2′-Fluoro modified nucleic acids: polymerase-directed synthesis, properties and stability to analysis by matrix-assisted laser desorption/ionization mass spectrometry

Tetsuyoshi Ono, Mark Scalf and Lloyd M. Smith*

Department of Chemistry, University of Wisconsin, Madison, WI 53706, USA

Received July 14, 1997; Revised and Accepted September 29, 1997

ABSTRACT

Fragmentation is a major factor limiting mass range and resolution in the analysis of DNA by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Protonation of the nucleobase leads to base loss and backbone cleavage by a mechanism similar to the depurination reactions employed in the chemical degradation method of DNA sequencing. In a previous study [Tang,W., Zhu,L. and Smith,L.M. (1997) Anal. Chem., 69, 302–312], the stabilizing effect of substituting the 2′hydrogen with an electronegative group such as hydroxyl or fluorine was investigated. These 2′substitutions stabilized the N-glycosidic linkage, blocking base loss and subsequent backbone cleavage. For such chemical modifications to be of practical significance, it would be useful to be able to employ the corresponding 2′-modified nucleoside triphosphates in the polymerase-directed synthesis of DNA. This would provide an avenue to the preparation of 2′-modified PCR fragments and dideoxy sequencing ladders stabilized for MALDI analysis. In this paper methods are described for the polymerase-directed synthesis of 2′-fluoro modified DNA, using commercially available 2′-fluoronucleoside triphosphates. The ability of a number of DNA and RNA polymerases to incorporate the 2′-fluoro analogs was tested. Four thermostable DNA polymerases [Pfu (exo–), Vent (exo–), Deep Vent (exo–) and UlTma] were found that were able to incorporate 2′-fluoronucleotides with reasonable efficiency. In order to perform Sanger sequencing reactions, the enzymes’ ability to incorporate dideoxy terminators in conjunction with the 2′-fluoronucleotides was evaluated. UlTma DNA polymerase was found to be the best of the enzymes tested for this purpose. MALDI analysis of enzymatically produced 2′-fluoro modified DNA using the matrix 2,5-dihydroxy benzoic acid showed no base loss or backbone fragmentation, in contrast to the extensive fragmentation evident with unmodified DNA of the same sequence.

INTRODUCTION

The development of alternative technologies for DNA sequencing is an area of substantial interest and activity worldwide (1). In one approach to this problem the separation of DNA strands produced in enzymatic (Sanger) extension reactions is performed by mass spectrometry rather than by conventional electrophoresis (2–8). There are two mass spectrometric techniques which are able to effectively analyze large biopolymers, electrospray ionization (ESI) (9) and matrix-assisted laser desorption/ionization (MALDI) (10). MALDI is better suited to the analysis of mixtures than is ESI, as it produces predominantly singly charged molecular ions, in contrast to the fairly complex charge distributions generated by ESI. Accordingly, most work on the analysis of Sanger sequencing reactions by mass spectrometry has employed MALDI.

These efforts have revealed both the strengths and the weaknesses of MALDI for DNA sequencing. The basic feasibility of sequencing by MALDI-MS has been demonstrated for the analysis of both Sanger reactions (2–8) and of the products of nuclease digestion of DNA (11–15) and RNA (16). Sensitivity and resolution have increased markedly due to improvements in both the instrumentation (17,18) and in the chemistry, particularly with respect to methods for purifying the DNA adequately prior to mass analysis (19–25). Notwithstanding these advances, substantial limitations in the approach have also been revealed. The longest sequences analyzed by MALDI are still only ~100 nucleotides in length (26); although longer RNAs have been MALDI analyzed [147 bases (27) and 461 bases (28)], both resolution and sensitivity were severely degraded compared to that obtained with shorter oligomers. These issues were particularly apparent in an analysis of mock sequencing reactions, where the signal intensity diminished by over a factor of 10 for a 41mer oligodeoxynucleotide compared to a 17mer in an equimolar mixture of six oligodeoxynucleotides of varying length (5). Thus, MALDI analysis of DNA faces at the present time a severe limitation in mass range, which has limited its utility to the analysis of fairly short oligomers.

