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The use of modified primers to eliminate cycle sequencing artifacts

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ABSTRACT

Cycle sequencing is the workhorse of DNA sequencing projects, allowing the production of large amounts of product from relatively little template. This cycling regime, which is aimed at linear growth of the desired products, can also produce artifacts by exponential amplification of minor side-products. These artifacts can interfere with sequence determination. In an attempt to allow linear but prevent exponential growth of products, and thus eliminate artifacts, we have investigated the use of primers containing modified residues that cannot be replicated by DNA polymerase. Specifically, we have used primers containing 2′-*O*-methyl RNA residues or abasic residues. Oligomers consisting of six DNA residues and 20 2′-*O*-methyl RNA residues, with the DNA residues located at the 3′-end, primed as efficiently as DNA primers but would not support exponential amplification. Oligonucleotides containing fewer DNA residues were not used as efficiently as primers. DNA primers containing a single abasic site located six residues from the 3′-end also showed efficient priming ability without yielding exponential amplification products. Together these results demonstrate that certain types of modified primers can be used to eliminate artifacts in DNA sequencing. The technique should be particularly useful in protocols involving large numbers of cycles, such as direct sequencing of BAC and genomic DNA.

INTRODUCTION

Cycle sequencing is a technique in which the DNA to be sequenced serves as the template for multiple rounds of primer extension (1). Cycling allows the production of more product than would be produced with the same quantity of template in a conventional sequencing reaction. Although the technique superficially resembles PCR, the goal is linear rather than exponential growth of the product.

Linear amplification techniques such as cycle sequencing are vulnerable to a particular type of artifact. Any DNA strand produced that contains both a primer sequence and the complement of a primer sequence in the appropriate orientation will be a template for exponential amplification. Even if such molecules are produced only as rare side-products, their exponential growth may bring them to high concentrations relative to the linearly growing desired product. The problem potentially worsens as the number of cycles is increased. Other factors, such as the simultaneous use of two primers, may aggravate the problem.

We have encountered such artifacts in cycle sequencing reactions. An artifact was first observed when two primers were used to sequence outward from a transposable element. Resulting sequence ladders were heavily obscured by products that were shown to result from exponential amplification. Two unusual features of the primer system, namely the simultaneous presence of two primers and the partially pallindromic sequences of the transposon ends, contributed to the problem. However, we have also observed this artifact when only one primer was used, and some templates yielded similar artifacts in reactions not involving transposable elements. Attempts to combat these artifacts by adjustment of temperature profiles and ionic strength did not lead to consistently artifact-free sequence. We therefore sought a more fundamental solution to the problem.

Exponential amplification, unlike linear amplification, relies on the fact that the product of one round of replication is a template for subsequent rounds. Part of this requirement is that the primer incorporated into a newly synthesized strand can later be replicated to form a primer-binding site. No such requirement exists for linear growth. The use of a primer that cannot be replicated by DNA polymerase might allow the desired linear amplification but prevent the undesired exponential growth of side-products.

Most DNA replication *in vivo* is initially primed by RNA (2), and RNA would not be a template for a strictly DNA-dependent DNA polymerase. RNA would therefore seem to meet the criteria of a molecule that can serve as primer but not as template. A potential pitfall of the use of RNA is its susceptibility to hydrolysis, both spontaneous and enzyme catalyzed. Hydrolysis-resistant derivatives of RNA have been developed. One such derivative is 2′-*O*-methyl RNA (2′-*O*-Me RNA). We report here the use of oligonucleotides consisting mainly of 2′-*O*-Me RNA residues as primers for linear amplification, including cycle sequencing. If a sufficient number of DNA residues are placed at the 3′-end of the oligonucleotide, linear amplification is efficient but artifacts due to exponential amplification disappear. We

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also show that another type of non-replicatable primer, a DNA oligomer containing one or more abasic residues, can be used to solve the same problem in a similar fashion.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides were synthesized from commercially available dA, dG, dC and dT phosphoramidites (Perkin Elmer), 2′-*O*-Me-A, 2′-*O*-Me-G, 2′-*O*-Me-C and 2′-*O*-Me-U phosphoramidites (Glen Research), and dSpacer phosphoramidites (Glen Research) with an Applied Biosystems 394 DNA/RNA Synthesizer (Perkin Elmer). The series of oligonucleotides used as primers is listed in Table 1. For studies of melting behavior, DNA oligomers complementary to the primers were synthesized: antiFK, ACCTCGCTACCTTAGGACCGTTATAG; antiFS, ACTAACACTTTACAGCGGCGCGTCAT; antiUP, TCGTGACTGGGAAAACCCTGGCGTTA; antiRP, CCTG-TGTGAAATTGTTATCCGCTCAC. The template for extension studies was KS52 (CTATAACGGTCCTAAGGTAGCGAG-GTACTAACACTTTACAGCGGCGCGTCAT).

