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Rapid Communication

Detection of Hepatitis B Virus DNA in Formalin-fixed, Paraffin-embedded Liver Tissue by the Polymerase Chain Reaction

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DNA isolated from formalin-fixed, paraffin-embedded liver tissues from nine patients with hepatocellular carcinoma and six control patients was screened for hepatitis B virus (HBV) DNA with surface (S) and core (C) gene-specific primers by a modification of the polymerase chain reaction–southern blot technique (PCR-SB). PCR–SB results were correlated with histologic, immunohistochemical, and serologic findings. All cases with an established HBV etiology were positive by PCR-SB, as were three cases with negative immunohistochemistry and serology. Often there was selective amplification with one primer set and, in two cases, smaller than expected HBV amplification products suggesting internal deletions. The presence of a potent PCR inhibitor in nucleic acid preparations from tissue blocks that can be removed by Sephadex G-50 chromatography was confirmed. PCR–SB will be a powerful method for the diagnosis and follow-up of patients with HBV infection and may provide new insights into viral hepatocarcinogenesis. (Am J Pathol 1990, 137:253–258)

Vast quantities of paraffin blocks from virally infected tissues are available but generally are unsuitable for classical molecular biologic approaches due to DNA and RNA degradation. A recently developed method, the polymerase chain reaction (PCR) circumvents DNA fragmentation due to fixation by allowing selective amplification of short, contiguous regions.[1] After amplification, target regions can be visualized by ethidium-stained, agarose gel electrophoresis.

Very low levels of target DNA (less than 1 femtogram) can be effectively detected by coupling southern blot (SB) hybridization to PCR (PCR–SB). Many viral pathogens have been studied by PCR such as human immunodeficiency virus (HIV),[2] cytomegalovirus (CMV),[3] human papilloma virus (HPV),[4] and hepatitis B virus (HBV).[5] Kaneko et al[5] demonstrated by PCR that chronic HBV carriers had circulating viral DNA despite negative serum HBV markers or conventional DNA hybridization analysis. Thus PCR may be very useful for a better understanding of the natural history of HBV infection as well as for the correct diagnosis and the appropriate management of infected patients.

In this study we have used PCR–SB on DNA extracted from formalin-fixed paraffin-embedded liver tissues to examine the prevalence of HBV DNA in hepatocellular carcinoma (HCC) and nearby, nontumorous liver tissue from individual patients. This method has been successfully used to detect low levels of viral sequences in nonhepatic routinely processed tissues.[3,6–8] Using four primer sets specific for the surface (S) or the core (C) genes, we find PCR–SB is extremely sensitive for the detection of HBV DNA in formalin-fixed tissues, although not all primer sets were equally effective. In addition, several smaller than expected amplification products were produced, possibly identifying HBV DNA deletions.

Materials and Methods

Tissues

Liver tissue removed at surgery from 15 patients was obtained from Istituto di Medicina Interna, Universita' di Mi-

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Table 1. *Summary of Data of 15 Patients*

Patient	Histologic diagnosis†	Immunostaining HBsAg/HBcAg		Serology HBsAg/anti-HBs/anti-HBc			PCR results S1/2	S3/4	C1/2	C3/4
1	HCC	−	−	+	−	ND	+	+	−	−
	PF	−	−				−	+	+	+*
2	HCC	−	−	+	−	ND	+	+	−	+*
	CIR	−	−				−	+	−	−
3	HCC	+	−	+	−	ND	+	+	+	−
	CIR	+	+				+	+	+	+
4	HCC	−	−	−	−	−	−	+	−	−
	CPH	−	−				−	+	−	−
5	HCC	−	−	−	+	+	−	+	−	−
	CIR	−	−				−	+	−	−
6	HCC	+	−	+	−	+	+	−	+	−
	CIR + CAH	+	+				+	−	+	−
7	HCC	−	−	−	−	−	−	−	−	−
	CIR + CAH	−	−				−	−	−	−
8	HCC	−	−	−	−	−	−	−	−	−
9	HCC	−	−	−	−	−	+	−	+	+
10	CIR	+	+	+	−	+	+	+	+	−
11	Steatosis	−	−	−	−	−	−	−	−	−
12	Chronic rejection	−	−	−	−	−	−	−	−	−
13	Fetal liver	−	−	ND	ND	ND	−	−	−	−
14	Paucity of bile ducts	−	−	−	−	−	−	−	−	−
15	Biliary obstruction	−	−	−	−	−	+	+	−	+