*To whom correspondence should be addressed. Tel: +1 608 263 2594; Fax: +1 608 265 6780; Email: smith@chem.wisc.edu
One possible explanation for this limited mass range is that the DNA molecules are fragmenting substantially in the course of the MALDI process. This might well be expected to have a greater effect upon longer DNA molecules than upon shorter ones, both because of the larger number of possible sites for fragmentation in longer molecules and because longer molecules spend more time in the source region of the spectrophotometer than do short molecules and thus have more time during which they can undergo gas phase reactions leading to fragmentation. Support for this hypothesis was obtained in studies of model synthetic oligonucleotides, which showed that substantial fragmentation is indeed occurring, and that the degree of fragmentation depends strongly upon oligonucleotide length and the field strength within the source region as well as upon the oligodeoxyxynucleotide sequence and the matrix employed (29). A model was developed for the fragmentation mechanism (30,31) in which the initiating step in oligodeoxyxynucleotide fragmentation during MALDI is protonation of the nucleobase moiety, which weakens the N-glycosidic linkage causing base loss with concomitant formation of a carbocation at the 1′ position of the deoxyribose moiety. A subsequent rearrangement leads to backbone cleavage at the carbon–oxygen bond. It was further shown that a hydroxyl or fluorine moiety substituted at the 2′ carbon stabilizes the DNA to fragmentation and thus extends the accessible mass range (32). This was attributed to the inductive effect of these substituents, reducing electron density at the 1′ carbon and hence destabilizing the putative carbocation intermediate. The fluorine group provides somewhat greater stabilization than a hydroxyl group, presumably because of its greater electronegativity (33).

In order for this stabilization of DNA to MALDI analysis to have utility for DNA sequencing or other nucleic acid analyses, it is essential that means be developed for incorporating such chemical modifications into the DNA analyte of interest. One reasonable approach to this is to synthesize the DNAs of interest by polymerase extension with 2′-fluoronucleotide triphosphates in place of the normal unmodified nucleoside triphosphates typically employed. This approach would enable both DNA sequencing and PCR amplification, as both techniques rely upon polymerase-directed synthesis. It is only necessary to have the 2′-fluoro substitution on the A, C and G nucleotides, as T is intrinsically resistant to fragmentation (27,34,35). Although 2′-fluoronucleotide homopolymers have been synthesized using polynucleotide phosphorylase (36–39), and 2′-fluoronucleotides have been incorporated into RNA with T7 RNA polymerase (40–42), methods for the polymerase-directed synthesis of nucleic acids substituted with 2′-fluoronucleotides have not been described.

In this paper we present methods for the polymerase-directed synthesis of 2′-fluoro modified mixed base nucleic acids. A number of polymerases were screened for their ability to incorporate the 2′-fluoronucleotides. The resultant chemically modified DNA molecules are shown to have a number of interesting properties, including resistance to nuclease digestion and stability in MALDI analysis. The resistance to nuclease digestion is exploited to provide a simple means of degrading unmodified primer and DNA template in the reaction, facilitating purification prior to MALDI analysis. These chemically modified and stabilized nucleic acids are likely to find many applications in molecular biology and molecular diagnostics, particularly in the area of MALDI-MS.

