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Elimination of Hepatitis C Virus RNA in Infected Human Hepatocytes by Adenovirus-Mediated Expression of Ribozymes

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Hepatitis C virus (HCV), a positive-strand RNA virus, is the major infectious agent responsible for causing chronic hepatitis. Currently, there is no vaccine for HCV infection, and the only therapy for chronic hepatitis C is largely ineffective. To investigate new genetic approaches to the management of HCV infection, six hammerhead ribozymes directed against a conserved region of the plus strand and minus strand of the HCV genome were isolated from a ribozyme library, characterized, and expressed from recombinant adenovirus vectors. The expressed ribozymes individually or in combination were efficient at reducing or eliminating the respective plus- or minus-strand HCV RNAs expressed in cultured cells and from primary human hepatocytes obtained from chronic HCV-infected patients. This study demonstrates the potential utility of ribozyme therapy as a strategy for the treatment of hepatitis C virus infection.

Hepatitis C virus (HCV) infection is the cause of more than 90% of chronic non-A non-B hepatitis worldwide (2). It has been estimated that more than 1% of the world population is infected with HCV. Although transmission is parenteral in the majority of cases, the incidence and mechanisms of nonparenteral transmission remain uncertain. Viral infection is variable; persistent viremia occurs in about 90% of cases, chronic hepatitis occurs in 60 to 70% of cases, and 20 to 40% of infected individuals ultimately develop cirrhosis, end-stage liver disease, and/or hepatocellular carcinoma (2). According to recent data, HCV infection is the leading indication for liver transplantation in the United States, and chronic hepatitis C is associated with more than 50% of hepatocellular carcinoma in the United States (6a). Orthotopic liver transplantation is performed in patients with end-stage liver disease, but viremia persists in 100% of patients, and ultimately, the transplanted organ becomes reinfected (11).

HCV is an enveloped positive-strand RNA virus with a genome size of approximately 9.4 kb (5, 13) which encodes a single large open reading frame of ~3,010 amino acids. The single polyprotein gene product undergoes proteolytic processing to multiple gene products, including a capsid protein, two or more envelope glycoproteins (E1 and E2), and six or more nonstructural gene products (NS2, NS3, NS4a, NS4b, NS5a, and NS5b) which are thought to be involved in viral assembly and replication, respectively (10). After the infection of hepatocytes, the genomic, positive HCV RNA strand is translated. The NS5 gene product, an RNA-dependent RNA polymerase, presumably catalyzes the synthesis of replicative, minus HCV RNA strands which represent the template for producing more plus strands for the production of progeny virus.

The 330-bp 5′-noncoding region (5′NCR) forms a characteristic secondary structure contained within the IRES (internal ribosome binding site), which permits an efficient, cap-independent translation of the HCV genome, most likely in the same way as was found for members of the family of *Picornaviridae* such as poliovirus and encephalomyocarditis virus (3, 29). Additionally, the HCV 5′ NCR region probably has functional importance for replication and packaging as well (4, 27). The 5′NCR is highly conserved (92 to 100%) among HCV isolates of widely varying geographic origins (4, 12). Based on sequence analysis, multiple genotypic variants (strains or types) of HCV appear to exist (only 67% homology). HCV isolates are classified in at least six main types (at least 15 subtypes) with different patterns of worldwide distribution (27). Subtypes 1a and 1b are the most common isolates in the United States and Japan, respectively.

The only current medical therapy for HCV infection is alpha interferon, where about 40% of patients show a biochemical and virological response to therapy. Unfortunately, only a small fraction of patients develop a sustained remission after therapy is completed (6). Thus, new therapies for HCV infection are of great clinical and economic importance. Because the optimal therapeutic agents will function against all or most of the different HCV strains, antisense strategies should be based on the most conserved regions of the virus.

Ribozymes are catalytic RNA molecules that cleave other RNA substrates. By flanking the catalytic sequences with sequences complementary to a target site, specific RNAs can be cleaved. Unfortunately, there have been a number of technical problems that have limited ribozyme efficacy in cells (20, 28). Recently, an expressed ribozyme directed against human growth hormone in an adenovirus vector was shown to eliminate over 96% of the mRNA from tissue from the livers of transgenic mice (17). In the current study, recently developed strategies for selection and expression of ribozymes were used to create RNAs with catalytic activities directed against highly conserved regions of HCV. The ribozyme activities specifically reduced or eliminated HCV RNA from an experimental cell line and from human hepatocytes isolated from two patients with advanced liver disease infected with two different HCV genotypes. These studies illustrate the feasibility of ribozyme therapy as a potential alternative approach for the treatment of hepatitis C infection.

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MATERIALS AND METHODS

T7 polymerase in vitro transcription. In vitro transcription was performed by incubating 2 μg of linear plasmid DNA template, 12.5 μl of TKB [20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.1 M KCl, 20% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 10 U of RNasin, 2.5 μl 5 mM nucleoside triphosphates (or 5 mM ATP, GTP, and UTP plus 20 μCi of ^{32}P[CTP] and 1 mM CTP), and 100 U of T7 RNA polymerase (New England Biolabs)] in a total volume of 25 μl at 37°C for 60 min. After digestion with 23 U of DNase I, the transcripts were purified twice with phenol-chloroform and ethanol precipitated. The *in vitro*-synthesized RNA was quantified in an ethidium bromide-stained agarose gel calibrated with concentration markers.

RNA extraction. Total cell RNA from liver tissue and cells for in vitro cleavage and in-solution hybridization was extracted by the guanidium-phenol method and quantified spectrophotometrically.

Selection of ribozymes. Potential accessible ribozyme cleavage sites around the AUG within the plus and minus strands of HCV RNA 1b were selected by a strategy described earlier (20). For in vitro cleavage, total RNA from HCV 1b positive livers, containing plus and minus strands, was incubated with the library of hammerhead ribozymes flanked by random sequences. The (RNA) ribozyme library (vaLRz library) was generated by T7 polymerase in vitro transcription of pGvaLRz (plasmid library). Five micrograms of HCV-infected total liver RNA was incubated with 50 μg of vaLRz library RNA or vaL RNA (control) in the presence of 10 mM $MgCl_2$ for 90 min at 37°C in 20 μl. vaL RNA (control) was generated from the same expression cassette but without the hammerhead ribozyme (pGvaL). HCV RNA after incubation with vaL RNA was used as a control for nonspecific RNA degradation for all the following steps. Five-microliter samples of the cleavage reaction mixtures were annealed with 10 μM HCV RNA-specific primer (see below) for 10 min at 70°C, and unbound primer was separated by centrifugation through a 30,000 MWCO spun filter (Millipore). Reverse transcription was performed with 200 U of superscript II reverse transcriptase (Bethesda Research Laboratories [BRL]) at 50°C for 1 h. To reduce free primers, cDNA-RNA hybrids were purified with the Gene Clean kit (Bio 101). Thirty microliters of the cDNA-RNA in H_2O was boiled for 2 min, and 3′ end tailing of cDNAs was carried out with 200 μM dGTP and 20 U of TdT (BRL) for 15 min at 37°C in a total volume of 50 μl. In order to analyze potential cleavage sites around the HCV AUG (nucleotide [nt] 342), specific HCV primers against the conserved region within the capsid protein (see Fig. 1B) were used for reverse transcription of plus-strand RNA cleavage fragments: C2+, 5′ (nt 872) GAAGATAGAGAAAGAGCA (nt 855), or C3+ 5′ (nt 747) ACCCCATGAG GTCGGCGAA (nt 728). The primer against minus-strand fragments was designed against a conserved sequence at the 5′ end of the nontranslated region: C1−, 5′ (nt 45) CTGTGAGGAACTACTGTC (nt 61). The C primer is specific against the G tail and extended with the appropriate restriction sites.

