Improved chemistry for oligodeoxyribonucleotide synthesis substantially improves restriction enzyme cleavage of a synthetic 35mer

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ABSTRACT

Two DNA duplexes of identical sequence and 35 nt in length were synthesized by an original and a highly improved version of phosphoramidite chemistry. By base composition analysis, DNA synthesized by improved chemistry (termed DMTS-imp) contained no detectable modified bases while DNA synthesized by the original chemistry (termed DMTS-std) had a large number of modifications. Under optimal reaction conditions, *Hha*I and *Rsa*I cleaved the DMTS-std duplex to 76-77% completion and the DMTS-imp duplex to 96-99% completion. Restriction analysis and piperidine treatment yielded estimates of ~3.0% modified nucleotides in DMTS-std and ~1.0% in DMTS-imp. Overall, the improvements in chemistry increased the restriction efficiency of synthetic DNA up to 10-fold.

INTRODUCTION

Synthetic oligodeoxyribonucleotides (oligomers) are being used increasingly in molecular biology for site-specific mutagenesis, DNA hybridizations and in assays for enzymes and proteins that interact with specific DNA sequences (1-7). For the success of many of these experiments, purified oligomers need to be free of modifications that would otherwise interfere with base pairing, cause helix distortion, and disrupt DNA-protein recognition. However, original versions of nucleoside phosphoramidite chemistry for solid phase synthesis have the potential for yielding DNA with modified bases. For example, methylation at the N3-position of thymine can occur if methoxyphosphoramidites are used and thiophenol deprotection is omitted (8). Additionally, modification at the O⁶-position of guanine has been reported in both methoxyphosphoramidite chemistry (9,10) and β-(cyanoethyl)phosphoramidite chemistry (11).

In the course of developing an assay for mammalian DNA methyltransferase using synthetic DNA, we found that only 60% of a duplex 35mer could be cleaved at a GCGC site by *Hha*I. Recently, however, improvements in solid phase phosphoramidite chemistry have made synthesis of DNA with very low levels of modified bases possible (11-13). For example,

if methoxyphosphoramidites are used, thiophenol deprotection time needs to be increased from the original 45 min (14) to eliminate any residual alkylated bases (13). Additionally, O⁶-guanine modification, which has been shown to produce 2,6 diaminopurine that can base pair with thymine thus causing transition mutations (15), can be virtually eliminated in both phosphoramidite chemistries by replacement of dimethyl-aminopyridine (DMAP), a capping catalyst, with N-methylimidazole (NMI) (11). Indeed, reports have appeared suggesting that the observed mutation frequency in cloning constructs may be related to the synthetic oligonucleotides used (16,17).

To determine whether reduced cleavage was related to the chemical authenticity of the oligonucleotides used here, new oligonucleotides were prepared using the highly improved phosphoramidite chemistry. While neither synthetic DNA was cleaved completely by the restriction enzymes tested as compared to the same oligomer cloned in *E. coli*, the improved chemistry yielded a duplex that was cleaved 96% or greater by *Hha*I and other enzymes. Thus, the improvements in chemical DNA synthesis have demonstrable biological relevance.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase, T4 DNA ligase, *Bam*HI and *Eco*RI were purchased from Promega; Klenow fragment of DNA polymerase I and *Pvu*II from International Biotechnologies, Inc.; *Rsa*I, *Sal*I and calf intestinal phosphatase from Boehringer Mannheim Biochemicals and *Hha*I (110,000 U/ml) by special arrangement from New England Biolabs. All chemicals were from Sigma except acrylamide (BioRad), piperidine (Fisher), dimethylsulfoxide (Fisher) and radioactive nucleotides (Amersham Corporation). Chemicals for synthesizing DNA oligomers were from Applied Biosystems, Inc. (ABI).

