Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8774–8778, November 1986
Microbiology

Structure and replication of the genome of the hepatitis δ virus

(viroids/virusoids)

PEI-JER CHEN*, GANJAM KALPANA*, JANET GOLDBERG*, WILLIAM MASON*, BARBARA WERNER†, JOHN GERIN‡, AND JOHN TAYLOR*

*Fox Chase Cancer Center, Philadelphia, PA 19111; †State Laboratory Institute, Massachusetts Department of Public Health, Jamaica Plain, MA 02130; and ‡Division of Molecular Virology and Immunology, Georgetown University School of Medicine, Rockville, MD 20852

Communicated by Baruch S. Blumberg, August 11, 1986

ABSTRACT **The hepatitis δ virus can be found in the serum and liver of some hepatitis B virus patients. We now report that the RNA genome of serum-derived δ virus is single-stranded and circular. Livers of infected chimpanzees or woodchucks contained as many as 300,000 copies of genomic strand RNA per average cell, and at least some of this RNA had a circular conformation. Also present in the livers were RNA species complementary to the virion RNA. The genomic RNA was 5–22 times more abundant than this antigenomic strand. Some of the antigenomic RNA was complexed with genomic RNA, as evidenced by the fact that at least 34% of the antigenomic RNA was resistant to digestion with either RNase A in 0.3 M NaCl or S1 nuclease. Some of the antigenomic RNA was in a circular conformation. These and other findings showed that the structure and replication of hepatitis δ virus are in many ways similar to those of the previously described plant viroids, virusoids, and satellite RNAs.**

The human hepatitis δ virus, as described by Rizzetto *et al.* (1), is considered to be a defective virus, in that its natural transmission has been detected only in the presence of hepatitis B virus. This dependence has been demonstrated by experimental transmission of the agent to chimpanzees chronically infected with hepatitis B (2, 3). It has also been extended by transmission studies to woodchucks in which the woodchuck hepatitis B virus provided the transmission function (4). Antibodies to the woodchuck hepatitis virus envelope have been used to show that the concomitant hepadnavirus infection provides the packaging for the δ viral genome. The characteristic antigen of the δ particle, the δ antigen, is packaged, with the virion RNA, inside a 36-nm-diameter lipoprotein envelope in which is incorporated hepatitis B surface antigen (2, 5).

Bonino *et al.* have previously reported that the RNA of the δ particle is a 1.7-kilobase (kb) single-stranded species (5). Denniston *et al.* used this RNA to make a cDNA clone in pBR322, designated pKD3, with a 166-base δ-specific insert (6). As presented here, we have derived an independent cDNA library, screened it with pKD3, made strand-specific probes, and used these probes to study both the virion and intracellular RNA from livers of infected chimpanzees and woodchucks. Our findings on the structure and replication of the δ genome support the previous conjecture (7) that the agent bears similarities to the viroids and virusoids of plant cells (8).

MATERIALS AND METHODS

Viral RNA. For most experiments, RNA was directly extracted from serum after dilution (1:20) into a buffer containing 10 mM Tris, 10 mM EDTA, 0.1% sodium dodecyl sulfate, and Pronase at 1 mg/ml. After digestion for 1 hr at 37°C, the sample was extracted once with phenol and twice with ether and then collected by precipitation with ethanol, using dextran as carrier. In some cases the virions were first purified by centrifugation through a sucrose cushion (5) or by isopycnic centrifugation in cesium chloride (5). RNA was then extracted as described.

Liver RNA. Liver samples were from either acutely infected chimpanzees (two; *Pan troglodytes*) or woodchucks (two; *Marmota monax*). The experimental infections have been previously described (3, 4). RNA was extracted with guanidinium isothiocyanate followed by equilibrium centrifugation in the presence of cesium chloride (9, 10). The RNA was then collected by precipitation with ethanol and used after an additional digestion with Pronase and extraction with phenol. Poly(A)-containing RNA was isolated, after denaturation at 68°C, by two passages of binding to oligo(dT)-cellulose (10).

cDNA Cloning. Total RNA from the liver of an infected woodchuck was preparatively analyzed by rate zonal sedimentation into a sucrose gradient. The virus genome-sized RNA was located and used for cDNA cloning in λgt11 by the method described by Sorge *et al.* (11) except that oligo(dT) primers were replaced by random oligonucleotides (12). We selected positive clones, using as a probe the purified insert of pKD3 of Denniston *et al.* (6). For the studies described below, we used only one such λ clone, which was found to have a 650-base insert.