Ten percent of the tailing reaction mixture was used for the first PCR with 2.5 U of *Taq* polymerase (Perkin-Elmer) for seven cycles (30 s at 95°C, 30 s at 42°C, 90 s at 72°C) in the presence of 15 μM C primer (5′GAGAATTCTAGAGGA TCCCCCCCCCCCC) and 1.5 mM $MgCl_2$. After a linear PCR with the dC primer corresponding to the poly(dG) tail, a second PCR (40 cycles) was carried out with an excess of specific primer amplifying the downstream cleavage product. The PCR conditions were optimized for each primer pair: (i) C primer (15 μM) and C3+ primer (120 μM), 2.0 mM $MgCl_2$ for 40 cycles (60 s at 95°C, 60 s at 65°C, and 60 s at 72°C); (ii) C primer (15 μM) and C1+ primer (50 μM), 1.5 mM $MgCl_2$ for 40 cycles (60 s at 95°C, 60 s at 60°C, and 60 s at 72°C); (iii) C primer (15 μM) and C1− primer (50 μM), 2.5 mM $MgCl_2$ for 40 cycles (60 s at 95°C, 58 s at 60°C, and 60 s at 72°C) (a 5% portion of this PCR was used to run the following PCR); (iv) C primer (15 μM) and C2− primer (50 μM), 1.5 mM $MgCl_2$ for 40 cycles (60 s at 95°C, 62 s at 60°C, and 60 s at 72°C). The sequences of primers C1+, C3+, and C2− are as follows: C1+, 5′ (nt 446) GTAAACTC CACCAACGAT (nt 429); C3+, 5′ (nt 747) ACCCCATGAGGTCGGCGAA (nt 728); C2−, 5′ (nt 65) CACGCAGAAAGCGTCTAGCC (nt 74).

The PCR products were separated by electrophoresis in a 3% NuSieve agarose and, after transfer to a nylon filter, hybridized with a ^{32}P-labelled HCV DNA probe containing 730 nt of the 5′ end of the HCV genome. The probe was labelled by random priming using a kit (GIBCO BRL). The specific activity of the probes was 5×10^8 cpm/μg, and 10^6 cpm/ml was added to Rapid Hyb (Amersham). HCV-specific cleavage products for plus or minus strands that appeared in the reaction with vaLRz but not after incubation with vaL were isolated, cloned into pGEM T II (Promega), and sequenced. The corresponding ribozyme genes containing 7- or 8-nt flanking regions were synthesized as oligonucleotides overlapping in the catalytic region, filled in with *Taq* polymerase, and cloned as *Xho*I-*Nsi*I fragments into the GvaL expression cassette. The correctness of all constructs was confirmed by sequencing from the T7 promoter.

The six expression cassettes (GvaLR) with the different HCV ribozymes were cloned as *Xba*I-*Nhe*I fragments (325 bp) as monomers and trimers into the *Xba*I site of pAd.RSV-bPA (14) under the control of the RSV promoter upstream of a bPA poly(A) signal (Fig. 3B).

Recombinant (E1-deleted) adenoviruses were generated (six with different ribozyme monomers [Ad.RzM] and six with different ribozyme trimers [Ad.RzT]). Individual plaques were amplified in 293 cells, and adenoviral DNA was analyzed by *Hin*dIII digestion. Adenoviruses with the correct restriction pattern were produced in large amounts and purified in a two-step CsCl gradient ultracentrifugation (14).

Generation of CHO cell lines stably expressing HCV RNA. For CHO cells expressing the positive strand of HCV type 1a (9.4 kb) [HCV(+) CHO] under the control of a T7 phage promoter, the plasmid pTET/HCV5′T7G3′AFL (16a) was cotransfected with pSV2neo (ratio, 20:1) into CHO cells. Stable colonies were selected with 600 μg of G418 per ml. The T7 promoter can also function as pol II promoter in eukaryotic cells (19) and was used to drive expression of HCV RNA in CHO cells. Approximately 100 colonies were pooled and analyzed for HCV RNA expression by in-solution hybridization.

A plasmid (pGasHCV) to express the minus strand of the HCV RNA was generated by cloning the HCV genome in an antisense orientation behind a T7 promoter in pGEM2. In order to delete the T7 promoter from the 5′ end of pTET/HCV+, a 579-bp fragment of the HCV 5′ end was amplified by PCR with following primers: 5′CGTCTAGAGCCAGCCCCCTGATGGGG *Xba*I 5′HCV and 5′AAGGGTACCCGGGCTGAGC *Kpn*I. The 579-bp *Kpn*I-*Xba*I PCR fragment and the 7,625-bp *Eco*RI-*Kpn*I fragment from pTET/HCV+ were cloned into the *Eco*RI-*Xba*I sites of pGEM2.

CHO cells stably expressing HCV minus-strand RNA [HCV(−) CHO] were generated as described for HCV(+) CHO.

CHO cells were infected with a multiplicity of infection of (MOI) of 1,000, a dose known to transduce 100% of the cells (21).

HCV-infected hepatocytes. Human liver specimens were obtained from HCV-infected liver transplant recipients with end stage liver disease. A 3- by 3- by 2-cm apical piece of the left liver lobe covered from three sites with capsule was perfused for 30 min with 120 ml of a preperfusion solution (Earle's balanced salt solution without Ca^{2+}) and for 20 min with collagenase D (Boehringer Mannheim). In comparison to protocols for the isolation of hepatocytes from normal liver (21), a higher collagenase concentration (0.6 mg/ml) was used for the highly fibrotic end-stage liver. Hepatocytes were separated from fibroblasts by three rounds of low-speed centrifugation. Up to 5×10^8 hepatocytes with >50% viability by trypan blue exclusion were obtained and plated at a density of 10^7 on collagen I-coated 10-cm dishes in Williams E (WE) medium (which represses growth of fibroblasts) with 10% fetal calf serum (FCS). After 5 h the WE-FCS medium was replaced with a hormonally defined medium, HMD (WE medium containing 10 μg of insulin, 400 ng of dexamethasone, 362 ng of hydrocortisone, 25 ng of human epidermal growth factor, and 10 ng of human hepatocyte growth factor per ml; 1 mM glutamine; and Pen/Strep). The number of plated cells was determined by counting. Plated hepatocytes were infected with adenovirus (MOI, 200) and collected for RNA extraction after 3 days.

In-solution hybridization. For hybridization, 10 μl containing total RNA isolated from 10^5 CHO cells or 10^6 hepatocytes was mixed with 20 μl of oligo-salt-mix containing 6 volumes of H_2O, 3 volumes of 10× hybridization salts (3 M NaCl, 100 mM Tris-HCl [pH 7.5], 20 mM EDTA), and 1 volume of the oligonucleotide (10,000 to 15,000 cpm) and incubated overnight at 45°C in an Ericomp thermocycler with cover heating. After hybridization, samples were diluted with 1 ml of S1 nuclease buffer (1 volume of 10× S1 buffer is 3 M NaCl, 0.3 M sodium acetate, 0.03 M zinc acetate [pH 4.5], 1 volume herring sperm DNA [1 mg/ml], and 8 volumes of H_2O). They were incubated for 1 h at 37°C with 8 to 24 U of S1 nuclease (Gibco). The S1-resistant nucleic acids were precipitated with 100 μl of 6 M trichloroacetic acid (TCA) for 1 h on ice, then collected on glass filters (Whatman GF/C), and washed three times with cold 3% TCA–1% $NaPP_i$ and once with 95% ethanol before being counted in a scintillation cocktail for 5 min on a Packard scintillation counter.