Synthesis and Characterization of Oligomers

Complementary oligomers were synthesized on either an ABI 380A or 381A DNA Synthesizer. The oligomer pair called DMTS-std were made with the 380A and synthesized on a 0.2 μ mol scale using ABI Standard cycle SSA003 (18) and Q-(methyl)-N,N-diisopropylphosphoramidites and deprotected as recommended (19). The oligomer pair called DMTS-imp were synthesized on the 381A at 0.2 μ mol scale using the 0.2 μ mol Standard Cycle (20) with version 1.23 software (21) and Q-(2'-cyanoethyl)-N,N-diisopropylphosphoramidites along with NMI-catalyzed capping (11) in place of DMAP, and iodine/water/pyridine oxidation solution (0.1 M/2%/20% in THF; ref. 12) in place of iodine/water/lutidine in

THF (11). Oligomers were purified on 20% polyacrylamide gels with 7 M urea and desalted on Sephadex G25-80 as described (19). Where indicated, oligomers were digested with snake venom phosphodiesterase, dephosphorylated, and analyzed by HPLC as described (22).

Radioactive Labelling and Duplex Formation

Synthetic oligomers were radioactively labelled at their 5' ends prior to annealing by T4 polynucleotide kinase (23) in the presence of [γ - 32 P]ATP (3000 Ci/mmol) and at their 3' ends after annealing by the Klenow fragment of DNA polymerase I (23) and [α - 32 P]-dATP or -TTP (3000 Ci/mmol). Duplexes were formed in 10 μ l of STE (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5), containing 2.0 pmol of each oligomer strand, by heating at 80°C for 15 min and cooling slowly to room temperature over 60 min. After labelling and annealing, one half volume of 7.5 M ammonium acetate, 10 μ g oyster glycogen and 5 volumes of ethanol were added to the DNA solution. DNA was isolated by centrifugation, washed three times with ethanol:water (5:1), dried and resuspended in STE at 0.05 pmol of duplex/ μ l.

Oligomer Cloning in *E. coli*

All restriction endonuclease digestions were done in buffer supplied by or using conditions recommended by the manufacturer and all procedures were from Maniatis *et al.* (23). pUC18 was digested to completion with SalI and dephosphorylated using calf intestinal phosphatase. DMTS-std duplex (2 pmol) was kinased as recommended for synthetic DNA linkers, ligated into the dephosphorylated pUC18 using T4 DNA ligase, and transformed into JM83. DNA from the resulting colonies was screened for the presence of inserts using XhoI and for number and orientation of inserts by linearizing the plasmids with BamHI, 3' end-labelling with the Klenow fragment of DNA polymerase I and [α - 32 P]-dATP, and digesting the DNA with HindIII for number and with MspI for orientation of inserts. A plasmid (p35) contained one insert in the desired orientation and was purified from a 500 ml culture by alkaline lysis and CsCl density gradient centrifugation.

The 35 bp insert (DMTS-p35) was purified from 50 μ g of p35 after digestion with SalI and 3' end-labelling with [α - 32 P]-dATP and the Klenow fragment of DNA polymerase I. After electro-phoresis on a 12% polyacrylamide gel in TBE, DMTS-p35 was eluted in 0.5 M ammonium acetate, 0.001 M EDTA, and acrylamide removed by centrifugation through glass wool. The oligomer was concentrated with n-butanol, ethanol precipitated, and resuspended in STE at 0.016 pmol/ μ l.

Restriction Analysis of Oligomer Duplexes and Longer DNA Fragments From p35

Restriction endonuclease cleavage of oligomers was done in buffers supplied by the manufacturers for 1 hr at 37°C containing additions as noted in each figure legend. After restriction, DNA was ethanol precipitated before gel electrophoresis as above and resuspended in loading buffer (80% formamide, 0.5 X TBE, 0.1% xylene cyanol, 0.1% bromophenol blue for the 35mer duplexes; 10% glycerol, 1 X TBE, 0.05% xylene cyanol, 0.05% bromophenol blue for longer DNA fragments). Cleavage products of the 35mers were assayed by electrophoresis on 20% acrylamide gels in 7 M urea and TBE at 45°C; longer DNA fragments were analyzed on non-denaturing 12% acrylamide gels in TBE at 25°C. After autoradiography, radioactive bands were excised and the amount of radioactivity determined by liquid scintillation counting.

