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Detection of Japanese Encephalitis Virus by Biotinylated cDNA Probe

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Biotin으로 표지된 cDNA Probe를 이용한 일본 뇌염 바이러스의 검색

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ABSTRACT: Japanese Encephalitis Virus (JEV) can be detected conveniently by the use of biotinylated cDNA probe. To prepare biotinylated probe aminoallyl dUTP was first synthesized chemically to reverse transcribe the virial RNA. The allylamine-labeled cDNA was then converted to the biotin-cDNA by the reaction with an activated biotin ester, NHS-ACA-biotin. The JEV genomic RNA was hybridized to the biotinylated cDNA probe on nitrocellulose filter and visualized colorimetrically by streptavidin complexes with alkaline phosphatase polymer. Sensitivity of the detection system was determined by estimating the amount of the JEV genomic RNA through comparison with signals generated from the biotinylated and ^{32}P-labeled probes. It was found that the biotin probe was as sensitive as ^{32}P-cDNA probe which can detect 50 pgs of the target RNA.

KEY WORDS □ Japanese Encephalitis Virus, Biotinylated Probe, Aminoallyl cDNA

Recently, the novel methods which lead to the direct diagnosis of infectious microorganisms, genetic abnormality, and cancer at the level of nucleic acid without the laborious immunological procedures have been developed. The nucleic acid probes used in these methods were commonly labeled with radioisotopes. However, there are serious limitations and disadvantages associated with the use of radioactively labeled hybridization probes, especially for routine application in clincal or diagnostic purpose. The short half-life of many radioisotopes, the isotope disposal problems, the personnel safety, the expense, make it desirable to have an alternative but equally sensitive methods for detecting, quantitating, or localizing specific nucleic acid sequences.

Cheung *et al.* (1977) have generated a fluoresent signal by coupling a latex sphere containing poly (U) and dansylated fluorochrome to polyadenylated mRNA probe. Tchen *et al.*(1984) have modified a nucleic acid probe chemically so that it could be detected by immunological methods. Langer *et al.*(1981) have synthesized the biotin-labeled nucleotide analog that can be incorporated into nucleic acid by enzymatic reactions. Hybridization signals can be visualized by indirect immuno-fluorescense, immuno peroxidase or immuno-colloidal gold following incubation with a primary anti-biotin antibody. Cytochemical methods that employ complexes of avidin and biotinylated enzyme complex can also be used to detect the biotin labeled probes.

In this report, we demonstrated the application of one of these techniques to clinical field, namely