Table 1. Oligonucleotides tested as primers in this study

S26-DNA	ATGACGCGCCGCTGTAAAGTGTTAGT
S26-12d	augacgcgccgcugTAAAGTGTTAGT
S26-10d	augacgcgccgcuguaAAGTGTTAGT
S26-8d	augacgcgccgcuguaaaGTGTTAGT
S26-6d	augacgcgccgcuguaaaguGTTAGT
S26-5d	augacgcgccgcuguaaagugTTAGT
S26-4d	augacgcgccgcuguaaaguguTAGT
S26-2d	augacgcgccgcuguaaaguguuaGT
S26d6-1	ATGACGCGCCGCTGTAAAG-GTTAGT
S26d6-2	ATGACGCGCCGCTGTAAA--GTTAGT
S26d6-3	ATGACGCGCCGCTGTAA---GTTAGT
S26d9-1	ATGACGCGCCGCTGTA-AGTGTTAGT
S26d9-2	ATGACGCGCCGCTGT--AGTGTTAGT
S26d9-3	ATGACGCGCCGCTG---AGTGTTAGT
K26-DNA	CTATAACGGTCCTAAGGTAGCGAGGT
K26-10d	cuauaacgguccuaagGTAGCGAGGT
K26-6d	cuauaacgguccuaagguagCGAGGT
K26d6-1	CTATAACGGTCCTAAGGTA-CGAGGT
K26d6-2	CTATAACGGTCCTAAGGT--CGAGGT
K26d6-3	CTATAACGGTCCTAAGG---CGAGGT
UP-DNA	TAACGCCAGGGTTTTCCCAGTCACGA
UP-10d	uaacgccaggguuuucCCAGTCACGA
UP-5d	uaacgccaggguuuucccaguCACGA
RP-DNA	GTGAGCGGATAACAATTTCACACAGG
RP-10d	gugagcggauaacaauTTCACACAGG
RP-5d	gugagcggauaacaauuucacACAGG

Lower case letters are positions with 2′-*O*-Me-phosphoramidites as discussed in the text. Dashes (–) indicate abasic DNA sites.

PCR assays

Amplification consisted of 25 cycles of 15 s at 96°C, 15 s at 60°C and 3 min at 72°C in an MJ Thermocycler. Reactions were carried out in 20 µl containing 100 fmol template, 10 pmol primer, 200 µM dNTPs, 1.4 µg modified *Taq* DNA polymerase (3), 0.01 U pyrophosphatase from *Thermoplasma acidophilum*, 2.75 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.2, and 100 mM KCl. The template for the UP/RP series of primers was pGEM-3Zf(+) and for the S26/K26 series was KS52. Reactions were analyzed on 3% Nusieve GTG gels (FMC).

Sequencing

Primers were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Unless otherwise noted, the thermal cycle sequencing reactions were performed as follows. Sequencing reaction mixes contained 25 fmol template, 1.25 pmol labeled primer, 2.75 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.2, 100 mM KCl, 0.01 U pyrophosphatase, and 1.4 µg *Taq* polymerase, 125 µM each dNTP and either ddATP, ddGTP, ddCTP or ddTTP at 1 µM in a total volume of 20 µl. Thermal cycling consisted of 25 cycles of 10 s at 96°C, a 1°C/s ramp to 50°C, 15 s at 50°C, a 1°C/s ramp to 60°C, and 4 min at 60°C. The template, pWD42a, is a 5.2 kb plasmid with an 8 kb insert and an RI origin of replication. Priming sites for the UP/RP primers flank the insert. They are oriented such that the primers extend toward each other and into the insert. This plasmid carries a $\gamma\delta$ transposon that provides priming sites for the K26 and S26 primers. These priming sites are located 368 bp apart and are oriented in opposite directions such that primer extension from the two sites is divergent. Products were separated on 6% acrylamide gels.