**MATERIALS AND METHODS**

**Materials**

2′-fluoro-2′-deoxyguanosine-5′-triphosphate (2′FdGTP), 2′-fluoro-2′-deoxyadenosine-5′-triphosphate (2′FdATP), 2′-fluoro-2′-deoxy-ctydine-5′-triphosphate (2′FdCTP) and 2′-fluoro-2′-deoxy-uridine-5′-triphosphate (2′FdUTP) were from Amersham Life Science (Arlington Heights, IL). dNTPs and ddTTP were from Pharmacia Biotechnology (Piscataway, NJ). Oligonucleotides (gel purified) were from Integrated DNA Technologies, Inc. (Coralville, IA), and in some cases included the fluorophores HEX and 6-FAM at the 5′ termini. Oligonucleotides used as controls for mass spectrometry were synthesized by the University of Wisconsin Biotechnology Center (Madison, WI) on an Applied Biosystems 394 DNA synthesizer. SequiTherm DNA Polymerase, TTh DNA polymerase, Th DNA polymerase, and rBst DNA polymerase were from Epicentre Technologies (Madison, WI). Thermo Sequenase DNA polymerase was from Amersham Life Science. AmpliTaq DNA polymerase, AmpliTaq DNA polymerase-Stoffel Fragment, AmpliTaq DNA polymerase-FS, and UItma DNA polymerase were from PE Applied Biosystems (Foster City, CA). Cloned Pfu DNA polymerase and recombinant exo− Pfu DNA polymerase were from Stratagene (La Jolla, CA). Vent DNA polymerase, Vent (exo−) DNA polymerase, Deep Vent DNA polymerase and Deep Vent (exo−) DNA polymerase were from New England Biolabs (Beverly, MA). SP6 and T7 RNA polymerases were from Promega Corporation (Madison, WI).

**Polymerase extension reactions from a 99mer template with 2′FdNTPs**

This extension reaction was used for screening DNA polymerases for their ability to incorporate 2′FdNTPs. It yields extension products labeled at the 5′ terminus with a single Rox dye. Buffers employed for polymerase extension reactions were those recommended by the manufacturer. Reactions (20 µl) were performed using 2 pmol of the oligonucleotide template d(ACTGACTACTGACTACTGACTACTGACTACTGACTACTGACTACTGACGCGCCCGCA), 4.5 nmol each of 2′FdATP, 2′FdTTP, 2′FdCTP, and 2′FdUTP, and 1–6 U DNA polymerase. The incorporation reaction was placed at 95 °C for 90 s, 55 °C for 30 s and 72 °C overnight. The reaction product was then centrifuged for 15 min at 16 000 g at 4 °C. The resulting sample pellet was washed with 200 µl of 70% EtOH, dried down by rotary evaporation in a Savant SpeedVac Concentrator and dissolved in 10 µl of formamide/EDTA loading buffer (9.5 ml 100% formamide, 0.2 ml 500 mM EDTA pH 8.0, 0.3 ml water, 0.1 g blue dextran). After heating at 95 °C for 5 min, 1 µl was applied to a 6% polyacrylamide gel and analyzed on an ABI 370A DNA Sequencer.

**Polymerase extension reactions from a 42mer template with 2′FdNTPs**

This extension reaction was used for preparing 2′-fluoro modified DNA for analysis by MALDI-MS. The template strand was
labeled in all cases with the fluorophore HEX. In some experiments the primer oligonucleotide was labeled with the fluorophore 6-FAM at the 5′ end. Internal labeling of the extension product was sometimes performed as well, using fluorescein-12-UTP (Boehringer Mannheim, Indianapolis, IN). Conditions employed for synthesis of full length (42 nt) extension product labeled internally with fluorescein were as follows: 20 μl reactions were performed using 20 pmol of the oligonucleotide template HEX-d(CCCCCCCCCCTGCCCTTGCCCTCCT-AGTGCTGCTTTTACA), 20 pmol primer 6-FAM-d(TGTAAAACGACGCTTGA), 4.5 nmol each of 2′FdCTP, 2′FdATP, 2′FdGTP, 1 nmol fluorescein-12-UTP, and 4 U Vent (exo−) DNA polymerase. The reaction buffer was 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris–HCl (pH 8.8), 4 mM MgSO₄, and 0.1% Triton X-100. The same conditions were employed for strand extension without an internal fluorescein label by substitution of the fluorescein-12-UTP with 4.5 nmol dUTP (yielding a 37mer as the extension product), and a 5′ phosphate primer was substituted for the 6-FAM primer in some experiments. A similar reaction was performed using Ultima DNA polymerase, except that the reaction buffer contained 10 mM Tris–HCl (pH 8.8), 10 mM KCl, 0.002% Tween 20 (v/v), 3.75 mM MgCl₂, 2 U Thø pyrophosphatase, thermostable (Boehringer Mannheim, Indianapolis, IN) and 6 U Ultima DNA polymerase. Reactions were performed for the times and temperatures described above. The reaction mixture was diluted to 500 μl with H₂O, and the products were desalted and concentrated by microcentrifugation in a Microcon-10 microcentrator (Amicon, Inc., Beverly, MA, 14 000 g, 35 min, 25°C).

