Title

Detection of Hepatitis B virus RNA in hematopoietic cell lines by polymerase chain reaction utilizing hemi-nested primers

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Detection of Hepatitis B virus RNA in Hematopoietic Cell Lines by Polymerase Chain Reaction Utilizing Hemi-Nested Primers

by

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Abstract

The Hepatitis B virus (HBV) is associated with hematopoietic cells of many chronically infected individuals. Its presence has been documented; the nature and activity of the virus in these cells remains unclear. HBV DNA was initially found associated with a bone marrow culture and peripheral blood mononuclear cells (PBMC) from infected individuals. Later data revealed transciptional activity and the possibility of viral replication within these cells. Our hypothesis is that low levels of HBV infection in hematopoietic cells is common and may play a role in viral persistence and/or hematologic complications associated with this disease.

To address viral activity in hematopoietic cells we developed a very sensitive assay detecting HBV mRNA with reverse transcription and Polymerase Chain Reaction . Novel sets of hemi-nested primers from conserved regions in the HBV genome coding for the surface antigen were developed. Control experiments demonstrated a PCR sensitivity of single copy HBV DNA. Further control experiments including reverse transcription indicated a sensitivity detecting 100 copies of HBV RNA seeded into in the RNA of 500,000 cells.

Using our protocol, we assayed cultured lymphocytic and monocytic cell lines (A3.01, Ach2, U937, and U1) after exposure to HBV. Sporadic HBV RNA activity was found in such cultures with no clear relationship to mitogenic stimulation or duration of culture. A preliminary experiment revealed HBV transcriptional activity after 7 days of culture with stimulation by LPS. With identification of conditions leading to consistent HBV transcription in hematopoietic cell lines, *in vitro* studies may provide useful information about the viral persistence and early cellular infection.

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Introduction

Until the early 1980's the hepatitis B virus (HBV) was thought to be strictly hepatotropic. Studies using light microscopy (1) and *in situ* hybridization (2) first demonstrated evidence of HBV in cells other than hepatocytes. Blum et al. used a complete HBV genomic probe to identify HBV *in situ* associated with hepatocytes and, in lower activities, bile duct epithelium, endothelial cells and smooth muscle cells (2). Later, using Southern blot techniques, Dejean et al. expanded the tissues associated with HBV DNA to include kidney and skin (3).

The first report of HBV associated with hematopoietic cells came in 1983 when Romet-Lemonne et al (4) and Elfassi et al (5) reported extrachromosomal forms of HBV DNA in a bone marrow culture. Infection of peripheral blood cells was then reported by Lie-Injo et al. (6) as well as others (7,8). These groups performed Southern blot hybridization using an HBV probe on cellular DNA before and after digestion with *EcoR1* and *HindIII*. These studies revealed HBV to be present in integrated as well as extrachromosomal forms. No replicative forms were demonstrated in PBMC's and the activity of HBV in these cells was unknown.

Work by Korba et al with the Woodchuck Hepatitis Virus (WHV) supported the possibility of viral replication within hematopoietic cells. WHV exists in PBMC's of animals chronically infected with the virus (9). Korba et al, using DNA and RNA blot hybridization, found lymphocytes from one infected animal had an increased viral transcription and increased levels of viral DNA when stimulated with lipopolysaccharide (LPS) (10). They later demonstrated these cells to shed infective WHV into the culture media (11).

Recent reports have confirmed HBV presence in hematopoietic cells and, with more sensitive techniques, demonstrated the presence of viral

DNA and the RNA transcripts for Surface and Core proteins. Roisman et al. found PBMC's to be positive for HBsAg using indirect immunofluorescence (12). Specifically, they noted B-cell and monocyte cell fractions had enhanced signal while T-cell fractions were depleted of HBsAg positivity (12). This association, however, could result from passive adherence of HBsAg or Dane particles to the blood cells.

More convincing evidence of virus within hematopoietic cells was presented by a study demonstrating polyadenlylated HBV RNA in PBMC using Polymerase Chain Reaction (13). PBMC's from two people with chronic hepatitis B were incubated in the presence or absence of LPS and PHA for 7 days. HBV RNA was detected by PCR prior to and after stimulation. Conventional hybridization techniques, however, identified viral RNA only after stimulation with LPS or PHA. These findings suggest a very low level of constitutive transcription which is inducible upon stimulation with LPS or PHA (13).

Further studies of cultured PBMC suggested possible viral replication. HBV DNA patterns in cells stimulated with PHA or Concanavalin A produced bands on a Southern blot suggestive of HBV replication(14). These replicative intermediates were only demonstrated in the stimulated PBMC's from one of twenty-five individuals studied. No evidence of Dane particles was found in the media of this culture. No other studies have reported clear evidence of HBV replication in PBMC or hematopoietic cell lines.

Recently, a common cellular receptor for HBV has been proposed for hepatocytes and PBMC. Antibodies directed against the [27-49]Pre-S1 protein blocked binding between recombinant hepatitis particles and PBMC (15). This study echoed earlier work addressing HBV binding to the HepG2 hepatoma cell line and human liver plasma membranes(16,17). Antibodies

against the same regions of the PreS protein blocked viral binding to hepatocytes in a similar manner(16).

This binding region was later defined as the [21-47]Pre-S1 protein segment of the HBV envelope protein(18). Similar HBV binding sites have been identified on Peripheral Blood Lymphocytes and hematopoietic cell lines of B cell lineage (19).

Very recent work by Neurath et al, demonstrated that Interleukin 6 contains recognition sites for the [21-47]PreS1 sequence and is found on stimulated monocytes and T-cell lines (20). The identification of putative viral receptors on established cell lines presents an opportunity to study viral activity in a more controlled and reproducible environment.

At Irwin Memorial Blood Bank, we have studied the role of HBV in human PBMC's and hematopoietic cell lines. HBsAg + donors were identified who had HBV DNA associated with their PBMC's. The PBMC's from one patient were harvested and cultured by the author to determine if the amount of HBV DNA increased during culture with or without stimulation. Using a PCR protocol which could detect 10 fold differences in HBV DNA, no increase in HBV DNA was found in PBMC cultures stimulated with IL-2, PHA, or LPS. Subsequent *in vitro* work with hematopoietic cell lines demonstrated an increase in HBV DNA after 12 days in a culture of Ach3 cells infected by exposure to high titer serum. This occurred in a single culture and was not repeated.

Currently we are using reverse transcription-Polymerase chain reaction to detect HBV mRNA as a qualitative signal for HBV transcriptional activity. An assay for HBV RNA discriminates between the presence of infective particles and viral activity in a way DNA studies cannot. Provided a RNA-free inoculum is used, the presence of RNA indicates viral transcription. For

valid DNA studies, a sound quantitative assay for HBV DNA is required to determine viral activity.

Past studies have indicated that, if active, the level of HBV transcription is very low in hematopoietic cells. Therefore, a very sensitive assay is required to address questions concerning the activity of HBV in hematopoietic cells. We chose hemi-nested primers with PCR because of publications demonstrating superior sensitivity when compared to regular primer pairs (21). We have used this assay to look at hematopoietic cell lines after exposure to HBV. Drawing from the work by Neurath et al. (20) we created conditions which would lead to an increase in viral receptors. In this report we describe our assay and its use in identifying HBV RNA in cell cultures.

Methods

Cell Culture

Cell lines A3.01, Ach 3, U937, and U1 were used for the following studies. A3.01 is a cell line derived from a female child with T cell lymphoblastic leukemia, cells are positive for Leu - 3, Leu - 8, and Leu-1. Ach-2 is a subclone of A3.01 and has latent HIV-1 (32). Ach-2 is positive for CD5, the transferrin receptor, Leu-1, and HIV-1 antigens. U937 is derived from a male child with diffuse histiocytic lymphoma and has monocyte-like morphology. U1 is a subclone of U937 with latent HIV-1 infection (33). Although the HIV-1 is latent or minimally constitutively active in Ach-2 and U1 cell lines, respectively, viral production can be stimulated with phorbol myristate and other mitogens (TNF-alpha for Ach-2) (32,33).