Hybridization Probes. The 166-base insert of the above-mentioned pKD3 plasmid was released as two fragments by digestion with *Pst* I. The large, 140-base, fragment of δ sequences was used as template for nick-translation. To make strand-specific probes, the 650-base-pair *Eco*RI fragment of the δ-positive λ clone was purified and inserted into phage M13 DNA. A pair of complementary M13 DNAs were purified and used to make strand-specific probes as previously described (13).

Agarose Gel Electrophoresis. Samples were treated with glyoxal and then subjected to electrophoresis in horizontal gels of 1.5% agarose in 10 mM sodium phosphate (pH 6.8) and 10 μM aurintricarboxylic acid to inhibit nucleases (10). After electrophoresis at 6 V/cm for 2 hr the samples were electrophoretically transferred out of the gel (6.7 V/cm, 2 hr) onto a Zeta-Probe nylon membrane (Bio-Rad), using an IBI apparatus and buffer of 10 mM Tris acetate. The filters were then baked at 80°C for at least 1 hr. Glyoxal was then removed from the sample by adding the filter to 20 mM Tris·HCl (pH 8.0) that had been heated to 100°C and allowing the solution to cool to room temperature. The filter was prehybridized and then incubated for 16 hr at 42°C in the presence of radioactive probe in a solution containing 50% (vol/vol) formamide (10). The filters, bearing the hybridized probe,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: kb, kilobase(s).

were then washed and autoradiographed as previously described (10).

Polyacrylamide Gel Electrophoresis. Samples were dissolved in a sample buffer containing 8 M urea and then heated at 100°C for 5 min. They were applied to a prerun vertical gel of 3% polyacrylamide made up in the same urea-containing buffer. Both the prerun and the electrophoresis were carried out at a current such as to maintain the gel temperature at 55°C. After electrophoresis the samples were electrophoretically transferred to Zeta-Probe, as described above.

HybriSlot Analysis. Samples of extracted RNA were glyoxalated (as for agarose gel electrophoresis, but without dimethyl sulfoxide) and then a series of twofold dilutions, beginning with 0.16 μg, were applied with suction to a Zeta-Probe membrane by using a HybriSlot apparatus (Bethesda Research Laboratories). Also applied was pKD3 double-stranded DNA, in a series of dilutions beginning with 0.025 μg. The filter was then treated as for standard hybridization (see above). The filter was first hybridized with the M13 probe for the genomic strand. After appropriate autoradiography the filter was stripped by heating and then rehybridized with the M13 probe for the antigenomic strand. Autoradiograms were scanned with an E-C densitometer coupled to a Hewlett–Packard HP3380 integrator. The signals obtained with the two M13 probes were normalized relative to those obtained with the double-stranded pKD3 standard.

RESULTS

The following experiment was undertaken to test the strand specificity of our M13 probes. Denniston *et al.* (6) have examined δ virion RNA by agarose gel electrophoresis under denaturing conditions followed by hybridization using a plasmid probe, pKD3. They detected an RNA species with an apparent length of 1.75 kb. Using agarose gel electrophoresis of glyoxalated virion RNA, we obtained a similar result, as shown in Fig. 1, lane 1. In addition, we found that of our pair of M13 probes, only one detected this RNA species (lane 2). No signal was detected by using the probe for the opposite strand (lane 3). Thus we concluded that our probes were specific and that only one polarity of δ RNA was packaged in virions. [For 18 separate human serum samples examined by slot hybridization and densitometric analysis, we found that the amount of this genomic strand was at least 30–80 times more than the antigenomic strand (data not shown)].