**Nuclease digestions**
The 42mer extension product prepared and purified as described above was subjected to a variety of nuclease digestions to remove/degrade unmodified primer DNA and/or template DNA. Conditions for these digestions were as follows.

*Lambda exonuclease.* Reactions (25 μl) were prepared using approximately half of the purified sample prepared as described above (~10 pmol), 67 mM glycine-KOH (pH 9.3), 2.5 mM MgCl₂, and 2 U lambda exonuclease (Pharmacia Biotechnology, Piscataway, NJ). Digestion was performed at 37°C for 60 min.

*DNase.* Reactions (50 μl) were prepared using approximately half of the purified sample prepared as described above (~10 pmol), 40 mM Tris–HCl (pH 8), 10 mM NaCl, 6 mM MgCl₂, 10 mM CaCl₂, and 2 U RNase-free DNase (Promega Corporation, Madison, WI). Digestion was performed at 37°C for 60 min.

*RNaseT1.* The resistance of the 2′-fluoro modified DNA to RNase digestion was determined by subjecting ~20 pmol of the 2′-fluoro modified DNA prepared by excess (15 U) DNase digestion as described above to RNase treatment. Reactions (100 μl) of 2′-fluoro modified DNA labeled with fluorescein-12-UTP in 50 mM Tris–HCl (pH 7.4), 2 mM EDTA (pH 8) were incubated with excess (2000 U) RNaseT1 (GIBCO BRL, Life Technologies, Grand Island, NY) at 37°C for 30 min. A control reaction using RNA (167 nt) synthesized by transcription from an SP6 promoter (Promega Corporation, Madison, WI) and internally labeled with fluorescein-12-UTP was performed using the same conditions as above.

**ddT ladder**
Reaction mixtures (20 μl) contained 10 mM Tris–HCl (pH 8.8), 10 mM KCl, 0.002% Tween 20 (v/v), 3.75 mM MgCl₂, 0.4 pmol ABI primer (−21M13), 1 μg ssM13mp18 phage DNA, 225 μM each of 2′FdCTP, 2′FdGTP, 2′FdATP, 45 μM ddTTP, 500 μM 2′,3′ddTT, 2 U Thø pyrophosphatase, thermostable, or 2 U thermostable inorganic pyrophosphatase (New England Biolabs, Beverly, MA) and 6 U Ultima DNA polymerase. Reactions were performed as follows: 95°C for 90 s, 55°C for 30 s and 72°C for overnight.

**MALDI analysis of 2′-fluoro modified DNA**
2′-Fluoro modified DNA treated with DNase to degrade unmodified DNA as described above was phenol-extracted once, desalted and concentrated with a Microcon-3 microconcentrator (Amicon, Inc., Beverly, MA) twice, and dried down in a Savant SpeedVac. The dried sample from 10 combined reactions was dissolved in 2 μl of H₂O and further desalting was accomplished by flat dialysis using a 0.025 μm filter (type VS, Millipore, Bedford, MA). The filter was floated on 50 ml of milliQ water and the sample was placed on top of the filter and left to equilibrate for 1 h.

Matrices for mass spectrometry were prepared as follows: saturated solutions of 3-hydroxypropionic acid (3-HPA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (Aldrich Chemical Co., Milwaukee, WI) were prepared in mixtures of 1:1:2 water:acetonitrile:0.1% acetic acid. The sample was spotted on a 2 mm diameter stainless steel probe tip and allowed to crystallize before analysis by MALDI-MS.

Mass spectra were obtained on a Bruker Reflex II time-of-flight mass spectrometer (Billerica, MA), equipped with a 337 nm N₂ laser and operated in linear, positive-ion detection mode with an acceleration voltage of 25 kV. Each spectrum consisted of the sum of 50 shots or fewer.