All cell lines were propagated in standard culture medium: RPMI 1640 containing Fetal Bovine Serum, heat inactivated (10-20%), Glutamine (2mM), 100 U/ml Penicillin, and 100 µg/ml Streptomycin. Cells were exposed to Dane particles (20µl Dane Particles, concentration 10*8/ µl, moi = 667), serum from an infected individual (MA high titer serum moi = 667), or culture media from 2.2.15 cell cultures.

The titers for 2.2.15 media were determined by the following protocol. Supernate from cell lines 2.2.15 at day 4 of culture were withdrawn and non-adherent cells pelleted with centrifugation. 150 µl of supernate was solubilized with 850 µl UNSET* buffer (8 M urea, 2% SDS, 0.15 M NaCl, 0.01M Tris pH 7.5, 0.001 M EDTA). To 880µl of the solubilized cells 2.0 µg calf thymus DNA (20µl volume) and 100 µl 3M NaOAc (pH 8.4) was added for a final volume of 1.00 ml. This was combined with 1.0 ml Isopropanol and left at room temperature for 1 hour. DNA was pelleted and resuspended in 90 µl TE' with 0.3 M NaOAc. DNA was pelleted a second time with 220 µl EtOH

and finally resuspended in 100 µl TE'. From this final volume, serial 4-fold dilutions were performed including 1:4, 1:16, 1:64, and 1:256. These dilutions were amplified according to the protocol described below.

Cultures were incubated at 37°C with 5% CO_2 in a cellular concentration of 1 million cells/ml. Culture media was changed every three days, no additional virus was added after the initial exposure. Cell samples were collected by suspending the cells with agitation, counting the concentration, and collecting enough media for approximately 1-2 million cells. Cells were pelleted at 1000 rpm and washed with Phosphate Buffered Saline. Cell pellets were stored at -70°C until nucleic acid separation.

RNA separation

RNA purification: Two detergent buffers were used to dissolve cells: UNSET* and Guanidinium Isothiocyanate (GITC).

The UNSET* buffer was made immediately prior to use. For acid UNSET* 0.1M NaOAc, pH 4.2 was included in the above solution. 2 million cells were solubilized in 630µL of UNSET*, 70 µL of 2M NaOAc, pH 4.2 was added and 700µL acid P/C* (5ml acid phenol, 1ml Chloroform:isoamyl alcohol [48:2], pre-equilibrated with acid UNSET* before extraction of RNA). Organic and aqueous phases were separated by centrifugation at 4°C for 30 minutes. 600µl of aqueous phase were re-extracted with P/C* and 450 of the next aqueous phase were precipitated with 2.5 volumes 100% ethanol. Cells were pelleted at top speed in a microcentrifuge for 30 minutes. This procedure was modified to a cellular input of 1 million cells/ ml UNSET* because of repeated DNA contamination(see results).

GITC buffer was subsequently used because of more reliable RNA/DNA separation. This method, described previously (22), reliably

isolates RNA with a single extraction. However, there may be a marginal decrease in sensitivity with GITC compared to UNSET* (21). In our protocol, 1 million cells were solubilized in 1ml of acid GITC buffer. 500 µl was used for RNA analysis. Acid Phenol:Chloroform extraction was performed as previously described (22): approximately 400 µl was recovered from the aqueous phase. RNA was precipitated using 2.89 volumes acid ethanol (100% ethanol with 0.8 M Sodium Acetate, pH 4.2). RNA was pelleted for 30 minutes at top speed in a microcentrifuge a 4°C.

DNase 1 digestion

Ethanol pellets were washed with 70% ethanol, pelleted a second time, and re suspended in 100µl DEPC-treated water. 50ml of DNase buffer was added and DNA digested for 60 minutes at Room Temperature with 50 units of RNase-free, DNase 1 (Bethesda Research Laboratories). The DNase buffer consisted of PCR II Buffer, 7.5mM MgCl, 6mM DTT, with or without 20 units RNasin (Bethesda Research Laboratories). After digestion, RNA was extracted with neutral Phenol/Chloroform/isoamyl alcohol (50:48:2) and precipitated as above.

***In Vitro* Transcription of HBV RNA**

In vitro transcription of HBV RNA was performed on a surface antigen containing plasmid (See Figure 6) with T7 RNA polymerase with a final concentration of approximately 20-30µg/100µl. This RNA was purified with GITC and treated with RNase-Free, DNase I according to protocol. The final RNA pellet was re suspended in 20µl TE' and the concentration was determined to be 28 ng/µl by spectrophotometry. With an approximate

weight of 1.2 million daltons the RNA was serially diluted in DEPC-treated water and used to standardize reverse transcription

Reverse Transcription (RT)

RNA pellets were diluted to approximately 200ng/μl based on OD 280 readings of similarly isolated RNA preparations. Each RT reaction contained approximately 2μg RNA, 400 units Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and 500μM dNTP's. The protocol involved combining 5μl RNA with 30 pmol random hexamers (p[dN]$_6$, Pharmacia-LKB Biotechnology), heating at 70°C for 2 minutes to melt tertiary RNA structure, cooling samples on ice, and adding 10.5μl RT mix (RT buffer, 28mM DTT, and 1.4mM (each) dNTPs). 10 μl were removed for RT negative controls and 400 units (2μl) M-MLV-RT were added to the remaining reaction mixture. Samples were incubated at room temperature for 10 minutes then at 37°C for 1 hour. After cDNA synthesis, the template RNA was degraded with 1.0 unit of RNase H (Bethesda Research Laboratories) for 1 hour at 37°C. Finally, samples were heated to 90°C to heat kill proteins for 5 minutes, pulsed in the microcentrifuge, and amplified according to the PCR protocol below.

PCR Protocol

Hemi-nested primers within the coding region for HBV surface antigen protein were used to amplify HBV cDNA. The initial primers (DB1, DB2, and DB4) were selected from highly conserved regions of the HBV genome coding for the surface antigen and synthesized at the UCSF Biomolecular Resource Facility using cyanoethylphosphophoramidite chemistry on the Applied Biosystems Inc. 380B Synthesizer. The details of

hemi-nested primers and their use has been described previously (21,23). Briefly, three primers are present in the PCR reaction mixture: two long outer primers (with the upstream primer in limiting concentration) and one short inner primer. During the initial 20 cycles of PCR, the hybridization temperature is kept prohibitively high for annealing of the shorter inner primer. Thus, only the longer, bracketing, primers are used for the amplification. These initial cycles are followed by 40 cycles of PCR with a thermal profile that includes a lower annealing temperature; the shorter, inner primer can anneal and be used for amplification. During these cycles, the short primer (DB4) is preferentially used because of its higher concentration compared to the longer upstream primer (DB2). Application of this procedure has been shown to be capable of amplifying a single copy of target DNA to levels detectable by ethidium bromide staining of amplified product in agarose gels (21,23).

Three different primer trios were used during the study. The first set included Primers DB1 (5'-AGAGTCAGACTCGTGGTGGACTTCTCTCAAT-3'), DB2 (5'-ACCAATAAGAAGATGAGGCATAGCAGCAGGAT-3') and DB4 (5'-GATAAAACGCCGCAGACACAT-3'). The location of these primers within the S antigen is noted in Figure 1. With further refining, two other primers were introduced with greater sensitivity (See Results). These were DB3 (5'-ACATCAGGATTCCTAGGACCCCTGCTCGTGTT-3' and DB5 (5'-CTGGTGGCTCCAGTTC(C/A)GGAACAGTAAACCCT. Figure 1 diagrams the locations of each primer and the resulting amplified product.