To study the mechanism by which this RNA is replicated in infected cells, we used the M13 probes to detect δ-related nucleic acid sequences as extracted from the livers of infected chimpanzees. In agreement with Hoyer *et al.* (7), we were unable to detect δ-related sequences in liver DNA (data not shown). In contrast, when these probes were applied to a gel of total RNA from the liver of an infected chimpanzee, they detected both genomic (lane 4) and antigenomic (lane 5) species. In the liver the major species appeared to be 1.7 kb in length. However, there was an additional band at the equivalent of about 3.5 kb. This band was detected both as genomic and antigenomic RNA, and in some experiments it could be resolved as two adjacent bands.

We quantitated the genomic and antigenomic RNA sequences by using slot hybridization. Results are summarized in Table 1, for the total liver RNA of two infected chimpanzees, for two infected woodchucks, and, as a negative control, one uninfected woodchuck. After normalization for the sensitivity of the two probes, we deduced that, in the infected liver, the genomic strands were present in a 5- to 22-fold excess relative to antigenomic RNA. Also, we deduced the average number of δ-related RNA species per average liver cell. There were the equivalent of 110,000–320,000 molecules of genomic δ RNA for each cell in the liver. [In contrast to these numbers, 18 δ antigen-positive human sera contained $0.3–25 \times 10^{10}$ molecules of genomic RNA per ml of serum (data not shown).]

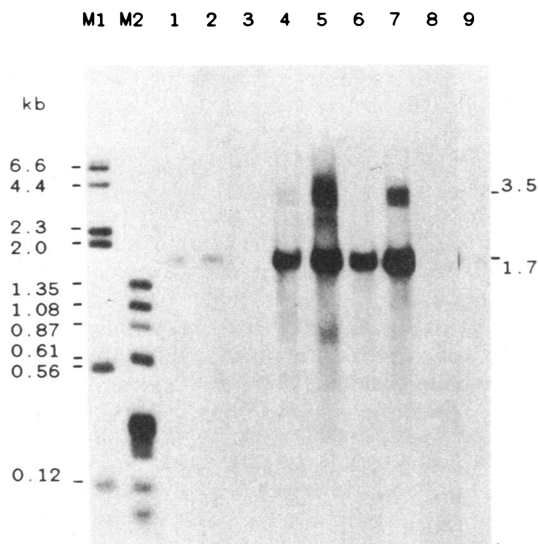

FIG. 1. Analysis of δ RNA by glyoxalation and electrophoresis into a gel of 1.5% agarose. Lanes M1 and M2 show restriction endonuclease *Hin*dIII fragments of phage λ DNA and *Hae* III fragments of phage ϕX174 replicative form DNA, respectively, electrophoresed after denaturation in the presence of glyoxal. The lengths of these single-stranded fragments are indicated at the left side. Lanes 1–3 represent virion RNA (extracted from 5 μl of serum) as assayed by three different probes: lane 1, double-stranded probe to pKD3; lane 2, an M13 probe detecting genomic RNA; and lane 3, a complementary M13 probe that does not detect genomic RNA and is therefore specific for antigenomic RNA. Lanes 4 and 5 represent total chimpanzee liver RNA (2 μg) as assayed with M13 probe for genomic and antigenomic RNA, respectively. Similarly, lanes 6 and 7 contain poly(A)-deficient species of chimpanzee RNA, and lanes 8 and 9 have poly(A)-containing RNA. In lanes 6 and 8, the M13 probe was for genomic RNA, and in lanes 7 and 9 the probe was for antigenomic RNA.

Previously Hoyer *et al.* reported that the δ virion RNA lacks poly(A) (7). We confirmed this (data not shown) and tested whether the same was true for the genomic or antigenomic RNA from livers of infected chimpanzees. Our finding, as shown in Fig. 1, was that the majority of genomic and antigenomic RNA in the liver lacked poly(A). [With an increased autoradiogram exposure, we were able to detect a minor component of the antigenomic RNA (1%) with a size of about 0.8 kb, that behaved as poly(A)-containing RNA.] As a positive internal control for these studies, we rehybridized the blot with a probe to human hepatitis B virus and showed that in the poly(A)-containing fraction there were, as expected (14), hepatitis B RNA species (data not shown).

