Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 323-328

A SENSITIVE COLORIMETRIC DETECTION OF VIRUS DNA AND ONCOGENE

Hayao Inoue, Kenji Gushi, Shuji Matsuura, and Yoshitsugu Sakata

Wako Pure Chemical Industries Ltd., Osaka Research Laboratory, 6-1 Takada, Amagasaki, Hyogo 661, Japan

Received December 18, 1986

Advantage of cloning probe DNA fragment in phage M13 DNA was taken to provide a larger single stranded DNA as a hybridization probe. High level of direct enzyme labels was introduced via the M13 DNA moiety as well as probe DNA. A highly sensitive colorimetric detection of virus DNA and oncogene was developed. © 1987 Academic Press, Inc.

Hybridization probes are commonly labelled with the radioisotopes such as ^{32}P, ^{125}I or ^{3}H. Recent developments offer many alternative methods to produce nonradioactive labellings. Among those are enzymatic labelling with biotinylated nucleotide analogs (1), and chemical methods with fluorescent compounds (2) or biotin derivatives (3). Another approach is to label nucleic acids with a protein such as histone (4) or enzymes (5). Particularly enzymes can be directly detectable colorimetrically by their substrates. This enabled the rapid detection of target DNA on nitrocellulose filters after hybridization.

Specific activity of the probe depends much upon the mass ratio of label to DNA. However, the extensive labelling of short nucleic acid probe with enzyme probably interferes with the recognition of sequence complementary to the probe. Phage M13 is small (about 7.2 kbp) and contains unique restriction sites in which DNA can easily be inserted (6). In

0006-291X/87 $1.50

this study we have taken advantage of cloning probe DNA in phage M13 to provide a larger single stranded DNA as a hybridization probe. High specific activity of the hybridization probe was obtained by introducing a large amount of enzyme labels via the M13 DNA moiety. Here we show a highly sensitive colorimetric detection of virus genes and oncogenes using M13 DNA probe as hybridization probes.

MATERIALS AND METHODS

The plasmid pHBV933 supplied by Dr. Y. Sugino, The Takeda Chemical Industries, Ltd., Osaka, is a pBR322 clone with a 3.2 kbp insert of genome DNA from hepatitis B virus (7). The plasmid pEB1 obtained from Dr. K. Toyoshima, Tokyo University, Tokyo, is a pBR322 clone with a 1.5 kbp insert of v-erbB oncogene (8). M13 mp18 RFI DNA and E. coli K12 strain JM103 were purchased from Pharmacia Fine Chemicals. The following enzymes were used: EcoRI and SalI (Wako Pure Chemical Ind.) . and T4 DNA ligase (Takara). Photobiotin kit was obtained from BRESA.

Preparation of peroxidase-polyethyleneimine conjugate (Labezyme)

Peroxidase-polyethyleneimine conjugate was prepared with the modification of the method of Renz and Kurz (5). Horseradish peroxidase (grade IC: Toyobo) (20 mg) was dissolved in 220 µl of 100 mM sodium phosphate, pH 6.0, followed with the addition of 60 µl of p-benzoquinone solution (30 mg/ml in ethanol). The reaction mixture was incubated at 37°C for 1 hour in the dark. Activated peroxidase was immediately separated from other reagents by gel filtration on a sterilized Sephadex G-50 column equilibrated with 0.15M NaCl. Fractions containing peroxidase (1.8 - 2.0 ml) were pooled. After pH was adjusted to alkaline condition with 200 µl of 1M $NaHCO_3$, 2.7 µl of polyethyleneimine (MW ca1400) solution was added. After the reaction mixture was incubated at 37°C for 18 hours in the dark, the conjugate was purified by gel filtration on a sterilized Sephadex G-50 column in stead of extensive dialyses (5). Peroxidase concentration in Labezyme was adjusted to give A_{403} = 0.12 - 0.15 for 1/200 diluted solution.