The S1 nuclease concentration was optimized for each oligonucleotide used for hybridization according to the procedure described in Durnham and Palmiter (7).

Each set of hybridizations included a standard curve with quantified amounts of a corresponding in vitro-transcribed RNA (1 to 150 pg per reaction) added to RNA isolated from either 10^5 control CHO cells or 10^6 control hepatocytes.

The oligonucleotides used for in-solution hybridization were HCV (+) RNA, (nt 409) 5′TGACGTCCTGTGGGCGACGGTTGGTG; HCV (−) RNA, (nt 251) 5′AGCCGAGTAGTGTTGGGTCGCGAAAGG; neomycin phosphotransferase RNA, (nt 42) 5′AGCGGCCGGAGAACCTGCGTGCAATC; and ribozyme RNA, 5′ TCGTTGGGTCCACACGCTGCACTGCAT (against the cloned loop). Hybridization was for 18 h at 45°C. For human alpha 1-antitrypsin (hAAT) RNA, (nt 157) 5′ TGTTGAAGGTTGGGTGATCCTGATCATGG was mixed with RNA preheated for 5 min at 85°C and then hybridized for 30 h at 55°C. Standard RNAs were generated by T7 polymerase in vitro transcription using linearized plasmids pTet/HCV5′T7G3′AFL PacI, pGasHCV XbaI, neomycin phosphotransferase pT7neo (18) HindIII, and ribozyme pAd.RSV Rz Nhe I. hAAT RNA was produced by T3 polymerase using pBShAATbPA(14), PstI.

HCV genotyping. HCV serum RNA quantification and genotype analysis were performed at the University of Washington Viral Hepatitis Laboratory by PCR methods as described elsewhere (11).

RESULTS

Isolation and characterization of six ribozymes against HCV. The following strategy, based on a previously described method (20), was used for the isolation and characterization of

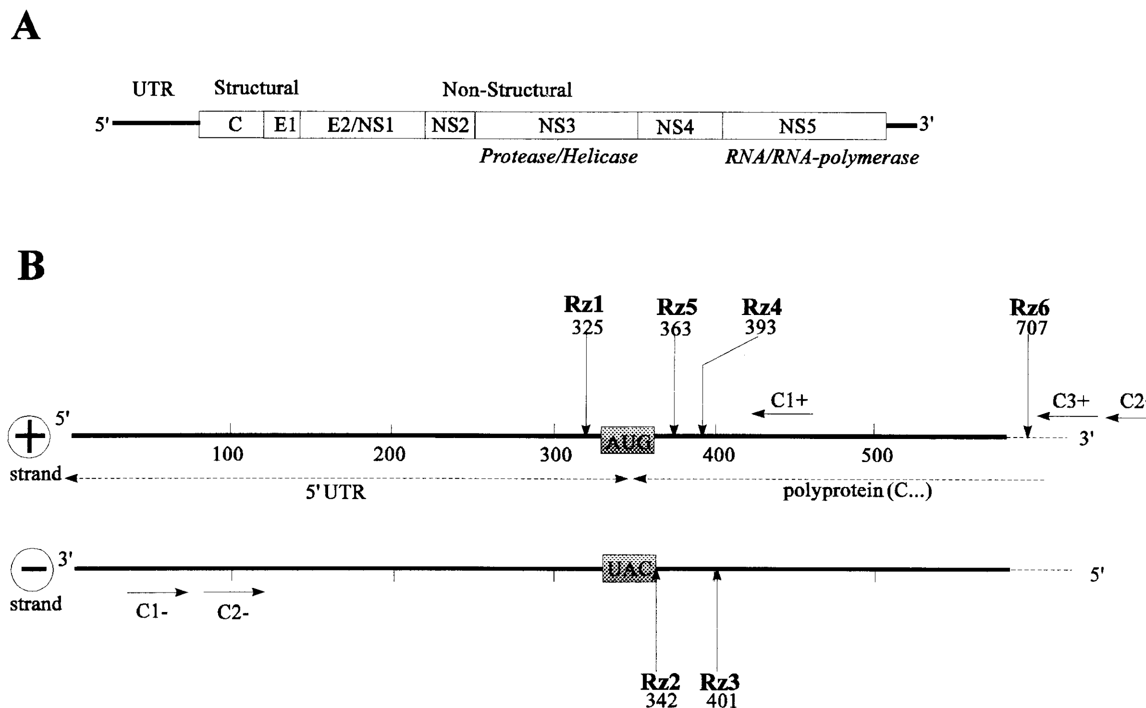

FIG. 1. HCV genome and cleavage sites. (A) An RNA map of the complete 9.4-kb HCV genome. The presumptive gene products are shown. (B) Scheme of the 5′ portion of the HCV plus- and minus-strand RNAs. The sites of ribozyme cleavage are indicated. The primers used for reverse transcription and PCR amplification (arrows) are described in Materials and Methods. (C and D) Secondary structure of the 5′ portion of HCV 1a plus-strand and minus-strand RNAs, respectively, obtained by GCG-(MFOLD). The sites of ribozyme cleavage corresponding to individual ribozymes are indicated numerically.

six ribozymes directed against HCV RNA. Total liver RNA from an HCV type 1b-infected patient was incubated with the in vitro-synthesized RNA from a hammerhead ribozyme library with random sequences in the target binding arms (vaLRz) or, in a parallel reaction, RNA generated from the same expression cassette without the hammerhead ribozyme (vaL). Cleavage products were reverse transcribed with primers specific for HCV plus and minus strands (Fig. 1A and B), tailed at the 3′ end with poly(dG), and amplified with oligo(dC) and nested HCV specific primers (Fig. 1B). The PCR products were separated by electrophoresis and, after transfer to a nylon filter, hybridized with a labelled HCV DNA probe containing 730 nt of the 5′ end of the HCV genome. An example of an experiment that was used to isolate ribozymes 1 and 4 is shown in Fig. 2A, and the conditions used to isolate the other four ribozymes are given in Materials and Methods. HCV-specific cleavage products for plus or minus strands that appeared in the reaction with vaLRz but not after incubation with vaL were isolated, cloned, and sequenced. For RNA fragments generated by specific Rz cleavage, the sequence following the dG tail will start immediately downstream of the NUH recognition site. Based on the sequence of the cloned downstream cleavage products for the six ribozymes (Fig. 2B), the site of cleavage in the HCV genome was determined (summarized in Fig. 1B).

In the initial screening process, 13 specific amplification products (9 for plus-strand RNA, 4 for minus-strand RNA) were identified as downstream ribozyme cleavage products following a GUC or CUC ribozyme recognition site. We selected the six potential cleavage sites (Fig. 1B) described above for further study based on the following criteria: (i) localization near the AUG, such that most of the untranslated region is deleted and/or the IRES secondary structure is disrupted, (ii) flanking regions and cleavage sites for ribozymes contained within highly conserved regions for HCV types 1a and 1b, and (iii) localization within single-stranded or loop regions in a secondary structure model of HCV RNA (Fig. 1C and D). At this time, the significance of this structure for predicting ribozyme cleavage sites which are accessible in a target cell is not known. As additional ribozyme sequences are studied, it may be easier to predict ribozyme function based on RNA modeling.

