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Cloned Fragment of the Hepatitis Delta Virus RNA Genome: Sequence and Diagnostic Application

K. J. DENNISTON,* B. H. HOYER, A. SMEDILE, F. V. WELLS, J. NELSON, J. L. GERIN†

Hepatitis delta virus (HDV) is a replication-defective etiological agent of hepatitis that requires hepatitis B virus (HBV) as a helper. A complementary DNA (cDNA) fragment of the RNA genome of HDV was cloned into the plasmid vector pBR322, and the primary nucleotide sequence and predicted protein products of the cDNA fragment were determined. This cloned cDNA fragment has been used as a sensitive radioactive probe for the detection of HDV RNA in the serum of patients with either acute or chronic HDV infections.

HEPATITIS DELTA VIRUS (HDV), a naturally occurring defective virus of man, depends on hepatitis B virus (HBV) for its replication and expression (*1, 2*). For the large population of chronic HBV carriers worldwide, HDV represents a serious medical problem because HDV infection frequently results in severe and progressive liver disease, delta hepatitis. Epidemics of delta hepatitis have occurred in the general population of underdeveloped countries where HBV is endemic. In developed countries the disease appears to be limited to certain high-risk groups, including intravenous drug addicts and hemophiliacs (*2*).

The viral nature of the HDV was established in chronically HBV-infected chimpanzees, an experimental model of acute HDV infection and disease (*3*). In acute phase serum, HDV circulates as a 36-nanometer particle having the HBV surface antigen (HBs) on its surface and the delta antigen (HDAg) and HDV RNA within the particle (*4*). The 1.75-kilobase (kb) HDV RNA is not polyadenylated, has considerable secondary structure, and appears to be single-stranded (*5*). The physical association of HDV RNA with HDAg (an established marker of HDV infection) within the viral particle (*1, 4, 5*), the lack of hybridization between complementary DNA (cDNA) transcripts of HDV RNA and HBV or host DNA's (*5*), and, most important, the near equivalence between the HDV RNA concentration and the HDV infectivity titer of an acute phase chimpanzee serum (*2, 6*) indicate that HDV RNA is the genome of this unusual viral agent. No information has been reported on the pri-

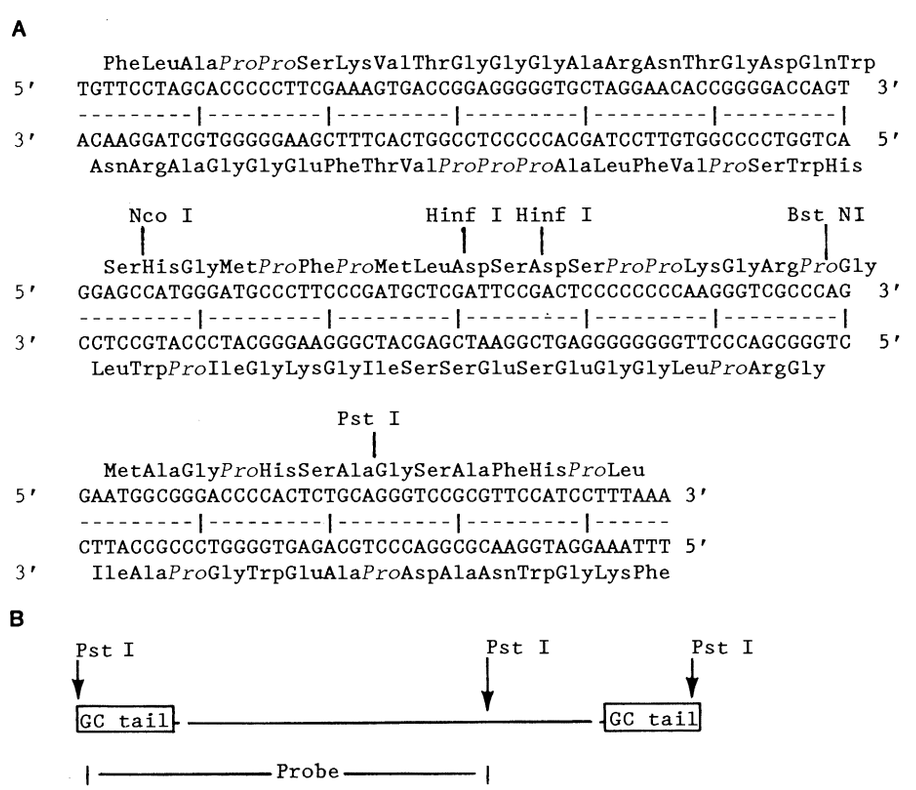

Fig. 1. (A) The primary nucleotide sequence of the 166-bp HDV RNA cDNA clone, pKD3. Above and below the nucleotide sequence are the predicted amino acid sequences of the single open reading frames in each strand. The sequence was obtained by the method of Maxam and Gilbert (*14*). Nucleotide sequence determinations were made from 3′-end-labeled Pst I termini and 5′-end-labeled Nco I, Hinf I, and Bst NI sites. These sites are indicated above the sequence. (B) The Pst I subfragment (139 bp + 14 bp GC tail) was used to make the hybridization probe.