DNA and cDNA samples were then subjected to PCR. Individual reactions in microcentrifuge tubes were set up using Ampliwax® "hot start" system (Perkin-Elmer Cetus Corp., Norwalk, CT) or mineral oil to inhibit evaporation during cycling. The Almpliwax® "hot start" system was used

according to manufacturers description with a final total volume of 100 µl per reaction. The final amplification mixture contained 3.0 Units Amplitaq (Perkin-Elmer Cetus Corp., Norwalk, CT), 5.0 pmol DB1, DB3, or DB5, 5.0 pmol DB4 , 0.04 pmol DB2, each dNTP 200 µmol/L, 10mmol/L Tris HCl (pH 8.3 at 25°C), 50 mmol/L KCl, and 2.5 mmol/L $MgCl_2$. For PCR with samples previously reverse transcribed, 150 mmol/L dNTP's was substituted in the lower reaction mixture due to dNTP's carried over in the cDNA sample.

Use of the mineral oil allowed reactions with a final volume of 50 µl. The same final concentrations of all substrates and buffers were used in the amplification process.

Samples were amplified using a Perkin-Elmer thermocycler, model 2.3. The specific cycling order used was: A. single cycle of 95°C-1 min (melt wax, hot start) and 72°C-40 seconds: B.19 cycles of 94°C-30 seconds, 30 second ramp time to 72°C-40 seconds then a 38 second ramp time back to 94°C: C. 40 cycles of 94°C-30 seconds, ramp of 77 seconds to 57°C-30 seconds, ramp of 30 seconds to 72°C-30 seconds: D. a single cycle at 72°C for 5 minutes: samples were then kept at -4°C until separation.

With each amplification PCR positive and negative controls were amplified simultaneously. Plasmid HBV DNA of known concentration was used for the positive controls. Generally, 5 and 50 copies were included as controls to assess specific trial sensitivity. 10µl of upper reaction mixture was used for the PCR negative controls.

DNA Separation

11µl loading buffer (20% ficoll 400, 0.1 M Na_2EDTA [pH 8], 1.0% Sodium dodecyl sulfate, 0.25% Bromphenol Blue) was added to each sample and 15 µl was loaded onto a 4% agarose gel with 0.5 µg/ml ethidium bromide.

Results

HBV Innoculum

Three different sources of infectious HBV were used in these studies. Initially, Dane particles of known concentration (Merck Co.) were introduced into the culture media of incubating cells. High titer serum from a chronically infected individual was used in a similar manner. Because of concern over the infectivity of both these methods when, after several days in culture, analysis of cells revealed no HBV RNA, the source of HBV changed to cell media from cultures of 2.2.15 cells. This cell line originates from Hep G2, is stably transfected with a plasmid carrying four 5'-3' tandem copies of the HBV genome, and produces infectious virus in variable amounts (24). The exact inoculum was unknown. Using our protocol we determined the approximate inocula to be 1.7X10e4/ml in media from cells on day 4 after passing with 50% confluence (Figure 2). For all subsequent inoculations, culture media from 2.2.15 was used.

Polymerase Chain Reaction: New Primers

The first set of Primers (DB1, DB2, and DB4) consistently gave single copy DNA sensitivity under the noted conditions within the limitations of the poisson distribution. This amplified product, a 145 base pair DNA fragment, was detectable using ethidium bromide staining after predigested Dane particles and plasmid pSAG were serially diluted down to a single copy (Figure 3). These primers consistently led to a strong primer dimer band and resulted in varying signal intensity from copy number 1 to 100 of target DNA. Two other primers were introduced in an attempt to decrease the primer dimers and increase the signal produced from 1 - 100 copies of target DNA.

Figure 4 demonstrates the comparison of three primer trios (DB1, DB2, and DB4; DB3, DB2, and DB4; and DB5, DB2, and DB4). Here, while strong and consistent bands are achieved with the new primers DB3 and DB5, the use of DB1 creates a noticeable difference between 10 and 100 copies. In pursuit of a very sensitive assay we began using DB3, DB2, and DB4 for all subsequent analysis.

RNA purification

Two detergents were used to purify RNA. Initially, UNSET* buffer was used under acid phenol/chloroform/isoamyl alcohol conditions to separate RNA from most of the cellular DNA. This method provided unreliable DNA clearance even with DNase I treatment. For this reason, GITC was subsequently used. Although the sensitivity is marginally decreased as reported previously (21), parallel purifications of HBV RNA after DNase treatment performed in our lab demonstrated UNSET* and GITC equally sensitive within a order of magnitude (Figure 5).

Reverse Transcription

The sensitivity of our reverse transcription was assumed to be approximately 10 copies of target HBV mRNA based on published experience (23). A control trial with mRNA of known copy number was performed to address this question. The source of HBV mRNA used for control was transcribed *in vitro* from plasmid HBV DNA (see Figure 6). The mRNA was purified with acid GITC extraction and DNase I treatment according to our protocol; the final concentration was measured by spectophotometry and found to be 28ng/μl. This sample was serially diluted and introduced into the protocol at reverse transcription with subsequent amplification. The signal

was lost between 23 and 4 copies (Figure 6) which is equivalent to the estimated sensitivity. This sensitivity was achieved in the absence of heterogeneous cellular RNA. Analysis of parallel processed serial HBV RNA dilutions revealed a maximum decrease in sensitivity of 10-fold in the presence of cellular RNA (Figure 7).

Cell Culture Infection Studies

Ach2 and A3

Ach2 or A3.01 cell lines were used in initial studies because of previous data suggesting increased HBV DNA in these cells after exposure to virus. As depicted in Table 1, no HBV mRNA was detected in these cell lines with exposure to Dane particles or high titer serum. In one parallel culture, PHA stimulation did not lead to the presence of HBV mRNA.

A3.01 cells were included in further studies using 2.2.15 cell media for HBV exposure. The results for this trial are presented in table 2. The samples demonstrated HBV mRNA at days 1 and 12 after exposure but were negative at days 2 and 5. The reverse transcriptase-negative controls were negative as were the PCR negative controls.

U937 and U1

The cell lines U937 and U1 were not exposed to Dane particles. They were incubated with 2.2.15 media in a similar manner as A3 and the results are included in Table 2. Again, in both cultures early signal was found indicating HBV mRNA. There was no persistence of this signal. Further results revealed no HBV RNA signal when samples were taken immediately after combination of cells and 2.2.15 media (data not shown). 2.2.15 media processed alone was free of HBV RNA. The reverse transcriptase-negative and PCR-negative controls were all negative.

In the presence of polybrene, a signal for HBV mRNA was detected in the U937 culture at day 2 whereas the polybrene (-) U937 culture was negative. Both cultures were negative by day 7.

U937, Human Serum, and LPS

U937 cells were exposed to either Human Serum, LPS, both or neither. Figure 8 depicts the outcome. HBV mRNA was found at day 7 of culture with U937 cells exposed to LPS without human serum with a cellular input of approximately 500,000. All other cultures were negative. All negative controls were negative.

Discussion

Methodology

The hemi-nested primer PCR we developed is very sensitive. For HBV DNA, we reliably detected a single copy. This is in agreement with previous experience with hemi-nested primer PCR and data to be published (21, 23). The early primer mix (DB1, DB2, and DB4) detected single copy input but gave a signal that was not at plateau; the stage where, regardless of increasing DNA input, the post amplification signal remains the same. The gradation in signal intensity for copy numbers 1 - 100 cannot be used to add a quantitative element to the assay. Repeated amplifications frequently led to fluctuations in the lighter bands. Occasionally the single copy of HBV DNA gave a stronger signal than 10 copies of similarly prepared DNA. By using the latter developed primer pair DB 3-2-4 we were able to consistently reach plateau with single copy HBV DNA.