Table 1. Quantitation of δ RNA in liver samples

Source of liver RNA	Ratio of genomic to antigenomic RNA*	Average no. of molecules of δ RNA per liver cell†	
		Genomic	Antigenomic
Infected chimpanzee A	21.7	176,000	8,100
Infected chimpanzee B	4.7	294,000	63,000
Infected woodchuck A	5.0	323,000	65,000
Infected woodchuck B	15.1	109,000	7,200
Uninfected woodchuck	—	<600	<200

*Deduced by HybriSlot analysis.
†The signal obtained with a known amount of pKD3 provided an absolute standard. It was assumed that each liver cell was infected and contained 25 pg of total RNA.

In our characterization of the δ RNAs in virions and in infected chimpanzee liver by electrophoresis into agarose gels, we sometimes observed that the 1.7-kb RNA species actually migrated as two adjacent bands of approximately equal intensity (data not shown). Since the electrophoretic mobility of linear molecules relative to linear markers should be independent of electrophoretic conditions, it was thus possible that the two species resolved by such electrophoresis differed not in length but in conformation. Others have found that significant effects of conformation on the electrophoretic mobility of single-stranded RNA species can be obtained by using polyacrylamide gels under denaturing conditions; this has been reported by those studying viroids (8), virusoids (8), and circular RNA splicing intermediates (15, 16). Under such conditions, circular RNAs migrate much slower than linear molecules of the same length. Therefore, such a gel system was used to examine the mobility of δ RNAs extracted from virions and from infected chimpanzee liver. As shown in Fig. 2, lane 1, some preparations of δ virion RNA migrated predominantly as a discrete species, designated C, with a mobility much less than that expected for a linear 1.7-kb molecule. (Other preparations contained increasing amounts, up to 50%, of a second component, designated L, with the mobility of a linear molecule.) This result was in contrast with that obtained with a 1.5% agarose gel, in which δ virion RNA behaved as a 1.7-kb linear species (Fig. 1, lane 2). This slower mobility of species C in the denaturing polyacrylamide gel allowed the hypothesis that it was circular. To test this hypothesis we subjected the virion RNA, prior to electrophoresis, to a limited treatment with RNase T1, to introduce a single nick into some of these molecules. As shown in lanes 2–4 of Fig. 2, as a consequence of the digestion we were able to detect the species designated L, which had a mobility expected for a linear 1.7-kb molecule. Our interpretation was that species C was converted to species L. Thus, we concluded that the majority (as much as 96% in our experiments) of the RNA in δ virions was circular.

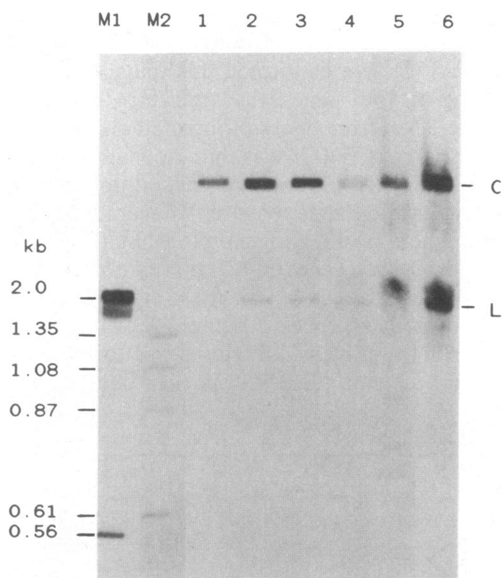

FIG. 2. Analysis of δ RNA by electrophoresis into a gel of 3% polyacrylamide containing 8 M urea. The markers and their indicated sizes are as in Fig. 1. Lanes 1–4 are virion RNA, as detected with an M13 probe for genomic RNA. Lanes 2–4 represent increasing extents of partial digestion with RNase T1. For this, virion RNA was mixed with 10 μg of carrier RNA and digested for 15 min at 50°C with 0.018, 0.036, and 0.072 unit (17). Lanes 5 and 6 represent chimpanzee liver RNA as assayed with M13 probes for genomic and antigenomic RNA, respectively.