Division of Molecular Virology and Immunology, Georgetown University Medical Center, Rockville, MD 20852.

*Present address: Department of Biological Sciences, Towson State University, Towson, MD 21204.
†To whom correspondence should be addressed.

mary sequence of the HDV genome or its gene products. We report the nucleotide and predicted amino acid sequences of a cDNA clone from a portion of the HDV genome and the use of this clone to detect HDV RNA by hybridization methods in serum of patients with acute and chronic HDV infections.

Cloning experiments were carried out with RNA purified from the serum of an experimentally infected chimpanzee, A-20 (*1*). Because the viral RNA is not polyadenylated, a 3′ poly(A) tail was synthesized with the *Escherichia coli* poly(A) polymerase (*5*). Reverse transcription was then carried out with oligo(dT) as a primer (*7*). From such cloning experiments a single recombinant plasmid, pKD3, was isolated. The plasmid showed positive hybridization in colony transfers (*8*) and in Southern blot gel transfers (*9*) with a radiolabeled HDV cDNA probe synthesized by reverse transcription of HDV RNA. The sequence of the cloned fragment is shown in Fig. 1A. The 166–base pair (bp) fragment is 63 percent G+C (G, guanine; C, cytosine). The first 48 bp can be represented as a hairpin having a 20-bp stem with one mismatch and an 8-bp loop. There is no basis to suggest that the three adenine residues at the junction between the cloned fragment and the GC tail represent the 3′ terminus of the HDV genome or the poly(A) tail added to the RNA. A 153-bp Pst I subfragment (Fig. 1B) was isolated, nick-translated, and then used as a probe to detect HDV RNA on a Northern blot transfer from a formaldehyde agarose gel (*10, 11*). To test the specificity of the cloned cDNA fragment, we hybridized lanes of HDV RNA from the same gel, using nick-translated vector, pBR322, or reverse-transcribed HDV RNA as probes (Fig. 2). The pBR322 probe did not hybridize with any RNA species (left panel); however, both the cloned pKD3 cDNA probe (middle panel) and HDV cDNA probe (right panel) hybridized to an RNA of approximately 1.75 kb. The cloned cDNA fragment did not hybridize to nucleic acid preparations from sera of normal or HBV-positive human subjects or to DNA from various normal mammalian sources. The specificity of the cloned fragment was further demonstrated by the analysis of serial serum samples from a chimpanzee with experimental acute HDV infection (*1*). The cloned cDNA fragment detected HDV RNA during the period of HD antigenemia. The serum concentration of HDV RNA paralleled that of HDAg. HDV RNA was not detected in serum after antibody to HDAg appeared (*1*).

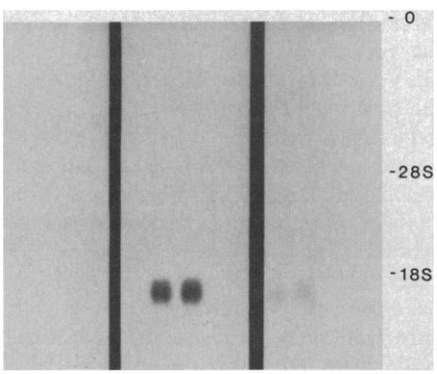

Fig. 2. Northern blot analysis of HDV RNA from the acute phase serum of an HDV-infected chimpanzee (*1*) was performed with three radioactive probes. HDV particles were purified from plasma by centrifugation through 20% sucrose in 0.02*M* Hepes and 0.1% bovine serum albumin, *p*H 7.5. After proteolytic digestion [proteinase K (1 mg/ml), yeast transfer RNA (50 μg/ml) in Hepes-HCl, *p*H 7.5, 50 m*M* EDTA, 200 m*M* NaCl, and 1% sodium dodecyl sulfate overnight at 37°C], RNA was purified by phenol-chloroform extraction. RNA was electrophoresed through a 2.2*M* formaldehyde–1% agarose gel (*10*) and transferred to nitrocellulose paper with 20× standard saline citrate (*11*). Duplicate samples were hybridized with (left lanes) ^{32}P-labeled pBR322, (middle lanes) ^{32}P-labeled cloned HDV cDNA fragment, or (right lanes) HDV RNA labeled with ^{32}P-dCTP with the use of avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku America). The cloned cDNA fragment used to make probe was the larger Pst I subfragment (see Fig. 1B) that had been purified by agarose gel electrophoresis before nick translation (New England Nuclear nick translation kit). The positions of the nonradioactive markers (18*S* and 28*S* ribosomal RNA) are indicated on the right.