Piperidine Treatment

DNA sequencing reactions were done on one strand of DMTS-imp labelled at its 5'-end as described (19). 3' end-labelled DMTS-p35 duplex labelled at its 3' ends and one strand of DMTS-std and DMTS-imp labelled at their 5'-ends were treated with piperidine by resuspending precipitated DNA in 20 µl of fresh 10% piperidine and incubating the solution at 90°C for 30 min. The solution was transferred to a new siliconized tube, frozen and dried in vacuo for 2 hr. DNA was resuspended in 40 µl water and dried twice, resuspended in denaturing gel loading buffer, and separated on a denaturing polyacrylamide gel as above. The amount of piperidine resistant DNA was determined by excising full length bands from control and piperidine-treated lanes after autoradiography.

RESULTS AND DISCUSSION

Restriction Endonuclease Cleavage of a 35mer Duplex Synthesized by the Original Chemistry

Two 35mers complementary at 31 nt were synthesized using an original version of methoxyphosphoramidite chemistry. The duplex, to be used for assaying mammalian DNA methyltransferase and termed DMTS (DNA methyltransferase substrate), contained several restriction sites including ones for HhaI and RsaI which were studied here. The sequence of the DMTS duplex is:

The methylase assay was designed so that transfer of a methyl onto the internal cytosine in the HhaI site by DNA methylase would inhibit HhaI cleavage. Providing that HhaI could cleave the unmethylated oligomer duplex

completely, the assay had the potential for being extremely sensitive. In unmethylated control reactions, however, ~40% of both strands of a DMTS duplex synthesized by the original chemistry (or DMTS-std) was not cleaved with *Hha*I or *Rsa*I at 200 units per pmol of duplex, a 700- to 1300-fold excess of enzyme (Figure 1A). Similar amounts of both strands were left uncut using other restriction enzymes (data not shown). A double digest of DMTS-std duplex with both *Hha*I and *Rsa*I reduced the level of uncut strands to ~14%, near the level expected if DNA molecules uncleaved by each enzyme were not a subset of molecules that are uncleavable at all sites. Hence, whatever inhibited the enzymes appeared to be random.

Results from several experiments also led to the conclusion that the oligomer itself was the source of the inhibition. For example, the inability of *Hha*I to cleave DMTS-std duplex was not an artifact caused by gel purification because unpurified DMTS-std duplex was also cleaved to the same extent (data not shown). Uncut molecules did not appear to arise from unhybridized single-stranded 35mers that cleave at a much lower rate than duplex DNA, because virtually all 35mers were in double-stranded form (Figure 1B). Additional experiments also showed that for the most part the extent of the reaction was unaffected by altering reaction times, enzyme-to-DNA ratio, and reaction conditions (i.e., salt, pH, temperature). However,

Figure 1. A. Restriction endonuclease cleavage of DNA oligomers synthesized by original chemistry. DMTS-std duplex was digested with *Hha*I (H), *Rsa*I (R) or both (H/R) in 20 μ l of buffer containing 0.05 pmol of DNA duplex (6.2×10^6 cpm/pmol) and 10 units of restriction enzyme. Two digests are shown for each enzyme; one with the bottom strand (1) and one with the top strand (2) 5' end-labelled.

B. Nondenaturing gel of DMTS-std. DMTS-std duplex 5'-end labelled on the top strand (lane 2) or the bottom strand (lane 3) and unannealed bottom strand (lane 1) were separated on a 15% acrylamide gel in TBE.

Figure 2. HPLC analysis of 35-base oligonucleotide (dC₁₁dG₁₂dT₆dA₆) after enzymatic digestion. The oligomers were made by either improved (A, DMTS-imp) or original (B, DMTS-std) phosphoramidite chemistry as detailed in Materials and Methods. Both UV254 and fluorescence (FI; EX=340 nm, EM>418nm) traces are shown. Peaks co-migrating with contaminants in the digestion blank are indicated by an asterisk (*).

a 16% improvement in cleavage efficiency was obtained by adding dimethylsulfoxide to 5% (v/v) and increasing the enzyme:DNA ratio to 600 U/pmol. Thus, under these conditions, which were used in the experiments described below, the DMTS-std duplex could be cleaved to 76% and 71% completion by *Hha*I and *Rsa*I, respectively.

