

Transcription of the Hepatitis B Surface Antigen Gene in Mouse Cells Transformed with Cloned Viral DNA

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Received 29 June 1981/Accepted 17 November 1981

Mouse L cells transformed with recombinant plasmids carrying hepatitis B virus (HBV) DNA fragments were used to study the transcription of the viral surface antigen gene (gene S). An HBV-specific, polyadenylated, 2.3-kilobase RNA was mapped on the HBV genome. This RNA hybridized with approximately 75% of the genome and excluded the region of the HBV core antigen gene (gene C). The 2.3-kilobase RNA species was present only in cell lines that produced hepatitis B surface antigen. An HBV-specific 2.3-kilobase RNA was also detected in human hepatoma cell line PLC/PRF/5 which produced hepatitis B surface antigen. A study of gene S expression in the transformed mouse L cells allowed us to localize the regions of initiation and termination of gene S transcription. Our results strongly suggest that the 2.3-kilobase RNA molecule is the mRNA of the major polypeptide of the envelope, which carries the viral surface antigen determinants.

Experimental transmission of hepatitis B virus (HBV) has been achieved only in chimpanzees, and attempts to propagate HBV in cell cultures have been unsuccessful. Such a narrow host specificity and the lack of an *in vitro* system for HBV propagation have greatly hampered our understanding of the molecular biology of this virus. Expression of the HBV genome has been studied recently in different systems. One of these systems consists of a human hepatoma cell line which was established by Alexander et al. (1) which excretes hepatitis B surface antigen (HBsAg) particles into the culture supernatant. Using this system, Chakraborty et al. (5) and Edman et al. (8) described transcripts which hybridize to the region of the genome which contains the HBsAg gene (gene S). Another approach has been to introduce the previously cloned genome into animal cells in culture and to look for viral antigen production. Using this mediated gene transfer technique, we found that mouse L cells transformed with two HBV genomes in a tandem head-to-tail arrangement excreted HBsAg as particles into the culture supernatant without lysis of the cells (7). Other viral markers were not detected. Using circularized HBV DNA, Hirschman et al. (11) observed virion-like particles in the supernatant of transformed human HeLa cells, and using a simian virus 40 hybrid containing an HBV DNA frag-

ment, Moriarty et al. (13) obtained HBsAg production in a monkey kidney cell line.

In this paper we describe further studies of gene S expression in HBV DNA-transformed L cells. A 2.3-kilobase (kb) viral transcript was mapped on the genome, and the region containing the gene S promoter was localized.

MATERIALS AND METHODS

Cell cultures and HBsAg detection. Mutant mouse LM cells (clone 1D, deficient in thymidine kinase [LTK⁻]) and PLC/PRF/5 cells (Alexander cell line) were grown in minimal essential medium (GIBCO Laboratories) supplemented with 10% calf serum and 10% fetal calf serum, respectively. LTK⁻ cells were transformed with DNA as described previously (7). The presence of HBsAg was detected with an AUSRIA II radioimmunoassay kit (Abbott Laboratories). Cellular clone b3 (7) is a TK⁺ clone that was obtained after cotransformation of the LTK⁻ cell line with plasmid pCP10 containing two cloned HBV genomes in a tandem head-to-tail arrangement and plasmid pAGO (6) containing the herpes simplex virus type 1 tk gene.

RNA extraction. For total RNA extraction, about 10⁸ cells were washed twice with phosphate-buffered saline, scraped off in 5 ml of the same buffer, harvested by centrifugation, and suspended in 5 ml of 10 mM sodium acetate (pH 5)–50 mM sodium chloride–0.5% sodium dodecyl sulfate. Then 1 volume of phenol saturated with water was added, and the suspension was agitated gently for 3 min at 65°C. The aqueous

phase was reextracted once with phenol. RNA was precipitated with 2 volumes of ethanol. Polyadenylated [poly(A)⁺] and non-polyadenylated [poly(A)⁻] RNAs were separated by two cycles of oligodeoxythymidylic acid cellulose column chromatography.