Ribozyme genes with 7- and 8-nt flanking regions were cloned into the pGvaL expression cassette (Fig. 3A). The ribozymes were embedded into the stem-loop structure which was part of an adenoviral vaI RNA with an internal pol III promoter (Fig. 3A) such that the catalytic sequences could theoretically be formed independently from surrounding RNA structures (20).

To confirm that the ribozymes embedded into this expression cassette would cleave RNA in a cell-free system, the isolated ribozymes (Rzs 1, 2, 3, 4, and 6) were studied in greater detail. In vitro RNA transcription products obtained from ribozyme genes 1, 4, and 6 cloned into GvaL were incubated with a radiolabelled, 730-nt HCV 1a (plus-strand) RNA generated by T7 polymerase in vitro transcription. For these three ribozymes, specific in vitro cleavage products of expected size corresponding to the cleavage sites were obtained (Fig. 4) and their cleaving ability was confirmed. As expected, Rzs 2 and 3 did not cleave HCV plus-strand RNA (Fig. 4).

Function of expressed ribozymes in tissue culture. The six ribozyme expression cassettes were cloned into recombinant adenovirus vectors (Fig. 3B). Recombinant adenovirus vectors can transduce virtually all hepatocytes with at least 15 to 30 genome copies per cell in animals (30), and we have previously demonstrated that a ribozyme directed against hGH mRNA