Base Composition Analysis

One strand of DMTS-std duplex (i.e., the lower strand, see above) was analyzed by enzymatic digestion followed by reverse phase HPLC (22). These values were compared to those obtained for the same strand of an oligomer duplex of identical sequence synthesized using the improved chemistry (DMTS-imp). The results are shown in Figure 2 and the data summarized in Table 1. It can be seen that virtually no modifications were detectable in DMTS-imp by optical density at 254 nm or by fluorescence. The deoxyinosine arises via an adenosine deaminase activity contaminating snake venom

Table 1.

Base composition analysis of the two oligomers synthesized by original and improved chemistry.

DMTS-std			DMTS-imp	
Assignment	Ret. time	% of total peak area*	Ret. time	% of total peak area*
dC	14.61	15.94	14.15	19.80
dI	29.80	0.57	28.06	1.09
dG	31.53	36.68	29.93	50.21
dT	34.47	7.66	33.17	10.55
dA	42.94	17.85	41.81	18.34
		78.7%	100.0%	
Extra peaks**		52	0	

*Peaks co-migrating with peaks in a digestion blank were omitted for the calculation. Benzamide (bz) present in DMTS-imp because a "crude" sample was used for analysis, was also omitted for the calculation. dI is present due to adenosine deaminase contamination in the snake venom phosphodiesterase (20).

**Peaks not assigned and not co-migrating with peaks in the digestion blank.

phosphodiesterase (22). Hence, 100% of the optical density was contained in dA, dT, dC, dG and dI. By contrast, DMTS-std contained 52 detectable peaks comprising 21.3% of the optical density with 78.7% of the absorbance residing in the expected 5 deoxyribonucleosides. Of this 21.3%, over half or 11.1% came from the peaks at 5-6 min peak which were absent from DMTS-imp. Of the remaining peaks, only a few are actually assignable as known modified deoxyribonucleosides, e.g., 1-methyldeoxyguanosine at 39.8 min (13); 2,6 diaminopurine at 40.5 min (11); N3-methyldeoxythymidine at 51.7 min (8,13); N⁶-methyldeoxyadenosine at 56 min (13); and 6-DMAP-deoxyguanosine at 63.6 min (11). Calculations of the empirical formula (22) for DMTS-std duplex based on these data gave an error rate of 5.8% per deoxynucleoside compared to theoretical values. The relationship between these data and those reported before (22), along with estimates of modification from restriction and piperidine treatment is discussed below.

Modification Levels Arising from Methyl Deprotection

Methyl transfer to N⁶-adenine, N¹-guanine, and N³-thymine is known to occur during methyl deprotection with thiophenol (11) and the three modified deoxynucleosides were found in DMTS-std at detectable levels. Without correction for differences in extinction coefficients, N⁶-methyl-dA occurred at ~1.1% of dA; 1-methyl-dG at 0.6% of dG; and N³-methyl-dT at 5.7% of dT.

Figure 3. Restriction endonuclease cleavage of DMTS-std, DMTS-imp and DMTS-p35 duplexes. DMTS-std (1), DMTS-imp (2) and DMTS-p35 (3) duplexes labelled at their 3'-ends were digested with *HhaI* and *RsaI* in 10 μ l of buffer containing 5% (v/v) dimethylsulfoxide, 0.033 pmol of DNA duplex (3.0 – 9.4×10^6 cpm/pmol) and 20 units of enzyme.

These values are consistent with a deprotection time of 5 min or less. The program for the early version chemistry used in the synthesis of this compound included a 30 min methyl deprotection step. Hence, the data imply an inadequate methyl deprotection step. How common this problem may have been using the older protocols is unknown, but was clearly substantial in this case. It should be noted, however, that it was previously reported (22) that methoxyphosphoramidites can be used successfully to synthesize chemically authentic DNA comparable to β -cyanoethyl-made DNA.