One advantageous characteristic of PCR is the coupling of sensitivity with specificity. Many procedures have to sacrifice sensitivity for specificity or vice versa. With PCR, by increasing the sensitivity, the specificity is increased as well. In the presence of positive signal, there was only a single incidence of non-specific signal(Figure 6, ¥). On the other hand, when no HBV DNA was detected, occasional non-specific DNA would be amplified. Figures 5 and 7 demonstrate amplified high molecular weight DNA fragments that are assumed to be nonspecific. Southern hybridization will be used to confirm the absence of amplified product homologous to HBV DNA within these signals.

The use of Ampliwax® significantly reduced non-specific activity in our amplifications. "Hot start" PCR abrogates early interactions between Amplitaq, Primers, and target DNA under non-stringent conditions.

Decreased specificity results from promiscuous annealing of the primers to partially homogeneous template sequences. The use of Ampliwax® combines Amplitaq® and Template DNA with dNTP and primers at a temperature prohibitively high for promiscuous annealing and elongation. For sample amplification, the use of Ampliwax® was standard.

The optimization of Reverse Transcription PCR proved more difficult. Our protocol had the expected sensitivity when tested with purified HBV mRNA without cellular RNA. Decreased sensitivity occurred when HBV mRNA was seeded into cellular RNA. In our control experiments, between and 5 and 10 fold decrease in sensitivity was introduced by the presence of cellular mRNA. This may be due to competition for random hexamers and MMLV. One control study led to a large amount of non-specific amplified product when known copy number HBV DNA was seeded into U937 cells (not shown). Further optimization will include using specific primers for reverse transcription. This has been demonstrated to increase the specificity and sensitivity of similar protocols looking for hepatitis C virus (21).

Assuming a 10 copy sensitivity of our RT-PCR without cellular RNA and as high as a 10 fold decrease in sensitivity when cellular material is added, we can detect approximately 100 copies of HBV mRNA within the cellular RNA from 500,000 cells (U937). With this information, figure 5 demonstrates a copy number of approximately 100 copies per 2 ng of cellular plus HBV mRNA from a transiently infected cell line: approximately 1000 copies of HBV RNA per 1000 Huh 7 cells transiently infected with pSAG. Although the copy number of HBV RNA produced with transient infection varies significantly, this level of activity is common (27).

The definitive control experiment with precisely known HBV mRNA copy number, seeded into HBV-naive cells, with subsequent analysis by our

protocol has not been performed. However, pending this result, our sensitivity agrees with what has been found in past experiments and leads to data confirming other known concentrations.

Cell Culture Infection Studies.

The initial studies with Ach3 and A3 cell lines did not detect HBV mRNA. This may indicate a true negative result; i.e. HBV inability to become transcriptionally active. It is not clear that HBV can become transcriptionally active in the A3.01 and Ach2 cell lines. PBMC studies have demonstrated increased HBV DNA within B-cell and monocyte enriched fractions but depleted HBV DNA within T-cell enriched populations (12,15).

A3 and Ach2 are derived from CD4+ cells that would mature to T-cells. Without stimulation, these cell lines may lack the receptor associated with HBV binding (19). Although T-cell subsets have been shown to demonstrate HBV binding sites with stimulation by Concanavalin A (20), no study has addressed stimulation with PHA. The increase in HBV DNA reported in PBMC stimulated by PHA may be due to stimulation of non-T cell populations; specific cell subsets were not studied during this investigation (14).

Data exist, however, indicating HBV DNA is present and may be active within T-cells. Studies in chimpanzees and woodchucks as well as human mononuclear cells have demonstrated hepadnavirus or HBV DNA associated with T-cells, respectively (25,26). Earlier work in our lab demonstrated an increase in HBV DNA in the Ach2 cell line. Subsequent work with A3.01 cells and a different innoculum also led to detection of mRNA signal after 12 days of culture.

The failure to detect HBV RNA in our early studies may be due to poor infectivity of the Dane particles used for innoculum or RNA activity below our sensitivity. The high titer serum and Dane particles used were old and repeatedly frozen in the past. Prior handling may have significantly reduced infectivity. HBV transcriptional activity below our assay's sensitivity leading to an increase in viral DNA would lead to a false negative result. HBV RNA has been detected without concurrent detection of HBV DNA (14) but it is unlikely that HBV activity leading to less than 100 copies of mRNA/100,000 cells could lead to a significant increase in HBV DNA.

The concerns about the infectivity of our innoculum led to the use of supernate from the 2.2.15 cell line. Early control studies with 2.2.15 cell supernate detected no HBV mRNA in 2.2.15 media alone or immediately after combination with cells (data not shown). Therefore, carryover of HBV mRNA in the innoculum does not account for cultures becoming positive after short incubations with 2.2.15 media.

Contamination of the samples with HBV mRNA is another possible explanation. These studies are performed in a lab free of concentrated HBV mRNA. The only source for contamination is diluted HBV mRNA samples used for positive reverse transcription controls. Any contamination significant enough (>100 copies) to be detected, would most likely lead to more consistent contamination of samples.

HBV DNA contamination potentially presents a more troublesome problem. A single copy would lead to a positive result. Early on, DNA persistence was a serious problem. Since the application of GITC buffer, DNA contamination is a rare occurrence. Reverse transcriptase-negative controls are run in parallel to detect DNA contamination. In the studies demonstrating HBV RNA, all reverse transcription-negative controls were

negative. Controls subjecting samples which demonstrated a signal for HBV RNA to RNase to ensure dissapearance of the signal were not performed.

Given the low likelihood of carryover RNA from innoculum, contamination by HBV RNA, or false positive results due to HBV DNA, the sporadic results remain to be explained. In fact, early work with HBV in PBMC's demonstrate inconsistent data regarding the presence or absence of HBV DNA and RNA and the ability to use mitogens to increase signal (see Table 2). These findings support the presence of very low levels of HBV DNA transcription in the cell cultures. Interpretation of our data reveals a transcriptional activity 1000 fold lower than transient infection Huh 7 cells with pSAG. To derive such a drop off we assumed detection of 100 copies mRNA in 100,000 U937 cells (Figure 8), or a single copy of HBV DNA per 1000 cells in contrast to a single copy of HBV DNA per Huh 7 cell as discussed above.

This low level of activity may be explained by the size of the inoculum. With a concentration of HBV Dane particles of approximately 1.7 X 10e4/ml, our procedure led to a virus to cell ratio of slightly greater than 1 to 100. Assuming 100% of viral particles get into cells (very generous), initially we would expect very low levels of viral transcription with such an inoculum; especially while studying a cell line outside of the virus's established tropism. If the virus could complete its replicative cycle and bud from these cells the amount of transcriptional activity would increase exponentially. Finding no evidence for such an increase after 14 - 17 days of culture argues for a block in HBV replication after early transcription. Our current studies do not allow a prediction as to where this possible block occurs.

With further optimization of our protocol, consistent patterns of HBV transcription may be described. Future changes we are considering include

using specific primers for reverse transcription rather than random hexamers and creating an internal control plasmid to help monitor variation between separate amplifications (21). Currently we are raising 2.2.15 cells in large numbers for eventual purification and concentration of Dane particles. Increasing the inoculum may lead to more consistent viral activity.

Our most encouraging results involve culturing U937 cells in the presence or absence of LPS and Human Serum, both or neither. This study was developed from results published by Neurath et al indicating increased HBV receptors on U937 cells with LPS stimulation (20). An increase in receptors may increase the likelihood of viral infection and eventual development of an *in vitro* system. The results agree with this hypothesis but have yet to be confirmed on a larger scale. Only the culture with LPS simulation and no Human Plasma was found to be positive for HBV RNA after 7 days. However, the level of RNA was at cut off as demonstrated by the failure to detect HBV RNA when fewer cells were processed (Figure 8). The effect of human serum is not clear. It is possible that, as a pooled serum sample, antibodies against HBV are present. These may decrease the frequency of cellular infection by disrupting virus-cell binding. Monoclonal antibodies against the HBV surface antigen have been demonstrated to block virus binding to hepatoma cell lines (28). The human serum is to be tested for such antibodies.

