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Chemical Cleavage Sequencing of DNA Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Narayana R. Isola, Steve L. Allman, Valerie V. Golovlov, and Chung-Hsuan Chen*

Photophysics Group, Life Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6378

In this paper, we report for the first time use of laser desorption mass spectrometry for measurement of chemical cleavage sequencing products of DNA. In this method, the target DNA was labeled with biotin and subjected to chemical modification and cleavage according to the Maxam−Gilbert sequencing protocol. The biotin-containing fragments were captured by streptavidin-coated magnetic beads and separated from the other fragments. The captured fragments were released by hot ammonia treatment, and the released fragments were analyzed by mass spectrometry. Potential applications of this method in resolving sequence ambiguities and sequencing repeat sequences as well as in the analysis of DNA−protein interactions are discussed.

DNA sequence determination is a very important research and diagnostic tool. Several methods are available for DNA sequence determination such as chain termination,[1] chemical cleavage,[2] sequence-based hybridization,[3] and exonuclease cleavage.[4] Sanger's chain termination method has become the most popular sequencing method in research and diagnostic laboratories due to its ease of application. This method requires enzymatic copying of DNA fragments and is subject to copying errors and DNA secondary structure problems. Some of these problems are alleviated by the use of diaza nucleotide triphosphates in the reaction and by using thermostable DNA polymerases for copying the template at high temperatures**.** Chemical cleavage sequencing is performed by fragmenting the original template molecule and not an enzyme copy of the template; hence, this method is not susceptible to sequencing errors related to secondary structure or enzymatic errors. To this day, the ability to probe DNA conformation and DNA−protein interactions remains a unique feature of the Maxam−Gilbert approach. The success of this approach depends on the specificity of the cleavage reactions that are usually carried out in two steps. In the first step, specific bases undergo chemical modifications. During the second step, the modified base is removed from its sugar and the phosphodiester bonds. These reactions need to be carried out under carefully controlled conditions to ensure that on average only one of the target bases in each DNA molecule is modified. Subsequent cleavage by piperidine yields a set of end-labeled molecules whose length ranges from one to a few hundred nucleotides. DNAs can be labeled with radioactive elements such as ^{32}P or fluorescent dyes. With MS for detection, DNAs are labeled with biotin for coupling to streptavidin-coated magnetic beads so that the labeled fragments can be separated from the unlabeled. The DNA sequence can then be obtained by the measurements of DNA ladders from the reactions of G, A + G, C and C + T. As originally described by Maxam and Gilbert, the chemical cleavage sequencing method is a time-consuming, multistep process that limits its use in most laboratories. However, commercial kits for Maxam−Gilbert sequencing are currently available so that the use of Maxam−Gilbert method for sequencing, especially for specific applications, is expected to have a significant increase soon. With mass spectrometry for DNA detection, no radioactive material or dye tagging is required, which should further simplify the use of the Maxam−Gilbert approach for sequencing. Several nonradioactive versions of the Maxam−Gilbert sequencing have been developed, most of which require gel electrophoretic resolution of the fragments. Methods of chemical sequencing as a solid-phase reaction[5] and automated methods for performing the chemistry[6,7] have been described. Chemical cleavage sequencing of phosphorothioate nucleic acids has also been reported.[8]

Sequencing of DNA by mass spectrometry has been proposed since the invention of matrix-assisted laser desorption/ionization methods.[9] Discovery of new matrix formulations[10−12] led to the

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development of several sequencing methods using MALDI spectroscopy. Several methods such as time-dependent sequential excision by exonuclease digestion,[13] collision-induced dissociation for Fourier transformation mass spectrometry,[14] and matrix-assisted selective fragmentation[15] have been developed. However, most efforts were directed toward using MALDI-TOF for sequencing DNA ladders with the conventional chain termination method. In 1993, Smith and his colleagues[16] used MALDI-TOF-MS to analyze mock Sanger sequencing reactions containing mixtures of synthetic oligonucleotide. In 1995, Shaler et al.[17] used MALDI to sequence ladders of a 45-mer ss-DNA which had been prepared enzymatically. Roskey et al.[18] reported the results of sequencing a synthetic DNA template of 50 nucleotides. Mouradian et al.[19] reported MALDI analysis from bacteriophage M13 and determined the sequence for samples up to 35 bases in length. Complete sequencing of a ss-DNA with 50 bases was demonstrated by Taranenko et al.[20] In 1996, Koster et al.[21] succeeded in sequencing a ss-DNA of 39 nucleotides using strepavidin-coated magnetic beads for DNA purification. Monforte and Becker[22] presented the sequencing of 100-mer templates in 1997. In 1998, Taranenko et al. reported success in sequencing DNA templates longer than 100 bases.[23] In this work, we have attempted to adapt the chemical sequencing reactions to read sequences by mass spectrometry in order to eliminate gel electrophoresis and enzyme-related errors in sequencing. This method is rapid and nonradioactive.