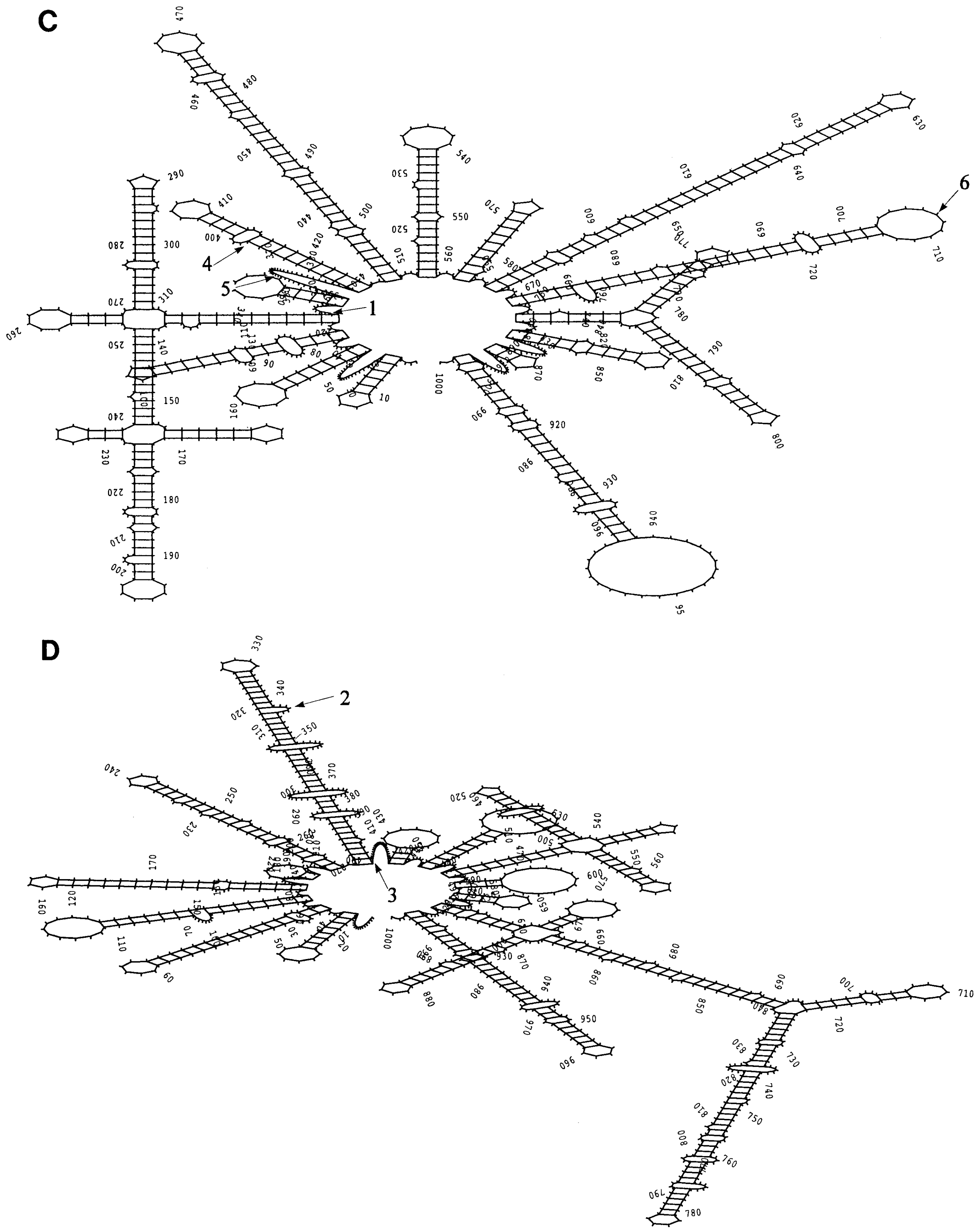

FIG. 1—*Continued.*

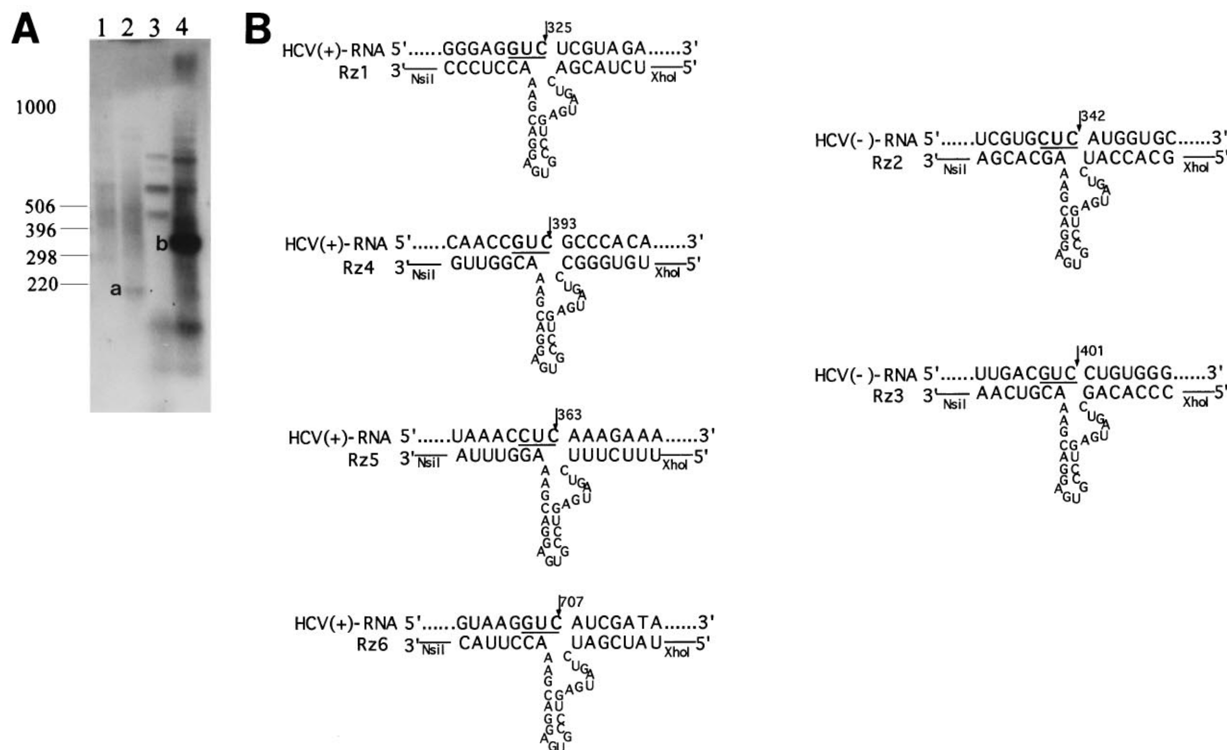

FIG. 2. Detection of HCV-specific RNA fragments after cleavage of RNA from HCV-infected livers in vitro by ribozymes from the library. (A) Detection of cleavage products. After incubation with the ribozyme library or control RNAs, 3′ cleavage products were reverse transcribed (rt), tailed with dG, and amplified by PCR with different primers. PCR products were separated on a 3% NuSieve agarose gel, transferred to a Nylon filter, and hybridized with a labelled HCV DNA probe containing 730 bp from the 5′ end of the HCV genome. See Materials and Methods and Fig. 1B for designation of primers. Lanes 1 and 2, rt primer C3+, PCR primers C and C1+; lanes 3 and 4, rt primer C2+, PCR primers C and C3+. Lanes 1 and 3, control (vaL RNA without ribozyme); lanes 2 and 4, ribozyme library. The sizes of the specific bands as determined by a DNA ladder, 180 nt (a) and 320 nt (b), correspond to the cleavage sites of ribozymes Rz1 and Rz4, respectively. Other cleavage patterns were obtained with different primer combinations and PCR conditions as described in Materials and Methods. (B) Sequences of the isolated ribozymes. The cleavage sites are indicated (arrows).

was efficacious when expressed from recombinant adenovirus vectors (17). The ribozyme genes were placed under control of the RSV long terminal repeat (LTR) promoter in one or three copies (Fig. 3B). The ribozymes contained as three copies can be transcribed from the pol II promoter either as one RNA containing three ribozymes or as three single RNAs from the internal pol III promoter (Fig. 3B).

In order to test the activity of the selected HCV ribozymes after adenoviral gene transfer in vivo, HCV(+) CHO or HCV(−) CHO cells that produced ~45 to 60 HCV genomes per cell were generated. The Ad.Rz vectors were infected into confluent HCV(+) CHO cells or HCV(−) CHO cells (at an MOI sufficient to transduce 100% of cells), and three days postinfection total cell RNA was analyzed for HCV RNA, ribozyme RNA, and neomycin phosphotransferase (control) RNA concentrations by quantitative solution hybridization. The sensitivity of the assay was at least 1 pg of RNA or less than one copy per cell (Fig. 5).

After infection with Ad.contr, nonspecific declines of about 30% in HCV and neo RNAs were detected (Fig. 6A, C, D, and F). All the expressed ribozymes directed against the positive strand of HCV RNA (Rz 1, 4, 5, and 6) reduced HCV plus-strand RNA in HCV(+) CHO cells (Fig. 6A and B); ribozymes 1 and 4 ablated HCV RNA in these cells. Ribozymes 2 and 3, selected for activity against the HCV minus strand, had no specific effect in HCV(+) CHO cells (Fig. 6A and B), whereas ribozymes directed against the plus strand (Rz 1, 4, 5, and 6) did not cleave the minus-strand RNA expressed in HCV(−) CHO cells (Fig. 6D and E). Ribozyme 2 reduced while ribozyme 3 eliminated the HCV minus-strand RNA completely. Fragments after ribozyme cleavage are rapidly degraded and not available for hybridization with the test oligonucleotide.

Each of the recombinant adenoviruses expressed about 8,000 to 15,000 ribozyme molecules per cell (Fig. 6B and E). Thus, a more than 100-fold excess of ribozymes over the target RNA was achieved, which allowed for the efficient diminution of HCV RNA in CHO cells. The ribozyme effect was specific for HCV RNA because the concentration of neomycin phospohotransferase was not influenced by high ribozyme concentrations (Fig. 6C and F).

Expressed ribozymes function in human hepatocytes. The CHO cell culture model may not reflect what occurs in an HCV-infected cell, because HCV production and replication do not occur in CHO cells. Specifically, in HCV-infected hepatocytes, positive and negative HCV RNA strands are simultaneously present and may form hybrids which are not accessible for ribozymes. Additionally, HCV RNA may be protected by HCV core proteins (or cellular proteins) or packaged into particles protecting them from ribozymes. Finally, ribozymes must be colocalized in the same cytoplasmatic and/or nuclear compartment as is HCV RNA.

Because no efficient cell culture system for propagation or infection by HCV is available (26), we used freshly isolated hepatocytes from patients with chronic HCV infection to test the ribozymes. Even in livers with advanced cirrhotic disease, most of the HCV-infected hepatocytes have normal morphol-