In conclusion, we have developed a sensitive assay for HBV RNA and have used it successfully to detect transcriptional activity in hematopoietic cell cultures exposed to HBV. We have yet to develop an in vitro model of HBV infection of hematopoietic cell lines that reliable leads to evidence of HBV transcriptional activity. Preliminary results with U937 cells in the presence of LPS are encouraging but require confirmation.

Creating an *in vitro* model of HBV infection of hematopoietic cells would facilitate the study of HBV infection. To date, no reproducible infection by HBV of stable cell lines has been described. The cell line 2.2.15, which produces infectious Dane particles, resulted from transfection of the virus and, though useful for the study of late viral activity, cannot be used to address early viral infection. One group has claimed the ability to infect HepG2 cells with HBV but their result have not been reproduced (29). With the establishment of an *in vitro* model, additional studies regarding the early infection of HBV and the effects of pharmacologic interventions will be possible.

HBV stable presence within hamatopoietic cells would also allow exploration of persistent viral infection, viral re-activation in hepatocytes, and re-infection of transplanted livers. HBV has an inhibitory effect on the growth of hematopoietic cell lines (30). Such an inhibition may play a role in viral persistence. Development of an infected hematopoietic cell line may greatly facilitate studies addressing the transient bone marrow suppression that occurs with HBV infection in humans. A better understanding of the stimulation of HBV transcription in hematopoietic cells would aid in addressing questions concerning viral re-activation in the human host. Finally, persistent HBV infection has been described in PBMC after liver transplantation (31). By demonstrating viral activity within these cells, the avenue for infection of the transplanted liver may be identified.

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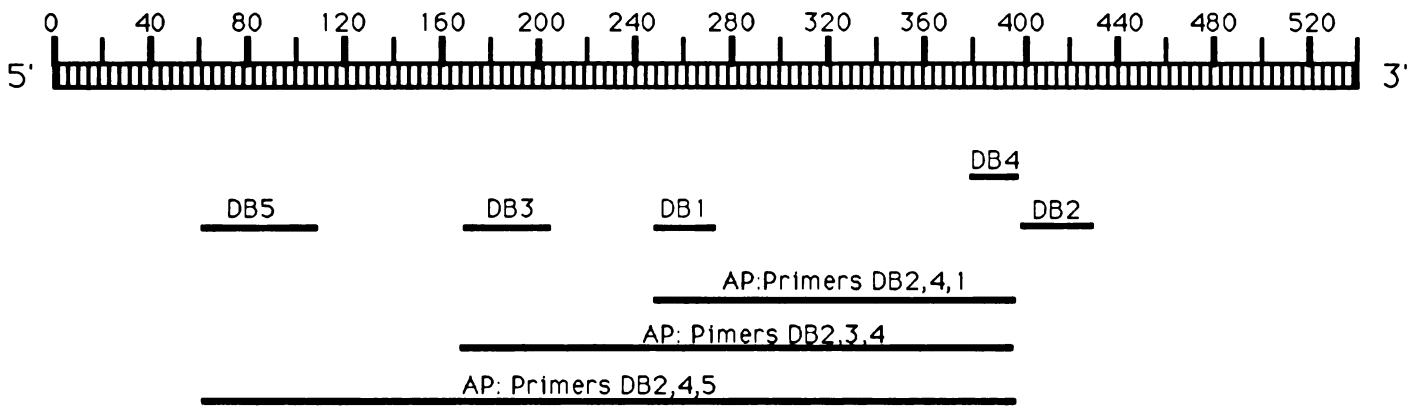
Figure 1: Location and sizes of primers and amplified product



1A: Schematic map of HBV genome with start at 5' EcoR1 endonuclease site.

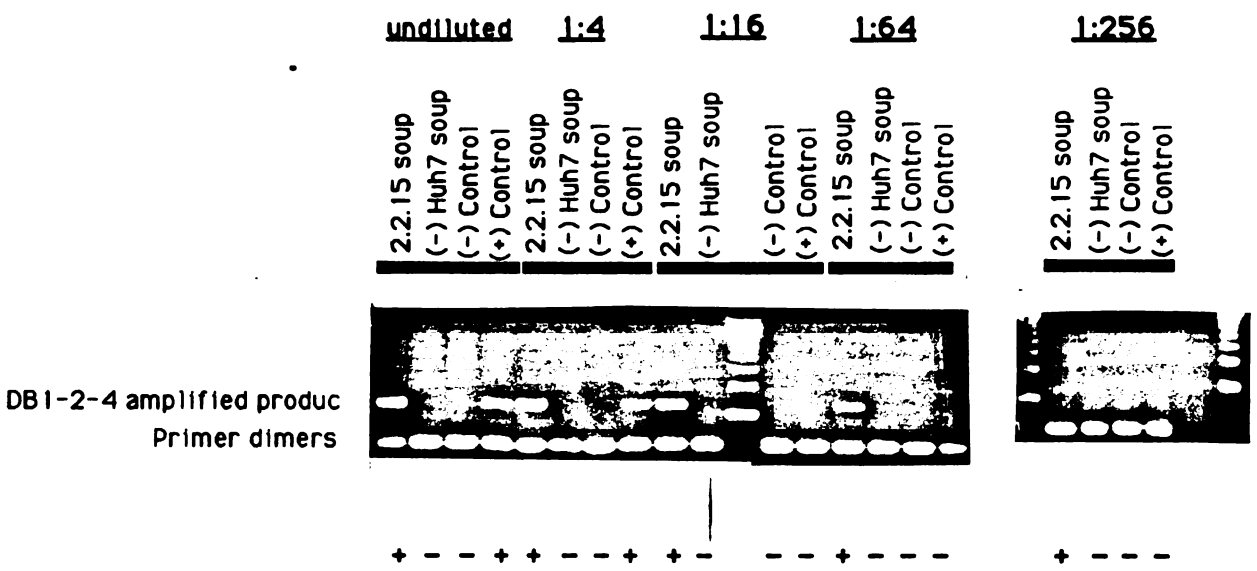
Primer	Location on HBV Genome
DB 1	245 - 276
DB 2	411 - 434
DB 3	169 - 200
DB 4	379 - 399
DB 5	62 - 93

1B: Specific locations of primers reported as number of base pairs upstream from EcoR1 restriction site

Primer Mix	Amplified Product
DB 1-2-4	145 base pairs
DB 3-2-4	231 base pairs
DB 5-2-4	338 base pairs

1C: Sizes of amplified products for primer mixes

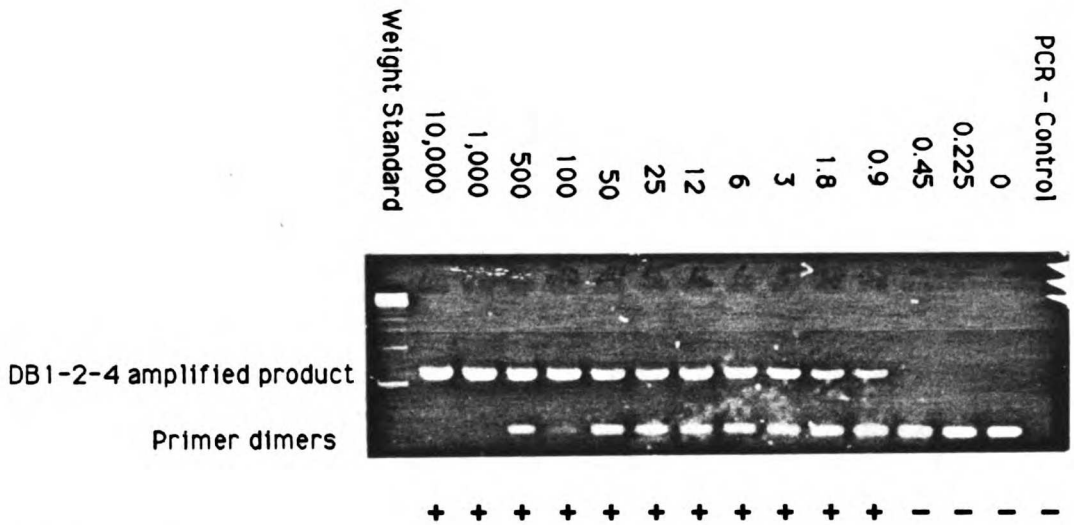
Figure 2: Titres of HBV in culture media from 2.2.15 cell line