M13 Probe Construction

EcoRI fragments of pHBV933 were inserted into EcoRI site of M13 mp18 RFI as described by Messing et al. (9) and E. coli JM103 was transfected with constructed DNA. Single strand HBV DNA inserted M13 DNA was isolated from phages in the supernatant of culture broth by the method of Kadonaga et al. (10). SalI fragments of pEB1 were also inserted into SalI site of M13 mp18 RFI and single strand erbB DNA inserted M13 DNA was prepared with the same manner. Approximately 1 mg of the single strand M13 probe DNA was obtained from 1l of culture broth. The probe DNAs were stored in TE buffer at 4°C.

Labelling of nucleic acid with Labezyme

One µg of single strand M13 probe DNA or DNA fragments was dissolved in 20 µl of 5 mM sodium phosphate buffer, pH 6.8 and denatured by heat (100°C, 3 min.). Twenty µl of Labezyme and 6 µl of 5% glutaraldehyde were added and incubated for 10 min at 37°C. Immediately the reaction mixture was added directly to the hybridization reaction containing nitrocellulose paper and hybridization solution. Labelling by photobiotin was carried out according to the method of Forster et al. (3).

Colony Blotting

After E. coli HB101 colonies transformed by the plasmid pHBV933 had grown on L-plates containing ampicilin, a nitrocellulose filter was placed on the surface of the agar medium in contact with colonies and peeled off. Colonies on the filter were successively contacted with filter paper sheets saturated with 0.5N NaOH, with 1M Tris-HCl, pH 7.5, and with 0.5M Tris-HCl, pH 7.5 containing 1.5M NaCl. The filter was then washed briefly with agitation in 6x SSC, air dried and baked for 2 hrs at 80°C for hybridization.

Hybridization and Staining

Nitrocellulose filters were prehybridized for 1 hr at 37°C in pre-hybridization mixture (10x Denhardt's solution, 4x SET, 0.1% SDS: 1x SET is 0.15M NaCl, 0.03M Tris HCl, pH 8.0, 1 mM EDTA). Filters were then soaked in blank hybridization mixture (50% v/v formamide, 2x Denhardt's solution, 4x SET, 0.1% SDS, 12% w/v polyethyleneglycol 6000 and 30 µg/ml yeast tRNA), and incubated with constant shaking for 1 hr at 37°C. Hybridization was carried out at 37°C for 4 - 20 hrs after the addition of the labelled probe. Filters were then washed with constant shaking in 50% v/v formamide, 0.4% SDS, 0.5x SSC (2x 30 min, 37°C) and in 2x SSC (2x 20 min, room temperature). For the detection of peroxidase activity, filters were incubated with the substrate solution (10 ml of 100 mM Tris HCl, pH 7.4, 10 mM imidazole, 2 ml of ethanol solution containing 6 mg of chromogen, 10 µl of 30% H_2O_2: 3,3', 5,5'-tetramethylbenzidine or 4-chloro-1-naphthol was usually used as chromogen). After staining, filters were rinsed and stored in water or Tris HCl buffer.

RESULTS AND DISCUSSION

Hepatitis B virus DNA (3.2 kbp) cloned in pBR322 (plasmid pHBV933) was detected by dot blot hybridization using M13 HBV DNA probe as shown in Fig. 1. Serially diluted plasmid samples were blotted onto nitrocellulose filters and hybridized with three different HBV DNA probes. Sensitivity of detection with labelled M13 HBV DNA probe was 0.4 pg (Fig. 1. lane 1) and far more superior to that with directly labelled HBV DNA (40 pg, lane 2). Biotin labelled M13 HBV DNA probe could detect 4 pg (lane 3). Serially diluted EcoRI digest of