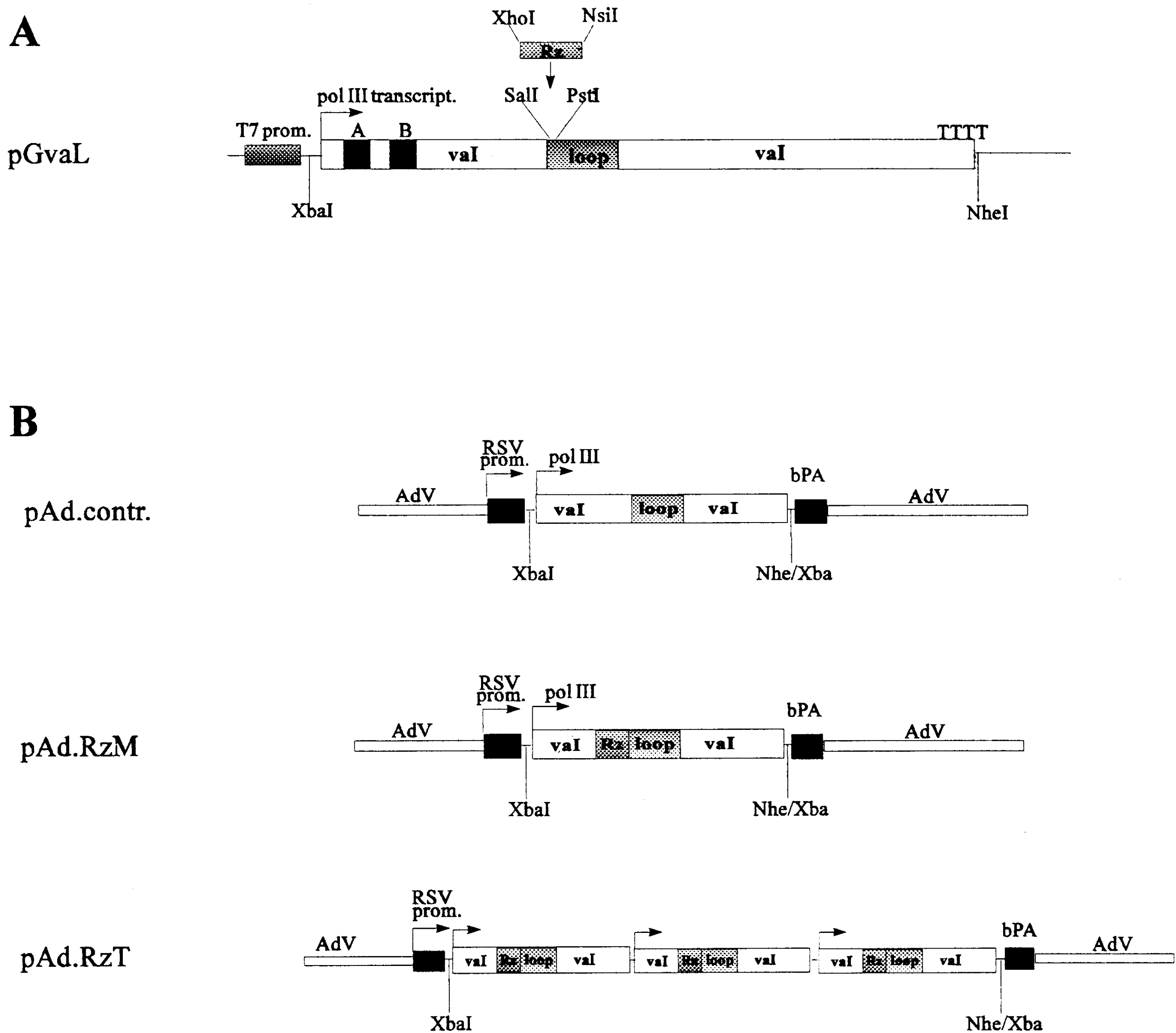

FIG. 3. Ribozyme expression cassettes. (A) Insertion of the ribozymes into the pGvaL plasmid vector is indicated. A and B, pol III promoter elements; vaI, virus-associated vaI RNA from human adenovirus. (B) The pGvaL sequences shown in panel A were cloned into plasmids used to produce recombinant adenovirus vectors. pAd.RzM contains a single, monomeric ribozyme expression cassette, while pAd.RzT contains the trimeric expression cassette.

ogy (23). In the first experiment (Fig. 6), hepatocytes were isolated from the explanted liver of a patient infected with HCV genotype 1b. The isolated hepatocytes showed normal morphology in culture. RNA was isolated from hepatocytes and culture media 3 days after infection with a mixture of adenoviruses containing each of the 6 ribozyme genes expressed as monomer (Ad.Rz1, Ad.Rz2M, Ad.Rz3M, Ad.Rz4M Ad.Rz5M, and Ad.Rz6M) or as trimer (Ad.Rz1T-Ad.Rz6T). The RNAs were quantitatively analyzed for HCV plus- and minus-strand RNAs; hAAT RNA which is expressed in differentiated hepatocytes served as a control for nonspecific effects of ribozyme treatment, and ribozyme RNA concentrations were determined by in-solution hybridization. In control hepatocytes, HCV plus-strand RNA was detectable at a concentration of about 20 copies per cell, although minus-strand HCV RNA was undetectable at a sensitivity of at least 0.1 copy per cell. The amount of minus-strand RNA in hepatocytes is thought to be about 1/100 to 1/1,000 that of plus-strand RNA (9, 33). No HCV RNA could be detected in tissue culture supernatants.

While control adenovirus had a minimal effect on HCV positive-strand RNA, infection with the adenovirus-ribozyme vectors reduced the HCV RNA concentration (Fig. 7A and B). The mixture of trimeric ribozymes Ad.Rz1T-Ad.Rz6T ablated HCV RNA from infected hepatocytes with a sensitivity of detection of at least 0.1 copy per cell. We presume that the greater reduction with trimeric expression cassettes was due to a twofold-higher ribozyme level (~700-fold excess over HCV RNA) in comparison to that obtained with the monomeric constructs (Fig. 6B).

In a second experiment (Fig. 8), hepatocytes from an HCV 1a RNA-positive liver were isolated and cultured. Positive HCV RNA (~10 copies per cell) was detected in control hepatocytes. RNAs from cultures infected with adenovirus vectors

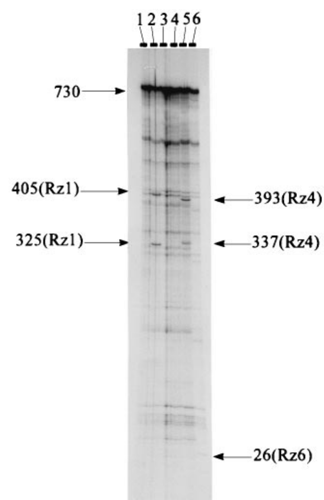

FIG. 4. In vitro cleavage activity of HCV ribozymes. A 730-bp radiolabelled HCV RNA generated by in vitro transcription with T7 polymerase of a *Cla*I-*Xba*I fragment of pTet/HCV5′T7G3′AFL was incubated with an equimolar (100 nM) amount of individual ribozyme RNA obtained from in vitro transcription. After cleavage, the RNAs were separated on a 6% denaturating polyacrylamide gel. The size of the fragments was determined by a DNA ladder. The specific sizes of the in vitro cleavage products are indicated. Lane 1, in vitro-transcribed HCV RNA (plus strand) with vaL control RNA; lane 2, Rz1; lane 3, Rz2; lane 4, Rz3; lane 5, Rz4; lane 6, Rz6.

Ad.Rz1T to Ad.Rz6T were analyzed separately. Among the ribozymes directed against the HCV plus strand, Rz1 and -4 were the most efficient at reducing HCV RNA concentrations (Fig. 8A). Interestingly, ribozymes 2 and 3 against the minus strand reduced the plus-strand level as well (Fig. 8A). One possibility is that the plus strand is produced by HCV replication in cultured cells, and cleavage of the minus strand would interrupt this process. The concentrations of expressed ribozymes in the second experiment were similar to the amounts detected in the first experiment (Fig. 8B). In both experiments

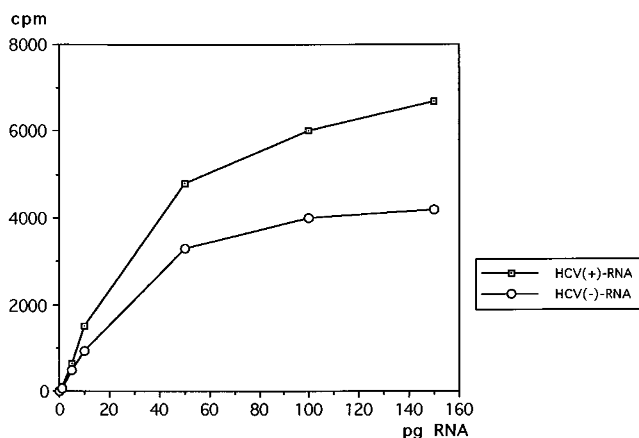

FIG. 5. In-solution RNA hybridization standard curve for HCV plus-strand and HCV minus-strand RNA quantitation. RNA isolated from 10^6 normal human hepatocytes or 10^5 CHO cells was mixed with varying amounts (1 to 150 pg) of in vitro-transcribed HCV plus- or minus-strand RNA. The curves obtained from the experiment using human hepatocytes are shown. The data are expressed as S1-resistant counts per minute. Sensitivities of detection: hepatocytes, 1 pg of RNA per 10^6 cells or 0.1 copy per cell; CHO cells, 1 pg of RNA per 10^5 cells or 1 copy per cell.

(Fig. 7C and 8C), the hepatocyte-specific hAAT mRNA concentrations were not affected by ribozyme expression.

