

A Fluorescent DNA Probe Prepared by the Direct Derivatization of the Sugar Moiety for Hybridization Assay

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The preparation of a fluorescent DNA probe based on the derivatization of the terminal hydroxyl group of the sugar moiety of a DNA primer and its applicability to the DNA hybridization assay are described. M13mp8 plasmid primer reacts with 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran in the presence of sodium azide to form the corresponding fluorescent probe, which can be used for the hybridization assay to the target DNA, M13mp8 plasmid vector. The detection limit of the DNA with the naked eye is 10 ng (approximately 300 fmol)/spot on filters for the hybridization assay.

Keywords fluorescent DNA probe; 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran; M13mp8; hybridization assay; membrane filter

We previously reported that 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl) reacted with a 5'-hydroxyl group of nucleotides in the presence of sodium azide to produce the corresponding OMB-carbamates.¹⁾ This paper deals with the preparation of a fluorescent DNA probe by the direct fluorescence derivatization of the terminal hydroxyl group (the sugar moiety of DNA primer) utilizing the above mentioned reaction and its applicability to DNA hybridization assay. Single strand M13mp8 plasmid vector and its primer [d(GTTTTCCAGTCACGAC)] were used as a model target DNA and for the preparation of a fluorescent DNA probe, respectively.

Experimental

Chemicals OMB-COCl, M13mp8 vector and the primer were obtained from Dojindo Laboratories (Kumamoto, Japan), Wako Pure Chemicals (Osaka, Japan) and Yuki Gosei Kogyo (Tokyo, Japan), respectively. Nitrocellulose membrane filters were purchased from Advantec Toyo (Tokyo) and polyvinylidene difluoride membrane filters (Immobilon-P, Immobilon-N and Immobilon-PSQ) were from Nihon Millipore (Tokyo) and used after wetting. Deionized water was passed through a Milli-Q II system (Nihon Millipore), which was sterilized by heating in an autoclave when required for use. All other chemicals were of reagent grade or were molecular biology reagents (Sigma, St. Louis, MO, U.S.A.).

Apparatus The high-performance liquid chromatography (HPLC) system and its operating conditions were essentially the same as described previously.¹⁾ The columns for size exclusion and reversed phase HPLCs were a TSKgel G3000SWXL (300 × 7.6 mm i.d.; particle size 5 μm; Tosoh) and a TSKgel ODS-80T_M (150 × 4.6 mm i.d., particle size 5 μm; Tosoh), respectively. The mobile phases were mixtures of acetonitrile and 50 mM phosphate buffer (pH 6.5) (1:9 and 1:1, v/v, respectively), both of which were pumped at a flow rate of 1.0 ml/min.

Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi F-2000 spectrofluorometer in 10 × 10-mm quartz cells; spectral bandwidths of 10 nm were used for both the excitation and emission monochromators. A PAN UV lamp (PUV-1) (UV range 250—400 nm; Tokyo Kogyo Kikai, Tokyo) was used for the observation of fluorescence with the naked eye.

Preparation of Fluorescent DNA Probe A mixture of the primer (1.3 nmol) and 0.15 M aqueous sodium azide (0.3 ml) placed in a screw-capped vial was lyophilized. To the lyophilizate was added a mixture of pyridine and 3 mM OMB-COCl in benzene (1:3, v/v) (0.5 ml). The vial was tightly closed and heated at 100 °C for 90 min in the dark. After cooling, the mixture was dried in a stream of nitrogen at 60 °C, and the residue was dissolved in a mobile phase (0.2 ml) for the size exclusion HPLC. A portion (100 μl) of the resulting solution was subjected to size exclusion HPLC. The HPLC procedure was repeated twice and the fractions containing the fluorescent product were combined and lyophilized, then the lyophilizate was dissolved in TE buffer (pH 8.0)²⁾ to

give an approximate concentration of the fluorescent product, 0.3 nmol/ml.

Dot-Blot Hybridization Assay M13mp8 vector solution (upto 100 μg/ml, 10 μl) was spotted onto a filter, and the following procedures for the baking (80 °C, 2 h), prehybridization (42 °C, 5 h) and hybridization (42 °C, 18—36 h) were carried out in the usual manner.²⁾ The fluorescence on the filter was observed with the naked eye under the irradiation of the UV light.

Results and Discussion

The optimal conditions of the derivatization reaction (reaction temperature and time, and concentrations of sodium azide, pyridine and OMB-COCl) were almost identical with those previously described.¹⁾ In the size exclusion HPLC, the fluorescent product [retention time (t_R), 8.5 min] was completely separated from the reagent blank peaks (t_R , 13—35 min). The excitation and emission maxima of the fluorescence of the eluate were at 340 nm and 420 nm, respectively, which are identical with those of the derivatives of mono- to tetra-nucleotides.¹⁾ The lyophilizate of the eluate, dissolved in TE buffer (pH 8.0), had identical excitation and emission maxima, and the fluorescence was stable for at least 72 h at room temperature (25 ± 2 °C) in the dark.

The approximate yield of the fluorescent product was calculated to be 46% by comparing the fluorescent intensity of the lyophilizate (concentration, corresponded to 300 pmol/ml of the primer) in the TE buffer with that of the OMB-carbamate of adenosine-3'-monophosphate¹⁾ (300 pmol/ml) in the buffer.

When the lyophilizate was dissolved in the mobile phase for reversed phase HPLC and treated under the conditions of the HPLC, a single peak (t_R , 43 min) and double peaks (t_R , 28 and 43 min) were found in the fluorescence and UV detections, respectively. This indicates that the lyophilizate is a mixture of the fluorescent product and the primer that remained unreacted (t_R , 43 and 28 min, respectively); it was tentatively subjected as a fluorescent DNA probe to the examination of the hybridization assay without separation. When the separated probe by the reversed phase HPLC was used in the hybridization assay, the sensitivity doubled.

The results obtained according to the hybridization assay procedure are shown in Table I. The fluorescence from the probe hybridized with the target DNA on Immobilon-PSQ, Immobilon-P and nitrocellulose membrane filters could be

TABLE I. Fluorescence Detection of M13mp8 Vector Immobilized on Various Membrane Filters^{a)}

Membrane filter	Amount of M13mp8 vector (ng on filter)					
	5	10	50	100	500	1000
Immobilon-PSQ ^{b)}	±	+	++	++	+++	+++
Immobilon-P ^{c)}	±	+	++	++	+++	+++
Immobilon-N	N.D. ^{d)}	N.D.	N.D.	N.D.	N.D.	N.D.
Nitrocellulose ^{e)}	±	+	++	++	+++	+++

a) The fluorescence detection with the naked eye: ± = detectable but unclear; + = detectable; ++ = strong; +++ = very strong. b) The fluorescence was observed from both the DNA immobilized side and the other side of the filter. c) The fluorescence was detectable provided that the UV light was irradiated from the other side of the filter. d) N.D., not detected.

observed. Of the three filters, Immobilon-PSQ provided the most clearly observable fluorescence. The other two allowed the fluorescence to be observed, provided that the UV light was irradiated from the other side of the filter: the irradiated UV light was reflected from the filter surfaces and

baldly interfered with the detection with the naked eye. Immobilon-N did not afford fluorescence for unknown reasons.

Although this hybridization assay is not so sensitive as those using either radioisotope- or enzyme-labelled probes,³⁾ the present method for the preparation of a DNA probe based on the direct fluorescence derivatization of the sugar hydroxyl group is very simple and rapid. The method should be applicable to the preparation of other fluorescent DNA probes for hybridization assay.

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