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A fluorescence-labeling method for sequencing small RNA on polyacrylamide gel

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ABSTRACT

A practical fluorescence-labeling method for sequencing small RNAs by the traditional 'direct read out' on polyacrylamide gel electrophoresis was established. The 3′ terminus of RNA was oxidized into dialdehyde by sodium periodate and then labeled with fluorescein-5-thiosemicarbazide through the condensation reaction between carbazide and aldehyde. The fluorescence-labeled RNA was partially degraded enzymatically and fractionated by polyacrylamide gel electrophoresis. The fluorescent bands were visualised by ultraviolet photography. A partial sequence of yeast 5S rRNA was determined. The result indicates that this method can be used in sequencing small RNAs rapidly, conveniently and safely.

Among the methods used in determining the primary structure of RNA, the classical procedure established by Holley *et al.* involves the identification of nucleotides by their ultraviolet absorption spectra, which is more time-consuming and usually requires large amounts of purified RNA (1). The prelabeling techniques developed by Sanger and his colleagues, suitable for the study of ^{32}P-labeled RNA, have great advantages both in the separation of oligoribonucleotide fragments and in the sensitivity of detection (2). Later, several postlabeling techniques for sequencing RNA by high resolution polyacrylamide gel electrophoresis were developed. These techniques include enzymatic digestion (3), chemical degradation (4,5) and the wandering-spot method (6). These methods are highly sensitive but need the use of radioisotopes and are also labor-intensive, time-consuming and fairly expensive. Since fluorescence-labeling for sequencing nucleic acid avoids the use of radioisotopes, many researchers have developed fluorescent DNA sequencing (7,8) which is widely used. However, no similar fluorescent method for sequencing RNA has been reported yet.

In the early 1980s, Liu *et al.* proposed sequencing RNA by fluorescence-labeling, and sequenced an oligoribonucleotide by step-wise degradation (9). A practical fluorescence-labeling method for sequencing small RNA by the traditional 'direct read out' on polyacrylamide gel electrophoresis has now been established and used to determine the sequence of the 3′ terminal 69 nucleotides (nt) of yeast 5S rRNA. The result is identical to the known sequence. This indicates that the fluorescence-labeling method can be used in sequencing small RNAs rapidly, conveniently and safely.

In this method, sodium periodate was used to oxidize the 3′ terminus of RNA into dialdehyde. The excess of the oxidant was removed by adding sodium sulfite, and then the fluorescent dye, fluorescein-5-thiosemicarbazide, was added to label the 3′ terminus of RNA through the condensation reaction between carbazide and aldehyde. The detailed procedure is as follows: 5 µg yeast 5S rRNA was dissolved in 10 µl redistilled water, then 10 µl of buffer (0.25 M sodium acetate, pH 5.6) and sodium periodate was added. The molar ratio of RNA and sodium periodate was 1/10 in a final volume of 40 µl. The oxidization of 3′ terminus of RNA was carried out at 25°C in the dark for 90 min. Then a 2-fold excess of sodium sulphite over sodium periodate was added to the system to remove the excess oxidant. The reaction mixture was incubated at 25°C for 15 min. Finally, fluorescein-5-thiosemicarbazide was added; the molar ratio of the fluorescent dye to the RNA was 30:1. After labeling for 3 h at 37°C in the dark, the RNA was precipitated by adding 1/10 vol of 8 M LiCl and 2.5 vol ethanol, standing at –20°C for 3 h, and then centrifuged (13 000 *g* at 4°C) for 20 min. The precipitate was washed with 75% ethanol several times to remove the free fluorescent dye and the labeled RNA was used for sequence determination. In these operations, no obvious degradation of RNA was observed.

The sequence of the fluorescence-labeling RNA was analyzed by enzymatic degradation as the same mainly as described for sequencing 5′ or 3′ terminal ^{32}P-labeled RNA (10). Partial digestion of the terminally labeled RNA with base-specific ribonucleases T_1, U_2, *Bacillus cerus* or *Phy*I is performed at elevated temperature (50–55°C) and in the presence of 7 M urea in the cases of ribonucleases T_1 and U_2 to avoid interference of the secondary/tertiary structure of RNA on enzymatic hydrolysis. Then the labeled RNA fragments, generated by the partial digestion of 3′ terminal labeled RNA, were separated according to their chain length by 15% polyacrylamide gel electrophoresis (containing 15% DMF for short RNA fragments).

An ultraviolet detecting device with short wavelength of ultraviolet light was constructed to detect the fluorescent bands in the gel. The total power of the ultraviolet light was 24 W and an ultraviolet glass plate of the detecting device was 20×15 cm. The ultraviolet glass plate was cleaned with redistilled water and kept wet. As soon as the electrophoresis finished, the glass plate on one side of the gel was taken away and the gel was peeled off carefully

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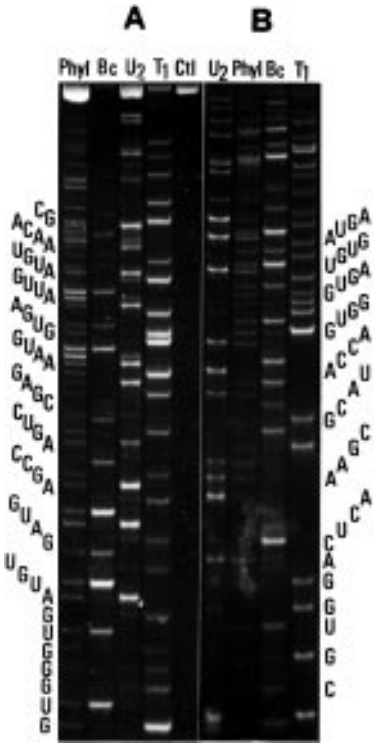

Figure 1. The electrophoretic pattern of fluorescence-labeling fragments of yeast 5S rRNA on polyacrylamide gel. The 3′ terminally fluorescence-labeled yeast 5S rRNA was digested with RNase T_1 (Gp↓N), RNase U_2 (Ap↓N), RNase *B.cerus* (Up↓N and Cp↓N) or RNase *Phy*I (Gp↓N, Ap↓N and Up↓N) and fractionated on the 15% polyacrylamide gel (36 cm × 20 cm × 0.4 mm, containing 15% *N*-dimethylformamide in (B). Electrophoresis was carried out at 30 W for 4 h (**A**) or 2 h (**B**). The fluorescent photographs were taken as described in the text for 8 min. The partial sequence from 3′ to 5′ end of yeast 5S rRNA is shown (from bottom to top) in the margin. T_1, RNase T_1; U_2, RNase U_2; *Phy*I, RNase *Phy*I; Bc, RNase *B.cerus*; Ctl, control.

from the glass plate and put directly on the ultraviolet glass plate. The gel was kept smooth on the surface of the ultraviolet glass plate with no intervening air bubbles. After exciting with short wavelength ultraviolet light and exposing a film for 8 min by a camera with color filter and black-and-white film insensitive to ultraviolet light, clear and sharp bands were obtained on the film (Fig. 1). The sensitivity of a single band of the fluorescent RNA fragment is <0.01 pmol.

ln this method, 4–6 nt at the very beginning of the 3′ terminus of RNA could not be read out, possibly because of the nonpolar property of the fluorescent dye. However, compared with the traditional radioisotope labeling method, this fluorescence-labeling and fluorescent photograph technique avoids the hazards of radioactivity and also decreases the diffusion of RNA fragments in the gel owing to the short time in the detecting procedure. The bands obtained by fluorescent photography are clearer and sharper than those by autoradiography because of less diffusion of RNA fragments and the focus effect of photography.

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