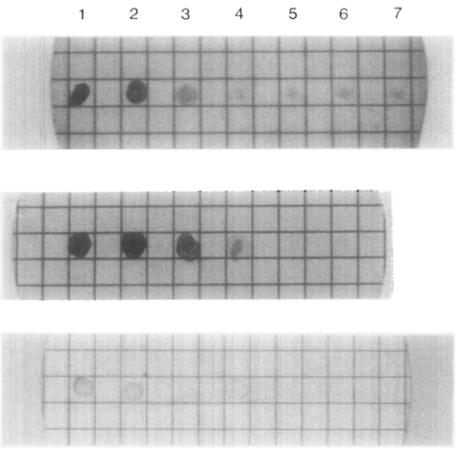

Fig.1. Dot Blot Hybridization of HBV DNA Fragments
HBV DNA fragments (1;400ng, 2;40ng, 3;4ng, 4;400pg, 5;40pg, 6;4pg, 7;0.4pg) were bound to nitrocellulose filters and hybridized with each probe. (Lane 1) Hybridization with ssM13-HBV DNA labelled with Labezyme and staining with TMBZ-H_2O_2. (Lane 2) Hybridization with dsHBV DNA fragment labelled with Labezyme and staining with TMBZ-H_2O_2. (Lane 3) Hybridization with ssM13-HBV DNA labelled with photobiotin, detection by alkalinephosphatase conjugated avidin and staining with BCIP and NBT (3).

pHBV933 (Fig. 2.A) and SalI digest of pEB1 (Fig. 3.A) were detected by southern blot hybridization (11). Detection sensitivity was 0.4 pg as same as dot blot hybridization using corresponding M13 DNA inserts.

Southern blot hybridization of HBV DNA from EcoRI digestion of pHBV933 was also carried out using M13 HBV DNA probe as shown in Fig. 2. While both HBV and pBR322 DNA bands were detected by labelled pHBV933 plasmid as a hybridization probe (Fig. 2.B). Only HBV DNA band was detected by M13 HBV DNA probe (Fig. 2.C). Furthermore various HBV DNA fragments obtained by digestion of pHBV933 with restriction endonucleases (EcoRI, BamHI, HincII, AvaI and AccI) were detected by southern blot hybridization. Corresponding DNA bands with each restriction endonucleases were specifically detected by M13 HBV DNA probe without crossreaction to pBR322 DNA.