Hybridization of HBV DNA fragments with a cDNA probe. HBV DNA fragments were electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized by using the blot hybridization technique of Southern (16), as modified by Wahl et al. (20). cDNA from poly(A)⁺ RNA was prepared by the method of Perricaudet et al. (15), using avian myeloblastosis virus DNA polymerase and oligodeoxythymidylic acid₁₂₋₁₈ as a primer. The second DNA strand was synthesized with the same enzyme. The double-stranded cDNA was radiolabeled by nick translation and hybridized to the HBV DNA fragments immobilized on the nitrocellulose filter.

Hybridization of RNA with HBV DNA probes. RNA was denatured as described by McMaster and Carmichael (12) and was electrophoresed on a horizontal 1% agarose gel in 10 mM sodium phosphate buffer at 90 V for 6 h with constant recirculation of the buffer. The gel was then stained for 30 min in sodium phosphate buffer containing 30 μg of acridine orange per ml. RNA transfer and hybridization were performed by the method of Thomas (17). The prehybridization and hybridization steps were performed at 42°C for 8 and 16 h, respectively. The HBV DNA probes were nick-translated by the method of Weinstock et al. (21)

(specific activity, 2×10^8 to 4×10^8 cpm/μg). The filter was autoradiographed by using preflashed X-Omat R film at -70°C and a Kodak intensifying screen.

Construction of the recombinant plasmids containing HBV DNA fragments. Plasmid pCP9 resulted from integration of one *EcoRI* HBV genome in the *EcoRI* restriction site of plasmid pAGO. The pAC plasmids were constructed as follows. Plasmid pCP10 was digested with *HindIII* and *PstI* restriction enzymes, and the DNA fragment containing the two HBV genomes in tandem was purified by gel electrophoresis and electroelution. This DNA fragment was then partially digested with *BglII* endonuclease. The restriction fragments were inserted into the *BamHI* restriction site of plasmid pAGO. *Escherichia coli* strain DP50 was transformed (7), and in situ hybridization was performed on the colonies by using the *XbaI-HhaI* restriction fragment which contains the S gene as a probe. The plasmids corresponding to 24 positive colonies were analyzed by the method of Birnboim et al. (3). Plasmid pAC1 contained *BglII* fragment A plasmid pAC2 contained *BglII* fragments A and C, and plasmid pAC3 contained *BglII* fragments A, C, and B. The pANC plasmids were constructed by partial digestion of plasmid pCP10 with *BamHI* endonuclease, purification of the 3.2-kb *BamHI* fragments, and insertion of these fragments into the *BamHI* restriction site of plasmid pAGO. After transformation of strain DP50 bacteria, plasmids were extracted from 24 tetracycline-sensitive bacterial clones and analyzed by the

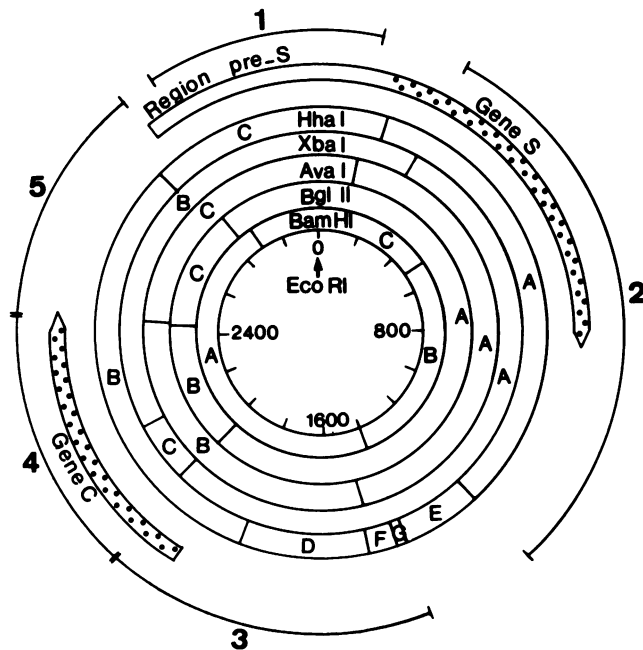


FIG. 1. Physical map of cloned HBV DNA. The positions of the restriction sites were deduced from the nucleotide sequence of the genome, which is 3,182 bp long (10). The single *EcoRI* site was used as the origin of the map. *EcoRI* endonuclease cleaves *BglII* fragment A into two fragments, A' (2 kb) and A'' (0.34 kb), and *AvaI* fragment C into two fragments, C' (0.78 bp) and C'' (0.13 bp). The positions of gene S, the pre-S region, and gene C are indicated (18). The outer circle shows the positions of the probes (numbered 1 to 5) used for blot experiments.

same method. Plasmid pANC1 contained one HBV genome inserted through its *Bam*HI restriction site at position 1,400.