Primer extensions

Primer extension experiments were carried out in two stages. First, an extension reaction was carried out in a 20 µl reaction volume using 10 pmol of one of the S26 series primers, 50 fmol KS52 template, 1.4 µg *Taq* DNA polymerase with 2.75 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.2, and 100 mM KCl in an MJ Thermocycler using 25 cycles of 96°C for 15 s, 60°C for 15 s, and 72°C for 2 min. A second extension was then carried out after addition of 1.4 µg *Taq* DNA polymerase, 5 pmol labeled K26-DNA primer, and buffer to maintain ion concentrations and bring the total volume to 30 µl. Conditions for this extension reaction were the same as for the first, but only one cycle was performed.

Melting temperature measurements

Melting curves were measured as follows. Absorption at 260 nm was monitored on a Beckman DU 7400 spectrophotometer over a range of temperatures. Equimolar amounts of sample and antisense oligonucleotide were placed in a buffer containing 2.75 mM $MgCl_2$, 10 mM Tris, pH 9.2, and 100 mM KCl. The temperature profile was 35–65°C, 1°C/min, 66–85°C, 0.5°C/min, and 86–94°C, 1°C/min. Melting temperature was taken to be the temperature at which the first derivative was highest.

RESULTS

PCR with modified oligonucleotides

As discussed above, inclusion of 2′-*O*-Me RNA residues or abasic sites in primers might prevent exponential amplification with *Taq* DNA polymerase (Fig. 1). To test this hypothesis, we attempted several PCRs with conventional and modified primers. The modified *Taq* DNA polymerase (3), referred to here simply as *Taq* DNA polymerase, was used throughout this

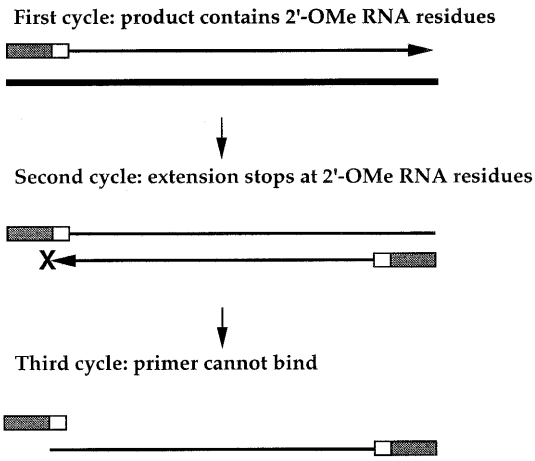

Figure 1. Model of the failure of PCR using primers containing 2′-*O*-Me RNA residues. Solid dark lines are the template. Boxed areas are primers. Open boxed areas are DNA residues of the primer. Shaded boxed areas are 2′-*O*-Me RNA residues of the primer.

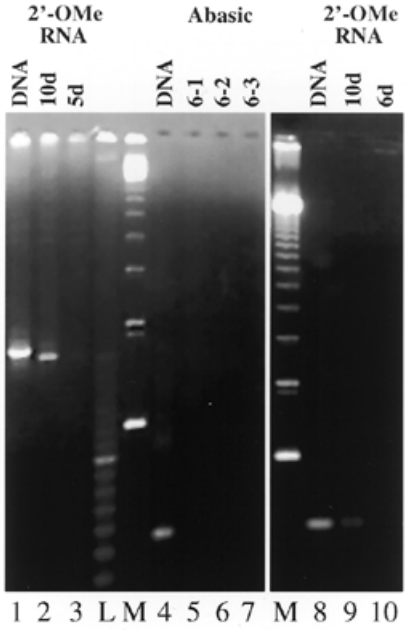

Figure 2. PCR reactions of RNA–DNA chimeric primers. The type of primer used in each PCR is indicated at the top. Three UP/RP primers with pGEM-3Zf(+) (Promega) and six S26/K26 series primers with KS52 as the template were used in a PCR then analyzed for products. Lane 1, UP/RP-DNA primers; lane 2, UP/RP-10d primers; lane 3, UP/RP-5d primers; lane 4, S26/K26-DNA primers; lane 5, S26d6/K26d6-1 primers; lane 6, S26d6/K26d6-2; lane 7, S26d6/K26d6-3; lane 8, S26/K26-DNA primers; lane 9, S26/K26-10d primers; lane 10, S26/K26-6d. L, 1 μg BRL 10 bp ladder; M, 1 μg BRL 123 bp ladder. Sizes of the ladders are indicated at the left.

