139.0, 145.1, 158.6; HRMS (FAB, DCM/NBA/PPG matrix) calcd for $C_{36}H_{33}O_5$ (M-1) 545.2298, found 545.2328.

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-pyrenyl)-α-D-ribofuranose (P)

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-pyrenyl)-α-D-ribofuranose (**P**) was obtained as a white foam in 50% yield, R_f = 0.23 (ethyl acetate:hexanes 1:2):^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.42 (1H, m), 8.32–8.01 (8H, m), 7.72–7.28 (9H, m), 6.94 (4H, d, J = 8), 6.26 (1H, unresolved m), 4.65 (1H, m), 4.55 (1H, m), 3.84 (6H, s) 3.54 (2H, m), 3.09 (1H, m), 2.25 (2H, m); ^{13}C NMR 400 MHz (CDCl3, p.p.m.) δ 43.5, 55.2, 64.9, 74.9, 77.1 (obscured by solvent), 85.3, 86.5, 113.3, 122.7, 124.7 (d), 125.1, 125.2 (d), 125.9, 127.0 (d), 127.3, 127.5 (d), 128.0 (d), 128.3 (d), 130.2, 130.3, 130.6 (d), 131.4, 136.2, 136.9, 145.1, 158.6; HRMS (FAB, 3-NBA matrix) calcd for $C_{42}H_{36}O_5$ (M+) 620.2577, found 620.2563.

General procedure for the preparation of 1′,2′-dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(aryl)-D-ribofuranose-3′-(cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidites

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(4-methylindolyl)-β-D-ribofuranose (0.311 g, 0.6 mmol) was dissolved in 3 ml methylene chloride under nitrogen. With stirring, *N*,*N*-diisopropylethylamine (0.040 ml, 2.3 mmol) was added via syringe followed by 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.19 ml, 0.85 mmol). The pale yellow solution was stirred at room temperature for 20 min and then was diluted with 10 ml ethyl acetate. Washing the solution with saturated sodium bicarbonate solution and brine before drying over anhydrous sodium sulfate gave a pale yellow oil. The oil was chromatographed on silica gel eluting with ethyl acetate:hexanes:triethylamine (40:55:5), R_f = 0.66, to give 90% yield of 1′,2′-dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(4-methyl-indolyl)-β-D-ribofuranose-3′-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (**M**) as an off-white foam. ^{1}H NMR ($CDCl_3$, p.p.m.) δ 7.47 (2H, m), 7.37 (4H, m), 7.27 (5H, m), 7.11 (1H, t, J = 6), 6.96 (1H, d, J = 6), 6.80 (4H, d, J = 9), 6.56 (1H, d, J = 3), 6.42 (1H, t, J = 6), 4.74 (1H, m), 4.26 (2H, m), 3.80 (6H, s), 3.67 (3H, m), 3.39 (2H, m), 2.71–2.55 (1H, m), 2.56 (3H, s), 2.49 (2H, t), 2.41 (3H, s), 1.23 (12H, t); ^{13}C ($CDCl_3$, p.p.m.) δ 11.2, 18.6, 20.2, 24.5, 39.5, 43.3, 46.0, 55.1, 58.4, 63.6, 73.5, 73.9, 85.2, 86.4, 101.4, 107.7, 113.0, 120.2, 121.8, 123.55, 126.5, 127.7, 128.5, 129.1, 130.1, 130.6, 135.7, 144.7, 158.4; ^{31}P (H_3PO_4, p.p.m.) 146.3, 146.7; HRMS (EI+) calcd for $C_{44}H_{52}N_3O_6P$ 749.3594, found 749.3582.

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-pyrenyl)-α-D-ribofuranose-3′-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (P)

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-pyrenyl)-α-D-ribofuranose-3′-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (**P**) was obtained as a white foam in 96% yield, R_f = 0.23 (ethyl acetate:hexanes 1:2):^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.50 (1H, m), 8.36–8.05 (8H, m), 7.76 (2H, m), 7.55 (4H, m), 7.44 (2H, m), 7.30 (1H, m), 6.96 (4H, m), 6.39 (1H, unresolved m), 4.94–4.80 (1H, m), 4.77 (1H, m), 3.85 (6H, s), 3.76–3.40 (4H, m), 3.34 (1H, m), 3.22 (1H, m), 2.44 (1H, m), 2.20 (1H, m), 1.15–1.05 (12H, m), 1.02 (2H, d, J = 6.7); ^{13}C NMR 400 MHz (CDCl3, p.p.m.) δ 20.0 (m), 24.5 (m), 43.0 (m), 55.2, 58.3 (m), 64.3 (d), 75.7 (m), 77.2 (d), 85.1 (d), 86.3, 113.2, 117.5 (d), 122.7 (d), 123.1 (d), 124.7 (d), 124.9 (m), 125.2, 125.8 (d), 126.8, 126.9, 127.1, 127.2, 127.4, 127.5 (d), 127.8, 127.9, 128.3 (m), 130.2 (m), 130.4, 130.6 (d), 131.3 (d), 136.1 (m), 136.8, 137.2, 145.0, 158.5; HRMS (FAB, 3-NBA matrix) calcd for $C_{51}H_{54}N_2O_6P$ (M+1) 821.3762, found 821.3720.