MATERIALS AND METHODS

Synthetic oligonucleotides were purchased from Oligos Etc. and were used without further purification. Chemical modification and cleavage reactions were performed using a Maxam−Gilbert chemical sequencing kit (Sigma Chemical Co., St. Louis, MO). Picolinic acid and 3-hydroxypicolininc acid were obtained from Aldrich Chemical Co (Milwaukee, WI). Streptavidin-coated magnetic beads were from CPG, Inc. (Lincoln Park, NJ). All other reagents from other manufacturers were molecular biology grade.

Synthetic oligonucleotide (5′-biotin-tac tcc cct gcc ctc cac aag atg ttt tgc) designed from exon 5 of human p53 gene and a 60 mer containing 5′-biotin-$(gaa)_{20}$ were used as templates. The chemical cleavage sequencing reactions were performed as described[2] with minor modifications. The concentrations of biotinylated DNAs for G and C reactions and for G + A and C + T reactions are 1 and 2 μM, respectively.

G + A Reaction. To 20 μL of DNA in water, 2 μL of 4% pyridinium formate was added and the mixture incubated for 30 min at 37 °C. The mixture was lyophilized to dryness using a Savant Speed Vac. The DNA was redissolved in 20 μL of sterile distilled water and lyophilized to dryness.

G Reaction. To 5 μL of DNA in water, 200 μL of DMS buffer (50 mM sodium cacodylate, 1 mM EDTA, pH 8.0) was added, and the G residue modification was initiated by the addition of 1 μL of dimethyl sulfate and incubation at room temperature for 20 min. The reaction was terminated by the addition of 50 μL of DMS stop solution (1.5 M sodium acetate, 0.5 M dithiothreitol, 100 μg/mL yeast tRNA, pH 7.0).

C + T Reation. To 20 μL of DNA in water, 30 μL of hydrazine was added and the resultant mixture was incubated at room temperature for 20 min. The reaction was terminated by the addition of 200 μL of hydrazine stop solution (0.3M sodium acetate, 0.1 mM EDTA, 25 μg/mL yeast tRNA, pH 7.0).

C Reaction. To 5 μL of DNA in water, 15 μL of 5 M sodium chloride and 30 μL of hydrazine were added, and the mixture incubated at room temperature for 20 min. The reaction was terminated by the addition of 200 μL of the hydrazine stop solution described above.

The G, C + T, and C reactions products were precipitated by the addition of 750 μL of absolute ethanol and incubated at −70 °C for 5 min. The mixture was centrifuged at 12000*g* for 15 min at room temperature, and the supernatants were discarded. The G reaction supernatant was discarded into 5.0 M sodium hydroxide; the C and C + T reaction supernatants were discarded into 3.0 M ferric chloride. The DNA pellets were redissolved in 250 μL of 0.3 M sodium acetate, precipitated by the addition of 750 μL of ethanol, and incubated at −70 °C for 5 min. The DNA precipitate was collected by centrifugation as described above and dried in a Speed Vac.

Cleavage Reactions. The modified DNAs were dissolved in 100 μL of freshly diluted (1:10) piperidine, and the tubes were incubated at 90 °C for 30 min. The mixture was dried using a Speed Vac, redissolved in 10 μL of sterile distilled water, and dried again using a Speed Vac.

Bead Capture. The cleavage products were dissolved in 100 μL of binding buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.0), 10 μL of streptavidin-coupled magnetic beads (CPG, Inc.) was added, and the mixture incubated at room temperature for 30 min with agitation. The beads were washed twice in binding buffer and twice in 70% ethanol and air-dried.