study. The primer pairs used were forms of either UP and RP, the common 'universal' and 'reverse' primers found in many vectors, or S26 and K26, both specific for primer-binding sites on a transposon (Materials and Methods). The primer sets were standard DNA oligomers, chimeric oligonucleotides with both DNA and 2′-*O*-Me RNA residues, or DNA oligomers containing one or more abasic sites. Each oligonucleotide contained a total of 26 residues. The first type of modified primers contained 2–12 DNA residues at the 3′-end, the remaining residues being 2′-*O*-Me RNA. These oligonucleotides were named according to the number of DNA residues at the 3′-end. S26-4d, for example, contains four DNA residues at the 3′-end, and the other 22 residues are 2′-*O*-Me RNA. The second type of modified primers contained a stretch of one, two or three abasic sites located six residues from the 3′-end. These oligonucleotides were named according to the number and position of abasic sites. S26d6-3, for example, contains six unmodified (ordinary DNA) residues at the 3′-end, followed, in the 3′→5′ direction, by three abasic residues and then 17 unmodified residues to complete the primer.

Figure 2 compares the results of PCR using nine pairs of oligonucleotides: two pairs of ordinary DNA primers and seven pairs of modified primers. The first set of primers, UP/RP, was tested with the template pGEM-3Zf(+). As expected, a PCR product of 212 bp was observed when the primers contained all DNA (lane 1). The next two lanes show the results when primers containing mostly 2′-*O*-Me RNA residues are utilized. When the UP/RP-10d primers were used (lane 2) the amount of product was reduced. The UP/RP-5d primer set does not yield any detectable product (lane 3).

PCRs using primers with abasic sites are shown in the next set of experimental lanes of Figure 2. The template for the S26/K26 primers in this experiment was a single-stranded oligonucleotide, KS52. The sequence of this 52mer corresponds to the K26 primer followed by the complement of the S26 primer. It should therefore serve as a template for PCR with S26/K26 primers. When S26/K26-DNA primers are used a band is present at the expected position (lane 4). The other primers contain one (lane 5), two (lane 6), or three (lane 7) abasic sites. There appears to be no product in any of the reactions using two primers with abasic sites.

The last set of lanes shows the results using S26/K26 primers with 2′-*O*-Me RNA residues. The template for these PCRs was KS52. When S26/K26-DNA primers were used a band was present at the expected position (Figure 2, lane 8). The other primers contain either 10 (lane 9) or six (lane 10) DNA residues at the 3′-end. There appears to be less product in lane 9, and very little product is visible in lane 10. These results demonstrate that the modified oligonucleotides do not support efficient exponential amplification.

DNA sequencing with chimeric primers

In order to determine the relative efficiencies with which the chimeric DNA/2′-*O*-Me RNA oligonucleotides could be used as primers by *Taq* polymerase, we attempted sequencing reactions. Each primer in the S26 2′-*O*-Me series was labeled with ^{32}P and used in a cycle sequencing reaction. As seen in Figure 3a, the 12d, 10d, 8d, 6d and 5d primers work as well as a conventional DNA primer. The 4d oligonucleotide is slightly less effective as a primer, and the 2d primer yields no detectable signal under these conditions. Figure 3b shows a similar test of chimeric oligonucleotides with the UP/RP primer series. The DNA and chimeric versions of the UP primer appeared to be equally effective as sequencing primers. The DNA and 10d versions of the RP primer yielded equal product intensities, but the 5d sequence ladders were slightly less intense.

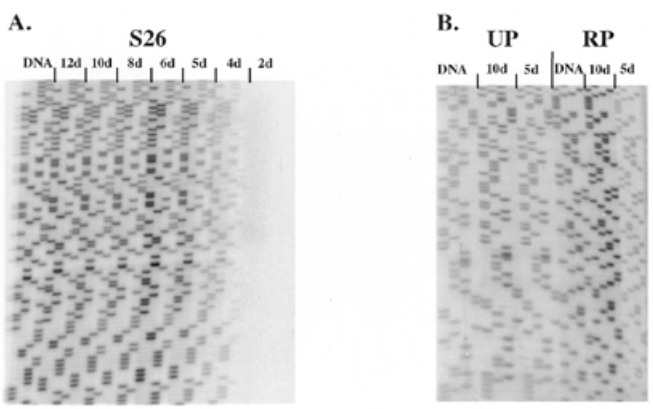

Figure 3. Sequencing with chimeric primers. (**A**) The S26 series of primers were used to sequence a plasmid template. The sequence of the primers used are listed in Table 1. (**B**) The UP/RP series of primers was used to sequence a plasmid template.