DISCUSSION

The development of therapies for hepatitis C infection has been hindered by the inability to grow the virus in culture and the lack of an animal model other than the chimpanzee (8). The ultimate goal of ribozyme therapy for HCV is to inhibit the expression and replication of viral genomic RNA. The current study uses a method developed earlier to isolate six ribozymes with potent ability to degrade HCV from experimental cell lines and from liver cells isolated from two patients with chronic HCV infection. Our study is important because it is the first to demonstrate that expressed ribozymes which cleave conserved regions of both the plus (genomic) and minus (replicative) strands are efficient at eliminating HCV RNA from infected human hepatocytes. Of high interest was our finding that ribozymes were active in human hepatocytes from different patients infected with two different genotypes. In one previous study, adding synthesized antisense nucleotides to fragments of HCV RNAs reduced translation in vitro by more than 95% (31). In a second study, adding very high concentrations of antisense phosphothioate oligodeoxynucleotides to cells expressing the 5′ portion of the HCV RNA fused to a luciferase reporter decreased protein expression more than 95% (1). Neither of these studies used expressed antisense molecules or tested their efficacy against complete genomic RNA or bona fide infected cells. We have previously demonstrated that using adenovirus vectors to express the ribozymes is advantageous because it allows targeting to the liver in vivo. We expressed hammerhead ribozymes from recombinant adenovirus vectors into the livers of mice and were able to eliminate over 96% of a target mRNA (17). These results, taken together with the findings from the present study, suggest that it may be possible to eventually treat HCV infection in humans by vector-mediated ribozyme therapy.

Towards the goal of eliminating HCV from infected hepatocytes, it may be possible to slow progression of the liver disease and improve the quality of life by a significantly decreasing viral load, as has been suggested for interferon therapy (22). It is not clear whether or not transient expression of ribozymes in all hepatocytes will result in permanent elimination of the virus, because it is not known if a source of nonhepatic HCV replication exists (16, 24). A second potential therapy involves patients with end stage liver disease who require orthotopic liver transplantation because it may be possible to express ribozymes in a recipient liver prior to transplantation to avoid HCV reinfection.

We used a combination of multiple ribozymes directed against conserved regions of HCV for several reasons. (i) There is less of a chance that a resistant quasispecies will survive and escape the therapy. Viral variants that escape immunologic surveillance are thought to contribute to high rates of HCV persistence (32, 34). Notably, selection of resistant quasispecies has been noted to occur when single ribozymes have been used against HIV in cultured cells (25). (ii) Individual ribozymes directed against specific regions may be more effective against different viral subgenotypes, while a combination of ribozymes is more likely to be effective against all the major genotypes. Our preliminary experiments demonstrate substantial in vivo activity against viral RNAs representing two major HCV genotypes, types 1a and 1b. However, in order to determine the spectrum of activity against HCV, additional livers from patients with different genotypes will need to be exposed to individual ribozymes. A correlation between viral

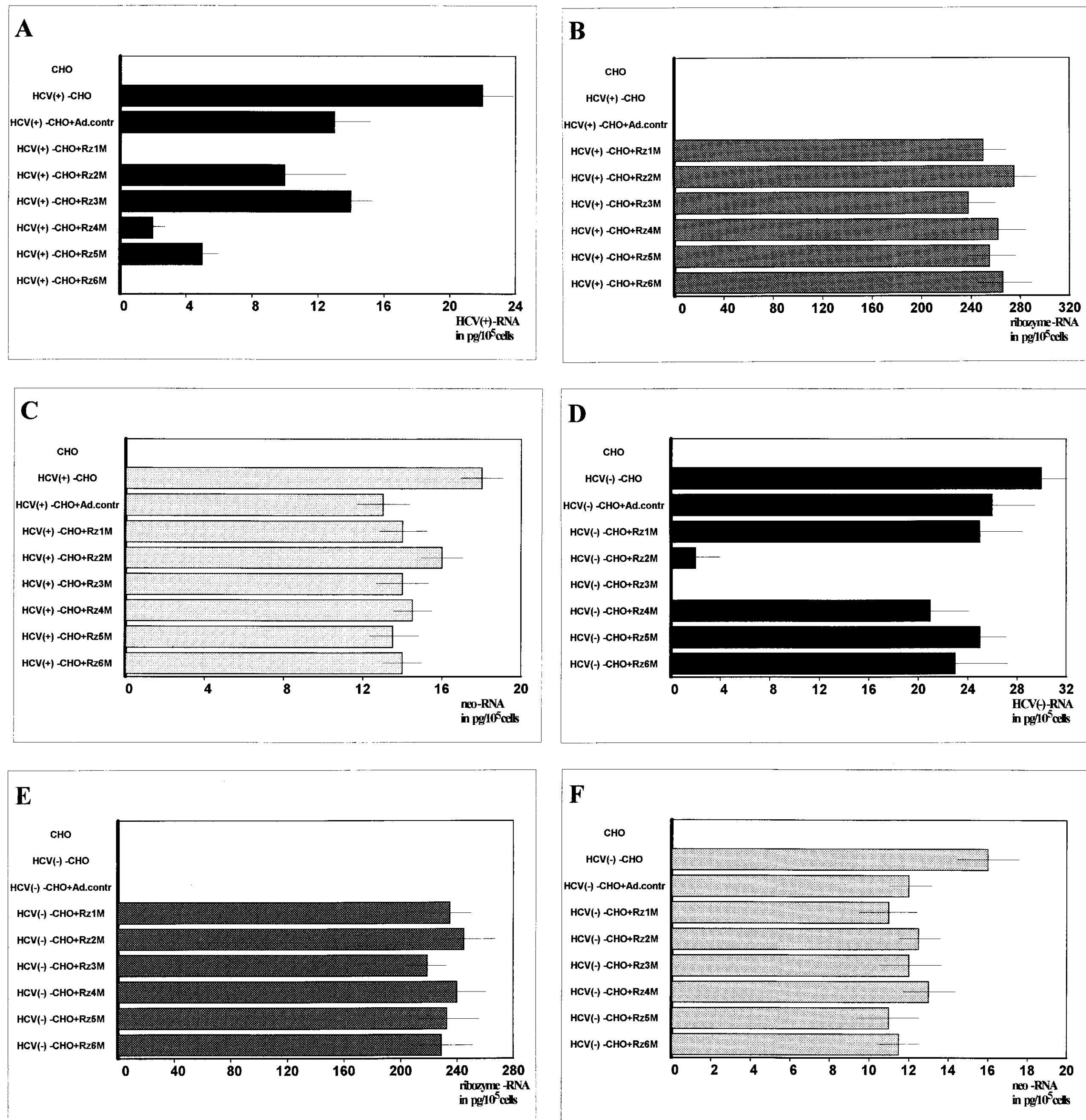

FIG. 6. Cleavage of plus (A to C)- and minus (D to F)-strand HCV RNAs expressed from CHO cells. CHO cells that express full-length HCV RNAs were infected individually with Ad.RSVRz1-6M or Ad.contr. Three days later, RNA was extracted and quantified by in-solution hybridization for plus (A)- or minus (D)-strand HCV RNA, ribozyme RNA (B and E), and neomycin phosphotransferase RNA (C and F). The values represent means and standard deviations for three experiments.

sequence and ribozyme effectiveness may be possible to obtain. (iii) Cleavage of the 5′ end of the viral genome should, in theory, eliminate translation of the polyprotein; hence, none of the individual viral proteins should be produced. Moreover, cleavage of the minus strand should abolish replication of full-length plus strands and may further inhibit packaging, replication, and translation of truncated plus strands. Thus, the simultaneous elimination of both strands may be synergistic in decreasing viral load in an infected individual.

The ribozymes characterized here have been shown to cleave HCV RNA in vitro; however, because of the instability of cleaved RNA, it has not been possible to detect the cleavage products in infected cells. Because of this, we cannot eliminate the possibility that some of the effects of the expressed ribozymes are not in part due to an antisense effect. We feel that this is unlikely for several reasons. First, maximal antisense effects usually require longer contiguous complementary sequences than the 8 and 7 nt present in the HCV ribozymes.