We then used the same electrophoretic conditions to examine the total RNA from the liver of an infected chimpanzee. Again, we were able to detect species C (lane 5). In addition, there was a significant proportion of linear molecules present, band L. Actually, under these electrophoretic conditions, linear DNA molecules ranging from 2 to 23 kb all migrated close to the cut-off position of 2-kb markers, as shown in lane M1. [A similar result was obtained with linear RNA species of 2–9.4 kb (data not shown).] In total liver RNA the circular δ-related molecules were present not only as the genomic strand but also as the antigenomic strand (lane 6). As considered in *Discussion*, these findings have significant implications for the structure and replication of the δ virus genome.

Since we had found species of both genomic and antigenomic RNA in the liver, we investigated whether or not they were associated in a replicative structure. An initial approach was to examine the nuclease sensitivity of the genomic and antigenomic RNA species. Thus, samples were tested either with RNase A, under high-salt conditions in which it is single-strand specific, or with the single-strand specific nuclease S1. Quantitation was done by slot hybridization using the strand-specific probes. As summarized in Table 2, no more than a few percent of the virion RNA was resistant to RNase A. A similar result was obtained for the genomic RNA in the chimpanzee liver. However, as much as 34% of the antigenomic RNA was resistant. The data summarized in Table 1 showed that in the liver of the same chimpanzee, B, there was an excess of genomic relative to antigenomic RNA of about 5 to 1. The RNase data in Table 2 thus allow the interpretation that some of the antigenomic liver RNA homologous to the probe was base-paired with an equal amount of genomic RNA and that the majority of the genomic RNA was not base paired.

Before pursuing the above interpretation further, it should be noted that, under our assay conditions, the S1 nuclease gave somewhat different results. We found that even virion RNA was about 30% resistant to S1 nuclease. Since the hybridization assays of Fig. 1 and Table 1 showed that virions contain genomic sequences and no antigenomic sequences, it was concluded that the virion RNA of itself possessed significant S1-resistant secondary structure in the region detected by the 650-base probe. Since at the same time this secondary structure was accessible to RNase A, our interpretation was that the RNase A digestion was more stringent than the S1 nuclease digestion and that there was some mismatching in the intramolecular base pairing.

We also examined the nuclease digestion products by agarose gel electrophoresis, after denaturation in the pres-

Table 2. Nuclease resistance of δ sequences

Sample (sequences analyzed)	% resistance to digestion	
	By RNase A	By nuclease S1
Virion (genomic)	<2	30
Infected chimpanzee liver (genomic)	5	31
Infected chimpanzee liver (antigenomic)	34	100
Single-stranded RNA, control	<6	<5
Double-stranded RNA, control	100	100

RNase A digestions were in 100 μl of 0.3 M NaCl/0.03 M sodium citrate for 30 min at room temperature, using 100 μg/ml (18), followed by extraction with an equal volume of phenol. Nuclease S1 digestions were carried out in 125 μl of buffer for 1 hr at 37°C, using 8000 units/ml (19). The liver RNA was from chimpanzee B of Table 1. Samples were then precipitated with ethanol in the presence of 10 μg of carrier dextran. RNA was then quantitated by using a HybriSlot apparatus, and the data were normalized to the double-stranded RNA standard.