Release of Captured DNA. The beads were resuspended in 100 μL of ammonium hydroxide (28−30%) and incubated at 65 °C for 10 min. The ammonium hydroxide, which contained the DNA, was aspirated into a fresh tube and dried using the Speed Vac. The resultant DNA was rinsed once with 70% ethanol and once with absolute alcohol. The DNA was then dissolved in 2 μL of the matrix solution.

Mass Spectrometry. The matrix solution was 3-hydroxypicolininc acid/picolinic acid/ammonium chloride (molar ration 9:1:1) in 50% aqueous acetonitrile. One microliter of the DNA in matrix solution was spotted onto the substrate and dried under a stream

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of air until the mixture was crystallized. Mass spectrometry measurements were performed using a linear time-of-flight (TOF) mass spectrometer (Voyager; PerSeptive Biosystems, Framingham, MA) equipped with a 337-nm pulsed nitrogen laser for desorption and ionization. The laser fluence was 45−65 mJ/cm^2, and the acceleration voltage was 28 125 V. A delayed pulse ion extraction device was installed to increase the mass resolution. The signals of negative ions were collected (typically for 128−256 pulses), digitized, and averaged by a digital oscilloscope (Tektronix 520A) controlled by a 486 PC.

Detection of Biotin. The presence of biotin was detected using streptavidin-linked alkaline phosphatase (Blu-Gene kit, Bethesda Research Laboratories, Bethesda, MD) as follows. The DNA was spotted onto nylon membranes (Zeta probe membranes from Bio-Rad) and was covalently bound by UV cross-linking. The membranes were incubated at 65 °C for 1 h in 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM sodium chloride and 3% (w/v) bovine serum albumin (BSA) to block the unreacted sites on the membrane. The membranes were incubated at room temperature for 15 min with streptavidin−alkaline phosphatase conjugate (1 μg/mL in 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM sodium chloride) and washed in 20−40-fold excess of buffer. The presence of alkaline phosphatase was detected by incubating the membranes in 100 mM Tris-HCl (pH 9.5) containing 100 mM sodium chloride, 50 mM magnesium chloride with NitroBlue Tetrazolium (NBT) and bichlorophenol indophenol (BCIP) until blue formazan precipitates were visible.

Gel Electrophoresis. Samples for denaturing gel electrophoresis were mixed with equal volumes of loading buffer (95% formamide, 0.01% Bromophenol Blue, 0.01% Xylene Cyanol) and heated at 95 °C for 2 min. Electrophoresis was performed using the Castaway (Stratagene Inc., La Jolla, CA) system. Precast gels 0.25 mm thick containing 12% acrylamide, and 7.0 M urea in tris−borate buffer were used. Three microliters of the sample was loaded into each lane and separated by electrophoresis at 2000 V for ∼1 h. After electrophoresis, the gels were soaked in 9:1 (water/glacial acetic acid) for 30 min and silver staining was performed.

Silver Staining. Silver staining of sequencing gels was performed using the Silver Sequence kit (Promega, Madison, WI).

RESULTS AND DISCUSSION

Determination of the Stability of Biotin. We have determined the integrity of biotin after performing the chemical modification and cleavage reactions. The preparation of biotin-labeled oligonucleotides subjected to chemical modification and cleavage reactions was described in the section Materials and Methods. The detection of biotin was also presented in the text above. No observable amount of biotin was found to be detached from DNAs. This indicates that biotinylated DNA is suitable for chemical degradation sequencing.

Purification of Informative Fragments for Sequence Determination. Chemical cleavage sequencing performed with end-labeled fragments does not require the removal of internal cleavage products since only the labeled fragments are detected. However, mass spectrometric determination of fragment sizes cannot distinguish the fragments on the basis of their origin, making it impossible to determine the sequence of the template molecule. Therefore, the attachment of biotin was used as a means to separate biotinylated DNA ladders from other fragments. Figure 1 shows a schematic for the chemical cleavage and the capture of end fragments.