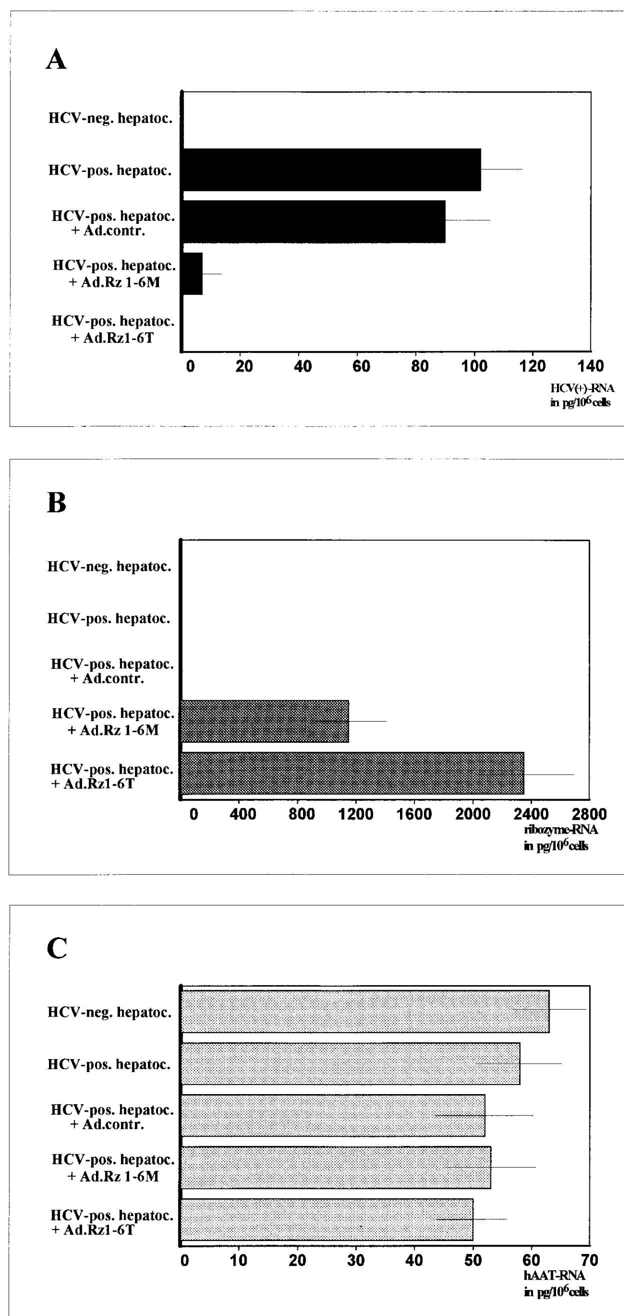

FIG. 7. Elimination of HCV RNA type 1b from infected human hepatocytes. Cultured hepatocytes from an HCV-infected individual were infected with a mixture of Ad.Rz1-6M, Ad.RzT 1-6, or Ad.contr. Three days later, isolated RNA was quantitated by in-solution hybridization for concentration of HCV plus-strand RNA (A), ribozyme RNA (B), or hAAT RNA (C). Values represent means and standard deviations for samples analyzed in triplicate.

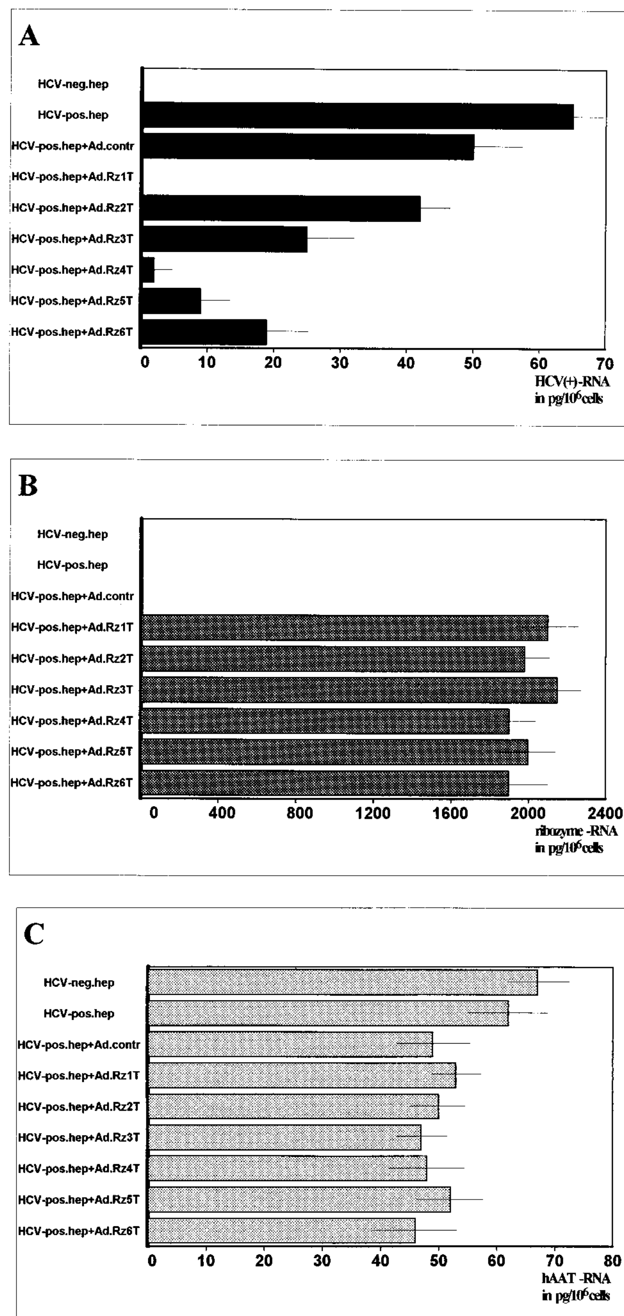

FIG. 8. Elimination of HCV RNA type 1a from infected human hepatocytes. Cultured hepatocytes from HCV-infected individuals were infected individually with Ad.Rz1-6T or Ad.contr. Three days later, isolated RNA was quantitated by in-solution hybridization for concentration of HCV plus-strand RNA (A), ribozyme RNA (B), or hAAT RNA (C). Values represent means and standard deviations for samples analyzed in triplicate.

Second, a similar (non-HCV) ribozyme isolated from the library, expressed in cultured cells, was ineffective when a mutation was placed in the catalytic portion of the molecule (20). Whatever the mechanism(s), the ribozymes described in this study clearly demonstrate efficacy.

There are several important issues regarding gene therapy for hepatitis C infection that need to be resolved before this approach can be considered in a clinical setting. Much of the hepatic damage associated with persistent HCV infection may in fact be due to immunologic responses (15). Thus, it is hoped but unproven that elimination of the virus from an infected organ will slow or eliminate immune-mediated liver disease. Additionally, for the therapy to have a chance for cure, virtually all hepatocytes must express the ribozymes for a currently undefined period of time. While recombinant adenovirus vectors may be able to achieve this goal, the current vectors induce

major inflammatory immune responses that limits the duration of gene expression. Newer generations of adenovirus or other novel vectors will be required to make this therapy feasible in humans. Nevertheless, the study presented here is an important first step towards demonstrating that expressed ribozymes can eliminate HCV RNA from infected hepatocytes.

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