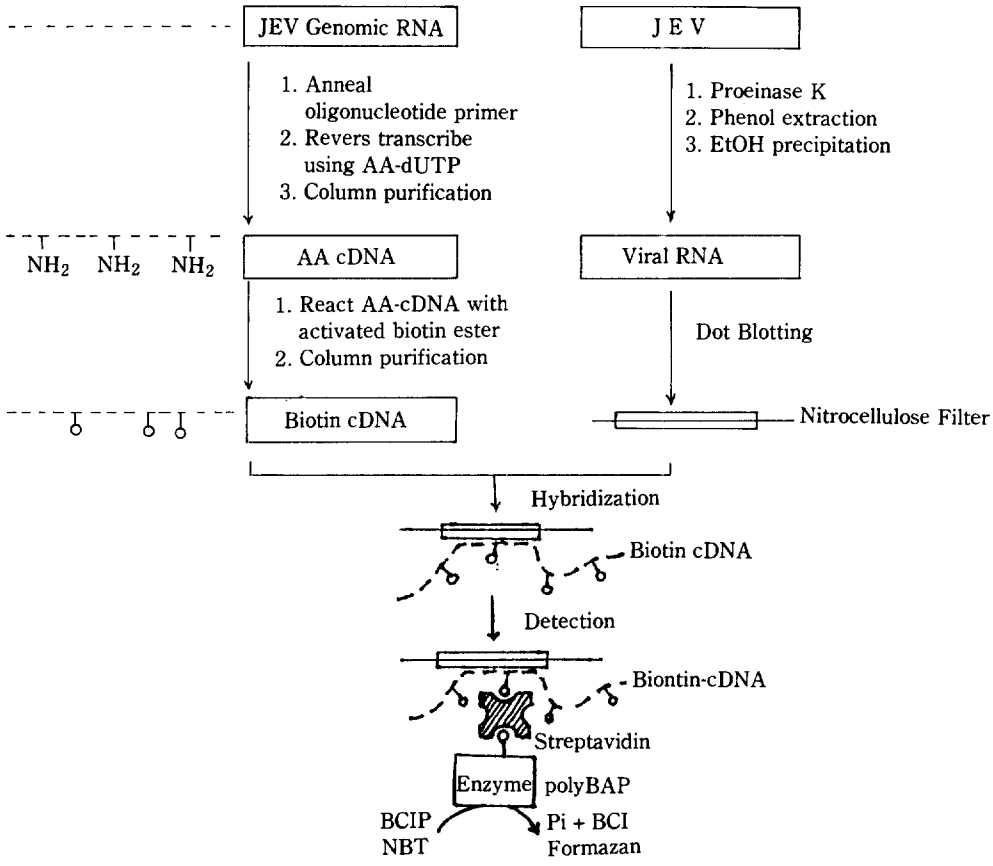

Fig. 1. *Strategies for the detection of Japanese encephalitis virus (JEV) with biotin-labeled cDNA probe.*

the detection of Japanese encephalitis virus. We used streptavidin and bacterial alkaline phosphatase polymer to visualize the hybridization signal generated by the biotinylated cDNA probe. The overall scheme of the experimental procedure is outlined in Fig. 1.

MATERIALS AND METHODS

Materials

AMV reverse transcriptase, oligonucleotide primer, Sephadex G 50 and DEAE Sephadex A 50 were obtained from Pharmacia. Dextran sulfate, ficoll, herring sperm DNA, bovine serum albumin, formamide, and cadaverin were procured from Sigma. DNA detection system, RNA ladder, and NHS-ACA-biotin were purchased from BRL. Disuccinanidyl suberate was obtained from Pierce and [α-^{32}P] dCTP was the product of Amersham.

Japanese encephalitis virus stock was a Nakayama strain isolated in 1936 and was maintained at the Korea National Institute of Health.

Preparation of allylamine-dUTP

Since AMV reverse transcriptase can not use biotin-dUTP as substrate, we synthesized allylamine-dUTP which could be incorporated into cDNA by the reverse transcriptase reaction. The allylamine-dUTP in not yet commercially and was prepared by the procedure of Langer *et al.*(1981).

Mercuration step: dUTP (100 mg, 0.181 mmole) in 18 m*l* of 0.1 M sodium acetate, pH 6.0, was treated with mercuric acetate (0.287 g, 0.9025 mmole). The solution was heated at 50 °C for 4 hr and then cooled in an ice-H_2O bath. Lithium chloride (70.758 mg, 1.62 mmole) was added and the solution was extracted six times with equal

volume of ethylacetate to remove excess $HgCl_2$. The nucleotide products in the aqueous layer were precipitated by the addition of 3 volume ice cold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol and once with ethylether and then air dried.

