Selective detection of hepatitis B virus RNA by PCR.

R Sallie

Genome Res. 1994 3: 376-377

Selective Detection of Hepatitis B Virus RNA by PCR

Richard Sallie

The Liver Diseases Section, National Institute of Diabetes, Digestive and Kidney Diseases, The National Institutes of Health, Bethesda, Maryland 20892

The hepatitis B virus (HBV) is a circular, partially double-stranded DNA virus of ~3.2 kb in length. The genome codes for at least three different-sized polyadenylated RNA species, each of which results in the synthesis of different proteins.[1] HBV RNA, as evidence for transcriptional activity, is often sought within tissues in the investigation of disease pathogenesis, but it is frequently difficult to obtain sufficient RNA from clinical samples (such as those obtained by needle biopsy) to perform Northern blot analysis or RNase protection assay. HBV, unlike some other viruses (notably cytomegalovirus), lacks introns; hence, detection of HBV RNA by reverse transcriptase polymerase chain reaction (RT–PCR) requires convincing demonstration of the complete elimination of HBV DNA by preliminary DNase digestion. In situ hybridization for HBV RNA is fraught with similar methodological difficulties. This paper demonstrates the application of random amplification of cDNA ends (RACE) methodology[2] to the detection of HBV RNA. The general principles of RACE have been described previously by other investigators,[2,3] but the specific application to the detection of HBV RNA is demonstrated schematically in Figure 1.

MATERIALS AND METHODS

RNA was purified from diagnostic needle liver biopsies performed on patients with chronic HBV (HBsAg positive, HBeAg positive) by guanidinium isothiocyanate.[4] First-strand cDNA was synthesized using AMV RT and oligo(dT)-based cDNA primer A (Fig. 1) as follows: Total liver RNA (100 ng) in diethylpyrocarbonate (DEPC)-treated water was added to a mixture containing 20 ng of primer A, 4 μl of 10× RT buffer (1× RT buffer = 50 mM Tris-HCl at pH 8.3, 50 mM KCl, 10 mM MgCl, 0.5 mM spermidine, 10 mM DTT), 200 μM dNTPs in a total of 20 μl, overlaid with two drops of mineral oil, and incubated at 70°C for 5 min. The mixture was cooled to 42°C prior to addition of 10 units of AMV RT, 20 units of recombinant RNase inhibitor (Promega), and DEPC-treated water to a total volume of 40 μl and incubated at 42°C for 1 hr. Nested PCR was then performed using hot start methodology[5] and 5 μl of the first-round mix in a PCR reaction containing 200 ng each of the primers described by Larzul et al.[6] [for the HBV (sense) component of the resulting chimeric molecule] or primers homologous to the oligo(dT)-based cDNA primer ($b\times c$, c = GGC GAC AC TCC ACC ATA GAT C, outer amplicon) or ($d\times e$, e = ATA GAT CGA ATT CGC GGC CGC, inner amplicon) and dNTPs at 50 μmoles, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl at pH 9.0, 2.0 mM MgCl, 0.1% Triton X-100), and 2 units of *Taq* polymerase (Biotech International, Bentley, Western Australia), which was added last with the reaction at 85°C. Twenty-five cycles of both outer and inner amplification were performed using 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min as the cycling parameters for both outer and inner nested amplifications. Non-nested PCR amplifications for albumin mRNA and HBV DNA were carried out using the same parameters (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min). In addition to positive and negative controls for amplification of HBV DNA (to demonstrate the presence of HBV DNA within the tissues examined), an internal control for the RT [primed by the locking-docking oligo(dT)-based primer A] and amplification of mRNA (albumin) was also run. Similarly, controls to confirm the importance of the oligo(dT)-based cDNA primer as well as the HBV-specific primers were also included in the experiments.

RESULTS

As demonstrated in Figure 2, RACE–PCR applied to HBV-infected liver tissue resulted in specific amplification of RNA owing to single bands of amplified product that is confirmed to be HBV sequence by Southern blot analysis. Omission of the oligo(dT)-based primer from the RT step or preliminary RNase digestion prior to cDNA synthesis resulted in failure of amplification, confirming that polyadenylated RNA was the template for amplification. In contrast, RACE–PCR performed with full-length plasmid HBV DNA as a template did not result in an amplified product, confirming that this technique is specific for HBV RNA. Figure 2 demonstrates specific amplification of HBV RNA and confirms that (1) HBV DNA is not amplified by this method even when target template is present in relatively high concentrations, and (2) the process is conditional on the use of the oligo(dT)-based cDNA primer confirming polyadenylation of