* The amplified HBV DNA sequence was smaller than expected.
† HCC, hepatocellular carcinoma; PF, portal fibrosis; CIR, cirrhosis; CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; ND, not done.

lano, Milano (5 patients), Tulane University Medical Center, New Orleans, LA (7 patients) and Mount Sinai Hospital, New York, NY (3 patients). The tissues were fixed in formalin and embedded in paraffin in 1988 and 1989, except for two cases (10 and 15, Table 1) that were obtained in 1984 and 1987, respectively. Nine cases were histologically diagnosed as hepatocellular carcinoma (HCC), one each as steatosis, cirrhosis, chronic allograft rejection, normal fetal liver, paucity of bile ducts, and extrahepatic biliary obstruction (Table 1). All cases of HCC but two had another paraffin block from uninvolved liver surrounding the neoplastic lesion. Five patients were positive for serum HBsAg, one was anti-HBs/anti-HBc positive, eight were negative for HBV markers, and in one case (fetal liver) serologic HBV markers were not available. The serologic tests were performed by radioimmunoassay (Abbott Laboratories, Chicago, IL).

Immunoperoxidase Staining

HBsAg and HBcAg were demonstrated by the avidin-biotin-peroxidase complex (ABC) method in serial sections from the same tissue blocks used for PCR–SB. After deparaffinizing and rehydrating, the liver sections were incubated in 1% H_2O_2, 0.5% saponin, and the appropriate blocking serum (normal goat serum for HBcAg and normal rabbit serum for HBsAg staining) before overnight incubation with primary antibodies goat anti-HBc and rabbit anti-HBs (Dako Corporation, Carpinteria, CA). Vectastain ABC kits (Vector Laboratories, Burlingame, CA) were used for the linking and labeling steps of the immunoperoxidase (IP) procedure. The slides were developed in Diaminobenzidine (DAB), dehydrated, and coverslipped. All washes were done in PBS and all incubations were at room temperature.

Oligonucleotide Primers

Oligonucleotide primers, specific for HBV S and C gene sequences and for beta globin gene, were synthesized on a Milligen/Biosearch Cyclone DNA synthesizer (Novato, CA) by phosphoramidite chemistry. Primers were deprotected by treatment with ammonium hydroxide for 5 hours at 55°C, vacuum dried, and resuspended in 100 μl water. After electrophoresis on native, 15% polyacrylamide gels, full-length oligonucleotides were visualized and appropriate gel pieces excised. Slices were finely minced and incubated overnight in water. Oligonucleotides were vacuum dried, resuspended in 200 to 500 μl of water, and quantitated by absorbance at 260 nm. Four primers each for the surface-gene region and the core-gene region were selected based on location and identity among different HBV subtypes. Figure 1 shows primer S1 (begins at map position 110 of the HBV adr genome sequence published by Fujiyama et al[9]), 5′-TACCACAGAGTCTAGACTCG-3′; primer S2 (map position 390R, where R denotes the complementary DNA strand), 5′-GCATGGTCCCGTGCTGGTAG-3′; primer S3 (map position 313), 5′-GTTCTTCTGGATTACCAAGG; primer S4 (map position 642R), 5′-CAGACTTGGCCCCCAATACC-