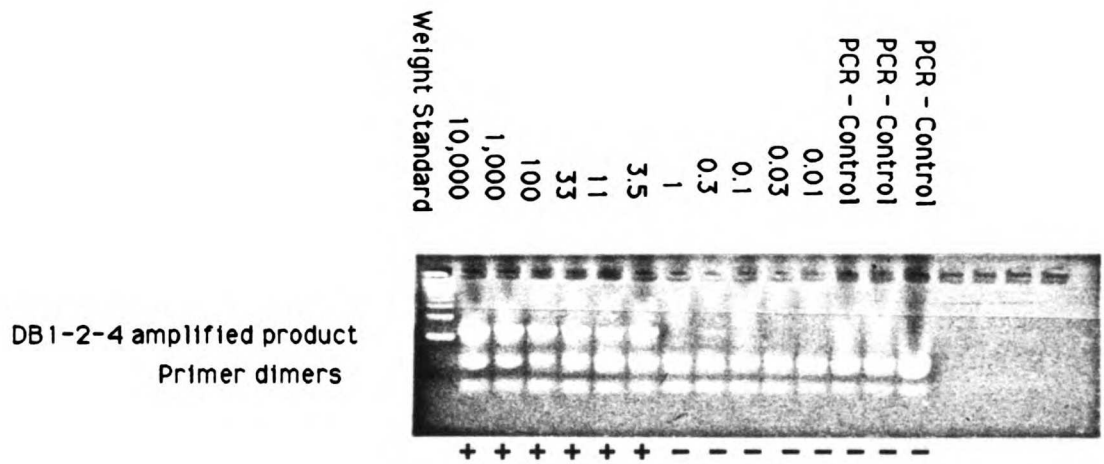
The supernate from cell lines 2.215 at day 4 of culture solubilized in UNSET* buffer and isopropanol precipitated as described in methodology. Serial 4-fold dilutions were performed including 1:4, 1:16, 1:64, and 1:256. Samples were amplified by PCR according to protocol.

Negative controls used were Huh7 cell supernate and RPMI 1640 + 10% FBS; the positive control was RPMI 1640 + 10% FBS + 20 µl HBV E2 (100 copies/µl). The final (+) input at each dilution was *200 (undiluted), *50 (1:4), *12.5 (1:16), *3.1 (1:64), and *0.8 (1:256). The compromised sensitivity of this trial leads to an approximation of the titre for 2.2.15. The presence of HBV DNA at approximately 1 copy per 10 µl at 1:256 dilution we estimate the titre to be 1.7 X 10e4/ml ignoring losses or inhibition of PCR.

Figure 3: PCR Sensitivity: Serial dilutions of HBV DNA

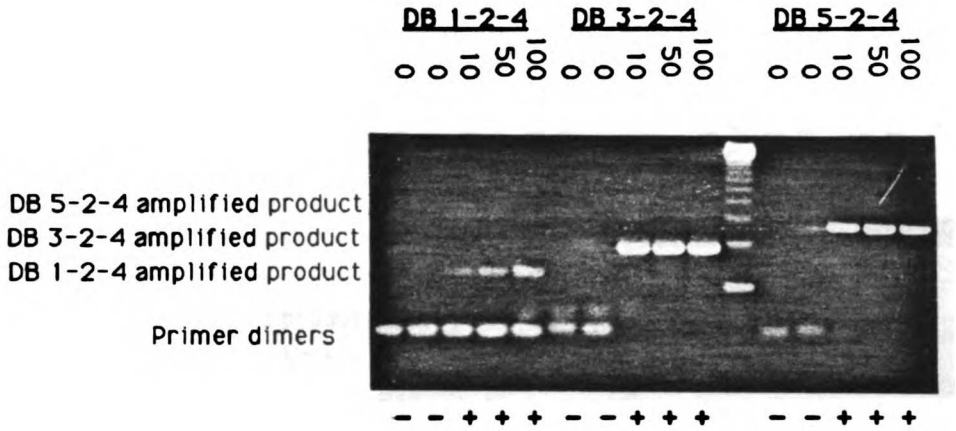


3A: The plasmid pSAG (2.56 µg/µl) was incubated with 140 units EcoR1 in a total volume of 100 with ReAct 3 as a buffer for 10 minutes at 37° C then 1.5 hours at Room Temperature. Assuming a weight of 5.5 Kb for pSAG, samples were diluted to a copy number as diagramed.



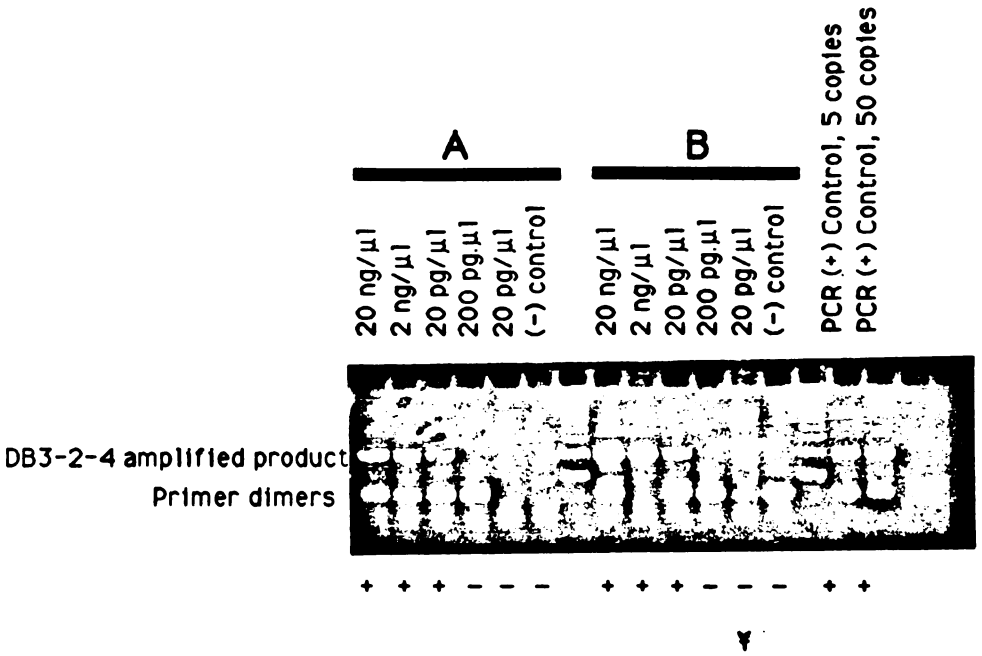
3B: Dane particles were solubilized in UNSET* Buffer. DNA was extracted by neutral phenol:chloroform:iso amy alcohol(50:48:2) and precipitated according to protocol. DNA copy number was derived by assuming 1 to 1 Dane particle to HBV DNA and minimal losses during extraction and precipitation.

Figure 4: Comparison of Primer mixes



Three primer mixes were made for DB1-2-4, DB3-2-4,and DB5-2-4 to compare sensitivities. All mixes had final concentrations of DB2 and DB4 of 0.04 pmol/l and 5.0 pmol/l, respectively. DB1, DB3, and DB5 were added to the respective mixes for a final concentration of 5.0 pmol/l. 0, 10, 30, and 100 copies of pSAG DNA were used to determine relative sensitivities.

