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Expression of Hepatitis A Virus cDNA in *Escherichia coli*: Antigenic VP1 Recombinant Protein

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The genome of hepatitis A virus (HAV) was reverse transcribed into cDNA and molecularly cloned. cDNA clones coding for the capsid protein VP1 that carries the major HAV antigen were cloned into the expression vector pUR290 and expressed in *Escherichia coli*. The recombinant fusion protein reacted in an immunoblot with rabbit anti-HAV serum, suggesting that it possesses HAV antigenicity.

Hepatitis A virus (HAV) is a member of the picornaviruses and is classified as type 72 in the genus *Enterovirus* (12). Propagation of HAV in various cell cultures (4, 6, 11, 15) yields only limited amounts of virus, and the pattern of replication is remarkably different from that of other picornaviruses. HAV grows in cell cultures without obvious cythopathic effects, and its replication is much slower than that of other picornaviruses; i.e., a significant titer increase generally cannot be detected before 1 to 2 weeks, and longer incubation periods are needed before reasonable amounts of viral antigen are obtained. These properties have hampered biochemical and, particularly, molecular studies to elucidate the antigenicity of HAV. Apart from the limited virus yields, another problem is that host cells must be disintegrated to recover the virus, and the virus must be purified from the cellular debris, making it difficult to produce pure viral antigens for diagnostic tests and vaccines. These problems led to the molecular cloning and biological amplification of the HAV genome in *Escherichia coli* (1, 10, 13, 18, 19).

In a previous paper (19), we reported incomplete cDNA cloning of the HAV genome; here we report cloning of the entire viral genome and expression of cDNA clones coding for the structural viral protein VP1 in *E. coli*, leading to a recombinant protein with HAV antigenicity (14).

Cloning. The MBB isolate (4) of HAV was propagated in a human hepatocellular carcinoma cell line (PLC/PRF/5), and the virus was purified essentially as described previously (19). RNA was extracted from virus particles by hot phenol and chloroform and was used as a template for reverse transcription either with oligo(dT) as primer or by primer extension with existing HAV cDNA fragments into single-stranded cDNA. For synthesis of double-stranded cDNA, Klenow polymerase was used, except for the 5′ end of the HAV genome. In this case, a short stretch of homopoly(C) was tailed to the 3′ end of the reverse-transcribed single-stranded cDNA, and then the double strand, primed by oligo(dG), was synthesized by using reverse transcriptase. For cloning of double-stranded cDNA the 3′ ends were tailed with oligo(dC) and inserted into the *Pst*I site of the dG-tailed plasmid pBR322, which was then grown in *E. coli*. Several thousand clones were obtained, all of which were subgenomic in size (about 1 to 3.5 kilobases). Inserts of these clones were screened for HAV specificity by Northern blot (RNA blot) hybridization to HAV RNA which had been extracted from highly purified virus and which had a size of 7.5 kilobases (32S), typical for HAV RNA (2, 17). HAV-specific cDNA clones were nick translated and used as molecular probes for screening additional clones by the technique of Grunstein and Hogness (7). HAV-specific cDNA clones were aligned along the HAV genome, beginning at the 3′ end of the RNA [identified by hybridization to poly(A)] and extending to the physical 5′ end of the genome. The mapping was checked by hybridization of various clones to each other by Southern blot hybridization analysis.

Nine representative cDNA clones, overlapping each other and covering the entire genome, and a map of restriction enzyme sites of the genome which were deduced from these clones are shown in Fig. 1.

Expression of VP1 cDNA clones in *E. coli*. Recent results from biochemical and immunological studies (3, 8, 9) indicate that, as in other picornaviruses, the capsid protein VP1 of HAV contains a major antigen. We reconstructed various HAV cDNA clones coding for part or all of VP1, inserted them into the expression vector pUR290 (16) (Fig. 2), and studied the expression of HAV VP1. The HAV cDNA clones were inserted into the plasmid vector pUR290 so that the HAV sequences were fused just before the 3′ end of the *lacZ* gene, thus yielding a fusion protein of the β-galactosidase and the joined HAV VP1 sequences.

E. coli cultures were transformed with each of the clones and grown to confluence, the promoter was induced by addition of isopropylthiogalactoside, and incubation was continued for 90 min. The bacteria were lysed and sonified, and the lysate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, either stained with Coomassie blue (Fig. 3A) or blotted to nitrocellulose (Western blot [immunoblot]), and then reacted with anti-HAV (5) (Fig. 3B) or preimmune rabbit serum (data not shown). The serum had been obtained by immunization of rabbits with purified HAV particles; it neutralized the infectivity of HAV and reacted with virus particles in an immunocompetition assay (a modified HAV antibody test; Abbott Laboratories, North Chicago, Ill.) and with purified VP1 in an immunoblot.