3:376–377©1994 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/94 $5.00

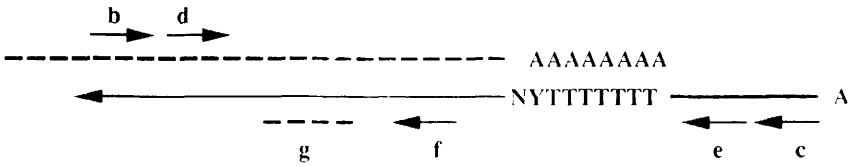

FIGURE 1 Schematic diagram of RACE–PCR as applied to the detection of HBV RNA. HBV polyadenylated RNA cDNA synthesis primed with oligo(dT) locking–docking primer A (GGCGACACTC CACCATAGAT CGAATTCGCGGCCGCTTTTT TTTTTTTTTTTYN, where Y = A/C/G and N = A/C/G/T) and followed by nested PCR [outer amplicon *b*×*c*, inner amplicon *d*×*e*, *d* = TGCCAACTGGATCCT(G/T)CGCGGGACGTCCT] of the resulting chimeric molecule, whereas HBV DNA was detected by *d*×*f* [*f* = GCGAAGCTTGTTCACGGTGG(AT)CTCCATG].

HBV RNA. Normal human liver, when subjected to RACE–PCR using HBV primers and run concurrently as a control for nonspecific amplification of constituent cellular mRNAs, failed to produce amplification products (data not shown).

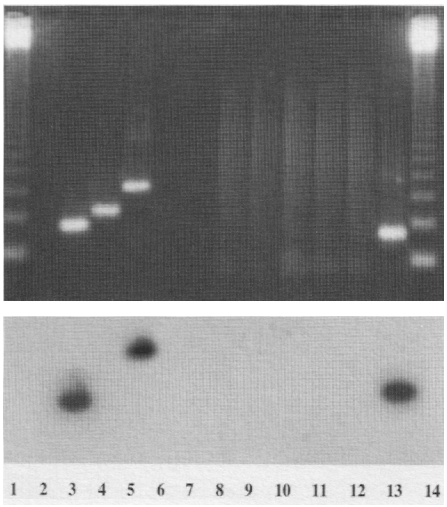

FIGURE 2 Gel electrophoresis and Southern blot analysis of HBV RACE–PCR. (Lanes *1,14*) 123-bp molecular weight ladder; (lane *2*) negative water blank control; (lane *3*) standard HBV DNA PCR control performed on sample using primers (*d*×*f*) to produce an amplicon of ~217 bp; (lane *4*) albumin mRNA PCR amplified with intron-spanning primers;[7] (lane *5*) HBV RNA amplified using nested primers (*a*×*b*, outer pair) and (*c*×*d*, inner pair) to produce a chimeric molecule of ~442 bp; (lanes *6*) PCR performed after digestion of sample with RNase prior to cDNA synthesis; (lanes *7–9*) PCR performed without primers A, *c*, or *d*, respectively; (lanes *10–12*) RACE–PCR with HBV DNA plasmid at 10, 50, and 100 ng per reaction using primers (*b*×*bc*, outer pair) and (*d*×*e*, inner pair); (lane *13*) standard PCR performed on HBV DNA at 10 ng/reaction primers (*d*×*f*). The specificity of both HBV RNA and DNA amplicons was confirmed by Southern blot analysis probed with HBV-specific oligonucleotide *g*, (*g* = TAC GTC CCG TCG GCG CTG AAT CC[TC] GCG GAC GAC CC-[CGT] TCT CGG G) as described by Larzul et al.[8]

DISCUSSION

This paper presents a detailed methodology for the selective detection of HBV RNA from small tissue samples by RACE–PCR. Distinction between RNA and DNA PCR template is generally achieved by one of two methods: (1) selection of PCR primers that span one or more introns of sufficient size to permit the RNA and DNA amplicons to be distinguished after electrophoresis, or (2) preliminary DNase digestion before RT–PCR of an RNA template. The genome of HBV, like other small viruses, does not contain an intron, and, hence, an intron-spanning amplification strategy is inapplicable. Although preliminary DNase digestion has been reported by some investigators for PCR amplification of HBV RNA, convincing demonstration of the destruction of the DBA template is difficult and requires that the DNA control PCR is negative. However, this constitutes "negative evidence," and complete elimination of a DNA template from the RNA reaction tubes can never be positively proved. The methodology described here ensures that only polyadenylated HBV RNA can act as a template for PCR amplification and, hence, obviates the need for removal of contaminating DNA by treatment with DNase prior to amplification. This RACE-based technique is especially suited to the detection of HBV transcriptional activity within small amounts of liver tissue obtained during percutaneous liver biopsy and is also applicable to the evaluation of viral activity in extrahepatic tissues.