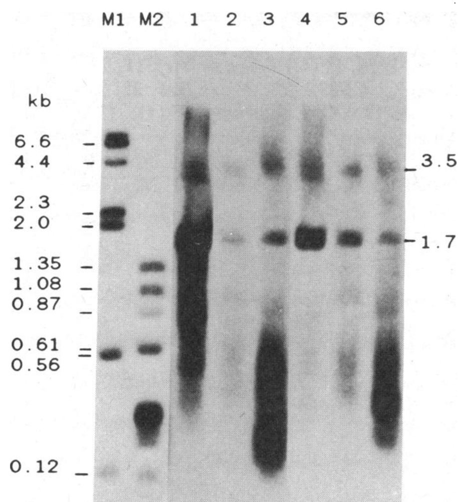

FIG. 3. Agarose gel electrophoretic analysis of δ sequences resistant to nuclease digestion. Chimpanzee liver RNA was analyzed along with markers M1 and M2 as in Fig. 1. Lanes 1–6 contain total chimpanzee liver RNA. The samples in lanes 2 and 5 and lanes 3 and 6 were digested with either RNase A or S1 nuclease, respectively, using conditions described for Table 2. Lanes 1–3 and 4–6 were assayed with M13 probes for genomic and antigenomic RNA, respectively.

ence of glyoxal. The RNA in the liver that was resistant to RNase A included both genomic and antigenomic sequences that migrated as 1.7 kb, as shown in Fig. 3, lanes 2 and 5, respectively. In addition, the 3.5-kb species were also largely resistant. These findings further supported the interpretation mentioned earlier, that some of the antigenomic RNA in the liver was base paired with an equivalent amount of genomic RNA. This interpretation also implies that the majority of genomic RNA in the liver would be free and thus sensitive to RNase A. As predicted, after an RNase A digestion, the majority of the genomic RNA could no longer be detected (lane 2). With S1 nuclease we were again able to confirm a nuclease-resistant core of 1.7- and 3.5-kb genomic and antigenomic RNA species (lanes 3 and 6, respectively). However, in addition, the S1 nuclease digestion of genomic RNA released a heterogeneous distribution of fragments that migrated at 0.2–0.5 kb. Such a result was consistent with the previous slot analysis in Table 2, which showed genomic RNA in liver and in virions to have significant resistance to S1 nuclease. Presumably such digestion occurred at only a limited number of sites on the virion RNA.

DISCUSSION

We have provided evidence suggesting that the δ-related RNA sequences in both virions and chimpanzee liver may exist as circular forms. We also detected in the liver antigenomic species, some of which appeared to be in a double-stranded RNA complex. These double-stranded species could be replicative forms of the genome, but we have not excluded the possibility that they are artifacts of RNA extraction. Most of the double-stranded RNAs were of the same length as the genome, but there were in addition species that appeared to be twice this length (the 3.5-kb species in Fig. 1, lanes 4 and 5). These features of the genomic RNA and of the putative replication intermediates are unusual for an animal virus system, and yet they are similar to what others have described for the transmissible disease-causing agents of plants known as viroids (8). These similar features allow the speculation that δ RNA, like a viroid, once inside the infected cell, can replicate without the help of a second virus. That is, the hepatitis B virus may simply provide the envelope proteins for transmission of the δ genome from cell to cell and from animal to animal. Nevertheless, there are important differences between δ and the viroids. One difference is that the δ genome is at least four times larger than the various viroids that have so far been reported (8). Also, in δ-infected cells, we have detected not only a genomic circular form but also an antigenomic circular form, something not yet detected for viroids (20). Thus, if δ replicates by a rolling-circle model similar to the models proposed for viroids, then an acceptable model would have to involve not one but two types of rolling circle, one of genomic and another of antigenomic RNA (21). Such a model is also consistent with our ability to detect what could be dimeric species of both genomic and antigenomic RNA in the infected liver.

δ virus also differs from viroids in that it is encapsidated. The δ RNA and antigen are found within the particle but not in a defined core structure as is found for a hepatitis B virus (5). Nevertheless, since the δ RNA is surrounded by a lipoprotein envelope containing the hepatitis B surface antigen, it can be considered as encapsidated. In this respect, δ may resemble two groups of encapsidated RNAs of plants, the satellite viruses and the satellite RNAs (20, 22). Both of these two groups need a helper virus for replication. The satellite viruses can encode their own coat protein, whereas the satellite RNAs depend upon the helper virus. A subgroup of the satellite RNAs, previously known as virusoids, have small circular genomes (less than 400 bases) that become encapsidated. The remainder of the satellite RNAs, together with the genomes of the satellite viruses, can be up to 1.2 kb in length. However, of all those studied so far, there is only one case in which a circular species has been detected in infected cells (23). The length of the δ genome, 1.7 kb, is more analogous to such satellites, and if δ codes for its own antigen, it would more closely resemble a satellite virus. Thus a critical question at this stage is to determine just how much help is provided for δ replication by the hepatitis B virus.