Analysis of DNA from transformed cells. The presence and the state of HBV DNA in the transformed cells were investigated as previously described (7).

Nomenclature. The physical map and nomenclature of the genetic organization of HBV DNA (see Fig. 6) were as reported by Tiollais et al. (18).

RESULTS

Transcription of HBV DNA in mouse L cells.

Gene S transcription was first investigated in HBsAg-producing L-cell clone b3 cotransformed with plasmid pCP10, which contains two HBV genomes in tandem, and plasmid pAGO, which contains the herpes simplex virus type 1 tk gene (see above). To localize the region of the genome which was transcribed, HBV DNA and pAGO restriction fragments were transferred to nitrocellulose and hybridized with the ³²P-labeled double-stranded cDNA corresponding to total poly(A)⁺ RNA. Strong hybridization signals were observed for the *Bgl*III A' and A'' and *Ava*I A, B, and C' HBV DNA fragments (Fig. 1 and 2). A weak signal was observed for the *Pvu*II A pAGO DNA fragment (this fragment corresponds to pBR322). No signal was obtained with the *Bgl*III B and C HBV DNA fragments.

To determine the number and size of HBV-specific RNAs, poly(A)⁺ RNAs from clone b3, PLC/PRF/5 cells, and pAGO-transformed L cells were denatured with glyoxal, electrophoresed, transferred to nitrocellulose, and hybridized with ³²P-labeled HBV DNA, as described above. The autoradiogram pattern for clone b3 RNA showed the presence of one major band at 2.3 kb and two weak bands at 4.3 and 0.9 kb (Fig. 3). The pattern for PLC/PRF/5 RNA showed the presence of a major band at 2.3 kb and a minor band at 1.0 kb. No bands were observed on the pAGO-transformed L-cell pattern. When HBV restriction fragments were used as probes (Fig. 1), the major 2.3-kb RNA species extracted from clone b3 and cell line PLC/PRF/5 were mapped on the viral genome by blot experiments. Among the restriction fragments used, only *Bgl*III fragments B and C (probes 4 and 5) did not hybridize (data not shown). The major 2.3-kb transcript covered approximately *Bgl*III fragment A.

Localization of the promoter region of gene S. The results of the hybridization experiments suggested that gene S transcription was initiated inside the HBV genome. In an attempt to localize the promoter region of gene S, we constructed different recombinant plasmids containing pAGO inserted at different distances from gene S (Fig. 4). For plasmid pCP9 construction, pAGO was inserted in the pre-S region at 155 nucleotides upstream from the ATG of gene S.

