MOLECULAR HYBRIDIZATION METHODS FOR DETERMINATION OF SERUM HBV-DNA

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Summary

Molecular hybridization methods for determination of hepatitis B virus DNA (HBV-DNA) in serum were studied. A simple method by which serum was treated with sodium hydroxide, followed by dot hybridization procedure on filter sheets provides a sensitive and direct result for detecting HBV-DNA. Another method in which DNAs extracted from Dane particle fraction were subjected to the molecular hybridization method on a filter membrane, provided similar results although this method is time consuming. The third method in which serum was directly spotted on filter sheets, followed by alkaline-treatment seems to be less sensitive. Three filter papers, NC filter, Zeta-Probe and Biodyne, on which molecular hybridization was performed, gave similar sensitivity.

Key Words: Dane particle, Dot hybridization, Filter sheets, HBV-DNA, Molecular hybridization.

Introduction

Molecular hybridization procedures have been employed to detect HBV-DNA at the picogram (10^{-12} gm) level which has enhanced the study of HBV-related liver disease through detection of specific HBV-DNA sequences in the serum^{1-4}. Recently more simplified spot methods for the determination of HBV-DNA in serum were reported^{5,6}.

HBV-DNA sequences in serum can be detected on filter membranes, such as NC-filter, Biodyne transfer or Zeta-Probe blotting membrane by modifying these methods^{1-6}.

Materials and Methods

Spot and dot techniques: The following 3 methods to detect HBV-DNA sequences in the serum positive for HBsAg were compared. 1. Dane particle associated DNA was extracted from 250 µl of serum, dissolved in 10 µl of water, and 1 µl of this solution was spotted on the filter sheet^{9}. 2. DNAs in 25 µl of serum were denatured with 0.5 N NaOH and placed in dots on the filter sheet (8 x 12 cm) with a Bio-Dot Apparatus (Bio-Rad Lab., Richmond, CA, USA). Aspiration was provided by a vacuum pump at a pressure of 20 cm Hg^{9}, and 3. Ten µl of serum were applied directly to the filter sheet and denatured with 0.5 N NaOH.
For the quantification of HBV-DNA sequences in the serum, standard serum was included in each sheet. The filter sheets were then soaked in a solution containing 0.3 M NaCl and 0.03 M Na$_2$ citrate, blotted with two sheets of filter papers (Whatman #3 filter paper, Whatman Ltd., Maidstone, England), dried at 60°C overnight, and subjected to the molecular hybridization analysis. Nitrocellulose (NC)-filter (0.45 µm Schleicher & Schuell, Dassel, W. Germany), Biodyne A transfer membranes (1.2 µm Pall Ultrafine Filtration Corp., Glen Cove, NY, USA) and Zeta Probe blotting membranes (0.45 µm Bio-Rad Lab.) were used as filter sheets for the analysis. HBV-DNA in the serum was expressed as the quantity of $^32$P-cloned HBV-DNA bound to the corresponding spots on the filter sheets in the hybridization procedures. Sera subjected to analysis were stored at -20°C until used.

Preparation of $^32$P-labelled HBV-DNA probe: [$\alpha$-$^32$P]-labelled cloned HBV-DNA was prepared by the nick-translation procedure. Cloned HBV-DNA (7.7 µg) provided by Dr. K. Matsubara, Osaka University, was incubated in 100 µl of 100 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$, 9 mM 2-mercaptoethanol, 100 µg of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) per ml, 0.83 M $\alpha$-$^32$P-labelled dCTP (New England Nuclear, Boston, MA, USA, 3000 Ci/m mol), 400 mM each of dATP, dCTP and dTTP and 5% glycerol in the presence of 50 pg of DNase I (New England Nuclear) per ml. Polymerization was then carried out for 120 min at 20°C after the addition of 5 units of E. Coli DNA polymerase I (New England Nuclear). The reaction was stopped by addition of 2 µl of 1 M NaOH. The labelled DNA was separated from unreacted nucleotides by Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) columns (1 x 20 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and specific activities of about $5 \times 10^8$ cpm/µg of DNA were usually achieved.

Hybridization: Nonspecific binding of DNA probe was minimized by preincubating the filter sheet at 42°C for at least 5 hr in a modified Deinhardt solution: 0.75 M NaCl, 0.075 M Na$_2$ citrate, 0.1% each of PVP 360 (polyvinyl-pyrrolidone, Sigma), Ficoll 400 (Pharmacia), BSA (Sigma) and SDS (sodium dodecyl sulfate, Wako Pure Chemicals Ltd., Osaka, Japan), 50 mM phosphate buffer, pH 6.7, 0.1 mg/ml heat-denatured salmon sperm DNA and 50% formamide. After the preincubation the filter sheets were blotted between two sheets of Whatmann #3 filter paper (Whatman).

Hybridization was done in the same solution by addition of $2 \times 10^7$ cpm/ml of heat-denatured $^32$P-labelled cloned HBV-DNA probe whose specific activity ranged from $2 \times 10^8$ to $5 \times 10^8$ cpm/µg DNA. Hybridization volume was 5 ml for 2 pieces of filter sheet in a polyethylene bag which was heat sealed and the incubation was carried out at 42°C for 18 hr. The filter sheets were then washed with solution containing 0.3 M NaCl and 0.03 M Na$_2$ citrate, solution containing 15 mM NaCl and 1.5 mM Na$_2$ citrate and then with the solution containing 0.05% SDS, 0.02% BSA and 0.1 M phosphate buffer, pH 6.7 until detectable labelled material was no longer released into the wash solution. The filter sheets were finally washed twice with solution containing 15 mM NaCl, 1.5 mM Na$_2$ citrate, 0.02% each of PVP 360, Ficoll 400 and BSA, and 0.01 M phosphate buffer, pH 6.7.

The air-dried filter sheets were autoradiographed at -70°C using preflashed Kodak XAR-5 film with an intensifying screen (Du Pont Cronex Lightening Plus, Boston, Mass., USA). The filter sheet region corresponding to a radioactive spot was punched out and counted in a toluene-PPO (2,5-diphenyloxal-