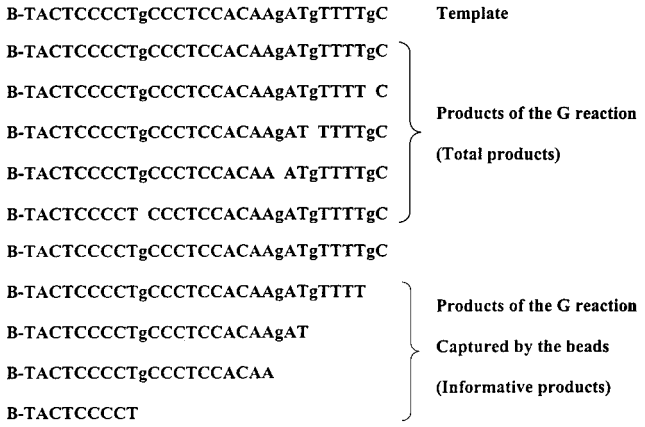

Figure 1. Schematic of the biotin capturing method for chemical cleavage sequencing.

A complete set of sequencing reactions for a 30 mer with biotin at the 5′ end is shown in Figure 2A,B and the sequence derived from the figure corresponds to the sequence of the original template molecule. The derived sequence from mass spectrometric measurement is shown in Figure 2C. The peaks in Figure 2 are in general quite broad. The mass resolution ($M/\Delta M$) was estimated as ∼30. The poor resolution can be due to the residue of salts. Further study on the cause of the poor resolution with the chemical degradation approach will be pursued. A 60 mer consisting of GAA repeats was analyzed by the above method, and the spectra are shown in Figure 3. These repeat sequences are notoriously difficult to sequence by conventional chain terminator methods. The C and C + T reactions showed no cleavage products as expected; the peaks at the low-m/z region are due to multiply charged ions. The G and G + A reaction products were difficult to resolve for fragments longer than 40 mer. Figure 3A represents the individual reactions, and Figure 3B represents the composite spectrum. The sequence derived from the spectrum and the actual sequence are shown. Although promising, the difficulty in obtaining sequencing longer than 40 mer indicates that further modifications in the technique as well as computer analysis methods are necessary for this method to become more robust. We also pursued gel electrophoresis for comparison with the results from MALDI. Results from the two different methods were in good agreement.

We have successfully adapted the chemical cleavage sequencing method for mass spectrometric determination of the products. This method should eliminate problems associated with electrophoretic methods such as G−C compressions, inability to read long runs of similar bases (polyA tracts), etc., as well as problems associated with repeat sequences in the template. We are currently analyzing such difficult templates, and we believe this method will have a number of applications in completing the human genome sequencing project. This method also has a potential application for detection of point mutations by chemical cleavage of hetero-