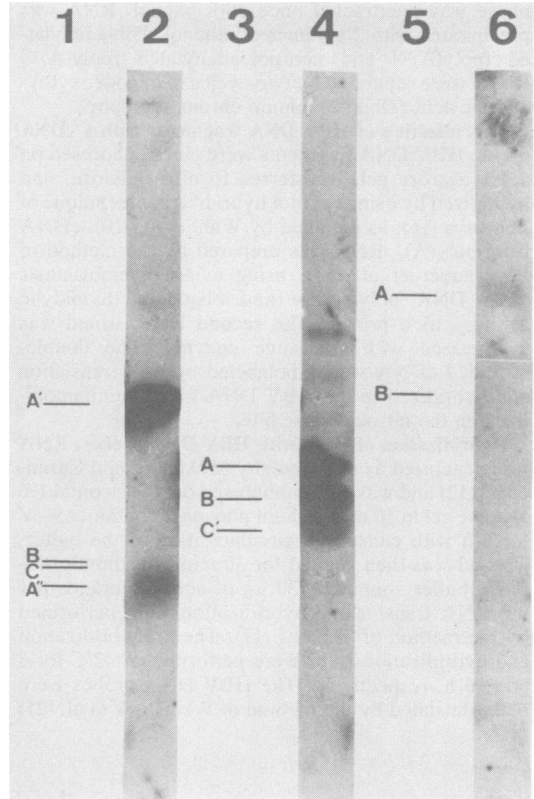


FIG. 2. Hybridization of HBV DNA fragments with [³²P]DNA complementary to clone b3 mRNA. DNA restriction fragments were electrophoresed on a 1% agarose gel and hybridized to 100 ng of labeled double-stranded cDNA (specific activity, 1.5×10^8 cpm/ μ g). The filter was autoradiographed for 3 days. Lane 1, Positions of the *Bgl*III-*Eco*RI HBV DNA fragments; lane 3, positions of the three largest *Ava*I-*Eco*RI HBV DNA restriction fragments; lane 5, positions of the two *Pvu*II pAGO DNA restriction fragments. Fragment A corresponds to linearized pBR322, and fragment B is the fragment containing the herpes simplex virus type 1 tk gene. Lanes 2, 4, and 6, Hybridization patterns of *Bgl*III-*Eco*RI HBV DNA fragments, *Ava*I-*Eco*RI DNA fragments, and *Pvu*II pAGO DNA fragments, respectively.

For pAC1 construction, pAGO was inserted just before the first ATG of the pre-S region. For the construction of pAC2, pAC3, and pANC1, pAGO was inserted at varying distances from the pre-S region. A 2- μ g portion of each plasmid and 10 μ g of salmon sperm DNA were used to transform 2×10^6 cells in a 25-cm² flask. After 2 weeks of growth in the selective medium, about 100 to 200 colonies per flask were observed; 3 weeks later, the colonies were confluent, and the presence of HBsAg in the cell supernatant was checked by radioimmunoassay. Only cells

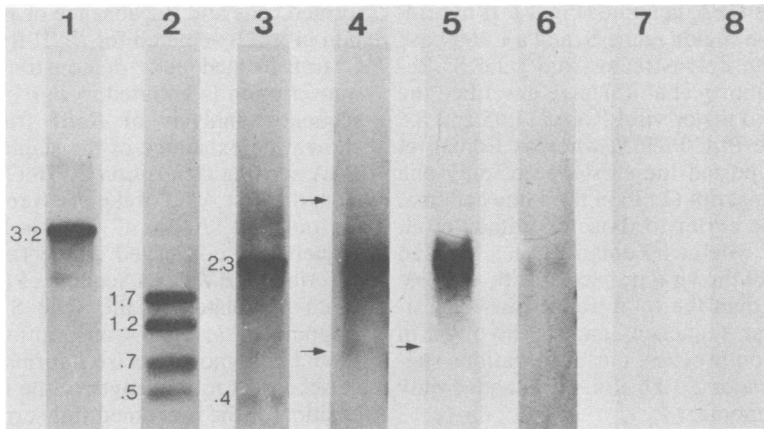


FIG. 3. Hybridization of poly(A)⁺ RNA from transformed cells with ³²P-labeled HBV DNA. A 20-μg sample of glyoxalated poly(A)⁺ RNA was electrophoresed on a 1% agarose horizontal slab gel and hybridized to 25 ng of ³²P-labeled HBV DNA (2.5 × 10⁸ cpm/μg) for 16 h at 42°C in 10 ml of hybridization mixture. The filter was autoradiographed for 1 week. Lanes 1, 2, and 3 contained 10 pg of pCP10 digested with *Eco*RI, *Hind*II, and *Bgl*II restriction endonucleases, respectively (size markers). The sizes of the DNA fragments are expressed in kilobases. Lane 4, Clone b3 poly(A)⁺ RNA; lane 5, cell line PLC/PRF/5 poly(A)⁺ RNA; lane 6, pAC2-transformed cellular RNA; lane 7, pANC1-transformed cellular RNA; lane 8, pAGO-transformed cellular RNA. The arrows indicate the weak bands at 4.3 and 0.9 kb (lane 4) and 1 kb (lane 5).

transformed with pAC2 and pAC3 synthesized HBsAg. To verify that the lack of HBsAg production was not due to the absence of HBV DNA, the transformed cellular DNA was analyzed by the transfer-hybridization technique with ³²P-labeled cloned HBV DNA as a probe. Bands that corresponded to high-molecular-weight DNA fragments which hybridized with HBV DNA were detected on the autoradiograms of both HBsAg-producing and HBsAg-nonproducing cells upon *Hind*III digestion (data not shown).

poly(A)⁺ RNAs from pAC2- and pANC1-

transformed cells were analyzed as described above for clone b3, using total HBV DNA as a probe. An RNA species corresponding to a 2.3-kb position was present in the pAC2-transformed cells, and no RNA was observed in the pANC1-transformed cells (Fig. 3).

