Identification of eIF2Bγ and eIF2γ as cofactors of hepatitis C virus internal ribosome entry site-mediated translation using a functional genomics approach

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The 5′-untranslated region of hepatitis C virus (HCV) is highly conserved, folds into a complex secondary structure, and functions as an internal ribosome entry site (IRES) to initiate translation of HCV proteins. We have developed a selection system based on a randomized hairpin ribozyme gene library to identify cellular factors involved in HCV IRES function. A retroviral vector ribozyme library with randomized target recognition sequences was introduced into HeLa cells, stably expressing a bicistronic construct encoding the hygromycin B phosphotransferase gene and the herpes simplex virus thymidine kinase gene (HSV-*tk*). Translation of the HSV-*tk* gene was mediated by the HCV IRES. Cells expressing ribozymes that inhibit HCV IRES-mediated translation of HSV-*tk* were selected via their resistance to both ganciclovir and hygromycin B. Two ribozymes reproducibly conferred the ganciclovir-resistant phenotype and were shown to inhibit IRES-mediated translation of HCV core protein but did not inhibit cap-dependent protein translation or cell growth. The functional targets of these ribozymes were identified as the gamma subunits of human eukaryotic initiation factors 2B (eIF2Bγ) and 2 (eIF2γ), respectively. The involvement of eIF2Bγ and eIF2γ in HCV IRES-mediated translation was further validated by ribozymes directed against additional sites within the mRNAs of these genes. In addition to leading to the identification of cellular IRES cofactors, ribozymes obtained from this cellular selection system could be directly used to specifically inhibit HCV viral translation, thereby facilitating the development of new antiviral strategies for HCV infection.

Although translation of most cellular mRNAs requires a cap-modification at the 5′-end of the RNA, some viral and cellular mRNAs use a cap-independent mechanism of ribosome binding mediated by an internal ribosome entry site (IRES) (1). Viral IRESs significantly differ in length, functionally important structures, translation efficiency, and the requirement for cellular cofactors (2). The 5′-untranslated region (UTR) of hepatitis C virus (HCV) is highly conserved in all known HCV strains and folds into a complex secondary structure that constitutes the IRES. HCV IRES binds directly to 40S ribosome subunits to form a stable binary complex at the site of the initiator AUG at position 342 (3, 4). The ternary eIF2/GTP/Met-tRNA^{Met} complex binds to the binary IRES/40S subunit complex at the AUG whereas eIF3 independently binds to the upstream stem-loop III of the IRES (5). This complex formation—in contrast to other IRESs—does not require additional eukaryotic translation initiation factors (eIFs) such as eIF1, eIF1A, eIF4A, eIF4B, eIF4E, or eIF4G (6). Additional cellular factors, such as La antigen (7), pyrimidine binding tract protein (8), and p25 (9), were shown to bind to the 5′-UTR of HCV. However, the functional relevance of these factors and the potential role of additional molecules involved in HCV IRES-mediated translation has not been characterized. Identification of these cellular factors should have important implications for the development of new antiviral therapies directed against HCV.

We have developed a reverse functional genomics approach for gene discovery based on the use of a randomized hairpin ribozyme library. Ribozymes are catalytic RNA molecules that bind to defined RNA targets based on sequence complementarity and enzymatically cleave these RNA targets. Typically, hairpin ribozymes cleave upstream of a GUC triplet in the target sequence. By randomizing the substrate binding sequences (Fig. 1*B*, 8 nt in helix 1 and 4 nt in helix 2 of the hairpin ribozyme), we generated a library of 1.7×10^7 ($=4^{12}$) different ribozyme molecules that can potentially cleave any RNA substrate containing a GUC. This library of ribozymes can be stably introduced into reporter cells via retroviral vector transduction on which these cells are selected for a given altered phenotype. Selected ribozymes that reproducibly confer phenotypic changes can be exploited to identify the gene(s) involved in this alteration by different approaches [e.g., BLAST search, 5′- and 3′-rapid amplification of cDNA ends (RACE)]. Here we report the development of a cell selection system to specifically identify cellular genes involved in HCV IRES-mediated translation. Our results, identifying two genes (eIF2γ and eIF2Bγ) in the same translational pathway, are consistent with what is known about HCV IRES function and validate this ribozyme-based technology of gene discovery. Furthermore, our data demonstrate that ribozymes directed against the translation factors eIF2γ and eIF2Bγ inhibit IRES-mediated translation of HCV core protein without changing general cap-dependent translation or cell growth.