Restriction Analysis of the Oligomer Synthesized by Improved Chemistry

Even though DNA synthesized by the improved chemistry contained no detectable modifications (see above), we cloned the oligomer duplex into the *SalI* site of pUC18 to obtain an oligomer duplex that would serve as a control. The plasmid p35 had one copy of the 35mer duplex, termed DMTS-p35, which was purified on a non-denaturing polyacrylamide gel after digestion with *SalI* and 3' end-labelling. Although the insert was not completely sequenced, five restriction sites were still intact. DMTS-std, -imp and -p35 duplexes were digested with *HhaI* and *RsaI* under optimal conditions (see above), and the results are shown in Figure 3 and summarized in Table 2. Although none of the DNAs was cut to completion by either enzyme, the extent of cleavage was substantially improved with DMTS-imp and -p35 duplexes as

Table 2.
Restriction endonuclease cleavage
of oligonucleotides.

DNA	Uncleaved DNA (%)	
	HhaI	RsaI
DMTS-std	24.5 ^a (24.2-24.9)	29.3
DMTS-imp	7.9 (7.7-8.0)	8.5
DMTS-p35	2.4 (2.3-2.7)	7.0 (6.6-7.2)

^a data is expressed as the percentage of the DNA that is uncleaved and is the average of three digests when given with a range ().

substrates. HhaI cut DMTS-std duplex to 76% completion, DMTS-imp duplex to 92% completion and DMTS-p35 duplex to 98% completion while RsaI cut DMTS-std duplex to 71% completion, DMTS-imp duplex to 91% completion and DMTS-p35 duplex to 93% completion. Normalizing the extent of cleavage to DMTS-p35 duplex indicated that HhaI and RsaI cut DMTS-std and -imp duplexes 76-77% and 96-99% as effectively as they cut DMTS-p35 duplex. Hence, instead of ~1 of 4 molecules remaining uncut, the optimized chemistry yielded DNA molecules in which only ~1 of 40 molecules were not cleaved by either enzyme.

Restriction Analysis of the Oligomer in Longer DNA Fragments

Because DMTS-p35 duplex was not cut to completion with either enzyme, incomplete cleavage of the synthetic oligomer duplexes could not have been caused entirely by modified bases. Incomplete cutting of the cloned oligomer may have been caused by impurities in the commercial enzyme preparation or inactive enzyme molecules bound to the cleavage site. However, other HhaI preparations gave comparable results on DMTS-std duplex and increasing or decreasing HhaI in the digests did not substantially alter the extent of cleavage (unpublished observations). Moreover, removing the enzyme by extraction with organic solvents after a first digestion did not alter the level of cutting the three oligomers duplexes in a second digestion.

It was also possible that both enzymes had reduced activity on the short DNA fragments or that the large number of palindromes in the oligomer "poisoned" the reactions. To test both possibilities, two HhaI-containing fragments were isolated from p35 (Figure 4). A 266 bp HindIII/PvuII fragment contained the 35mer sequence 39 bp from the HindIII site; it was labelled at the HindIII site and assayed cutting at the HhaI site in the oligomer. A 201 bp BamHI/PvuII fragment lacked the 35mer but contained a "natural" pUC18 HhaI site 40 bp from the PvuII end; it was end-labelled at

Figure 4. Restriction map of oligomer in pUC18. The portion of p35 from the HindIII site in the polylinker through the oligomer to the PvuII site in the lacI' gene is shown. HindIII (D), SalI (S), HhaI (H), BamHI (B), EcoRI (E), PvuII (P).

the BamHI site and assayed cleavage at a pUC18 HhaI site. Because both HhaI sites are on fragments longer than the oligomer and lie at nearly identical distances from each end, the two fragments effectively test the effect of fragment length and oligomer sequence on cleavage. As shown in Table 3, HhaI sites in the oligomer and pUC18 were cut to similar levels and neither to completion. Uncut DNA did not appear to come from DNA damaged during isolation since the 266 bp fragment was cleaved to >99% completion by EcoRI. Hence, HhaI was unable to cut the DMTS-p35 duplex to completion for reasons other than the length of the fragment and sequences surrounding the HhaI site in the oligomer. Rather it reflects an inherent property of the enzyme.

Piperidine Sensitivity of the Synthetic DNAs

The improved chemistry clearly produced a synthetic oligomer duplex with substantially improved substrate cutting efficiency. Nonetheless, it fell slightly short of the cleavage levels seen for DMTS-p35 duplex. It was

Table 3.
Restriction endonuclease cleavage
of sites in DNA fragments from p35.