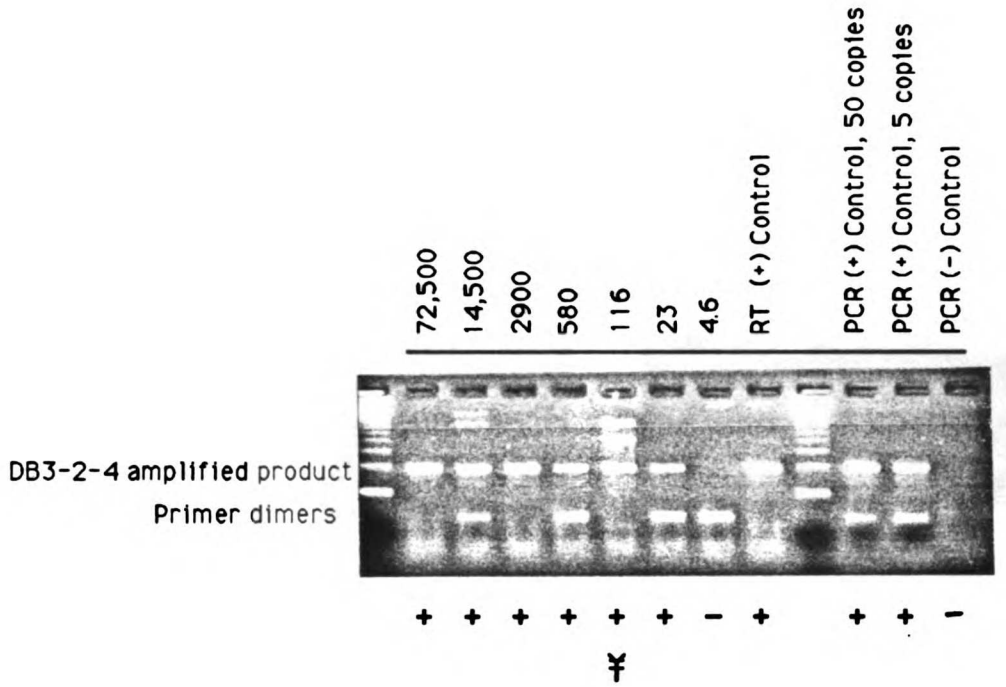
Figure 5: UNSET* v. GITC as detergents for mRNA purification.



RNA from a transient transfection of Huh7 cells by pSAG was treated with DNase and serially diluted. The initial input concentration of RNA was 1µg/10µl DNase buffer. Two parallel samples were solubilized with GITC or UNSET* detergents and extracted with neutral phenol:chloroform:isoamyl alcohol. Samples were serially diluted by a factor of 10, subjected to reverse transcription, and amplified. Signal was lost after 1000 fold dilutions in both samples indicating less than a ten fold difference between the two detergents.

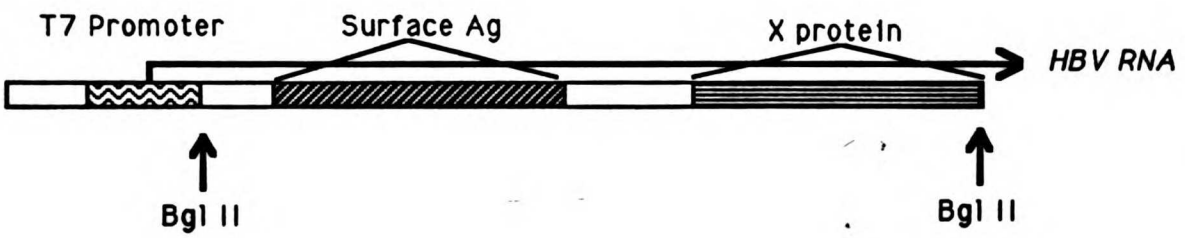
¥ Non-specific high molecular weight band of unknown significance - sample read as negative

Figure 6: Absolute sensitivity of Reverse transcription and PCR amplification



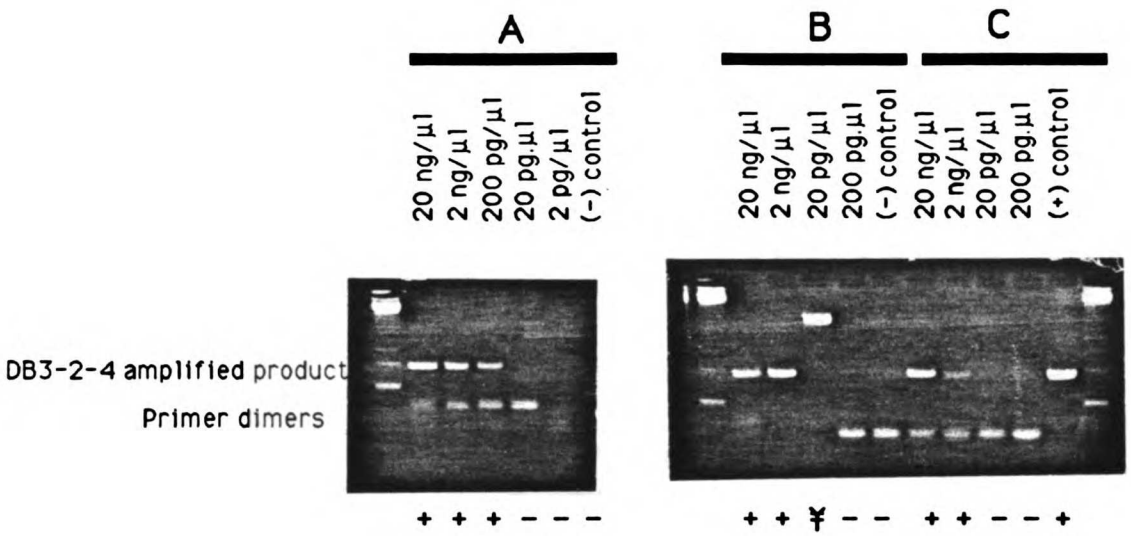
6A: *In vitro* HBV RNA from transcription with T7 RNA polymerase of the plasmid diagramed below was purified and diluted as described. RNA from a transient infection of Huh7 by pSAG was used as the reverse transcription (+) control. pSAG DNA of known concentration was used for the PCR (+) control.

¥ - these high molecular weight bands are of unknown significance but do not affect results.



6B: Plasmid used as source for *in vitro* HBV RNA

Figure 7: Reverse Transcription PCR sensitivity with and without U937 cellular RNA.

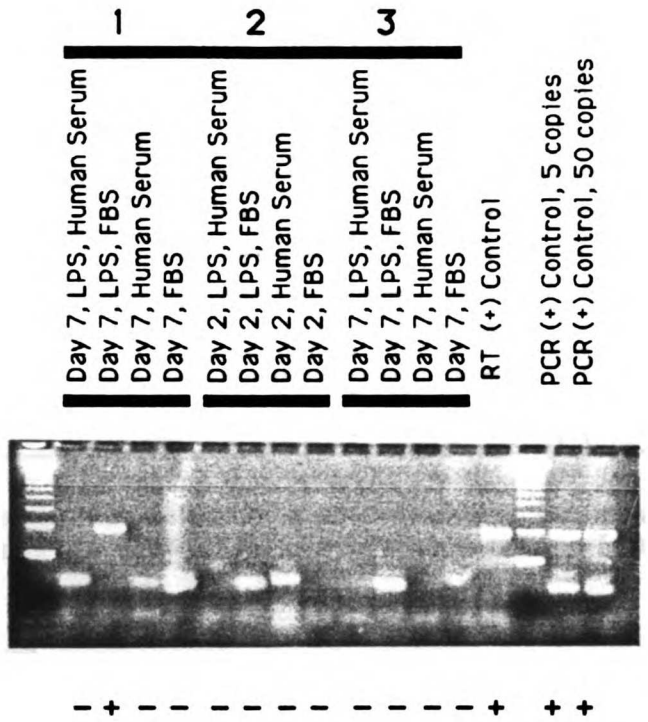


RNA from a transient transfection of Huh7 cells by pSAG was treated with DNase and serially diluted*. The initial input concentration of total RNA was 20 ng/µl assuming minimal losses during DNase treatment and RNA purification with GITC. The five dilutions initially tested were 20 ng/µl, 2 ng/µl, 200 pgm/µl, 20 pgm/µl, and 2 pgm/µl. Initially 5 µl of each dilution was introduced into the reverse transcription step without additional cellular RNA. These results are presented in A; all negative controls were negative (not shown). 10 µl of the first four dilutions (20 ng/µl, 2 ng/µl, 200 pgm/µl, 20 pgm/µl) were then introduced into 500,000 U937 cells solubilized in GITC solution and taken though the entire protocol described in methodology. The results are presented in B and indicate a 10 fold decrease in sensitivity. If the same dilutions are seeded into U937 RNA from 500,000 cells just prior to reverse transcription and amplification a similar 10 fold drop in sensitivity is noted indicating that the cellular RNA is causing the drop in sensitivity rather than losses during purification and DNase I treatment.