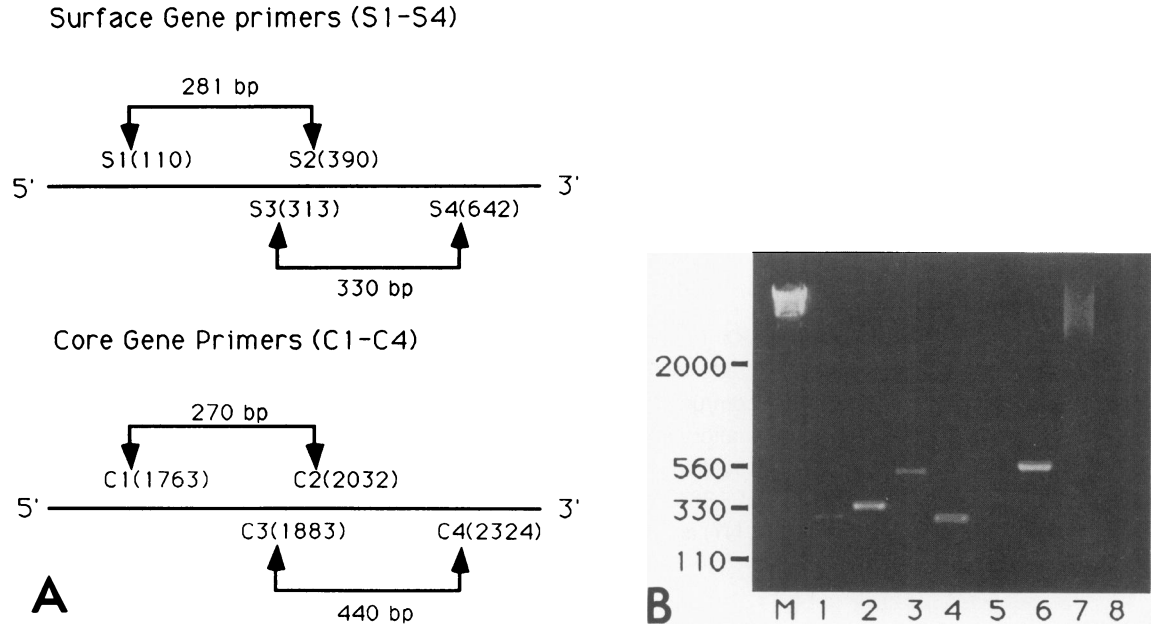

Figure 1. **A:** *Surface- and core-specific oligonucleotide primers used for PCR. Numbers in parenthesis refer to the HBV genome nucleotide position of the primers. The size of the expected amplified regions is shown between primers.* **B:** *Ethidium bromide-stained agarose gel of PCR products. In lanes 1–4 and 6, 0.1 pg HBV cDNA was amplified. In lane 5, DNA was omitted. In lane 7, salmon sperm DNA was amplified, while in lane 8 the globin gene was amplified from 1.5 μg genomic DNA isolated from formalin-fixed, paraffin-embedded tissue. Lambda Hind III markers (lane M), S1-S2 primers (lane 1), S3-S4 primers (lane 2), S1-S4 primers (lane 3), C1-C2 primers (lane 4), primers C3-C4 without DNA (lane 5), C1-C4 primers (lane 6), S3-S4 primers with salmon sperm DNA template (lane 7), beta globin primers (lane 8, showing faint band only). Molecular weights are shown along the left (base pairs).*

3′; primer C1 (map position 1763), 5′-GCTTTGGGGCATGGACATTGACCCGTATAA-3′; primer C2 (map position 2032R), 5′-CTGACTACTAATTCCCTGGATGCTGGGTCT-3′; primer C3 (map position 1883), 5′-ATATCGGGAGGCCTTAGAGTCTCCGGAACA-3′; primer C4 (map position 2324R), 5′-CTAACATTGAGATTCCCGAGATTGAGATCT-3′. The nucleotide sequence of the beta globin primers was obtained from published data.[1]

