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Chemical sequencing of restriction fragments 3′-end-labeled with [^{35}S]dATPαS

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Abstract

Although the phosphorous radioisotope ^{32}P is routinely used in Maxam–Gilbert sequencing, it presents disadvantages that adversely affect safety and ease of use, resolution, and DNA degradation rates. Here, we introduce a new protocol utilizing the alternative isotope ^{35}S for 3′-end-labeling DNA restriction fragments. In our method, plasmid DNA is labeled with [^{35}S]dATPαS and T7 Sequenase™ Version 2.0. We have shown that bands on Maxam–Gilbert sequencing gels are sharp with extremely low background. In addition, a single labeling reaction produces DNA sufficient for 80 sequencing lanes, and the labeled DNA can be utilized for prolonged periods of time without significant degradation. We have further demonstrated the utility of our ^{35}S-end-labeling procedure by successfully mapping the sequence-specificity of DNA damage induced by photoexcited riboflavin. Overall, we have shown that ^{35}S can be used as a safe and practical alternative to ^{32}P in the 3′-end-labeling of DNA restriction fragments.

Keywords: Sulfur radioisotopes; End-labeling; Maxam–Gilbert sequencing

Most investigators employ ^{32}P-nucleotides for end-labeling DNA that is sequenced by the Maxam–Gilbert chemical method [1,2]. Unfortunately, the use of ^{32}P in DNA sequencing has a number of disadvantages [1,3]. Aside from safety concerns, the

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt.

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high-energy β emissions of ^{32}P (1.71 MeV) produce DNA strand breaks that can result in unacceptably high levels of background on sequencing gels. In our experience, DNA end-labeled with ^{32}P must be discarded after about a week and new DNA must be radiolabeled and purified. In addition, high β emission scattering produces diffuse autoradiographic bands, which can be difficult to read depending upon their location in the sequencing gel.

In general, ^{35}S confers some practical advantages over ^{32}P in sequencing experiments [1,3–5]. Because of its lower β-particle emission energy (0.167 MeV), ^{35}S is easy to use and no special shielding is required. In addition, bands on autoradiograms are sharper and less DNA damage is produced, substantially reducing background. The lower levels of DNA damage and the longer half-life of ^{35}S (87.4 days) compared to ^{32}P (14.3 days) make it possible to store ^{35}S-labeled DNA for much longer periods. In addition, ^{35}S offers clear advantages over recently introduced ^{33}P-nucleotides. Although the β-particle emission energy of ^{33}P (0.249 MeV) resides between ^{32}P and ^{35}S, its primary disadvantages are its high cost and relatively short half-life (25.4 days).

Although widely used in dideoxy enzymatic DNA sequencing, there has been great resistance to employing ^{35}S as an alternate labeling strategy in the Maxam–Gilbert method due to the low β-particle emission energy of this radioisotope [1,4]. In dideoxy sequencing, many ^{35}S-nucleotides are incorporated into DNA during the radiolabeling step [6]. However, in Maxam–Gilbert sequencing, DNA is end-labeled, severely limiting the number of radiolabeled nucleotides that can be incorporated [2]. Although many commercially available kits for 3′-end-labeling DNA have been introduced, the procedures to produce homogeneous 3′-ends suitable for Maxam–Gilbert sequencing entail the use of terminal deoxynucleotidyl transferase with either [α-^{32}P]3′-dATP (cordycepin 5′-triphosphate) [7] or [α-^{32}P]ddATP [8]. Both of these approaches permit the addition of only one nucleotide per DNA end. Although [^{35}S]ddATPαS [5] has been introduced as a safe alternative to the above ^{32}P-nucleotides, it is often overlooked by investigators because it is assumed that specific activity of the labeled DNA is too low. Nevertheless, we envisioned that the use of ^{35}S in Maxam–Gilbert sequencing would have many practical advantages over both ^{32}P and ^{33}P. This motivated us to develop chemical sequencing protocols to optimize efficient 3′-end-labeling with ^{35}S-nucleotides.