Materials and Methods

Plasmid Constructs. The hygromycin B phosphotransferase gene was amplified from pIRES-hygro (CLONTECH) by PCR with oligonucleotide primers P1 (5′-ggatgatgaagacatacaaggagacgaccttccatggatagatccggaaagcct-3′) and P2 (5′-gtcggcatgtcgactattcctttgccctcggacg-3′), then was digested with *Bbs*I and *Sal*I and was used to replace the puromycin-resistance gene in pPur-HCV (10) to generate pHyg-5′C. The herpes simplex virus thymidine kinase

Abbreviations: HCV, hepatitis C virus; UTR, untranslated region; IRES, internal ribosome entry site; Rz, ribozyme, RACE, rapid amplification of cDNA ends; GCV, ganciclovir; eIF, eukaryotic initiation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSV-*tk*, herpes simplex virus thymidine kinase gene.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. nkit318828 (human eIF2Bγ sequence) and AF257077].

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gene was amplified by PCR with primers P3 (5′-cgatcgtagaattcaggtctcgtagaccgtgcaccatggcttcgtacccctgccatcaacacgcgtctgcgttcgaccggct-3′) and P4 (5′-gtacccgattatgatctcagttagcctcccccatctcccg-3′) from pcHy-*tk*, and was inserted into pHyg-5′C to generate pHyg-5′*tk*. The Moloney retroviral genome-based plasmid pLHPM-RzLib contains a ribozyme (Rz) expression cassette with eight random nucleotides in helix 1 and 4 random nucleotides in helix 2 driven by the $tRNA^{val}$ promoter. This results in a possible complexity of the library of 1.7×10^7 ($=4^{12}$) different ribozyme molecules. The neomycin-phosphotransferase gene confers resistance against G418. The retroviral library plasmid pLHPM was constructed by inserting a *Cla*I-*Xho*I fragment (containing a cassette with $tRNA^{val}$ promoter and the sequence of an unrelated Rz) into pLNL-PUR-HCV vector (10) in place of *Bst*BI-*Xho*I. The library insert was generated by annealing three chemically synthesized and 5′-end phosphorylated oligonucleotides Lib1, 5′-cgcgtaccaggtaatataccacaacgtgtgtttctctggtnnnnttctnnnnnnnnnggatcctgtttccgcccggttt-3′, Lib2 5′-cgttgtggtatattacctggta-3′, and Lib3 5′-cgaaaccgggcggaaacagg-3′ (IDT, Coralville, IA). To maintain randomization of the 12 target recognition nucleotides and to achieve efficient cloning of the ribozyme library sequences, the annealed oligonucleotides were ligated overnight with 20 μg of vector, and the ligation mixture was electroporated (GenePulser, Bio-Rad) into DH12s cells (GIBCO). Master stock and working stocks of bacteria containing several complexities of vector library were used to obtain the library plasmid DNA (Qiagen, Chatsworth, CA). Randomness of the Rz library insert was demonstrated by sequencing 60 individual bacterial colonies by using the primer NL6H6 (5′-ctgactccatcgagccagtgtagag-3′). In addition, *in vitro* cleavage of several different short RNA transcripts by a comparable ribozyme library confirmed the randomness (data not shown). Plasmid pLHPM-BR1 (expressing a ribozyme directed against human hepatitis B virus), pLHPM-CR2A (directed against position 323 within the HCV 5′-UTR), as well as target validation ribozymes were constructed by annealing of overlapping ribozyme-specific oligonucleotides as described (11). Plasmids gag-pol (retroviral helper functions) and VSV-G (vesicular stomatitis virus G-protein) for retroviral production were a kind gift of Ted Friedman (University of California, San Diego).

Cell Culture and Reagents. Human cervical carcinoma cells (HeLa) and canine thymus cells (CF2) were obtained from the American Type Tissue Culture Collection. Cells were cultured at 37°C, in a 5%CO_2 humidified incubator, in DMEM supplemented with 10% heat-inactivated FCS (HyClone), 1% penicillin-streptomycin, 1 mM sodium pyruvate, and nonessential amino acids (all from GIBCO). Ganciclovir was obtained from Syntex (Palo Alto, CA).