We thank Laura Coates for technical assistance and W. Thomas London and Jesse Summers for critical comments on the manuscript. This work was supported by Grants CA-22651, CA-06927-20, and RR-05538 from the National Institutes of Health, Grant MV-7 from the American Cancer Society, and an appropriation from the Commonwealth of Pennsylvania.

1. Rizzetto, M., Canese, M. G., Arico, S., Crivelli, D., Bonino, F., Trepo, C. G. & Verme, G. (1977) *Gut* **18**, 997–1103.
2. Rizzetto, M., Hoyer, B., Canese, M. G., Shih, J. W.-K., Purcell, R. H. & Gerin, J. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6124–6128.
3. Rizzetto, M., Canese, M. G., Gerin, J. L., London, W. T., Sly, D. L. & Purcell, R. H. (1980) *J. Infect. Dis.* **141**, 590–602.
4. Ponzetto, A., Cote, P. J., Popper, H., Hoyer, B. H., London, W. T., Ford, E. C., Bonino, F., Purcell, R. H. & Gerin, J. L. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2208–2212.
5. Bonino, F., Hoyer, B., Shih, J. W.-K., Rizzetto, M., Purcell, R. H. & Gerin, J. L. (1984) *Infect. Immun.* **43**, 1000–1005.
6. Denniston, K. L., Hoyer, B. H., Smedile, A., Wells, F. V., Nelson, J. & Gerin, J. L. (1986) *Science* **232**, 873–875.
7. Hoyer, B., Bonino, F., Ponzetto, A., Denniston, K., Nelson, J., Purcell, R. & Gerin, J. L. (1983) in *Viral Hepatitis and Delta Infection*, eds. Verme, G., Bonino, F. & Rizzetto, M. (Liss, New York), pp. 91–97.
8. Riesner, D. & Gross, H. J. (1985) *Annu. Rev. Biochem.* **54**, 531–564.
9. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
10. Hsu, T. W., Taylor, J. M., Aldrich, C., Townsend, J. B., Seal, G. & Mason, W. S. (1981) *J. Virol.* **38**, 219–223.
11. Sorge, J., West, C., Westwood, B. & Beutler, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7289–7293.
12. Taylor, J. M., Illmensee, R. & Summers, J. (1976) *Biochim. Biophys. Acta* **446**, 324–330.

13. Mason, W. S., Aldrich, C., Summers, J. & Taylor, J. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3097–4001.
14. Cattaneo, R., Will, H. & Schaller, H. (1984) *EMBO J.* **3,** 2191–2196.
15. Zaug, A. J. & Cech, T. R. (1986) *Science* **229,** 1060–1064.
16. Chu, F. K., Maley, G. F., West, D. K., Belfort, M. & Maley, F. (1986) *Cell* **45,** 157–166.
17. Donis-Keller, H., Maxam, A. M. & Gilbert, W. (1977) *Nucleic Acids Res.* **4,** 2527–2538.
18. Baltimore, D. (1968) *J. Mol. Biol.* **32,** 359–368.
19. Taylor, J. M., Faras, A. J., Varmus, H. E., Levinson, W. E. & Bishop, J. M. (1972) *Biochemistry* **11,** 2343–2351.
20. Diener, T. O. (1983) *Adv. Virus Res.* **28,** 241–283.
21. Branch, A. D. & Robertson, H. D. (1984) *Science* **223,** 450–455.
22. Francki, R. I. B. (1985) *Annu. Rev. Microbiol.* **39,** 151–174.
23. Lindhorst, H. J. M. & Kaper, J. M. (1984) *Virology* **137,** 206–210.