Isolation of DNA

Five sequential 10-μm–thick sections were cut from paraffin blocks and placed in a 1.5-ml Eppendorf tube. The sections were deparaffinized with 1.0 ml of xylene at room temperature for 10 minutes on a rocking platform followed by centrifugation for 5 minutes at 16,000 rpm. The xylene was decanted and its residue was removed with 1.0 ml of 95% ethanol, followed by mixing, centrifuging, and decanting as above. The tissue pellet was desiccated under vacuum for 5 minutes before resuspension in 100 μl of distilled water. The samples were boiled for 30 minutes, centrifuged through a 1.0-ml Sephadex G 50 mini-column, and the nucleic acid concentration was determined by spectrophotometric absorbance at 260 nm. The samples were stored at −20°C until use.

PCR Amplification

The nucleic acid samples were boiled for 10 minutes and 1 μg of nucleic acid was amplified in 50 μl reaction volume containing 2.5 U of Taq polymerase (Promega Corp., Madison, WI), 1.5 mmol/l (millimolar) of each dNTP, 500 ng of each primer, 8 mmol/l of $MgCl_2$, 17 mmol/l ammonium sulfate, 67 mmol/l TRIS HCl (pH 8.0), 10 mmol/l 2-mercaptoethanol, 170 ng/ml of bovine serum albumin, and 0.1% Triton x-100. The reaction mixture was covered with 100 μl of mineral oil. The amplification was performed for 37 cycles on a programmable DNA thermal cycler (NOLA Scientific, New Orleans, LA). Each cycle consisted of heating at 93°C for 1 minute (denaturation), followed by cooling at 48°C for 1 minute (annealing) and heating again at 72°C for 30 seconds (amplification). Positive and negative controls were included in each experiment.

Analysis of Amplified DNA

Fifteen microliters of the reaction volume was electrophoresed on 2% agarose gels and transferred onto Zeta Probe (Bio-Rad Laboratories, Richmond, CA) by vacuum alkaline blotting (Vacugene; LKB-Pharmacia, Piscataway, NJ). Hepatitis B virus sequences were detected with full-length cloned HBV-DNA radiolabeled with ^{32}P-dCTP by

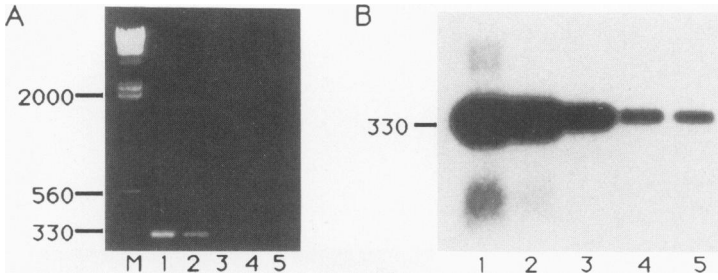

Figure 2. *Polymerase chain reaction amplification of HBV cDNA with S3-S4 primers.* **A:** *Ethidium bromide-stained agarose gel of PCR products from 1 pg HBV cDNA (lane 1), 0.1 pg (lane 2), 10 fg (lane 3), 1 fg (lane 4), and 0.1 fg (lane 5). Lambda Hind III markers are in lane M and sizes are shown along the left.* **B:** *Southern blot hybridization of gel shown in* **(A)** *with HBV cDNA radiolabeled probe. Lanes are as described for panel* **(A).** *Molecular weight is shown on the left (base pairs).*

nick translation (Promega-Nick Translation Kit (Promega Corp., Madison, WI) used per manufacturer's instructions) to a specific activity of 2-5 $\times$ 10^8 cpm/ug DNA. Prehybridization, hybridization and washes after hybridization were performed following Bio-Rad instructions. Autoradiography of the films was performed at −80°C with Kodak Xomat x-ray film (Kodak, Rochester, NY) and two intensifying screens.