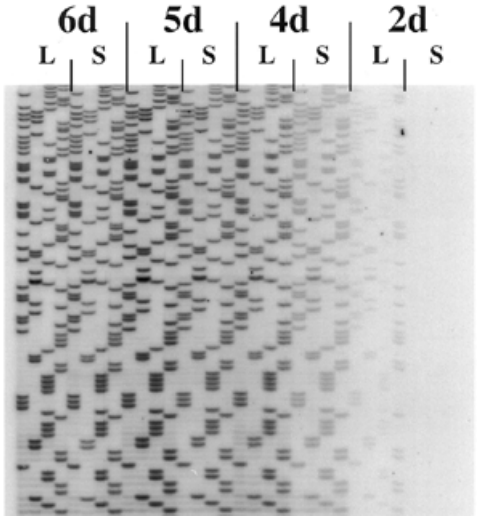

Figure 4. Long and short primer extension times. The four S26 series chimeric primers were used in a sequencing assay. Reactions were performed as described in Materials and Methods except for the cycling conditions. The L lanes refer to a cycling profile of 15 s at 94°C, 4 min at 60°C, 4 min at 72°C. The S lanes refer to a cycling profile of 15 s at 94°C, 15 s at 60°C, 1 min at 72°C.

The primers with fewer than five DNA residues yielded light (S26-4d) or undetectable (S26-2d) sequencing ladders. This was presumably the result of inefficient extension of an unnatural substrate by *Taq* polymerase. If this were the case, increasing the annealing and extension times might increase the product yield. Figure 4 shows a comparison using the S26-6d, -5d, -4d and -2d primers with two cycling protocols. The long (L) cycling conditions increased the annealing time from 15 s to 4 min and the extension time from 1 to 3 min as compared to the short (S) cycling conditions. The longer hybridization and extension times had no effect on the S26-6d and -5d primers,

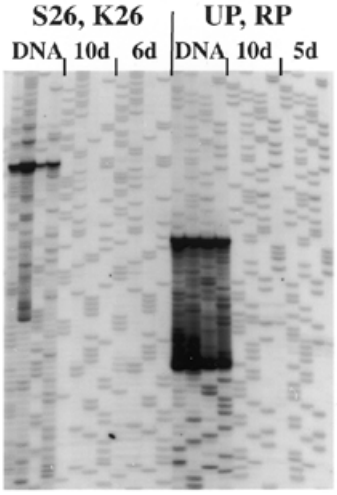

Figure 5. Two-primer sequencing reactions. Sequencing reactions were performed as detailed in Materials and Methods except that an unlabeled primer was added. In each reaction with the S26/K26 series the K26 primer was unlabeled. In each reaction with the UP/RP series the RP primer was unlabeled.

which perform as well as DNA primers even when cycle times are short. However, Figure 4 shows that the S26-4d primer yields more product under the longer times. More striking is the case of the S26-2d primer, which yields no visible product under the short times but a clearly visible, albeit light, ladder under the longer hybridization and extension times.

Artifact elimination with chimeric primers

The results presented above suggest that the use of chimeric DNA/2′-*O*-Me RNA primers in a sequencing reaction might yield good sequencing ladders while preventing artifacts that are due to exponential amplification. We tested this possibility using several primer pairs on a single template. Two primers were used in each experiment. Each primer could hybridize to the template, but only one primer was labeled. Two informative sequencing ladders may be produced, but only one is visualized.

The left side of Figure 5 shows the results from using the K26 and S26 primers together in a sequencing reaction. The template for the reaction was one that had led to a variety of artifact bands using conventional DNA primers. In this experiment the ^{32}P-labeled S26-DNA primer and unlabeled K26-DNA primer were combined with pWD42a in a sequencing reaction. The sequencing ladder can be partially read but is greatly obscured by a number of undesired products. However, when ^{32}P-labeled S26-10d or -6d primers were used with unlabeled K26-10d or -6d, respectively, the undesired products were eliminated and the entire sequence ladder could be clearly seen (the S26, K26 10d and 6d ladders in Fig. 5).