Generation of an HCV IRES Reporter Cell Line (HeLa 5′*tk*). HeLa cells were electroporated with pHyg-5′*tk* (20 μg) by using a Bio-Rad electroporator at a setting of 880V/25 μF. The cells were selected with 250 μg/ml hygromycin B. Single cell clones of stable herpes simplex virus thymidine kinase gene (HSV-*tk*) expressing cells were obtained according to standard techniques and were functionally characterized for ganciclovir (GCV)-mediated cell killing. Individual clones were seeded at 0.4×10^4cells/cm^2 in cell culture flasks (Costar), and, after exposure to GCV for 24–96 h, cells were cultured for 21 days in the presence of hygromycin B (250 μg/ml), were fixed with methanol, were stained with Coomassie blue, and were counted.

Viral Vector Production. Retroviral particles were produced on CF2 cells by triple transfection (*Trans*IT-LT1, PanVera) of pLHPM-Rz (expressing individual Rzs) or pLHPM-RzLib (expressing Rz library), with plasmid gag-pol (expressing retroviral helper function) and plasmid VSV-G expressing the vesicular stomatitis virus glycoprotein (VSV-G). Retroviral particles were collected every 24 h after addition of fresh media and were filtered through 0.4 μM filters. Viral titers of G418-resistant polyclonal cell populations were estimated by using a standard titration assay performed on HeLa cells.

Retroviral Ribozyme Vector Transduction, Ganciclovir Selection, Ribozyme Rescue, PCR Amplification, and Ribozyme Sequence Analysis from GCV-Resistant Cells. Retroviral library transduction was performed on clonal HeLa 5′*tk* cells (plated at 0.8×10^4/cm^2 in sixteen 225-cm^2 cell culture flasks by using a total of 460 ml of nonconcentrated retroviral supernatant (titer 4×10^5 colony-forming units/ml determined on HeLa cells, multiplicity of infection of 2). Control retroviral transduction (total volume 40 ml, titer 2×10^4 colony forming units/ml) was performed in two 225-cm^2 cell culture flasks. After selection with G418 (500 μg/ml), library transduced cells were plated in two independent experiments at a density of 0.4×10^4/cm^2 (twenty-five 15-cm-diameter cell culture dishes per experiment, four 15-cm dishes for the pLHPM-BR1 control, respectively). Cells were exposed to GCV at a concentration of 40 μM for 24 h (experiment A) or 40 h (experiment B) and were subsequently cultured under hygromycin B selection (250 μg/ml). GCV-resistant colonies were counted after 21 days, were harvested, and were further expanded under selection with hygromycin B. Genomic DNA was isolated from cultured cells (QIA amp DNA extraction kit, Qiagen) and 1 μg of genomic DNA was used to amplify the integrated $tRNA^{val}$ Rz cassette in a PCR amplification with primers LHPM-2878 (5′-ggcgggactatggttgctgactaat-3′) and 5′MFT2 (5′-ggttatcacgttcgcctcacacgc-3′). *Bam*HI- and *Mlu*I-digested PCR fragments were cloned into retroviral vector pLHPM. Bacterial colonies were pooled and grown up, and plasmid DNA was extracted to obtain retroviral vector for the production of new retroviral particles. Individual colonies were analyzed for ribozyme sequence by standard sequencing techniques using a primer close to the Rz cassette (NL6H6). Single ribozyme transduced cells were seeded at a density of 0.4×10^4/cm^2 and were exposed to GCV (10 μM) for 4 days, and were subsequently cultured under hygromycin B selection (250 μg/ml) and counted after 21 days.

Identification and Cloning of Potential Target Genes using Ribozyme Sequence Information. 5′-RACE was used with reverse transcription–PCR to amplify mRNAs (2 μg) extracted from HeLa 5′*tk*. Double-stranded cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and a modified lock-docking oligo(dT) primer (Marathon cDNA amplification kit, CLONTECH). 5′-RACE amplification was performed according to the manufacturer's protocol. Amplification using AP1 and RzHCV1 binding sequence (5′-ggatccttcttattgacnagct-3′) revealed a partial nucleotide sequence of human eIF2Bγ. Using the BLAST algorithms (12), the sequence was aligned with overlapping human expressed sequence tags (dbEST) and rat eIF2Bγ (GenBank accession no. U38253) (13). Primer heIF2Bγ-1 (5′-gctgtgatctgataacagacgttgcctac-3′) was used to amplify a 1.2-kb 3′-part of the human eIF2Bγ gene from Marathon cDNA (generated from the parental cells 5′*tk*). The 5′ terminus of the gene was cloned by using a SMART amplification technique (CLONTECH). PCR-amplified cDNA fragments were cloned into T/A-type PCR cloning vectors (pCR2.1; Invitrogen) and were sequenced.