Results

Application of the PCR requires selection of conserved target sequences as starting points for cycles of denaturation, annealing, and primer extension. To avoid negative results due to chance deletions or mutations at the primer binding sites, we amplified two regions of both the S and C genes. The selected sequences were unique within the HBV genome, as determined by homology search. The primers, their annealing sites, and anticipated amplification products are shown in Figure 1A. Polymerase chain reaction with a variety of combinations of these primers using 0.1 pg HBV cDNA as a target produced clearly visible fragments of the anticipated size (Figure 1B). Southern blot hybridization of the amplification products with both end-labeled, internal oligonucleotide probes or full-length HBV cDNA yielded identical data and confirmed that the products were HBV DNA (not shown). In general, as shown in Figure 2A, amplification of less than 0.1 pg of target DNA could not be reliably detected by ethidium bromide staining. However PCR–SB extended the detection limit from 0.1 pg to 100 attograms, or approximately five copies of viral DNA.[10] Therefore, for maximal sensitivity and specificity, we performed Southern blotting on the PCR material. In all cases, HBV cDNA was used as a positive control. Hepatitis B virus-negative DNA samples and DNA free controls were also used to ensure that the samples were not inadvertently contaminated with HBV DNA. As a PCR control, we amplified a 110-bp fragment of the beta-globin gene. All the samples were positive with globin primers as visualized on ethidium bromide-stained agarose gels (Figure 1B).

Several investigators have described the presence of potent inhibitor(s) of Taq polymerase in samples of human genomic DNA from ancient tissue,[11,12] human blood,[13] and paraffin sections.[14] Our results confirmed these observations: we were unable to amplify HBV-related sequences in some unpurified paraffin-extracted DNA samples known to be positive for HBV DNA. The nature of this inhibitor(s) remains unknown, but it may be removed by boiling and centrifuging genomic DNA samples through Sephadex G-50.[13] In our experiments with unpurified DNA from formalin-fixed paraffin-embedded liver tissue blocks, this procedure increased PCR performance and therefore was used routinely for all samples. To demonstrate that PCR efficiency was not impaired by formalin fixation, we isolated DNA from viable and formalin-fixed, paraffin-embedded HBV-positive, hepatoblastoma cells 2.2.15.[15] Polymerase chain reaction was equally effective on equivalent amounts of nucleic acid isolated from these two samples (not shown). The amplification efficiency was reduced if more than approximately 2 μg of target DNA was used. We quantitated the nucleic acid concentration in each sample and found 1 μg per reaction yielded consistent results with PCR.

We analyzed a variety of cases by PCR and correlated these findings with the histologic diagnosis, serologic HBV markers, and immunohistochemical findings (Table 1). A concise summary of the overall trends is shown in Table 2. PCR–SB analysis was positive for all cases with documented current or past HBV infection (6/6). All tissues that were immunohistochemically positive for HBsAg (5/5) or HBcAg (3/3) were positive by PCR. Polymerase chain reaction also detected HBV DNA in several cases that were negative for HBV markers in serum and tissues. However amplification was not observed in all cases with all primer sets. For example, of the five HBsAg-positive tissues, only three of five were detected with the S3-S4 primers while the S1-S2 primers were positive for all five. Similar differential results were seen with the core

Table 2. *Comparison of PCR and Serologic Data in 15 Patients*

Diagnosis*	No. of blocks	Serology			+PCR results
		HBsAg	α-HBc	α-HBs	
CLD/HCC	9	+	+	−	9
CLD/HCC	2	−	+	+	2
CLD/HCC	6	−	−	−	3
MLD	5	−	−	−	1

* CLD, chronic liver disease; MLD, miscellaneous liver disease; HCC, hepatocellular carcinoma.