An additional experiment tested the UP/RP primer series with the same template. The first lane in this series uses ^{32}P-labeled UP-DNA primer and unlabeled RP-DNA primer. This combination yields intense artifact bands, preceded by a double sequencing pattern. The result is a severely obscured sequencing ladder. The other two ladders on the right of Figure 5 show similar experiments with the UP/RP-10d and -5d primer pairs. In both

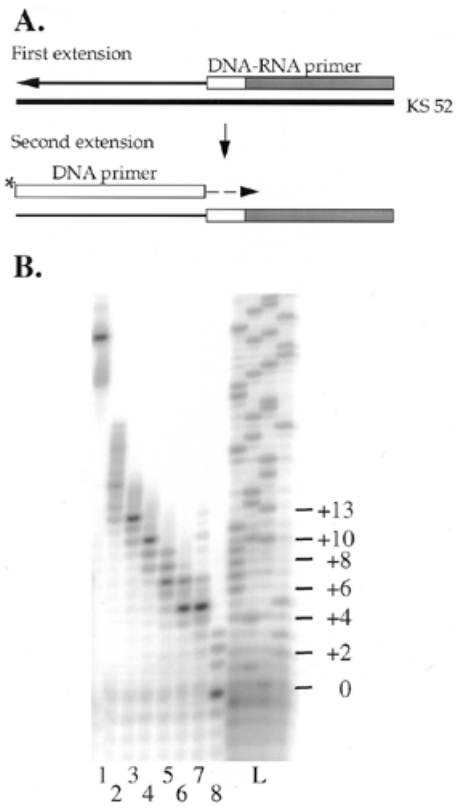

Figure 6. *Taq* polymerase extension products. (**A**) A schematic of the two-step extension experiment. Primers represented by a bar with both DNA sections (open) and 2′-*O*-Me RNA sections (shaded). Location of the γ-^{32}P label is indicated by the asterisk. (**B**) A labeled K26-DNA primer was extended into a template containing 2′-*O*-Me RNA. The template was made from S26-DNA (lane 1), S26-12d (lane 2), S26-10d (lane 3), S26-8d (lane 4), S26-6d (lane 5), S26-5d (lane 6), S26-4d (lane 7) or S26-2d (lane 8) primer. L, sequencing ladder from UP-primed M13. Length of extension from the end of the primer is noted at right.

cases it is the UP-type primer that is labeled with ^{32}P. Neither of these lane sets shows any artifact but they do show good quality sequence ladders.

Primer extension assays

The above results demonstrate that some chimeric DNA/2′-*O*-Me RNA primers can both prevent exponential amplification and efficiently prime sequencing reactions. The ability of the chimeric primers to prevent exponential amplification is probably due to the inability of *Taq* polymerase to use 2′-*O*-Me RNA as a template. The polymerase might stop at the junction between DNA and 2′-*O*-Me RNA residues, or it might polymerize a few nucleotides into the 2′-*O*-Me region. In order to determine how the polymerase was behaving we performed primer extension assays.

The primer extensions were performed in two stages. First, one of the S26 series primers was extended using KS52 as template (Fig. 6A). The expected product would contain a priming site for the K26 primer. This would be followed, in the direction of K26 extension, by either all DNA residues or, if a chimeric primer was used in the first stage, several DNA residues

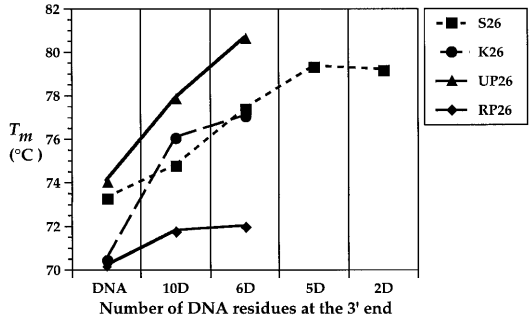

Figure 7. Melting temperatures (T_m, °C) of DNA and modified primers.

and then 2′-*O*-Me RNA residues. This product then served as a template for extension of ^{32}P-labeled K26 (DNA) primer through the DNA region and potentially into the 2′-*O*-Me RNA residues.

The results of these experiments (Fig. 6B) show that although *Taq* polymerase can usually extend partway into the 2′-*O*-Me RNA region of a template, it stops within a few bases of the DNA/2′-*O*-Me RNA junction. Each DNA/2′-*O*-Me RNA template yielded a range of products in the second extension, but one predominant band. The full-length product from the all-DNA control is clearly seen (Fig. 6B, lane 1). With one exception (the experiment using 2d), when templates containing 2′-*O*-Me RNA were used the DNA primer was extended at least up to the DNA/2′-*O*-Me RNA junction, and usually beyond. When the template incorporated the 4d, 5d, 6d, 8d, 10d or 12d oligonucleotide, the primer was extended 1, 0, 1, 2, 2 or 3 bases beyond the DNA/2′-*O*-Me RNA junction, respectively. Some of this variation must represent local sequence effects, although the amount of upstream DNA may also have some influence on extension. When the template contained only two DNA residues past the priming site, little primer extension was observed (Fig. 6B, lane 8). This result suggests that *Taq* polymerase cannot efficiently initiate synthesis two bases upstream of the 2′-*O*-Me RNA, despite the fact that it can apparently extend up to the 2′-*O*-Me RNA, or beyond, if it has initiated synthesis further upstream.