Synthesis of 5-(3-amino)allyl dUTP: The mercurated nucleotides were dissolved in 0.1M sodium acetate, pH 5.0, and adjusted to 20 mM (A_{267}, 200 units/m*l*). 0.878 m*l* of the neutralized allylamine stock (2M) was added to 7.3 m*l* of nucleotide solution. 1.17 m*l* of K_2PdCl_4 (163 mg/4 m*l* stock soln) was then added to initiate the reaction. After standing at room temperature for 20 hrs, the reaction mixture was passed through a 0.45 um membrane filter to remove most of the remaining metal precipitate. The yellow filtrate was diluted 1:5 with H_2O and applied to a DEAE-Sephadex A-25 column (3 × 12 cm). After washing with on column vol. of 0.1 M sodium acetate, pH 5.0, the products were eluted by linear gradient (0.1-0.6 M) of sodium acetate, pH 8.5. Final purification was achieved by reversed-phase high pressure liquid chromatography on a column of μ-Bondapak C-18 (30 × 0.4 cm I.D., 10 μm paticles). 0.5 M $(NH_4)HCO_3$, pH 7.8 (Isocratio elution was carried out with 0.5 M $(NH_4)HCO_3$, pH 7.8 at a flow rate of 2.0 m*l*/min. The chromatography was performed with a Waters 6000A pump and a U6K injector).

Preparation of biotinylated cDNA probe

From infected mouse brains JEV was purified by protamine sulfate precipitation (0.25 mg/m*l*) polythylene glycol precipitation (8%) and ultracentrifugation through sucrose gradient. Genomic RNA was phenol extracted from the pelleted virus, ethanol precipitated and used as the template for reverse transciptase to synthesize complementary DNA. AMV reverse transcriptase could not use biotin-dUTP efficiently as substrate (Langer *et al.* 1981). Therefore, we made allylamine-cDNA in the reaction with NHS-ACA-biotin.

To prepare allylamino cDNA viral RNA (5 μg) was first incubated in a 50 μl reaction mixture containing 50 mM Tris·HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 500 μM dCTP, dATP, dGTP, 250 μM AA-dUTP, 250 μM dTTP, 0.1 mg/m*l* BSA, 20 μg of oligonucleotide primer and 50 units of AMV reverse transcriptase. The control reaction mixture contained the same composition as above except that AA-dUTP was replaced by dTTP. To monitor the time course and efficiency of cDNA synthesis for each experiment, small amount of [α-^{32}P]dCTP was added to the reaction mixture (specific radioactivity, 1.07 Ci/mmole). While incubating the reaction mixture at 42 °C, small aliquots (3 μl) were taken out at the regular time intervals and the radioactivity incorporated into cDNA. Two uls of NHS-ACA-biotin (5 mg/m*l* in dimethylformamide) was added to aminoallyl-cDNA (in 50 μl of 0.1M sodium borate buffer, pH 8.0) and the mixture incubated for 2 hrs at room temperature. Biotinylated cDNA was purified from the reaction mixture by the use of Sephadex G 50 spin column.

Hybridization

Dot blotting was carried out as described by Patricia S.T. (1983). RNA was incubated in 1M glyoxal-10 mM phosphate buffer, pH 6.5-7.0, at 50 °C for 1 hr. The reaction mixture was cooled on ice and dilutions were made with sterile distilled H_2O. The pretreated RNA sample and dilutions were spotted directly onto dry nitrocellulose paper that had been pretreated with H_2O, equilibrated with 20X NaCl-citrate. After all samples had been spotted, the blot was dried under a tungsten lamp, baked for 2 hrs at 80 °C and treated with 20 mM Tris Cl, pH 8.0 for 5-10 min at 100 °C.

Prehybridization and hybridization were carried out as described by Wahl *et al.*(1979) except that the concentration of formamide was reduced from 50% to 45% to compensate for the reduced Tm of the Biotin-probe target hybrid. The posthybridization washes were performed as follows: Twice with 2X SSC, 0.1% SDS, 2-3 min for each wash at room temperature and twice with 0.2X SSC, 0.1% SDS under the same condition. Filters were washed twice again with 0.16X SSC, 0.1% SDS for 15 min at 50 °C. Filters were then rinsed

briefly in 0.16X SS 0.1% SDS at room temperature and air-dried. When the ^{32}P-cDNA was used as probe, the hybridization procedure was followed as described by Maniatis *et al.*(1982).