Northern Blot Analysis. Total RNA (15 μg per lane), extracted from cultures grown to 80% confluency (RNeasy mini kit, Qiagen), was separated by electrophoresis in a formaldehyde/1% agarose gel (2.2 M formaldehyde/20 mM Mops buffer) and was transferred onto a nylon membrane (Zeta-Probe, Bio-Rad). Membranes were incubated at 65°C in hybridization solution (QuickHyb, Stratagene) with cDNA of hepatitis C core, HSV-*tk*, human eIF2Bγ, eIF2γ, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled with α-^{32}P-dCTP by random

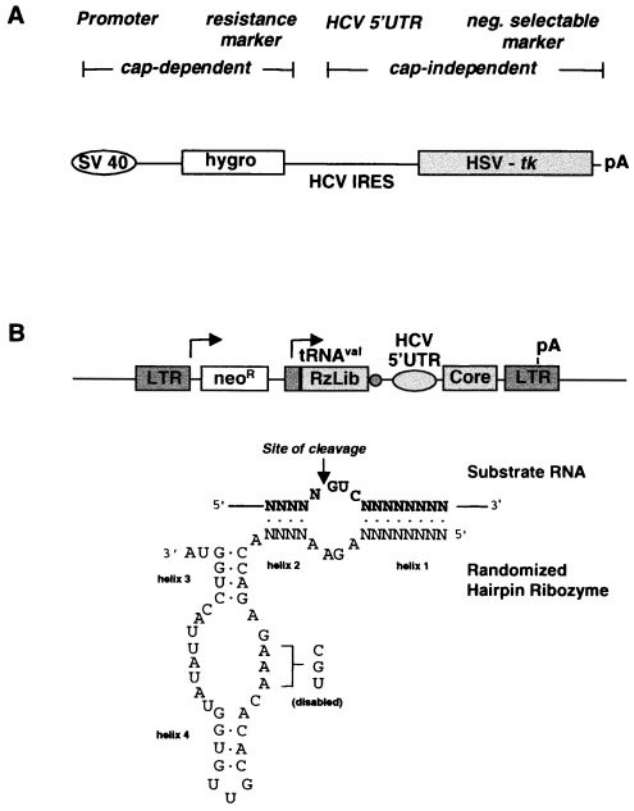


Fig. 1. Schematic representation of the reporter construct and the randomized hairpin ribozyme library for the discovery of HCV IRES cofactors. (*A*) A bicistronic construct is transcribed from the SV 40 promoter, allowing for cap-dependent translation of hygromycin B phosphotransferase (hygro) and cap-independent HCV IRES-mediated translation of HSV-*tk*. (*B*) The hairpin ribozyme library (RzLib) was constructed such that 12 positions of the substrate binding domains were randomized (8 nucleotides in helix 1, 4 nucleotides in helix 2). The ribozyme preferentially recognizes a GUC triplet within the substrate RNA and cleaves 5′ of the G residue as indicated. A hairpin ribozyme can be disabled by changing the bases AAA to CGU (as shown), without changing the substrate binding ability of the ribozyme. A ribozyme library cassette under control of the $tRNA^{val}$ promoter was cloned into a murine retrovirus vector (pLHPM) to generate pLHPM-RzLib. The LTR transcript expresses a neomycin-resistance gene (neo) to allow for selection of stable transductants, and the 5′-UTR of HCV, which directs the translation of the authentic HCV core (nucleotides 342–874). Arrows depict transcriptional start sites. Polyadenylation signal is indicated as pA.

priming method (high prime DNA labeling kit, Boehringer Mannheim), respectively. Membranes were washed twice in 2 × SSC/0.1% SDS at room temperature and once in 0.1 × SSC/0.1% SDS at 60°C for 30 min, and were exposed to Kodak X-Omat AR films. Quantitation was achieved by PhosphorImager analysis (Molecular Dynamics) and computer-assisted densitometry (IMAGEQUANT software).