**RESULTS AND DISCUSSION**

**Screening of polymerases for incorporation of 2′FdNTPs**

Several RNA and DNA polymerases were screened for their ability to incorporate 2′FdNTPs in overnight extension reactions. The overnight reaction time was chosen because it was found that longer reaction time gave longer extension products in the case of SequiTherm and also showed decreased amounts of false terminations using Vent (exo−) (data not shown). Although both SP6 and T7 RNA polymerases were able to incorporate a single 2′FdNTP (that is, one of the four dNTPs employed in the reaction was modified with a 2′ fluorine group), and some extension was seen with two 2′FdNTPs, little or no extension was obtained when all three (2′FdATP, 2′FdCTP and 2′FdGTP) were employed (data not shown). As better results were obtained with some of the DNA polymerases examined (see below), these were the focus of subsequent work.
Figure 1. Screening of polymerases for incorporation of 2′FdNTPs. SequiTherm, Thermo Sequenase and AmpliTaq FS have no exonuclease activities and the latter two enzymes contain the F667Y mutation. Bst has both 5′→3′ and 3′→5′ exonuclease activities. AmpliTaq, Tth and Tth have 5′→3′ exonuclease activity. Pfu and U Taq have 3′→5′ (proofreading) exonuclease activity.

Figure 2. Effect of lack of exonuclease activity. Four wild-type DNA polymerases and their exo– variants were employed. Lane 1, 18 nt DNA primer labeled with Rox at the 5′ end; lanes 2, 4, 6 and 8, extension products made by wild-type DNA polymerases; lanes 3, 5, 7 and 9, extension products made by exo– variant DNA polymerases; lanes 2 and 3, AmpliTaq DNA polymerases; lanes 4 and 5, Pfu DNA polymerases; lanes 6 and 7, Vent DNA polymerases; lanes 8 and 9, Deep Vent DNA polymerases. The presence of exonuclease activity in each DNA polymerase is shown as + (present) or – (absent).

Effect of lack of exonuclease activity

A potentially important factor in the apparent extension efficiency of the polymerase is the presence or absence of 3′→5′ exonuclease activity, which might excise a modified nucleoside which had been incorporated (45,46). Figure 2 shows the results of a similar 99mer extension study comparing the efficiency of incorporation of 2′FdNTPs by four DNA polymerases which had been engineered to remove exonuclease activity. Mutated polymerases lacking 5′→3′ exonuclease activity showed better incorporation of 2′FdNTPs compared to the wild-type enzyme, with AmpliTaq DNA polymerase Stoffel fragment (AmpliTaq 5′→3′ exo–) giving some full length (99 nt) extension product. The improved incorporation was much more dramatic for the mutated forms of Pfu, Vent and Deep Vent lacking 3′→5′ exonuclease activity. The 99mer full-length extension product constituted the major product of these reactions, in contrast to the very inefficient incorporation obtained with the wild-type enzymes.

Effect of Mg²⁺

In order to improve efficiency of 2′FdNTP incorporation by wild-type enzymes possessing proofreading activity, the concentration of Mg²⁺ in the extension reactions was increased.
It has been shown that loss of proofreading activity increases misincorporation in PCR (45,46). An increase in misincorporation in PCR is also seen when additional Mg$^{2+}$ or Mn$^{2+}$ cations are present (47,48). Therefore, we hypothesized that increased concentrations of Mg$^{2+}$ in the extension reactions might decrease proofreading activity and allow better incorporation of 2′FdNTPs by the wild-type enzymes still possessing proofreading activity. UITma, which has inherent proofreading activity, was used to study the effects of additional Mg$^{2+}$. At low concentrations of Mg$^{2+}$ (1.25 mM) UITma does not incorporate 2′FdNTPs. However, higher concentrations of Mg$^{2+}$ (2.00 and 3.75 mM) do permit effective incorporation of 2′FdNTPs. The amount of full length extension product made increases with increasing concentrations of Mg$^{2+}$. Similar results were obtained with Pfu, Vent and Deep Vent. Of these four enzymes, UITma gave the most full-length extension product with the least false terminations. This strong Mg$^{2+}$ dependence was not observed in control reactions employing unmodified dNTPs (data not shown).