In our protocol, we utilize [^{35}S]dATPαS and Sequenase™ Version 2.0 (Amersham Pharmacia Biotech), a modified T7 DNA polymerase devoid of 3′–5′ exonuclease activity. By digesting plasmid DNA with *Eco*RI (New England Biolabs), the overhang 3′-TTAA-5′ is generated, and two radiolabeled nucleotides can be incorporated. Use of the dideoxy analog ddTTP (Amersham Pharmacia Biotech) in the labeling reaction prevents the formation of "ghost bands" by the residual terminal transferase activity of T7 Sequenase™. Our procedure avoids the high cost associated with [^{35}S]ddATPαS and the safety concerns associated with ^{32}P-nucleotides employed in terminal deoxynucleotidyl transferase 3′-end-labeling protocols. In addition, we have employed a streamlined chemical sequencing method [9] that minimizes the loss of DNA by eliminating the standard ethanol precipitation step used in Maxam–Gilbert sequencing. With this combined approach, we have found that a single ^{35}S-end-labeling reaction generates enough labeled DNA to load 80 sequencing lanes. Autoradiographic bands are consistently sharp with extremely low background, even 1 month after labeling.

In the typical ^{35}S-labeling reaction, 40 μg of pUC19 plasmid DNA are digested with *Eco*RI. The linearized plasmid (22.9 pmol) is 3′-end-labeled in a total reaction volume of 50 μl, containing 26 U of T7 Sequenase™ Version 2.0, 10 μl of 5× Sequenase™ reaction buffer, 2 μl of 0.1 M DTT, 10 μl of 10 μM Redivue™ [^{35}S]dATPαS (specific activity > 1000 Ci/mmol; Amersham Pharmacia Biotech), and 1 μl of 10 mM ddTTP. The reaction proceeds at 37 °C for 30 min, after which an additional 13 U of Sequenase™ Version 2.0, 1 μl of 10 mM dATP, and 1 μl of 10 mM ddTTP are added. The incubation is then continued for 30 min at 37 °C and is stopped by the addition of 10 μl of 0.5 M EDTA pH 8.0 and 40 μl of H_2O. Unincorporated nucleotides are removed with a Quick Spin™ Sephadex® G-50 column (Roche Molecular Biochemicals). The DNA is then digested with *Fsp*I (New England Biolabs) to produce a restriction fragment 138 bp in length. The fragment is resolved on a 2.0% agarose gel, excised, and DNA is eluted by spinning the gel slice through siliconized glass wool. DNA is precipitated from the eluate with 0.3 M sodium acetate pH 5.2 in 2.5 volumes EtOH, washed with 70% EtOH, dissolved in deionized, distilled water, and then desalted on a NICK® column (Amersham Pharmacia Biotech). The purified DNA is stored in a total volume of 400 μl of deionized, distilled water at −20 °C.

The streamlined G, G + A, and T chemical sequencing reactions were conducted according to the method of Williamson and Celander [9]. Position of C's can be inferred from the absence of bands in all three lanes. For each reaction, 10–40 μl (0.4–1.6 pmol) of the 3′-end-labeled DNA were used. DNA was first concentrated *in vacuo* and then reacted in 9 μl of TE 7.5 buffer as described [9].

In our first experiment, we ran serial dilutions of the G reaction on an 8.0% polyacrylamide gel. Lane 3 in Fig. 1a shows that 1/80th of the [^{35}S]dATPαS end-labeled DNA produces a well-resolved sequencing ladder. Therefore, ^{35}S-labeled DNA generated in one labeling reaction can be used to load a total of 80 sequencing lanes.

As alternatives to ^{35}S in 3′-end-labeling of DNA restriction fragments, we also evaluated three commercially available flourescent nucleotides (data not shown). Unfortunately, we found this approach to be impractical due to extremely low incorporation efficiencies of the modified nucleotides and consistently high flourescence background on sequencing gels.

The ability to precisely map sequence-specific DNA damage at nucleotide resolution represents an important application that distinguishes the Maxam–Gilbert chemical method from other sequencing techniques [3]. One example involves photoexcited riboflavin, which has been shown by Maxam–Gilbert sequencing of ^{32}P-end-labeled DNA to induce the formation of piperidine labile lesions at guanine bases [10]. In order to demonstrate the versatility of our ^{35}S-end-labeling procedure, we ran the G, G + A, and T chemical sequencing reactions on an 8.0% polyacrylamide gel, adjacent to ^{35}S-end-labeled DNA that had been irradiated with visible light in the presence of riboflavin. The ^{35}S-end-labeled DNA was 1 month old at the time of the experiment.