REFERENCES

1. Tiollais, P., C. Pourcel, and A. Dejean. 1985. The hepatitis B virus. *Nature* **317:** 489–495.
2. Frohman, M., M. Dush, and G. Martin. 1988. Rapid production of full length cDNAs from rare transcripts: Amplification using gene specific oligonucleotide primer. *Proc. Natl. Acad. Sci.* **85:** 8998–9002.
3. Borson, N., W. Salo, and L.A. Drewes. 1992. A locking-docking oligo(dT) primer for 5′ and 3′ RACE PCR. *PCR Methods Applic.* **2:** 144–148.
4. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
5. Chou, Q., M. Russell, D.E. Birch, J. Raymond, and W. Bloch. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.* **20:** 1717–1723.
6. Larzul, D., D. Chevrier, V. Thiers, and J.L. Guesdon. 1990. An automatic modified polymerase chain reaction procedure for hepatitis B virus DNA detection. *J. Virol. Methods* **27:** 49–60.
7. Sallie, R., A. Rayner, B. Portmann, A.L. Eddleston, and R. Williams. 1992. Detection of hepatitis "C" virus in formalin-fixed liver tissue by nested polymerase chain reaction. *J. Med. Virol.* **37:** 310–314.
8. Larzul, D., F. Guigue, J.J. Sninsky, D.H. Mack, C. Brechot, and J.L. Guesdon. 1988. Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. *J. Virol. Methods* **20:** 227–237.

Received October 5, 1993; accepted in revised form March 11, 1994.

Instructions for Authors

Submission of Papers

PCR Methods and Applications welcomes high-quality research papers that describe improvements in PCR methodology, new amplification methods, or the results of PCR application. The journal also publishes review and commentary articles, technical tips, and reader correspondence. All submissions to the journal will be peer-reviewed.

The journal accepts primary research papers and technical tips that present original research which has not previously been published. Submission to the journal implies that a paper is not currently being considered for another journal or book. It is also understood that investigators who submit research papers to the journal are prepared to make available to qualified academic researchers materials needed to duplicate their research results.

Review articles are commissioned. Authors wishing to submit review articles should first contact the Editor.

Contributors should submit their papers to:

Judy Cuddihy, Editor
PCR Methods and Applications
Cold Spring Harbor Laboratory
POB 100, 1 Bungtown Road
Cold Spring Harbor, New York 11724-2203
USA
Phone 516-367-8492
FAX 516-367-8532

One original and two copies of the manuscript should be submitted. Original photographs should be supplied with each copy.

Manuscript preparation

Papers accepted by the journal will occupy between 2 and 10 journal pages. A manuscript of 5 to 25 typed, double-spaced pages total (including methods, references, and figure legends) will translate to this length. Computer printouts should be of letter quality, and each page should be labeled with the first author's name and a page number. All figures should be labeled with the first author's name, the figure number, and an indication of the top. The size of figures will be adjusted to fit the journal format; therefore, please try to keep labels, symbols, and other call-out devices in proportion to the figure size and detail. Figures should be supplied as high-quality glossy prints. Authors wishing to publish four-color art must pay part of the costs; price estimates will be provided on acceptance of a paper.

The following order of manuscript sections is preferred: Title page, abstract, introduction, methods, results, discussion, acknowledgments, references, tables, figure legends. The methods presented should be detailed enough to allow any qualified researcher to duplicate the results. References are cited by number in the text and the reference list should be numbered in the order the references are cited in the text. Bibliographic information should be supplied in the following order. For journal articles: Authors, year, article title, journal title, volume inclusive page numbers. For books: Authors, year, chapter title, book title, editors' names, volume, inclusive page numbers, publisher, city of publication.

Accepted manuscripts

Accepted manuscripts should be supplied on 3 1/2- or 5 1/4-inch discs to expedite typesetting. Please supply the manuscript as an ASCII file if possible. If a word-processing file is being sent, please do not use any underscoring, italic, or boldface; spell out special characters (Greek, math); use two carriage returns at the end of each paragraph, subheads, and list items. Indicate on the disc: computer brand name, type of file (text or word-processing), name of software, and disc format.

Proofs are considered the final form of the paper and correction can be made only in the case of factual errors. If additional information must be added at this stage, it should be in the form of "Note added in proof," subject to the approval of the editors.

Reprints may be ordered; a form will be included with the proofs.