* As the number of HBV RNA transcripts is not accurately known with this transfection only relative comparisons can be made in this study.

¥ Non-specific high molecular weight band of unknown significance - sample read as negative

Figure 8: U937 cells exposed to 2.2.15 cell media grown in the presence of LPS and/or Human Serum



1 million cells were incubated at 37° C and 5% CO2 in RPMI 1640, Glutamine, and Pen/Strep with the four following manipulations: A) 5µg/ml LPS, 10% human serum and 10% FBS were added to the first culture; B) 5µg/ml LPS and 20% FBS were added to the second culture; C) No LPS, 10% human serum, and 10% FBS were added to the third culture; and D) No LPS and 20% FBS were added to the fourth. Cultures were kept for seven days. 200µl of media and cells were harvested at T= 0, 2, 4, 6, 24, and 50 hours as well as days 3 and 6. On day 7, cell cultures were terminated and approximately 1 million cells were collected. For the above gel, the cellular input was 500,000 cells for "1", 100,00 cells for "2", and 100,00 cells for "3". The presence of a signal at Day 7 with a cellular input of 500,00 but ansence of a signal with and input of 100,000 cells indicates we are at the cut off point of our assay. All negative controls performed contemporaneously were negative.

Table I: Cell lines A3.01 and Ach2 exposed to Dane particles (D) or High titre serum (MA)

Day	A3	Result	Ach	Result
D2	*(+/- PHA)	(-)	*(+/- PHA)	(-)
D3	*(+/- PHA)	(-)	*(+/- PHA)	(-)
D4			*	N.D.
D6			*	N.D.
D7	*(+/- PHA)	(-)	*(+/- PHA)	(-)
D10	*	N.D.	*	N.D.
D12			*	N.D.
D13	*	(-)	*	(-)
D14			*	N.D.
D17			*	(-)

500,000 cells were collected on noted days and analyzed for HBV DNA. Parallel cultures with and without PHA stimulation were performed and tested for HBV DNA for days 2, 3, and 7.

Key: * = cells collected
+/- PHA = parallel cultures with or without PHA stimulation were performed
(+) = positive for HBV mRNA
(-) = negative for HBV mRNA
N.D. = not determined, sample was not processed

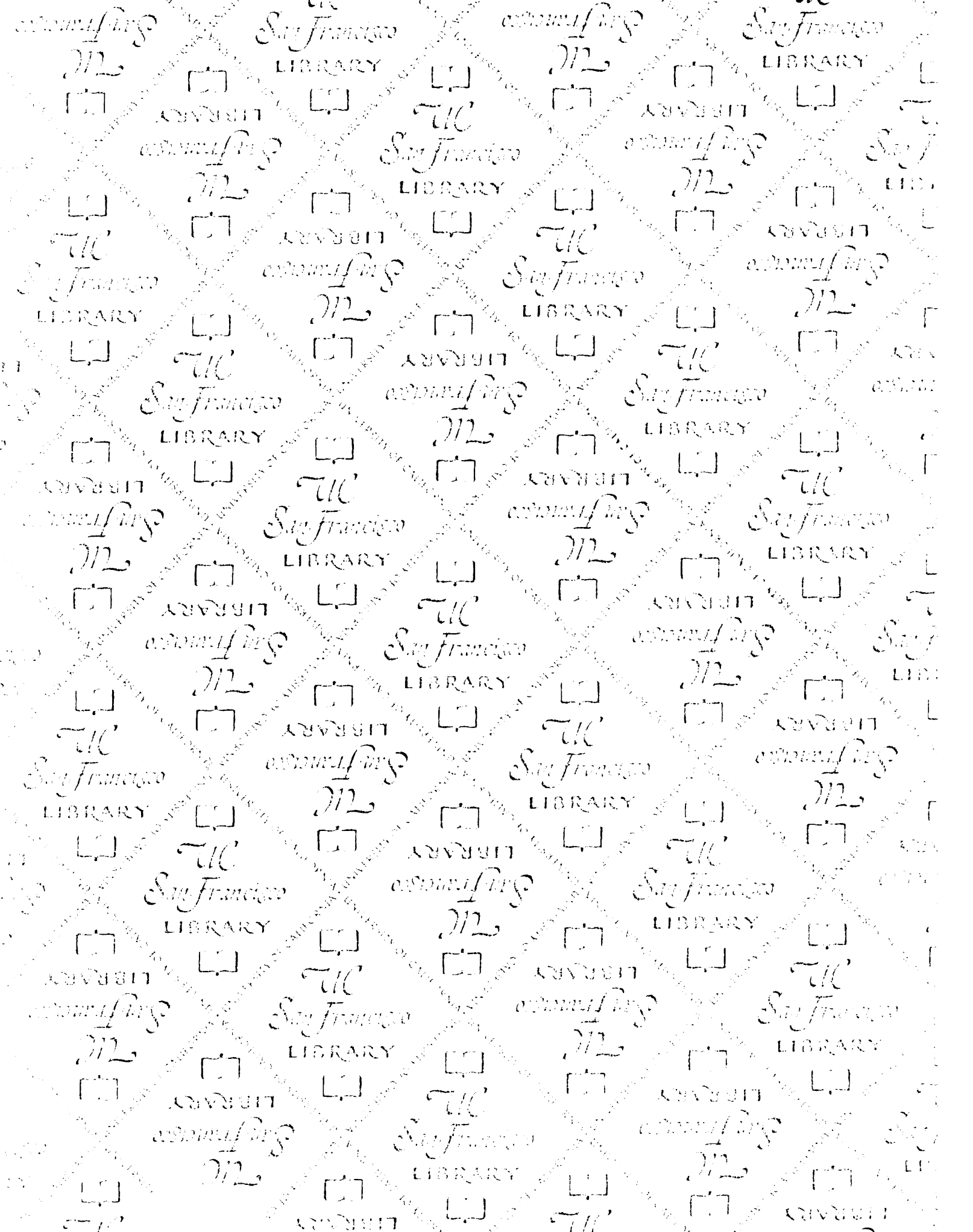
Table 2: Cell lines A3.01, U937, and U1.1 exposed to 2.2.15 cell supernate

Day	A3	Result	U1	Result	U937	Result	A	B
D1	*	(+)¥	*	(+)¥				
D2	*	(-)	*	(+)	*		(-)	(+)
D5	*	(-)						
D7			*	(-)	*		(-)	(-)
D12	*	(+)						
D14			*	(-)				

Approximately 500,00 cells were collected on noted days and tested for HBV mRNA. U937 cells were incubated in the presence (B) or absence (A) of polybrene. The percentage of cell viability of both U937 cultures remained equal. However, the culture with polybrene had a faster growth rate as demonstrated by cell count.

¥ Samples of 2.2.15 media and respective cell lines were negative for HBV RNA at Day 0.

Key: * = cells collected
(+) = positive for HBV mRNA
(-) = negative for HBV mRNA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