Restriction Enzyme ^a	Origin of Site	Sites uncleaved ^b (%)
HhaI	pUC18	7.0 (6.0-7.6)
HhaI	DMTS-std	6.0 (5.2-6.5)
EcoRI	pUC18	0.3 (0.02-0.4)

^a restriction digests were done in 10 μ l of buffer containing 0.033 pmol DNA duplex and 20 units of enzyme

^b data is expressed as the percentage of the DNA that is uncleaved and is the average of three digests when given with a range ().

likely, therefore, that DMTS-imp duplex still contained a low level of modification. Piperidine treatment was used to assess this possibility. The lower strand of each duplex (see above) was labelled at its 5' end and treated with or without piperidine at 90°C for 30 min. The results are illustrated in Figure 5 with both short and long exposures. Note that for DMTS-p35, it was not possible to isolate separate strands so the 3' ends were labelled by extending 4 nt with the Klenow DNA polymerase I and labelled dNTP. Hence, the uncleaved DMTS-p35 band contains both strands each 4 nt longer than the oligomers.

In the untreated controls, a small amount of both oligomers were contained in fragments smaller than the undegraded strands. The source of this partial degradation is unknown but may reflect in part radiation-induced cleavage because both strands were labelled to the same specific activity and partially fragmented to the same extent. However, DMTS-p35 did not show significant degradation at comparable specific activity and storage times (not shown). Nonetheless, DNA synthesized by original chemistry was

Figure 5. Piperidine treatment of DNA synthesized using original and improved chemistry. 5'-end labelled DNA synthesized using improved (Imp.; 1.3 pmol single-stranded DNA) and original chemistry (Std.; 1.8 pmol single-stranded DNA) and 3' end-labelled DMTS-p35 duplex (35; 0.5 pmol duplex DNA) were treated with fresh 10% piperidine for 30 min at 90°C. After drying and washing equal amounts of radioactivity from untreated (-) and piperidine-treated (+) DNAs were separated on a 20% acrylamide gel in 7 M urea. A long (15 hr; left side) and a short (3 hr; right side) exposure of the synthetic DNA lanes is shown. DNA synthesized by improved chemistry was used in G and G+A sequencing reactions for markers.

significantly more sensitive to piperidine cleavage (36% of the molecules completely resistant) than DNA synthesized by improved chemistry (64% of the molecules resistant). The DMTS-p35 duplex was virtually undegraded as observed by others for natural DNAs (24).

On the average, cleavage products for the DMTS-imp strand were also longer than for the DMTS-std strand reflecting a lower frequency of modified sites per strand. From Figure 5 it appears that many fewer counts from piperidine-treated DMTS-std oligomer were retained on the filter after fixing and drying the gel, suggesting that many short fragments were generated by the treatment. It also appears from the cleavage products that all 4 bases were piperidine-sensitive to varying extents. Piperidine treatment alone cannot, however, distinguish between a modified base that is piperidine-sensitive versus a preexisting apurinic site. Hence, what the sensitivity to piperidine means in strict chemical terms is not known.

If modification occurred during synthesis of the oligomer, modified bases would be more prevalent at the 3'-end of the DNA. However, each base occurred in the piperidine ladder at approximately equal intensity suggesting that the modified bases are distributed evenly throughout the DNA molecule. This is also consistent with the observation that *HhaI* and *RsaI* cut both strands of DMTS-std duplex with equal efficiency (Fig. 1A) despite the fact that their cleavage sites were not centrally located. Therefore, modification could have occurred after synthesis (e.g., during cleavage from the solid support and base deprotection), and/or some randomizing process could have occurred during synthesis. For example, bases modified early in the synthesis could depurinate (or depyrimidate) in subsequent cycles and never be found in a full-length oligomer purified at the end of the synthesis.