DISCUSSION

A major 2.3-kb HBV-specific poly(A)⁺ RNA was detected both in mouse L cells transformed with cloned HBV DNA and in human hepatoma cells (cell line PLC/PRF/5). This RNA was

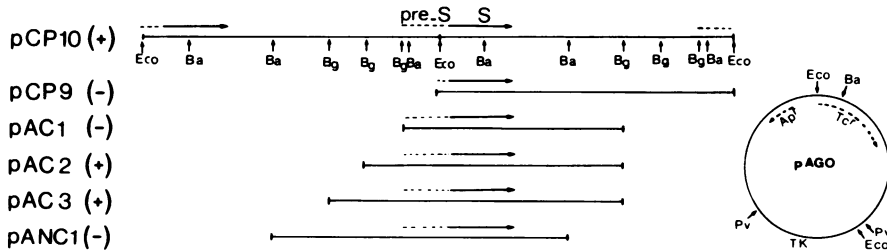


FIG. 4. HBV DNA fragments present in the recombinant plasmids used to transform L cells. Plasmid pCP10 contained two complete *Eco*RI HBV genomes in tandem head to tail. Gene S (solid horizontal arrow) and the pre-S region (dashed line) are indicated. The HBV DNA tandem was used to generate the DNA fragments of the other recombinant plasmids. The HBV DNA insertions had *Eco*RI (pCP9), *Bgl*II (pAC1, pAC2, and pAC3), or *Bam*HI (pANC1) extremities. The plus and minus signs indicate production and absence of production of HBsAg in the transformed L-cell supernatant, respectively. *Eco*, *Eco*RI restriction site; *Bg*, *Bgl*II restriction site; *Ba*, *Bam*HI restriction site; *Pv*, *Pvu*II restriction site. The physical structure of plasmid pAGO (6) is shown on the right. In plasmid pCP9, the HBV DNA fragment was inserted in the *Eco*RI site of pAGO. In plasmids pAC1, pAC2, pAC3, and pANC1, the HBV DNA fragments were inserted in the *Bam*HI site of pAGO.

mapped on the HBV genome (Fig. 5). It hybridizes to the pre-S region, gene S, and a 1,100-base pair (bp) region downstream from gene S. Recently, Chakraborty et al. (5) have described the presence of two major viral RNAs (3.05 and 2.5 kb) in cell line PLC/PRF/5, whereas Edman et al. (8) have reported the existence of only one major viral transcript (2 kb) in the same cell line. Our results are closer to those of Edman et al. Nevertheless, whereas Edman et al. observed hybridization of the viral transcript with an HBV DNA smaller than the total transcript, suggesting that cellular sequences may be involved in the transcription process, our observations suggest that the major 2.3-kb RNA is encoded only by the viral genome.

A series of recombinant plasmids carrying different HBV DNA fragments were constructed and used to transform L cells, and HBsAg production was tested in the different clones. The 2.3-kb RNA was found only in the HBsAg-producing cells. This and the hybridization results strongly suggest that the 2.3-kb RNA is the messenger of the major polypeptide of the viral envelope encoded by gene S. HBsAg production in pAC2 (deleted for *Bgl*III fragment B) trans-

formed cells and the absence of HBsAg production in pAC1 (deleted for *Bgl*III fragments B and C)-transformed cells demonstrate that gene S transcription is initiated in *Bgl*III fragment C. A sequence analysis of *Bgl*III fragment C has shown the existence of the sequence 5'-TATA-TAA starting at position 2,776 (72 bp upstream from the first ATG of the pre-S region), which is the only TATA box (2) in this fragment. This sequence is conserved in the three previously described HBV DNA sequences (10, 14, 19) and could be related to the gene S promoter. S1 mapping or *in vitro* transcription experiments should give more precise information.