Our analysis of the recombinant protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining showed that β-galactosidase was produced as a predominant protein after induction of the *lac* promoter of pUR290 (control) without an HAV insert (Fig. 3A). Clones containing the HAV sequences showed less of the

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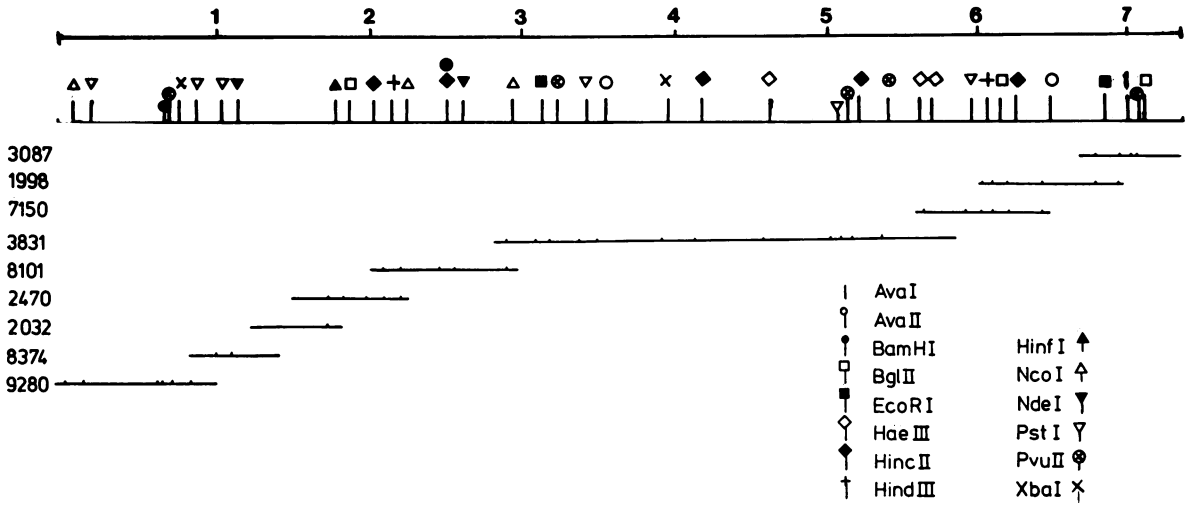

FIG. 1. Schematic representation of the HAV cDNA clones and their restriction enzyme map. The HAV cDNA sequence is numbered (in kilobase pairs) from the 5′ end to the 3′ end.

β-galactosidase band but had additional polypeptides that were larger than β-galactosidase (i.e., fusion proteins). (The intermediate-sized products, larger than β-galactosidase but smaller than full-length fusion protein, were probably either breakdown products or early-terminated products of the fusion protein. This phenomenon is sometimes observed with recombinant fusion proteins.) In the Western blot, the recombinant proteins of clones 290-17 and 290-17-7 (Fig. 2) reacted with anti-HAV serum, whereas those of clone 290-5 and the pUR290 vector without insert (control) did not react; none of the clones reacted with the preimmune rabbit serum (data not shown). It is unclear why clone 290-17-7 exhibited two bands and clone 290-17 exhibited a broad band and minor bands smaller than fusion protein in the Western blots. We think these polypeptides are either breakdown intermediates or early-terminated fusion polypeptide chains (as partly seen also in Coomassie blue-stained blots [Fig. 3A]); they were not detected in the noninduced lysate.

It is interesting that whereas the fusion proteins of clones 290-17 and 290-17-7, which contained the N-terminal third of the VP1 sequence, reacted with anti-HAV, the recombinant proteins of pUR290 and 290-5-2 did not, indicating that HAV antigenicity might be determined within the N-terminal amino acids of VP1 (*Nco*I to *Bam*HI, about 60 amino acids [Fig. 2]).

The solubility of the antigenic recombinant protein was determined by pelleting the crude bacterial lysate and analyzing the pellet and the supernatant fraction separately by polyacrylamide gel electrophoresis (data not shown). About 10 to 20% of the antigenic protein in the lysate was soluble, but most of the antigenic protein was present in the pellet fraction, as seen with other recombinant proteins (16). Attempts to analyze the antigenicity of the recombinant protein in an enzyme-linked immunosorbent assay were unsatisfactory because of the high degree of insoluble antigenic material that could not be coated to the plate.

Our recombinant proteins reacted with rabbit anti-HAV serum but not with human convalescent-phase serum; it is possible that convalescent-phase serum only recognizes intact virion structures, whereas our rabbit serum also recognizes disintegrated (i.e., VP1) antigen structures. Experiments to immunize rabbits with the recombinant protein and analyze the antiserum for HAV-neutralizing activity are in progress.