Analysis of the sequence of the cloned fragment (Fig. 1A) revealed on each strand a single reading frame that is open through the entire sequence. Each strand encodes an unusual peptide. Both predicted peptides have nine proline residues. Overall, each has 63 percent alpha helix–destabilizing amino acids. There are few basic amino acids (13 percent on the top coding strand and 9 percent on the bottom coding strand). Whether these predicted peptides represent a portion of HDAg is unknown, but synthetic peptides prepared from these sequences can be examined for HDAg activity.

Chronic delta hepatitis is a frequent outcome of HDV infection of chronic HBV carriers (*2*). The diagnosis of chronic HDV infection often requires the demonstration of intrahepatic HDAg in biopsy tissue. Serologically, chronic infection is indicated by a high titer of total antibody to HDAg and persistent immunoglobulin M specific for HDAg (*12*); HDAg is not detectable by radioimmunoassay. Experiments were performed to determine whether the Pst I subfragment of pKD3 could be used as probe in a diagnostic hybridization-based assay for HDV RNA in the serum of patients with HDV infection. RNA's were purified from the serum of a patient with acute HDV infection and a patient with chronic delta hepatitis. Results of Northern blot hybridization experiments with these RNA's and with RNA from an acutely infected chimpanzee are compared in Fig. 3. In each case a band of hybridization of 1.75 kb was obtained. Only 10 μl of serum from an acutely infected chimpanzee (Fig. 3, lane 1) and 1 μl of serum from the acutely infected patient (Fig. 3, lane 2) were required for detection of a strong band of hybridization in 3 days. In contrast, 0.5 ml of serum from the chronic HDV carrier (Fig. 3, lane 3) was required for a comparable signal. This 50- to 500-fold difference in volume probably reflects the lower concentration of HDV in the serum of HDV carriers. The serum requirements for this type of analysis, however, are readily obtain-

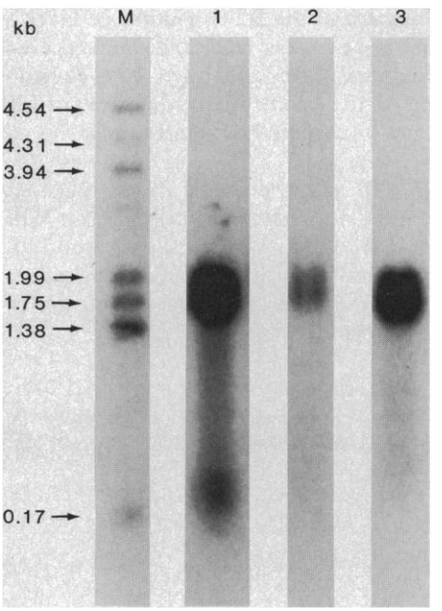

Fig. 3. Detection of HDV RNA in the acute phase serum of a chimpanzee experimentally infected with HDV (C57, lane 1), the acute phase serum of a patient with natural HDV infection (lane 2), and serum from a patient with chronic delta hepatitis with intrahepatic HDAg (lane 3). RNA samples were prepared, subjected to electrophoresis, transferred to nitrocellulose, and hybridized as described in Fig. 2. RNA from 10 μl, 1 μl, and 0.5 ml of serum were electrophoresed in lanes 1, 2, and 3, respectively. The molecular weight marker (lane M) is a mixture of five restriction endonuclease digestions of pKD3. Eco RI, Bgl I, Hinc II, Rsa I, and Hinf I digestions were carried out according to the conditions specified by the supplier (Bethesda Research Laboratories). After inactivation of the enzymes by phenol-chloroform extraction, the five digests were mixed and 10 pg of this marker mix were used. The sizes of the fragments are indicated on the left.

able in practice, and a probe of the cloned Pst I cDNA fragment from pKD3 readily detects HDV RNA in serum of patients with acute or chronic HDV infections (*13*).

The nucleic acid hybridization technique with this cloned cDNA fragment as probe represents a useful noninvasive diagnostic assay for the identification of chronic HDV carriers who are at high risk of serious and progressive liver disease. The cDNA probe can be used for studying the replication of HDV in infected liver tissues, and synthetic oligonucleotides of the cDNA probe will be useful as cDNA primers.