According to the length of the mRNA and the position of the presumed transcription initiation start, the polyadenylation site should be located around position 1,900. A sequence analysis of this region has shown the existence at position 1,916 of the sequence 5'-TATAAA, which is related to the classical polyadenylation sites 5'-AATAAA and 5'-ATTAAA. The localization of the end of transcription in this region is not in agreement with the results of Edman et al., who did not observe hybridization with HBsAg mRNA between positions 1,500 and 1,900. The

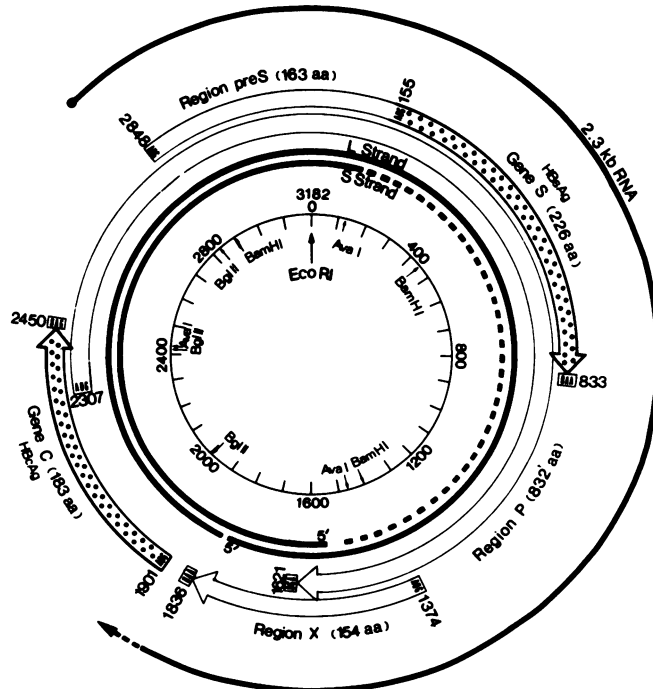


FIG. 5. Position of the 2.3-kb RNA with regard to the HBV genome. The genetic organization of the genome is as described by Tiollais et al. (18). The dashed line indicates the variable single-stranded region. The four potential coding regions, namely, S (divided into pre-S and gene S), C, P, and X, surround the genome. The numbers of amino acids (aa) (in parentheses) correspond to the lengths of the hypothetical polypeptides. The 2.3-kb RNA is indicated, and it covers approximately the entire *Bgl*III A fragment. HBcAg, Hepatitis B core antigen.

absence of HBsAg production in the cellular clone transformed with pANC1 could be due to the lack of an appropriate polyadenylation site.

The hybridization of the 2.3-kb RNA to the pre-S region proves that this region is transcribed and raises the question of its possible translation. The conservation of the open pre-S region in the three previously described HBV DNA sequences and the existence of an analogous pre-S region in the woodchuck hepatitis virus genome (9) are strong arguments in favor of the translation of this region. A search for a polypeptide precursor in HBsAg-producing cells would be interesting.

It was shown previously that L cells transformed with two complete genomes in tandem did not produce hepatitis B core antigen (7). Studies of the HBV-specific RNA produced in these cells demonstrated the absence of gene C transcription and showed that in this system the expression of gene C is regulated differently than the expression of gene S.

All of these studies on HBV gene expression were performed with a cellular system containing viral DNA sequences integrated in cellular DNA. The results obtained cannot be extrapolated a priori to the biology of natural liver infections in humans. It would be interesting to perform similar studies on infected liver samples.

ACKNOWLEDGMENTS

This work was supported by grant CR 22R29 from the Faculté de Médecine Lariboisière Saint-Louis, Université de Paris VII, grant 338 C from the Délégation Générale à la Recherche Scientifique et Technique, grant ATP 72.79.104/029 from the Institut National de la Santé et de la Recherche Médicale, and a grant from the Fondation pour la Recherche Médicale (subvention triennale).

We thank Louise-Marie Da and Simon Wain-Hobson for their contributions to the preparation of the manuscript.

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