The autoradiogram in Fig. 1b demonstrates that photoexcited riboflavin induces cleavage of ^{35}S-end-labeled double-helical DNA predominantly at guanine bases and that the presence of ^{35}S does not affect the photolysis reaction. Clear, sharp bands appear after a 24-h exposure of the dried gel to X-ray film (Kodak XAR 5; Kodak) and upon a 12-h exposure to a storage-phosphor screen (Molecular Dynamics). The back-

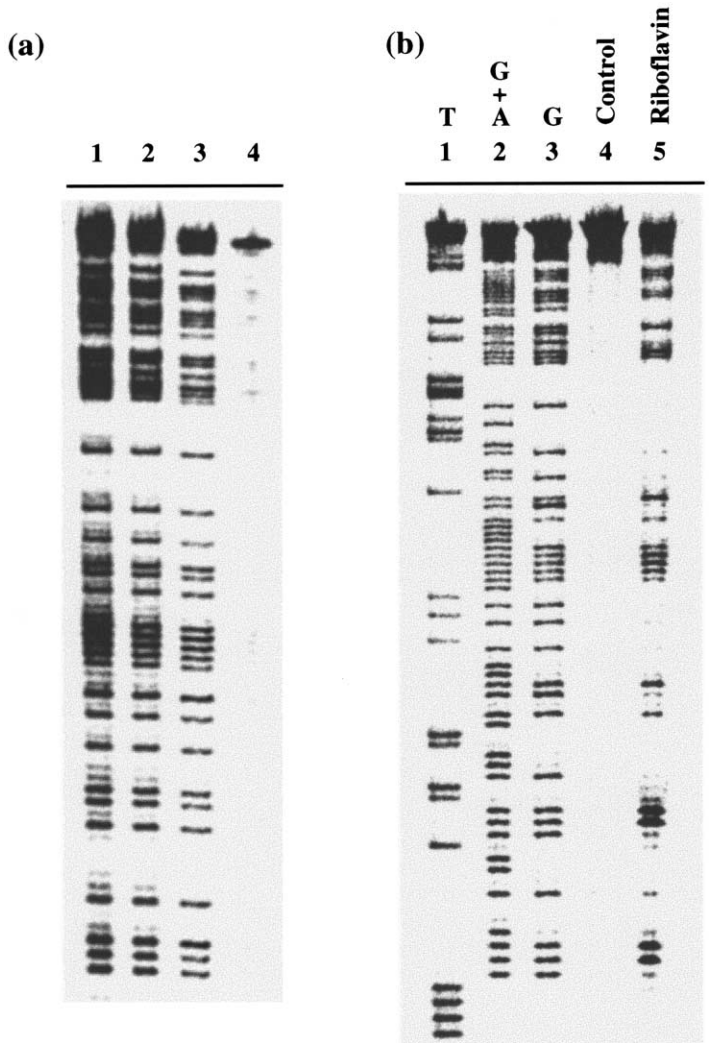

Fig. 1. Storage-phosphor autoradiograms scanned with a Storm™ 860 gel imaging system (Molecular Dynamics). The 138 bp restriction fragment was 3′-end-labeled with [^{35}S]dATPαS and then sequenced. (a) Serial dilutions of the G sequencing reaction. Lanes 1–4: 1/20th, 1/40th, 1/80th and 1/160th of DNA from one ^{35}S-end-labeling reaction. (b) Sequencing reactions indicating the sequence specificity of DNA cleavage mediated by photoexcited riboflavin. Lanes 1–3: T, G–A, and G reactions, respectively; lane 4: DNA control; lane 5: DNA and 100 μM riboflavin in 100 mM potassium phosphate buffer, pH 7.7, irradiated at 22 °C for 20 min with an NEC daylight T5 FL4D fluorescent lamp (NEC). The DNA control and the riboflavin reactions were treated with 1.0% piperidine at 90 °C for 30 min.

ground on the sequencing gel is extremely low, even though the ^{35}S-labeled DNA was prepared 1 month in advance. In addition, the plastic wrap was left on the gel during exposures, no intensifying screens were employed for X-ray film, and it was not

necessary to enhance β-particle emission by treatment of the gel with acetic acid/methanol.

Maxam–Gilbert chemical sequencing is optimal for DNA that cannot be accurately sequenced by the dideoxy method. Examples include small oligonucleotides [1,3] and DNA sequences that produce premature termination due to strong secondary structure [1]. In addition, Maxam–Gilbert sequencing can be used to map DNA modifications such as alkylation and cleavage produced by carcinogens, anti-cancer drugs, and other DNA-targeting agents [3,10–17]. In chemical and enzymatic footprinting experiments, the technique permits the precise identification of specific DNA sequences that bind to small molecules and proteins [18,19]. Therefore, new protocols that enhance the ease of use of Maxam–Gilbert sequencing are beneficial to a wide variety of applications in molecular biology and biochemistry. We believe that [^{35}S]dATPαS is an attractive and practical alternative to ^{32}P and ^{33}P for Maxam–Gilbert chemical sequencing of 3′-end-labeled DNA.

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