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Differences in Adrenergic Recognition by Pancreatic A and B Cells

F. C. SCHUIT AND D. G. PIPELEERS

The adrenergic control of glucose homeostasis is mediated in part through variations in the release of pancreatic hormones. In this study, purified pancreatic A and B cells were used to identify the recognition and messenger units involved in the adrenergic regulation of glucagon and insulin release. Catecholamines induced β-adrenergic receptor activity in A cells and α_2-adrenergic receptor activity in B cells. The two recognition units provoked opposite variations in the production of cellular cyclic adenosine monophosphate, the β-adrenergic unit enhancing the nucleotide's permissive effect on amino acid–induced glucagon release and the α_2-adrenergic unit inhibiting that upon glucose-induced insulin release. In both cell types, catecholamines interact powerfully with the synergistic control of hormone release by nutrient- and (neuro)hormone-driven messenger systems.

CATECHOLAMINES PLAY A MAJOR role in the rapid adjustment of various metabolic pathways to physiologic and pathologic demands (*1*). Their function is partly accomplished within the endocrine pancreas, where the hormonal secretion is under tight adrenergic control (*2–3*). The mechanisms whereby epinephrine and norepinephrine regulate insulin and glucagon release are still controversial (*4–5*), their investigation being hampered by the heterogeneous cell composition of the experimental models used so far. In vitro, the secretory response of unpurified islet tissue is markedly influenced by locally released hormones and other intercellular interactions (*6–8*), which stresses the need to investigate the regulation of pancreatic hormone release in purified islet cell preparations. This requirement is even more crucial in analyzing the effects of catecholamines, because these compounds may interact with various islet cell types (*2–5*) and because they are, in addition, stored in intact islet tissue (*9*). For these reasons, we examined the effects of epinephrine, norepinephrine, and adrenergic agonists and antagonists on purified islet A and B cells.

By using the technique of autofluorescence-activated cell sorting, we separated islet cells on the basis of their ability to vary the redox state of their flavin and pyridine nucleotides according to the extracellular glucose concentration (*10, 11*). Such isolated cell suspensions are more than 95 percent pure and vital (*11*) and can be used in vitro to study the stimulus-secretion coupling within pancreatic A and B cells (*7, 8, 12*). With this method, it was previously shown that both the glucagon and the insulin release process undergo a synarchic regulation by nutrient- and hormone-driven control units (*7, 8*). The hormone-induced message appeared to be mediated by adenosine 3′,5′-monophosphate (cAMP), which was attributed a permissive role in the nutrient regulation of pancreatic hormone release (*7, 8, 12*). We have now studied the sites where catecholamines influence the secretory process of islet A and B cells and hence regulate glucose homeostasis.

Department of Metabolism and Endocrinology, Vrije Universiteit Brussel, 1090 Brussels, Belgium.

The addition of epinephrine to purified A cells provoked a dose-dependent increase in both cellular cAMP content and nutrient-induced glucagon release (Fig. 1). Both effects were detected from $10^{-8}M$ on, reached maximal amplitudes between 10^{-7} and $10^{-6}M$, and were, at $10^{-7}M$, comparable to those measured at $10^{-7}M$ norepinephrine (Fig. 2). In purified B cells, both catecholamines lowered basal cAMP content by 46 ± 12 percent (mean ± SE, $n = 4$). This effect was not associated with a further reduction in the secretory activity of isolated B cells, as may have been expected from cells whose impaired secretory function had already been attributed to a cAMP deficiency in the absence of catecholamines (*7*). In the presence of $10^{-8}M$ glucagon, isolated B cells regained the cAMP levels and secretory activity of intact islet tissue (*7, 12*); under this condition epinephrine provoked a dose-dependent and parallel inhibition of both cAMP formation and glucose-induced insulin release (Fig. 1). These suppressive effects were detected from $10^{-9}M$ on and reached an 80 percent inhibition at $10^{-7}M$; norepinephrine at $10^{-7}M$ induced a 75 percent inhibition (Fig. 2).

Our data illustrate that catecholamines interact directly with both pancreatic A and B cells, exerting a stimulatory action on glucagon release without necessary participation of other hormones, while their inhibitory effect on insulin release was noted only in the presence of an adenylcyclase activator such as glucagon. Isolated islets, with their intercellular interactions and different adrenergic recognition units, can thus not be expected to provide accurate information concerning the adrenergic effects on a particular cell type. With the use of pure B cells, the adrenergic inhibition of insulin release could be attributed to a decreased cAMP production over the entire concentration range tested, which makes it unnecessary to postulate cAMP-dependent and -in-