Hybridization of DNA/2′-*O*-Me RNA oligonucleotides to DNA

Modifications to the backbone of an oligonucleotide will affect its hybridization properties. In order to assess the effect of 2′-*O*-Me RNA residues, we determined the melting temperatures (T_m) of duplexes consisting of a primer (DNA or chimeric) hybridized to a complementary DNA strand. Figure 7 shows the measured T_m for each chimeric oligonucleotide used in this study. The melting temperatures for both the UP and K26 series rise by ~7°C when 20 of the DNA residues are replaced with 2′-*O*-Me RNA residues. The increase in T_m for the S26 series is not quite as large, but it does increase and levels off at the point where 21 DNA residues have been replaced by 2′-*O*-Me RNA residues. For the RP series, the T_m increased by only ~2°C, several degrees less than for the other series. The T_m generally increases with the number of 2′-*O*-Me RNA residues present in the oligonucleotide.

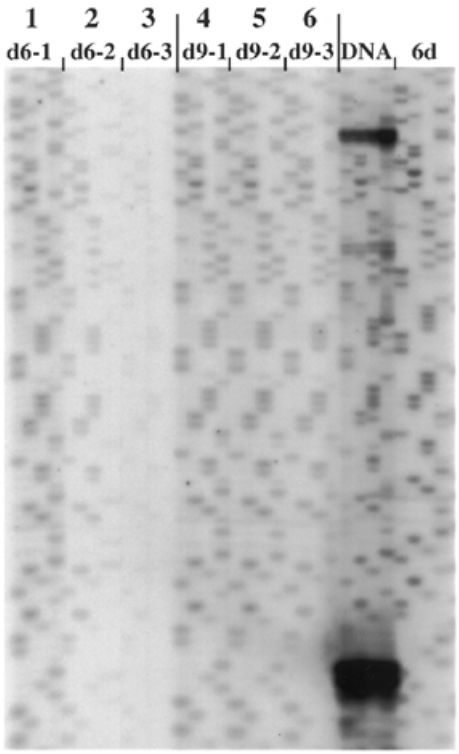

Figure 8. Sequencing with primers containing abasic residues. Sequencing reactions were performed as detailed in Materials and Methods. Lanes 1–3, two-primer reactions with the primer pairs designated above the lanes; lanes 4–6, sequencing ladders from only one primer. The DNA lane is a control with two DNA primers. The 6d lane contains the S26-6d and K26-6d primer pair.

Sequencing with primers containing abasic sites

Because of the success of the 2′-*O*-Me primers, we have not explored the use of abasic primers extensively. However, we have tested their ability to prime sequencing reactions and eliminate artifacts. Figure 8, lane 1 shows that oligomers containing a single abasic site located six bases from the 3′-end work effectively as sequencing primers. Furthermore, the artifact is eliminated by the use of these primers (compare lanes 1 and 7). Addition of more abasic sites results in a lower yield of sequencing products (lanes 2 and 3). When the abasic sites are placed further (nine residues) from the 3′-end, even oligonucleotides containing two (lane 4) or three (lane 5) abasic sites appear to work as well as unmodified DNA primers. We have not tested these in two-primer sequencing reactions, but presume that they would not yield artifacts.

DISCUSSION

We have demonstrated that oligonucleotides containing 2′-*O*-Me RNA residues or abasic residues can serve as efficient primers for DNA synthesis by *Taq* DNA polymerase in cycle sequencing reactions. Moreover, the use of these primers prevents the appearance of the artifacts that we set out to eliminate.

The ability of the 2′-*O*-Me RNA oligonucleotides to prime DNA synthesis was dependent on the inclusion of a few DNA residues at the 3′-end of the molecule. With six or more DNA residues, priming efficiency was indistinguishable from that observed with conventional DNA primers, although other experiments might reveal differences. As the number of DNA residues is lowered below six in some cases, or five in others, the efficiency of primer extension falls off. This result can be reconciled with the crystal structure of *Taq* polymerase complexed with duplex DNA (4), in which the protein contacts the primer strand on only the first five residues of its 3′-end.