Western Blot Analysis. Cell pellets were resuspended in Tris-glycine sample buffer (Novex), and were boiled for 15 min at 95°C. After quantitation (Bradford protein assay reagent, Bio-Rad), 15 μg of protein for HCV core or 5 μg of protein for β-actin was separated by gel electrophoresis under reducing conditions on 14% Tris-glycine polyacrylamide gels (Novex), and was transferred to a nitrocellulose membrane (Immobilon P, Millipore) by Semidry transfer (*Trans*-Blot semidry transfer cell, Bio-Rad) for 2 h at 14 V. The membranes were incubated with primary antibody for 3 h by using an enhanced chemiluminescent Western blotting kit (Novex). Anti-HCV core monoclonal antibody (6C7) was generously provided by Harry Greenberg (Stanford University). Blots were exposed to film for 1 sec or 10 sec for quantitation of β-actin or 20 sec or 60 sec for quantitation of HCV core protein, respectively. Signals for β-actin were used to normalize the core protein signals. Band intensities were measured by using IMAGEQUANT or NIH IMAGE software.

Table 1. Selection of GCV-resistant cells transduced with ribozyme vector library

	Round	
Transduction	1	2
No ribozyme	0	0
Control ribozyme	8	72 ± 4
Ribozyme library	38 ± 12	873 ± 228

Hygromycin B and GCV-resistant HeLa 5′*tk* cells were obtained after transduction with the retroviral hairpin ribozyme vector library or a control ribozyme vector. For additional rounds of selection, the ribozyme genes were PCR-amplified and were recloned into retroviral viral vectors for transduction of fresh HeLa 5′*tk* cells. Numbers are given as GCV-resistant colonies/150 cm^2.

Results

An HCV-IRES Reporter Cell Line Allows for Selection of Ribozymes Targeting Cellular Cofactors of HCV IRES-Mediated Translation. A bicistronic reporter gene was constructed with the SV40 promoter

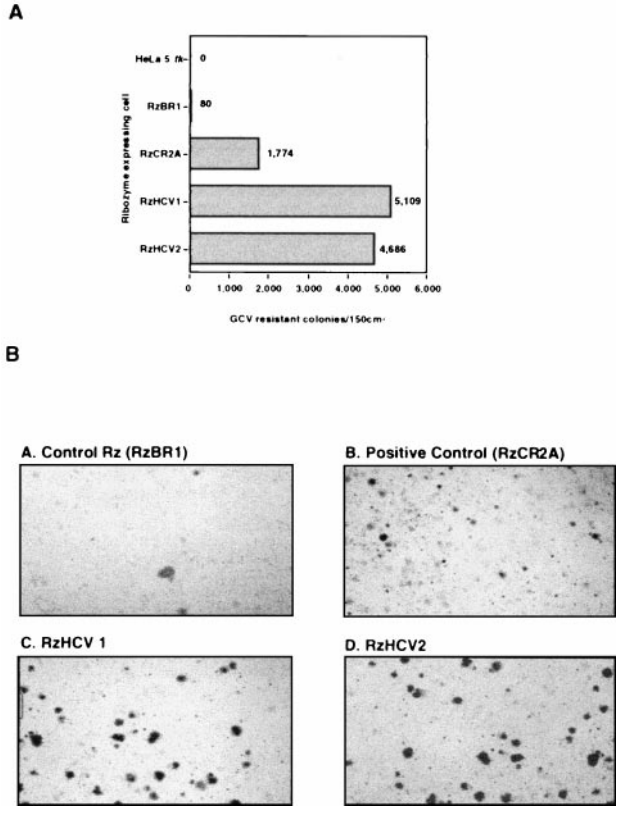
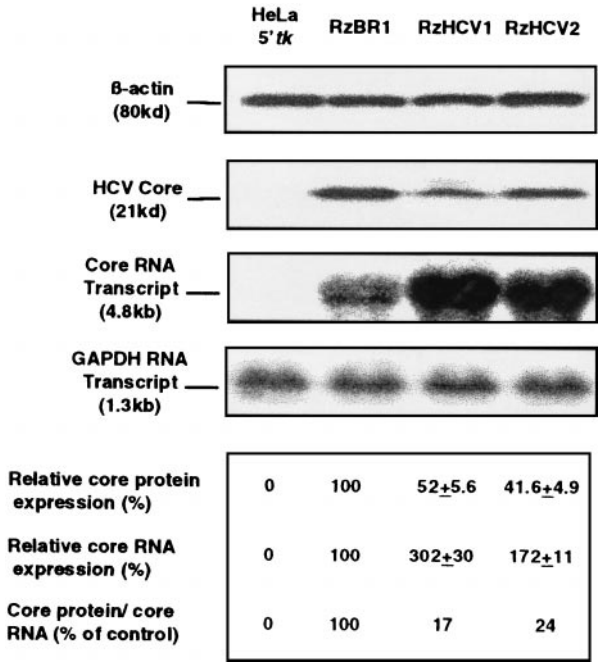