Our data suggest that a probable site of HAV antigenicity is in the region of the first 60 amino acids of the N-terminal part of the VP1 protein. This agrees with a report (3) of a comparative surface structure analysis of the HAV VP1 sequences and immunization studies with a synthetic oligopeptide; this report concluded that the antigenic site that probably induces HAV-neutralizing antibodies is lo-

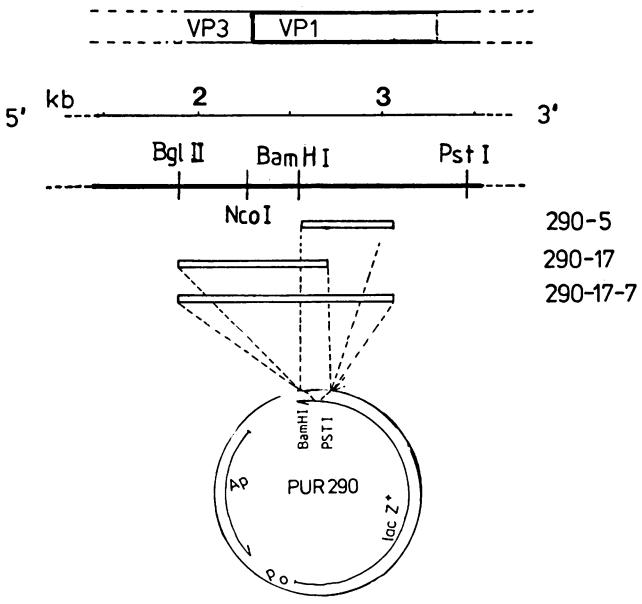

FIG. 2. Construction of expression clones from existing HAV cDNA clones. The DNA fragments shown, representing part of the VP1 gene and/or a few adjacent sequences, were inserted into the linker of plasmid pUR290 close to the 3′ end of the *lacZ* gene by cutting the 5′ ends of the HAV cDNA fragments with either *Bam*HI or *Bgl*II and ligating to the *Bam*HI site of the polylinker. The 3′ ends of the inserted HAV cDNA fragments are their natural ends, and hence were cleaved by *Pst*I from the pBR322 vector and ligated into the *Pst*I site of the polylinker of pUR290. kb, Kilobase pairs.

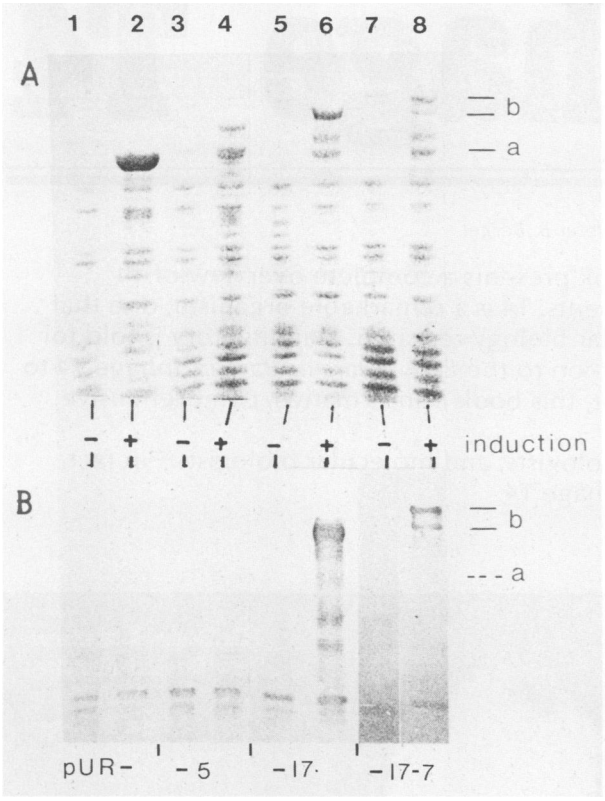

FIG. 3. Analysis of the recombinant fusion protein by polyacrylamide gel electrophoresis and Western blot. (A) Bacterial lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Lanes: 1 and 2, pUR290 without HAV insert; 3 and 4, clone 290-5; 5 and 6, clone 290-17; 7 and 8, clone 290-17-7. Lanes 1, 3, 5, and 7 were noninduced; lanes 2, 4, 6, and 8 were induced with isopropylthiogalactoside. Band a, β-galactosidase; band b, fusion proteins of galactosidase and HAV VP1 peptides. (B) A sodium dodecyl sulfate-polyacrylamide gel parallel to that in panel A was blotted to nitrocellulose (Western blot) and then reacted with rabbit anti-HAV. Lanes and band positions are as in panel A.

cated at VP1 between amino acids 5 and 114. These data together with ours suggest that the HAV VP1 carrying the (or one of the) antigenic site(s) responsible for inducing neutralizing antibodies is closer to the N-terminal end of VP1 than in other picornaviruses.

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