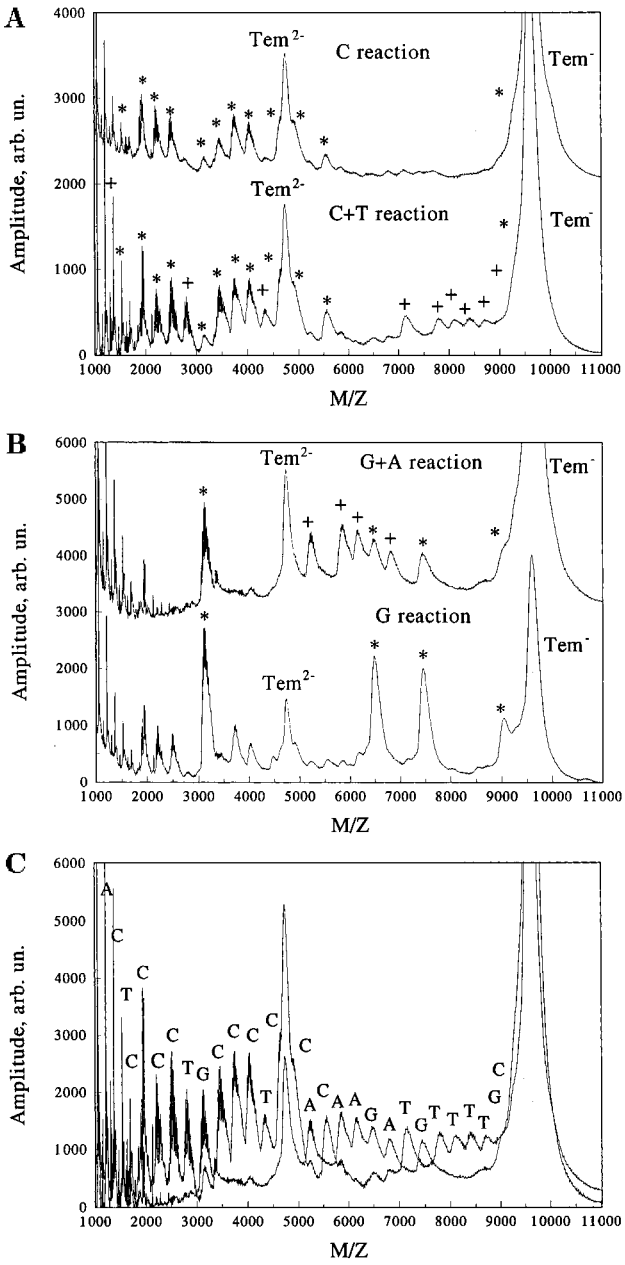

Figure 2. (A, B) MALDI-TOF spectra of chemical cleavage reaction products captured by streptavidin magnetic beads. ∗ and + represent the determination of C and T, respectively. (C) A composite of the spectra and the derived sequence of the template are shown.

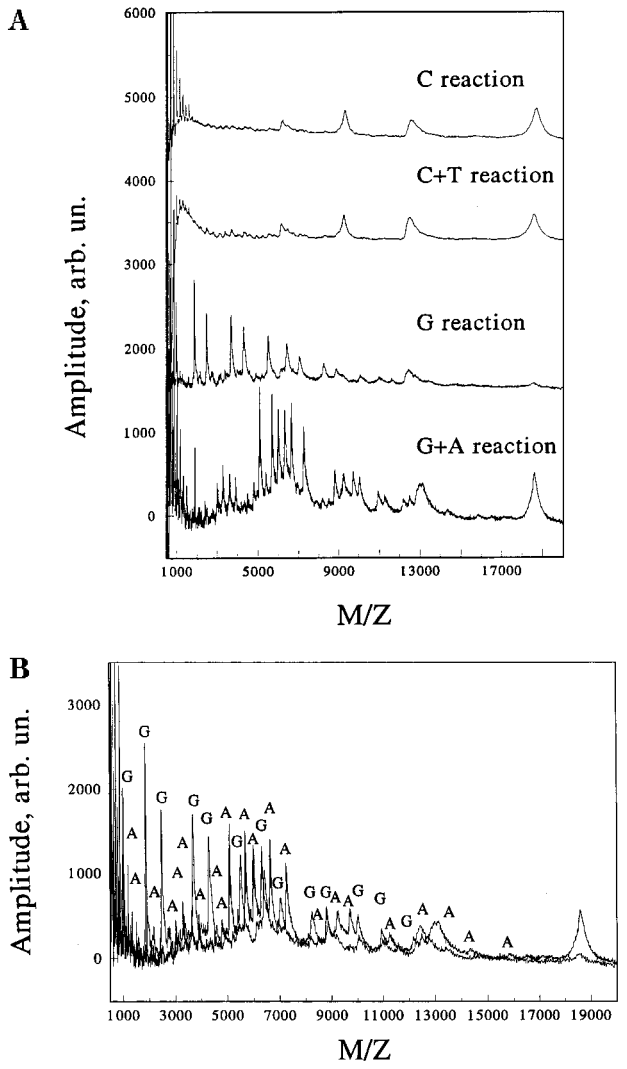

Figure 3. Chemical cleavage sequencing of 60-mer GAA repeat. (A) Spectra from the individual sequencing reactions. (B) a composite of the spectra. Template:5′-AAG-AAG-AGA-AAG-AGA-AAG-AAG-AGA-AAG-AGA-AAG-AAG-AGA-AAG-AGA-AAG-AAG-AGA-AAG-AGA-3′. Derived sequence from the spectra: 5′-**G-AAG-AGA-AAG-AGA-AAG-AAG-AGA-**G-AG*- AAG-**G-AG*-***-***-A**-***-A*-A*-A*-***-3′, where * represents the base that cannot be clearly decided by the experimental results.

duplexes[24] as well as in DNA−protein interaction analysis such as footprinting.

CONCLUSION

We have demonstrated the successful adaptation of a chemical cleavage sequencing method for mass spectrometric analysis. This method should prove useful in filling in the gaps of the complete genome sequencing projects since most genomes contain regions notoriously difficult to sequence by the enzymatic method. After the achievement of mass resolution improvement, this method would also be applicable for point mutation detection in amplified fragments by using the chemical cleavage of heteroduplexes described.[24]

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