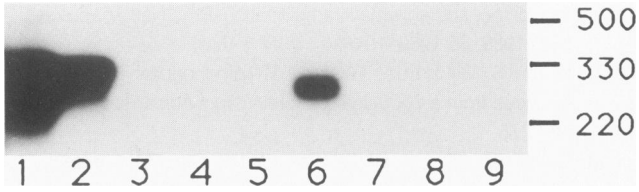

Figure 3. *Autoradiogram of PCR-SB, amplified with primers C3-C4 on DNA extracted from routinely processed liver tissues. Southern blot hybridization was performed with ^{32}P-labeled HBV cDNA. Molecular weights (base pairs) are shown along the right. Lanes 1 and 2 represent positive controls (HBV cDNA at 1 and 0.1 pg, respectively); lane 3 negative DNA control; lanes 4–9 DNA from patient 5 (HCC), 1 (HCC), 2 (HCC), 2 (CIR), 4 (CPH), and 3 (HCC), respectively, as described in Table 1.*

primers. In two cases (Figure 3), a significantly smaller than expected fragment was amplified with the C3-C4 primer pair.

Discussion

In the study reported here, a modified PCR–SB technique is used to detect HBV DNA sequences in formalin-fixed paraffin-embedded liver tissue without extensive DNA purification. It is important to streamline the procedure and to reduce the chances of contamination. We estimate PCR–SB can detect as few as five copies of the viral DNA target. Therefore PCR on fresh or fixed tissues is comparable in sensitivity. However the DNA isolated from formalin-fixed paraffin-embedded liver tissues appears to contain an inhibitor of PCR. While the identity of this substance is not known, others have reported PCR inhibitors in DNA isolated from peripheral blood[13] or paraffin blocks.[14] In our experience, samples boiled in and chromatographed through Sephadex G-50 were readily amplified by PCR with comparable sensitivity to highly purified DNA. This method is simple, rapid, reproducible, and inexpensive.

Using four sets of primers, two specific for the surface gene (S1-S2, S3-S4) and two specific for the core gene (C1-C2, C3-C4), we probed 22 paraffin blocks from 15 patients with different liver diseases for HBV DNA. We detected HBV sequences by PCR–SB in all cases with serologic or immunohistochemical evidence of HBV infection. The presence of HBV target DNA could only be assessed by Southern blotting because ethidium bromide-stained gels did not demonstrate amplification products. In particular, all five HBsAg-positive patients, as well as one anti-HBs/anti-HBc–positive patient and three HBV marker-negative HCC patients were positive by PCR–SB. These two patients are likely to be HBV carriers with low HBV replication. It is possible that a significant number of HBsAg-negative HCC cases will be HBV related using very sensitive diagnostic tests such as PCR–SB.

Despite its great sensitivity, we observed a poor correlation between PCR and immunohistochemical results. Only one of three cases with HBcAg in liver was positive by PCR with both core primer sets, while in two samples we amplified only the C1-C2 region. Similar data were observed with the two surface primer sets. One possible explanation is that primer target sites were altered or missing. Single mutations within three bases of the 3′ end of primer-target duplexes are sufficient to inhibit PCR.[16] While stringent annealing conditions can prevent primers with internal, single base mismatches from annealing to the target sequence,[1] our annealing temperature of 48°C may not have detected single base changes. HBV DNA integrated into the genome of hepatocytes has been shown to harbor mutations.[17] Because immunostaining in our cases was positive, ample viral antigen was clearly present. Thus mutations, if present, must have maintained the reading frame of the core or surface genes as well as the antigenicity of the proteins. Sampling error probably does not play a role because PCR analysis and immunoperoxidase staining were performed on serial tissue sections, but HBV DNA fragmentation during tissue processing (formalin fixation and paraffin embedding) must be considered. These data demonstrate the need to use multiple primers from different regions in PCR analysis of integrated HBV DNA.

Our finding in two of 22 tissue samples of smaller than expected amplification products was unexpected. Both shorter PCR products were produced by the C3-C4 primer pair and were reduced in size by approximately 30 to 40 base pairs. As shown by Southern blotting, the smaller fragments were HBV-specific sequences. In addition normal-sized PCR products were produced with other sets of primer pairs with this DNA, diminishing the possibility that technical problems were responsible. The integrated HBV genomic DNA usually shows multiple mutations.[18,19] Therefore a possible explanation for our observations is that a deletion between the C3-C4 primer set occurred in the two cases.

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