Visualization

Dry filters were incubated at 42 °C for 15 min in a 3% (w/v) solution of BSA in AP 7.5 buffer (0.1 M Tris·HCl, pH 7.5, 0.1 M NaCl, 2mM $MgCl_2$, 0.05% (v/v) Triton X-100) air dried, baked at 80 °C for 30 min, and then rehydrated in the albumin solution at 42 °C for 10 min. Filters were exposed to streptavidin (2 μg/m*l* AP 7.5 buffer, 5 m*l* per 100 cm^2 of filter paper) for 10 min, and rapidly washed three times in 250 m*l* of AP 7.5 buffer. Filters were exposed to biotinylated alkaline phosphatase polymer (1 μg/m*l* AP 7.5 buffer, 5 m*l* per 100 cm^2 of filter paper) for 10 min, and washed three times in 250 m*l* of AP 7.5 buffer and twice in AP 9.5 buffer (0.1 M Tris·HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$). Filters were incubated at room temperature in AP 9.5 buffer containing NBT (0.33 mg/m*l*) and BCIP (0.19 mg/m*l*), developed in the dark for 4 hrs or longer. The developed blots were washed in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and then stored dry or in heat-sealed bags containing a small amount of 20 mM Tris·HCl, pH 9.5, containing 5 mM EDTA.

RESULTS AND DISCUSSION

In order to purify the chemically synthesized allylamine-dUTP we applied crude solution of allylamine-dUTP to DEAE-Sephadex A-25 column. When the column was washed by a linear gradient (0.1-0.6M) of sodium acetate buffer, pH 8, a major UV-absorbing peak containing AA-dUTP was eluted at 0.37 M sodium acetate concentration (Fig. 2). Final purification was achieved by reversed-phase high-pressure liquid chromatography an a column of μ-Bondapak C-18 using 0.5 M $(NH_4)HCO_3$, pH 7.8 as eluent. AA-dUTP was the last peak eluted from the column and this was chromatograpically identical to the AA-dUTP (Fig. 3). Spectral analysis of the AA-dUTP shows that the purified sample has maximum UV absorption at the wavelength of 288 nm, 240 nm and

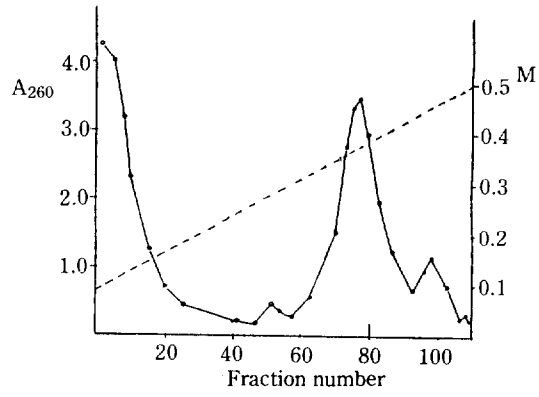

Fig. 2. *Ion exchange chromatography of AA-dUTP on a column of DEAE-Sephadex A-25.*
The column was washed with 0.1 M sodium acetate, pH 5.0 (100 m*l*), and eluted with a linear gradient (0.1-0.6 M) of sodium acetate buffer, pH 8.0. The major A_{260} peak appearing at 0.37M sodium acetate is allylamine dUTP

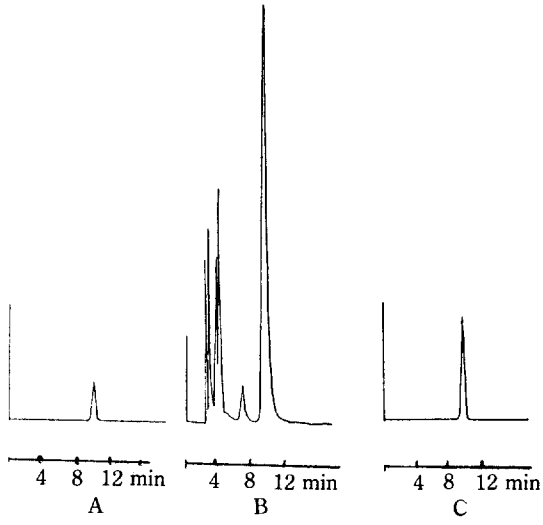

Fig. 3. *Purification and identification of allylamine dUTP by high performance liquid chromatography.*
A. Standard allylamine-dUTP
B. The fraction from DEAE-Sephadex A-25 column
C. Purified AA-dUTP fraction collected from B.
The reversed-phase liquid chromatography was carried out as described in the "Materials and Methods".