**Dideoxy T reaction**

The work described above showed that four DNA polymerases [Pfu (exo−), Vent (exo−), Deep Vent (exo−) and UITma] were able to efficiently incorporate 2′FdNTPs in strand extension reactions. The next capability sought for the enzyme was the ability to efficiently incorporate dideoxynucleotides from a nucleotide mixture containing both 2′FdNTPs and a ddNTP (i.e., a Sanger dideoxy reaction). This was explored using ddTTP. Vent (exo−), Deep Vent (exo−) and UITma, which were able to efficiently make the 99 nt extension product, were selected for study. For this analysis, a 42mer template with only a single A base in the extension region was employed (Fig. 3). In this model system, a strand extension reaction using ddTTP and no dTTP should completely terminate at the A site on the template. UITma indeed gave a clean termination product at the A site. However, both Vent (exo−) and Deep Vent (exo−) showed only minimal stoppage at the site of ddT incorporation and gave full length extensions as the major product. This result showed serious misincorporation by these two exo− polymerases under the conditions employed. Misincorporation events of this type, in which an incorrect deoxynucleotide is inserted at a given position, have little consequence in normal electrophoresis-based DNA sequencing, as the length of the termination products is not changed. However, in mass spectrometric sequencing, such misincorporations will change the mass of the termination product and hence lead to multiple and/or broadened peaks.

**ddT ladder**

Once it was possible to perform satisfactory dideoxy T reactions on the 42mer model system using 2′FdNTPs, the ability to prepare Sanger dideoxy termination ladders from an M13 template was examined. Figure 4 shows a gel analysis of fluorescently tagged T ladders made using UITma, incorporating 2′FdNTPs both with and without the addition of pyrophosphatase (PPase). The inclusion of pyrophosphatase is known to be important for obtaining good quality sequencing reactions using Thermo Sequenase, and was accordingly evaluated here (49). Two commercially available thermostable PPases were tested for this purpose. Each PPase gave the same results (Fig. 4). Control conventional dideoxy reactions (A,C,G,T) made using Vent (exo−) without addition of pyrophosphatase are also shown for reference. For UITma, addition of PPase dramatically lowered the incidence of false terminations, providing a recognizable T-ladder pattern. Some problems remain, however, as manifested by both a very weak second band for two consecutive Ts, and false terminations in a couple of cases (see < symbols, Fig. 4, lane 7). Further work will be required to address these remaining problems with the sequencing enzymology.

**Nuclease resistance of 2′-fluoro modified DNA**

A major goal of this work was to further evaluate the behavior and stability of 2′-fluoro modified DNA in MALDI analysis. Two issues arise in the preparation of DNA for MALDI analysis by the methods described above. First, if a normal unmodified DNA primer is used in the primer extension reactions, then all of the strand extension products made will contain the unmodified primer at the 5′ terminus. As unmodified DNA is not stable to MALDI analysis, fragmentation is likely to occur, obviating the advantage of the 2′-fluoro modification. In the case of dideoxy terminator reactions, a similar concern applies to the terminal deoxynucleotide, as 2′-fluoro-3′-deoxy nucleoside triphosphates are not available. Second, common to all MALDI analyses, it is very important to purify the extension products well, removing salts, buffer components and other polynucleotides from the sample. Such purification procedures are essential to obtain high quality MALDI results.

The simplest way of handling the problem with primer oligonucleotide stability would be to employ a 2′-fluoro modified primer oligonucleotide. Although the synthesis of such modified polynucleotides has been described in the literature (50), a lack of commercial availability made this avenue difficult to pursue. As an alternative approach, the ability to selectively destroy normal DNA by enzymatic digestion, without damaging the 2′-fluoro modified DNA extension product, was investigated. This would permit unmodified primer oligonucleotide to be destroyed subsequent to the strand extension reactions. Destruction of unmodified primer and template oligonucleotides by enzymatic digestion would also
facilitate purification of the extension product. This possibility was examined in the model system described above. In this system, template and primer oligonucleotides were labeled respectively with the dyes HEX or FAM, permitting them to be distinguished spectrally by fluorescence-based gel analysis. The strand extension reaction was performed using UlTma in conjunction with PPase to yield a 42mer primer extension product. This system was employed to examine the resistance of 2′-fluoro modified DNA to digestion with DNase, lambda exonuclease and RNase.