Fig. 2. Individual ribozymes selected from the randomized library confer GCV resistance after transduction of HeLa 5′*tk* reporter cells. (*A*) Two individual ribozyme candidates, RzHCV1 and RzHCV2, derived from sequence analysis of the resistant colonies after four rounds of GCV selection were cloned into retroviral vectors. Reporter cells were transduced with retroviral Rz vectors, and GCV-resistant colonies were selected. The negative control expresses a ribozyme that targets hepatitis B virus, and a ribozyme that directly targets the HSV-*tk* reporter transcript (RzCR2A) was used as a positive control for this assay. (*B*) Ganciclovir-resistant 5′*tk* cells stained with Coomassie blue 21 days after the transduction with single ribozyme candidates.

driving the expression of hygromycin B phosphotransferase and the HCV 5′-UTR (nucleotides 38–341) allowing for IRES-mediated translation of HSV-*tk* (Fig. 1*A*). A reporter cell line was generated by transfection of HeLa cells followed by hygromycin B selection and single cell cloning. HeLa cells stably expressing this construct (HeLa 5′*tk*) can be selected for intact transcription as well as cap-dependent translation by hygromycin B and for inhibition of HCV IRES-directed translation by ganciclovir (GCV). Retroviral vectors expressing a neo^R marker and either a control ribozyme or a library of ribozymes with randomized target recognition sequences (Fig. 1*B*) were used to stably transduce clonal populations of these reporter cells, which were then subjected to GCV selection. Cells that express ribozymes whose target mRNAs encode proteins necessary for IRES-dependent translation will no longer synthesize HSV-*tk* and therefore will survive GCV selection. After 2 weeks of G418 selection followed by four days of GCV selection, a 3- to 6-fold enrichment in the number of GCV-resistant colonies was observed in the library transduced cells over the control (Table 1). The ribozyme genes in these resistant cells were rescued and re-introduced into the reporter cells for additional rounds of selection. After two rounds, a 9- to 15-fold enrichment was achieved (Table 1), but analyses of 200 integrated Rz genes still revealed a high degree of sequence diversity (data not shown). However, after four rounds of selection, only nine distinct ribozyme sequences were detected in the GCV-resistant colonies, most of which had already been detected in previous rounds of selection. Two of these candidates (RzHCV1 and RzHCV2) were individually tested for their ability to confer GCV resistance after transduction of fresh reporter cells with retroviral vectors expressing each ribozyme alone. Compared with the control Rz transduced cells (RzBR1), both Rz candidates generated a 60-fold increase in the number of GCV-resistant colonies (Fig. 2 *A* and *B*). This result was not attributable to a reduction in HSV-*tk* mRNA levels because Northern blot analysis revealed either unchanged or actually increased steady state levels of this mRNA (data not shown). A ribozyme that directly targets the HSV-*tk* reporter transcript (RzCR2A) was used as a positive control for this assay.

The Selected Ribozymes Inhibit HCV IRES-Mediated Core Protein Expression. To ensure that the selected ribozymes did not merely interfere with the transport or metabolism of GCV, we evaluated their effects on IRES-mediated HCV core protein translation from the cassette within the retroviral vector (pLHPM) (Fig. 1*B*). We observed a reduction in core protein expression of 48 and 58% in GCV-resistant cells derived from RzHCV1- and RzHCV2-transduced cells, respectively, compared with control RzBR1-transduced cells (Fig. 3). Northern blot analysis revealed an increase in core transcript in RzHCV1- and RzHCV2-transduced cells. Therefore, the reduction in the "translatability" of the HCV core RNA, as measured by the ratio of core protein/core RNA levels, was actually greater than the apparent reduction in protein levels (Fig. 3). We observed a reduction of HCV IRES-dependent translatability of 83 and 76% for HCV1 and HCV2, respectively.