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-naphthyl)-α-D-ribofuranose-3′-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (N)

1′,2′-Dideoxy-5′-(4,4′-dimethoxytrityl)-1′-(1-naphthyl)-α-D-ribofuranose-3′-cyanoethyl-N,N-diisopropylphosphoramidite (**N**) was obtained as a white foam in 92% yield, R_f = 0.35 (ethyl acetate:hexanes 1:2):^{1}H NMR ($CDCl_3$, p.p.m.) δ 8.06 (1H, m), 7.95–7.80 (2H, m), 7.66–7.42 (9H, m), 7.38 (2H, m), 7.35 (1H, m), 6.92 (4H, m), 6.05 (unresolved m), 4.83–4.63 (1H, m), 4.58 (1H, m), 3.84 (6H, s), 3.63 (1H, m), 3.60–3.40 (3H, m), 3.37 (1H, m), 3.05 (1H, m), 2.42 (1H, m), 2.37 (1H, m), 1.18 (6H, d, J = 6.6), 1.11 (3H, d, J = 7), 1.05 (3H, d, J = 7); ^{13}C NMR 400 MHz (CDCl3, p.p.m.) δ 19.9 (m), 24.4 (m), 41.8 (m), 43.0 (m), 55.1, 58.1 (m), 64.2 (d), 75.6 (m), 77.2 (obscured by solvent), 84.8 (m), 86.2, 113.1, 117.5, 122.4, 123.4 (d), 125.3, 125.5 (d), 125.8, 126.7, 127.4, 127.6, 127.8, 128.3 (d), 130.2 (d), 130.3, 133.7 (d), 136.0 (d), 136.2 (d), 138.7, 139.0, 145.0, 158.5; HRMS (FAB, 3-NBA matrix) calcd for $C_{45}H_{52}N_2O_6P$ (M+1) 747.3590, found 747.3563.

Oligonucleotide synthesis

Oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer using β-cyanoethyl phosphoramidites. The coupling time used for the non-polar phosphoramidites was the same as for conventional Watson–Crick bases. Intact incorporation of residues **M**, **N**, **P** was confirmed by synthesis of short oligomers of sequence T-X-T (where X = **M**, **N**, **P**); proton NMR (400 MHz) indicated the presence of the intact structures with the expected integration. The 5′ dimethoxytrityl group was removed at the end of the synthesis, and the oligomers were deprotected with concentrated NH_4OH at 55°C overnight. Following lyophilization, oligonucleotides were purified by electrophoresis on 2.5 mm denaturing (8 M urea) 15 or 20% polyacrylamide gels. After elution with Milli-Q H_2O and desalting with a Waters C-18 Sep-Pak column, the purity of the oligonucleotide product was checked by 5′-end-labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase and then using analytical (0.4 mm) denaturing gel electrophoresis. Oligomers with terminators at their 5′-end ran one nucleotide slower than their unterminated control oligomer with the same sequence. Most sequences were gel purified twice to reduce the contaminating *n*–1 oligonucleotide to <10 % of full-length synthesis product; most had no *n*–1 product visible after purification.

Enzymatic RNA synthesis and quantitation

T7 RNA polymerase reactions were run in 10 µl volumes (final concentrations are listed). One µM each of the template and the 18-nt T7 promoter (Fig. 1B) were mixed in transcription buffer [40 mM Tris–HCl (pH 8.3 at room temperature), 6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine] and Milli-Q H_2O for 5 min (heating the promoter–template duplex to 80°C for 5 min to anneal had no effect on the yield or product distribution of the transcription reaction). Following addition of 0.5 mM rNTPs and 10 µCi [γ-^{32}P]GTP, [α-^{32}P]ATP or [α-^{32}P]UTP (New England Nuclear), the polymerization reaction was initiated by adding T7 RNA polymerase (New England BioLabs) to 5 U/µl [decreasing the cold (unlabeled) concentration of the rNTP carrying the α-radiolabel increased the specific activity of products but did not affect product distribution]. Increasing Mg^{2+} and DTT concentrations to 20 mM and using 1 mM each of the rNTPs increased the yield of the transcription reaction but did not affect the distribution of product oligonucleotides. Reaction time was 30 min at 37°C.

Both RNA transcription and DNA elongation reactions were stopped by adding 8 µl gel loading buffer [80% formamide, 1× TBE buffer (89 mM Tris–borate, 2 mM EDTA), 0.1% xylene cylanol, 0.1% bromophenol blue]. Reactions were assayed by analytical (0.4 mm thickness) denaturing gel electrophoresis (15 or 20% polyacrylamide). Following autoradiography to visualize reaction products, the intensities of various product bands were quantified with a PhosphorImager (Molecular Dynamics Model 400 Series).

Enzymatic DNA synthesis

Reactions with the large fragment of *E.coli* DNA polymerase I [Klenow fragment, both normal (wild type) and *exo*$^-$ mutant lacking the 3′–5′ exonuclease activity, US Biochemical] were also run in 10 µl volumes. The 18-nt T7 promoter was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified via denaturing gel electrophoresis (0.4 mm thickness). Following elution from gel slices with Milli-Q H_2O and dialysis to desalt, the radiolabeled oligomer (~5 nM) was mixed with cold template (0.2 µM), Klenow buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mg/ml bovine serum albumin), and Milli-Q H_2O for 5 min at room temperature. After addition of cold primer (0.18 µM) and another 5 min room-temperature incubation, Klenow was added to 0.3 U/µl (0.2 µM) and the reaction mixture was incubated 10 min at 37°C. Elongation of the DNA primer was initiated by the addition of 20 µM dNTPs. Reaction times are 2 and 15 min at 37°C.

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