minimum UV absorption at 262 nm (Fig. 4).

The purified JEV genomic RNA was electrophoresed on agarose gel after denaturation and the size of RNA was estimated to be 14 kb (Fig. 5).

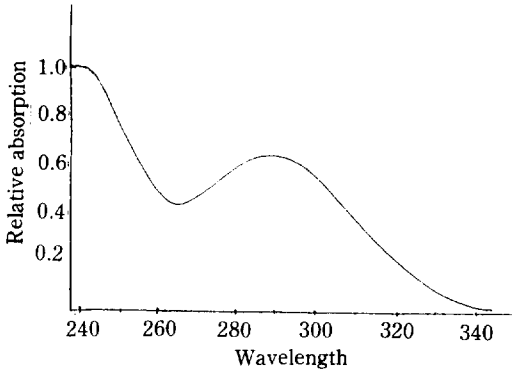

Fig. 4. *UV absorption spectrum of the allylamine-dUTP purified from H.P.L.C.*
The absorption spectrum of the standard AA-dUTP was identical to the chemically synthesized material.

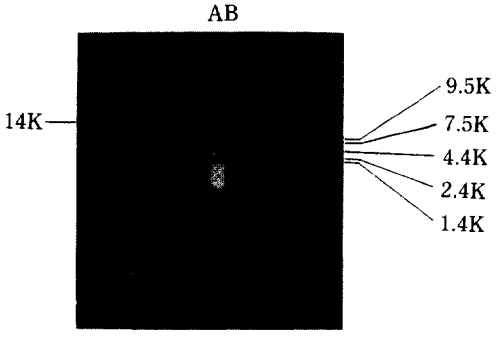

Fig. 5. *Electrophoresis of purified JEV genomic RNA on 1% agarose gel*
Lane A: Purified JEV genomic RNA
Lane B: Nucleic acid molecular weight standards, RNA ladder.
The RNA ladder is a series of six poly(A) tailed RNA transcripts synthesized and distributed by Bethesda Research Laboratories (BRL). Agarose gel electrophoretogram showed partial degradation of the commercially purchased material, however, the M.W. of the purified JEV RNA was estimated to be about 14 kb.

Using this genomic RNA as template, we prepared biotin-cDNA probe. The time course and effeciency of allylamine labeling reaction was monitored through [α-^{32}P]dUTP incorporation. It was found that the time required to achieve maximal yield of the cDNA synthesis under the reaction condition discribed in "Materials and Methods" was about 60 minutes (Fig. 6). The comparison of the reactions which contained AA-dUTP and the

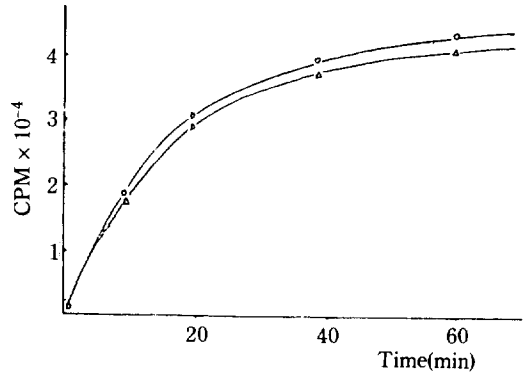

Fig. 6. *Time course of the cDNA synthesis.*
AA-cDNA was synthesized by using equimolar amount of AA-dUTP and dTTP, as substrate (△-△). The control reaction contained no AA-dUTP (○-○). To monitor the time course and efficiency of both reactions, [α-^{32}P]dCTP (specific radioactivity, 1.07 Ci/mmole) was added. The specific radioactivity of cDNAs synthesized at 60 min time point were app. 7.3×10^5 cpm/ug.