The eIF2Bγ Gene Is a Potential Target of RzHCV1. Using the 16 nt (8 nt in helix 1, 4 nt in helix 2 and NGUC) derived from the ribozyme binding sequences of RzHCV1 to search various databases (e.g., NCBI nonredundant and human EST databases), we failed to identify perfect matches to potential target genes. Therefore, 5′-RACE amplification was performed by using HeLa mRNA or cDNA as templates and ribozyme binding sequences as primers. We identified a 580-bp 5′-fragment sharing homology to the rat eIF2B gamma subunit gene (eIF2Bγ) (GenBank accession no. U38253). Based on this sequence information, we successfully cloned the entire human eIF2Bγ gene (GenBank accession no. nkit318828), which encodes a protein of 452 amino acids, with 91% identity



Relative core protein expression (%)	0	100	52±5.6	41.6±4.9
Relative core RNA expression (%)	0	100	302±30	172±11
Core protein/ core RNA (% of control)	0	100	17	24

Fig. 3. Immunoblot and RNA analysis of GCV-resistant HeLa-5′*tk* reporter cells after transduction with single ribozymes. Relative levels of HCV core protein, normalized to β-actin, present in protein lysate (15 μg) from ganciclovir-selected single Rz-transduced reporter cells. Signals for β-actin were used to normalize the core protein signals. The activity of the RzBR1 control (lane 2) was set as 100%. Parental cells expressing the bicistronic vector 5′*tk* (lane 1) lack core protein expression derived from transduction with the retroviral ribozyme construct. Fifteen micrograms of total RNA isolated from GCV-resistant cells were analyzed by Northern blotting. HCV core RNA was normalized to GAPDH, and the ratio of core protein/core RNA transcript is expressed as percent of the negative control. The data are presented as mean and SEM of three experiments.

to the rat protein (13). The putative RzHCV1 binding site contains one mismatch and two G-U base pairs in helix 1 of the Rz binding domains (Fig. 4*A*).

Additional Ribozymes Targeting Human eIF2Bγ mRNA Conferred GCV Resistance to HeLa 5′*tk* Cells. To further confirm eIF2Bγ as a target gene involved in GCV resistance and HCV core protein expression, we designed five "validation" ribozymes (VRz1–5) directed against additional GUC sites in the human eIF2Bγ mRNA (Fig. 4*B*). These were cloned into retroviral vectors for transduction of HeLa 5′*tk* cells. VRz1–5 conferred a >10-fold increase in GCV-resistant colonies compared with the control (Fig. 4*B*) whereas catalytically disabled RzHCV1 had no effect (data not shown), indicating that the RNA cleaving activity of the ribozyme was essential for the observed GCV resistance. When these GCV-resistant colonies were analyzed for levels of core protein and core RNA relative to the control, the results again indicated a significant reduction in the translatability of HCV core RNA (Fig. 4*B*). Interestingly, we again observed not only a reduction in core protein levels but also an increase in RNA levels in the GCV selected cells. It is possible that, to survive GCV selection through a ribozyme-dependent mechanism, cells with multiple copies of the active ribozyme gene are selected for, resulting also in an increase in the number of copies of the gene for HCV core protein.

A Second Protein (eIF2γ) in the Same Functional Pathway Is Independently Targeted by RzHCV2. We were intrigued to find that the binding sequence of RzHCV2 partially matched a sequence within the gamma subunit of human eIF2 (Fig. 4*C*). Because eIF2B is responsible for recycling eIF2, it is reasonable to speculate that eIF2 is a