Estimating DNA Modification Levels

The data on both piperidine sensitivity of the synthetic DNA and *HhaI* cleavage of DMTS-std and -imp duplexes can be used to estimate the extent of modification of the DNA. A *HhaI* site contains eight bases and if a single modified base can inhibit the enzyme, then the fraction of unmodified *HhaI* sites (F_{gcgc}) is equal to the fraction of unmodified bases (F_{nt}) raised to the 8th power, or $F_{\text{gcgc}} = (F_{\text{nt}})^8$. For the data in Table 2, after correction for the fraction of unmodified sites not cleaved by the enzyme (2.4%), DMTS-std and DMTS-imp duplexes contained 3.1% and 0.7% modification at each base, respectively. A similar calculation can be made from the data on the piperidine sensitivity of the 35mers. In this case, the fraction of

piperidine-resistant 35mer (F_{35mer}) equals the fraction of unmodified bases (F_{nt}) raised to the 35th power, or $F_{35mer} = (F_{nt})^{35}$. By this estimate, DNA synthesized by original and improved chemistries are 2.9% and 1.3% modified at each base, respectively.

These estimates for DMTS-std are about 2-fold lower than the level of modification (5.8% per base) estimated from base composition analysis (Figure 2 and Table 1), where greater than 20% of the absorbance occurred in the extra peaks. Only a few of these extra peaks have actually been assignable as modified deoxynucleosides. Some peaks may reflect incomplete digestion of the oligomer. For example, they could be phosphorylated deoxynucleotides or short oligomers (di- and trimers) that are not modified but arise because of modification on other bases in the oligomer that inhibit the enzyme at that point. Furthermore, some modifications may not inhibit activity of the restriction enzymes, thus underestimating the extent of modification.

Nonetheless, an important question concerns how changes between the early version and the improved chemistries led to improvement in cleavage at both HhaI and RsaI sites. Both sites contain two bases that could have been modified during synthesis in the original chemistry. HhaI sites contain two G's and RsaI sites contain a single G and a single T. Guanine underwent modification in the older chemistry during capping and methyl deprotection giving rise to 6-DMAP-deoxyguanosine and 1-methyldeoxyguanosine, respectively, while T underwent modification during methyl deprotection giving rise to N3-methylthymine. All three modified bases were reduced to undetectable levels in the improved chemistry in which β -(cyanoethyl)phosphoramidites replaced of methoxyphosphoramidites, NMI replaced DMAP during capping, and iodine/water/pyridine in THF replaced iodine/water/lutidine in THF during oxidation of trivalent phosphorous. Hence, the reduction of the three modified bases to undetectable levels most likely had a significant effect on the improvement in cleavage efficiency.

It is not possible, however, to account for the improved cleavage efficiencies by just these three modified bases. As noted above, the levels of cleavage were not easily correlated with the levels of the individual modified bases. Furthermore, the newer chemistry also eliminated the large number of other undefined peaks seen by HPLC of DMTS-std deoxynucleosides. It is also worth noting that the improved substrate still contained substantial levels of sites that were piperidine and heat sensitive. Indeed, all 4 bases in the RsaI site were modified to some extent. Hence,

modified Gs and Ts cannot be singled out as the only elements contributing to the inhibition of cleavage efficiencies. What the nature of the heat and piperidine-sensitive sites are is unknown, but it is likely that they have not been identified by HPLC given their lack of UV absorbance. They might include modified phosphoesters, abasic sites, or other products arising from deprotection of the exocyclic amines during ammonolysis and acid hydrolysis.

Concluding Comment

It is important to emphasize that oligomers synthesized by the improved chemistry manifest a greater chemical authenticity than those synthesized by an earlier version. Furthermore, this improvement in chemical authenticity yields enhanced biological fidelity. In the present examples, for instance, the background level of uncut DNA has been reduced to only a few percent for DMTS-imp as opposed to ~25% for DMTS-std, and the level of modification is at most one in a hundred bases. For short DNA fragments, therefore, most molecules are undamaged and will have little, if any effect, on most experiments. Nonetheless, it appears that some modifications are still introduced in the newer chemistry which are undetectable by HPLC analysis alone. In the future, improvements in phosphoramidite chemistry such as those discussed herein will insure high levels of biological fidelity, both *in vitro* and *in vivo*, thus expanding the utility, value, and success of synthetic oligonucleotides.

Nonetheless, it is important to note that the problems encountered in our early version oligomers may also apply to others synthesized under identical conditions. Given that oligonucleotides are usually synthesized in large excess over actual laboratory needs, those having oligonucleotides synthesized by similar chemistries should use them with caution.

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