RNA (without DNA residues) is known to be an efficient primer for many DNA polymerases, including *Escherichia coli* DNA polymerase I, a homolog of *Taq* polymerase (2). *Taq* polymerase appears to use RNA less efficiently as a primer (5), though a single 3′-terminal RNA residue does not interfere with PCR (6). 2′-*O*-Me RNA is even more different chemically from DNA, and probably presents more of a problem for primer extension. The need for DNA residues at the 3′-end of the primer is likely enhanced by methylation of the 2′-hydroxyl.

It is known that *Taq* polymerase possesses some reverse transcriptase (RTase) activity (7). This activity might compromise the beneficial effects of RNA primers. However, the RTase activity of *Taq* is weak, unlike that of the related *Tth* polymerase, and both enzymes show significant RTase activity only in the presence of manganese (8). If RTase activity were a potential problem, the use of 2′-*O*-Me RNA would likely have solved it: if a 2′-hydroxyl group makes for a bad replication template, methylation of this hydroxyl group probably decreases replication efficiency further. We expect, however, that the use of unmodified RNA would also prevent artifact amplification.

Oligonucleotides containing DNA and 2′-*O*-Me RNA residues were found to form more stable duplexes with DNA as the number of 2′-*O*-Me RNA residues increased. Inoue *et al*. (9) found that DNA/2′-*O*-Me RNA hybrids could be more or less stable than the corresponding DNA/DNA hybrids, depending on sequence. In contrast, we observed a stabilizing effect of 2′-*O*-Me RNA using four series of oligonucleotides with unrelated sequences, perhaps because of differences in the hybridization conditions. The largest difference in melting temperature between an oligonucleotide containing 2′-*O*-Me RNA and the corresponding all-DNA oligonucleotide was 7°C. This difference is smaller than the variation in melting temperatures among DNA primers of different sequences. The difference in melting behavior could be accommodated by a change in annealing temperatures or eliminated by adjustment of ionic strength or magnesium ion concentration. We have not, however, found it necessary to alter our cycle sequencing protocol in order to use the modified primers.

Some modified primers of another type, DNA oligomers containing abasic sites near the 3′-end, were also capable of priming DNA synthesis efficiently. The primers containing one to three abasic sites located nine bases from the 3′-end all primed synthesis as effectively as a molecule with no abasic sites. However, when the abasic sites were located closer to the 3′-end, priming efficiency was in some cases lowered. Molecules containing two or three abasic sites located six residues from the 3′-end yielded very little sequencing product. Although these sites would not directly contact the *Taq* polymerase (4), the modification must alter the primer–template structure enough to lower polymerization efficiency. In contrast, a single abasic site located six residues from the 3′-end did not lower priming efficiency. This modification did, however,

prevent exponential amplification and therefore eliminated the artifact.

There is much latitude in the choice of primer types from the families we have investigated. With DNA/2′-*O*-Me RNA oligonucleotides, as few as five or six DNA residues are sufficient for highly efficient priming, yet inclusion of as many as 10 DNA residues does not lead to artifact amplification. An entirely different type of modification, the inclusion of abasic sites, may also be used. Enzymes other than *Taq* polymerase can likely be accomodated, and other modified oligonucleotides, or molecules with non-nucleotide linkages such as peptide nucleic acids, can probably play the same role as oligonucleotides containing 2′-*O*-Me RNA or abasic sites.

The use of non-replicatable primers appears to be a practical method of eliminating cycle sequencing artifacts. This method is both cost-competitive and convenient in that it requires no change in sequencing protocols other than the substitution of one primer for another. Although artifacts were more of a problem when two primers were used together, they also arose when just one primer was used. The effect of using two primers may be template specific; the addition of a second primer increases the combinatorial possibilities for exponential amplification by only a factor of four. Furthermore, two-primer systems are in use, and developments in fluorescent dye technology may lead to increased use of such systems (10). Modified primers should be particularly useful in applications where it is desirable to perform a large number of cycles, such as direct sequencing of microbial genomic DNA (11) and direct sequencing of bacterial artificial chromosomes (BACs). The latter is essential to strategies involving BAC end sequencing, which have become important in the human genome project (12–14). By allowing increases in number of cycles without accompanying artifact generation, modified primers may allow further advances in sequencing and related methodologies.

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