Digestion of the reaction products with DNase, an enzyme which randomly degrades single-stranded or double-stranded DNA to produce 3′-hydroxyl and 5′-phosphate oligonucleotides (51), yielded a unique fragment of length 24–25 nt on the basis of its electrophoretic mobility (Fig. 5a). The length was estimated by comparison to dye labeled oligonucleotide standards (18, 19 and 42 nt) on the same gel. The template strand was also destroyed, as judged by the disappearance of the 42mer HEX-labeled oligonucleotide from the gel electropherogram.

Lambda exonuclease, a 5′→3′ exonuclease that requires a 5′ phosphate, was also tested for digestion of unmodified primer (Fig. 5a). Intact template and two products derived from the extension products were evident after lambda exonuclease digestion. The major digestion product appeared to be one base shorter than the DNase-resistant product, and a minor product present was the same length as the DNase-resistant product. These results show that lambda exonuclease efficiently digested unmodified primer but is not suitable for obtaining a single modified product.

The susceptibility of the 2′-fluoro modified DNA to digestion with RNaseT1 was also examined. Resistance of 2′-fluoro DNA to RNase is important for stability and ease of handling under laboratory conditions. RNaseT1 from Aspergillus oryzae is an endonuclease that specifically hydrolyzes RNA after G residues. Cleavage occurs between the 3′-phosphate group of a guanine ribonucleotide and the 5′-hydroxyl of the adjacent nucleotide. No digestion of the 24mer 2′-fluoro modified extension product was observed, under conditions where a 167 nt RNA was completely degraded (Fig. 5b). These results showed...
that 2'-fluoro modified DNA is resistant to degradation by all three enzymes examined.

**Analysis of 2'-fluoro modified mixed base oligonucleotides by MALDI-MS**

As treatment with DNase yielded a fairly homogeneous product and also destroyed the template DNA strand, DNase treatment, followed by purification using a Microcon microconcentrator was chosen for the preparation of samples for MALDI analysis. The procedures employed are detailed in Materials and Methods. MALDI studies of the 2'-fluoro modified DNA product produced using ddTTP in the extension reaction were performed in two matrices, 3-HPA and 2,5-DHBA. 3-HPA is the most widely used and successful matrix for the analysis of nucleic acids, whereas 2,5-DHBA is a matrix employed extensively in protein analyses, and 3-HPA and 2,5-DHBA consistent with a product sequence of PO₃-d(TGfGfGfGfCfAfGfGfGfGf)ddT. (52).

In the matrix 2,5-DHBA (Fig. 6), no fragmentation is evident for the 2'-fluoro modified DNA, in contrast to the extensive base loss observed for the control unmodified DNA. This is an important result, as it demonstrates the ability of the 2'-fluoro modification to stabilize DNA to MALDI analysis in matrices 2,5-DHBA and 3-HPA (Figs 6 and 7). This mass is consistent with the known behavior of DNase (51).

**CONCLUSION**

A suitable DNA polymerase (UITma) for preparing 2'-fluoro modified DNA in both polymerase-directed synthesis and Sanger-type sequencing reactions was found. The resultant chemically modified DNA was shown to be resistant to digestion by lambda exonuclease, RNase and DNase. The resistance to DNase was exploited to degrade the unmodified primer and DNA template and thereby facilitates purification of the 2'-fluoro DNA prior to MALDI analysis. MALDI results obtained using the matrix 2,5-DHBA support the hypothesis that substitution of an electron withdrawing group at the 2'-position of deoxyribose stabilizes DNA to MALDI analysis. The resultant degradation pathway for 2'-fluoro nucleosides (52). Alternatively this peak may reflect a low degree of misincorporated nucleotides.

**ACKNOWLEDGEMENTS**

We thank Professor James E. Dahlberg for materials and assistance in preparing RNA by in vitro transcription. This work was supported by Department of Energy grant # DE-FG02-91ER61130 and NSF Award # 9520868.

**REFERENCES**