E. coli HB101 colonies transformed by pHBV933 plasmid were detected by hybridization assay with M13 HBV DNA probe

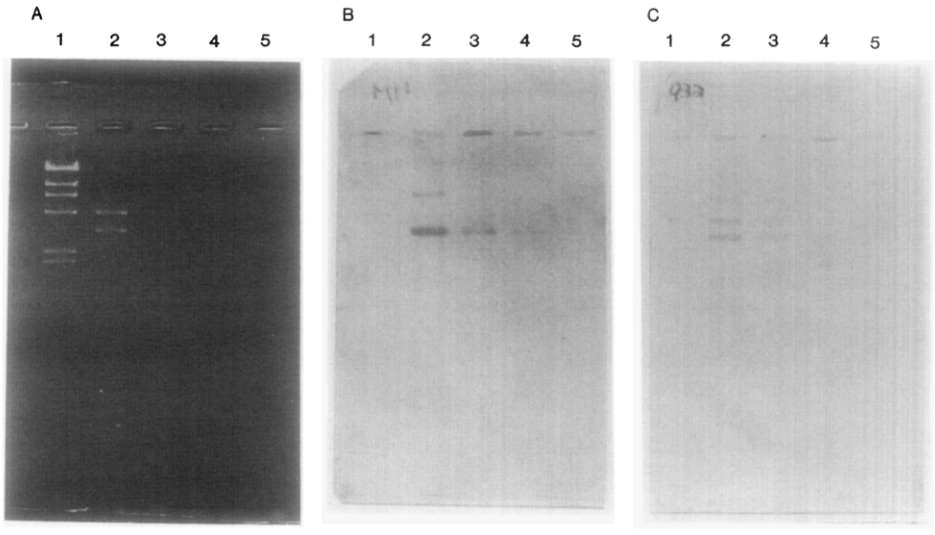

Fig.2. Southern Blot Hybridization of HBV DNA Fragments
A pHBV933 plasmid was digested with EcoRI which generated two fragments:HBV DNA, 3.2 kbp and pBR322, 4.6 kbp. Serial dilutions(10ng, 1ng, 100pg and 10pg) were electrophoresed, blotted onto nitrocellulose filters and hybridized with 1ug of each probe. (A) Agarose gel electrophorogram (ethidium bromide stained). (B) Hybridization with pHBV933 plasmid labelled with Labezyme and staining with TMBZ-H_2O_2. (C) Hybridization with ssM13-HBV DNA labelled with Labezyme and staining with TMBZ-H_2O_2.

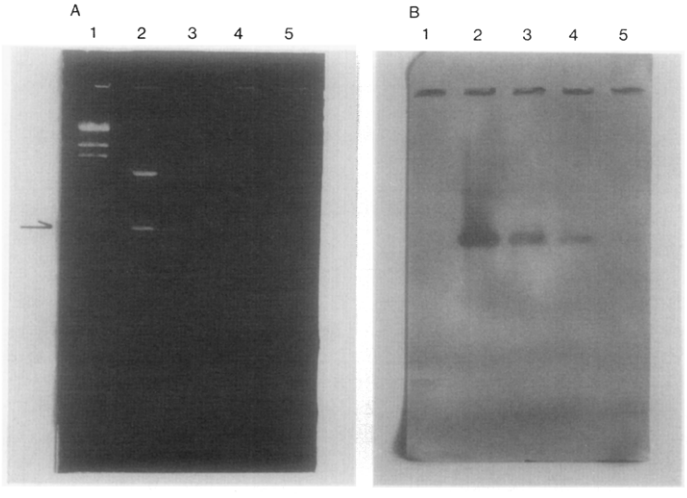

Fig.3. Southern Blot Hybridization of erbB Fragment
A pEB1 plasmid was digested with SalI which generated two fragments:erbB DNA, 1.5 kbp and pBR322, 4.6 kbp. Serial dilutions (100ng, 5ng, 250pg and 12.5pg) were electrophoresed, blotted onto nitrocellulose filters and hybridized with 1ug of the probe. (A) Agarose gel electrophorogram (ethidium bromide stained). (B) Hybridization with ssM13-erbB DNA labelled with Labezyme and staining with TMBZ-H_2O_2.

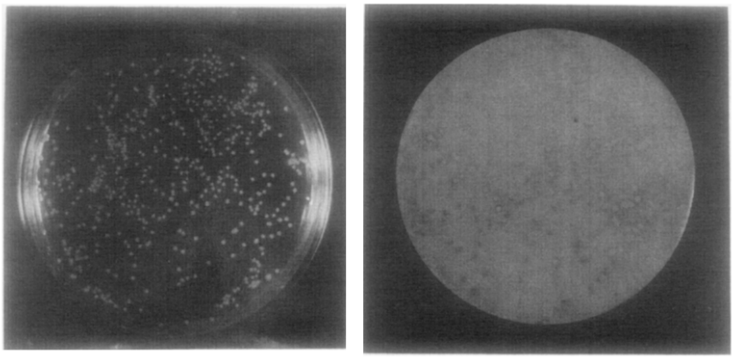

Fig.4. Screening of HB101 with a Plasmid pHBV933 by Colony Hybridization using POD Labelled ssM13-HBV DNA Probe
Left : HB101 colonies on a L-plate. Right : A replica filter was hybridized with ssM13-HBV DNA labelled with Labezyme and stained with 4-chloro-1-naphtol and H_2O_2.

(Fig. 4.). Positive colonies determined by hybridization assay were found to contain pHBV933 plasmid.

In conclusion, the highly sensitive DNA detection system presented here is comparable to tranditional method in its sensitivity, but superior to its speed. Adaptation of this system may be valuable in routine medical diagnosis as well as in basic researches.

ACKNOWLEDGEMENT

We are grateful to Drs. Y. Sugino and K. Toyoshima for supplying the plasmids.

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