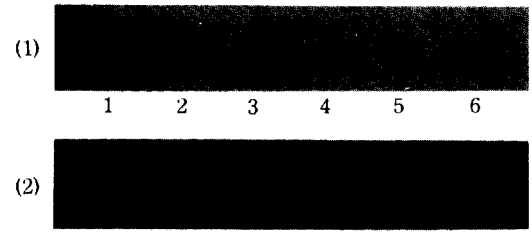

Fig. 7. *Detection of the JEV RNA using biotin-cDNA probe and ^{32}P-cDNA probe.*
Each spot contained the indicated amount of target RNAs(spot 1, 100ng; 2, 50ng; 3, 1.0ng; 4, 500pg; 5, 50pg; 6, 10pg).
Dot blots were hybridized
1) with biotin-cDNA probe for 20 hrs in hybridization buffer containing 45%(V/V) formamide. The signals were visualized by A poly BAP system.
2) with ^{32}P-cDNA probe (7.3×10^5 cpm/μg cDNA) for 20hrs in hybridization buffer containing 50% (V/V) formamide. X-ray film was exposed for 3 days.
The probe concentrations were 1.0 μg/ml.

normal substrate dUTP revealed that the amount of cDNA synthesized was nearly the same in both cases (Fig. 6). Therefore, we concluded that AA-dUTP could be incorporated efficiently into cDNA by the action of AMV reverse transcriptase.

To compare the sensitivity of bio-cDNA probe

with ^{32}P-cDNA probe, we prepared biotin-labeled cDNA in such a way that 50% dTTP was replaced by AA-dUTP. ^{32}P-labeled cDNA was prepared to give the final specific radioactivity of ^{32}P-cDNA to 7.3×10^5 cpm/μg cDNA. After adjusting the concentration to 1 μg/m*l*, we estimate the sensitivity of both probes by hybridizing them with the serial dilutions of target JEV genomic RNA. As shown in figure 7, the detection limit of the biotinylated probe was as sensitive as ^{32}P-labeled probe. In this expensiment, it took 3 days to visualize the ^{32}P-cDNA probe-target RNA hybrids, however in the case of the biotinylated cDNA, only 3-4 hours were required to visualize the hybrids.

When JEV RNA was prepared directly from infected mouse brain through protamine sulfate treatment, PEG precipitation, proteinase K, 1% SDS treatment and phenol extration, the amount of the viral RNA obtained from one mouse brain was sufficient to be deteced by this biotinylated cDNA probe (Fig. 8).

Fig. 8. *Detection of JEV genomic RNA from two infected mouse brains by the use of biotin labeled cDNA probe.*
(spot 1, 1X; 2,1/3X; 3,1/9X; 4,1/12X; 5, control)

적 요

Biotin으로 표지된 cDNA probe를 사용하여 일본 뇌염바이러스를 검색하였다. Bio-probe는 먼저 뇌염 virus의 genomic RNA를 화학적인 방법으로 합성한 aminoallyl-dUTP와 함께 역전사 시켜 aminoallyl group으로 표지된 cDNA를 만든 뒤 활성화된 biotin ester인 NHS-ACA-biotin ester와 반응시켜 제조하였다. 이 cDNA probe를 viral RNA와 nitrocellulose 상에서 hybridization 시킨 뒤, A poly BAP(Avidin-biotinylated bacterial alkaline phosphatase polymer) system으로 탐지한 결과 biotin-cDNA probe는 핵산수준에서 방사성 동위원소 ^{32}P로 표지된 cDNA probe 만큼 민감하여 수십 picogram의 target RNA를 검색할 수 있었다.

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