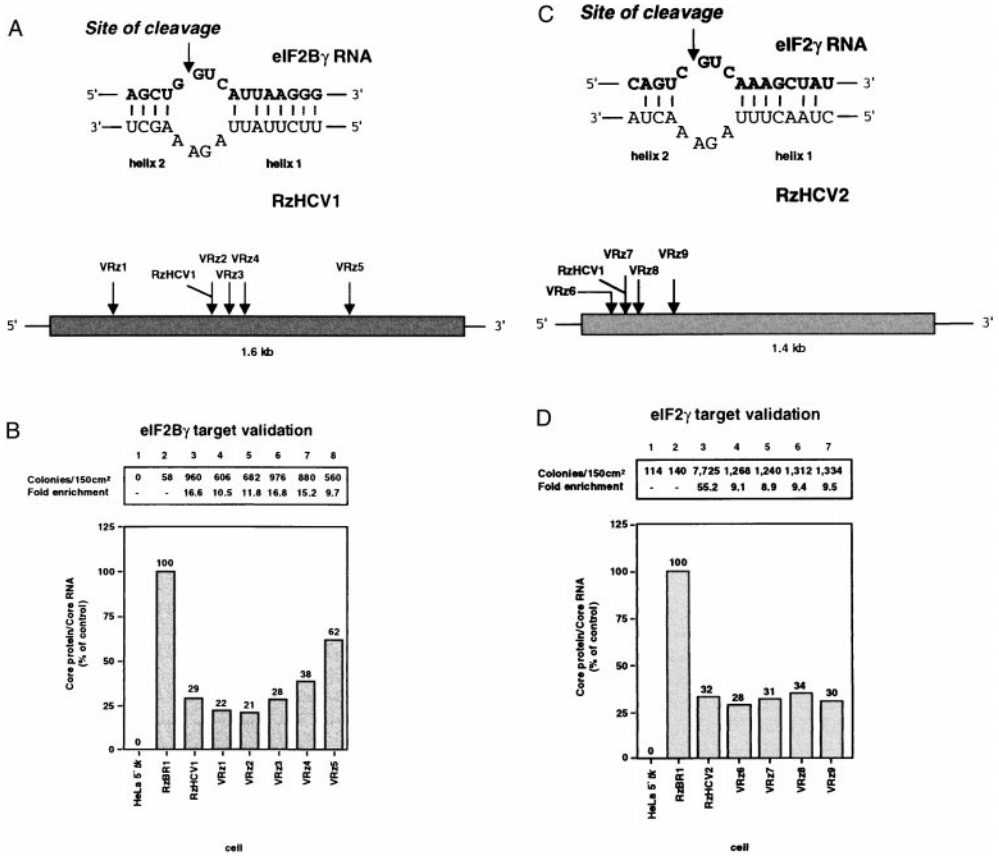


Fig. 4. Ribozyme-mediated identification and validation of eIF2Bγ and eIF2γ. (*A*) Ribozyme RzHCV1 targets human eukaryotic initiation factor 2B gamma subunit (eIF2Bγ) mRNA. The binding of ribozyme RzHCV1 to its target site (position 643) within the human eIF2Bγ mRNA is schematically illustrated, and the cleavage site is indicated (arrow). Partial sequence information of eIF2Bγ mRNA was obtained by using the sequence of the substrate binding arms (flanking the NGUC cleavage site) as oligonucleotide primer binding sites in a 5′-RACE amplification. (*B*) Five validation ribozymes (VRz1–5) were designed directed against GUC sites at position 253 (VRz1), 643 (VRz2, perfect match to RzHCV1 site), 695 (VRz3), 759 (VRz4), and 1203 (VRz5) within the human eIF2Bγ mRNA. HeLa 5′*tk* cells were stably transduced with retroviral particles from individual ribozymes. GCV-resistant colonies were counted 21 days after exposure to GCV and were pooled and analyzed for HCV core and GAPDH RNA transcript levels by Northern blot analysis. (*C*) RzHCV2 binds to eIF2γ mRNA (GenBank accession number NM001415). (*D*) Validation of eIF2γ by four ribozymes directed against GUC sites at positions 143 (VRz6), 172 (VRz7 perfect match corresponding to RzHCV2 site), 199 (VRz8), and 379 (VRz9).

downstream target also involved in IRES-mediated translation. Although the RzHCV2 sequence shows one mismatch in helix 1 and two mismatches in helix 2 to eIF2γ mRNA (Fig. 4*C*), *in vitro* cleavage assays confirmed the ability of RzHCV2 to cleave synthetic eIF2γ target RNA (data not shown). To determine whether eIF2γ was indeed a relevant target of RzHCV2, we constructed validation ribozymes against four potential cleavage sites in the eIF2γ mRNA (VRz6–9). Transduction of HeLa 5′*tk* cells with retroviral vectors expressing these ribozymes induced a ≥9-fold increase in the number of GCV-resistant colonies relative to the control (Fig. 4*D*). Again, we observed not only a reduction in the HCV core protein levels but also an increase in HCV RNA levels in the GCV-resistant colonies (Fig. 4*D*).

Expression of Functional Ribozymes Does Not Inhibit Cap-Dependent Protein Translation or Cell Growth. GCV- and hygromycin B-resistant cells derived from transduction with the functional ribozyme vectors or validation ribozymes were analyzed for cap-dependent translation and cell growth. We did not observe significant changes in cap-dependent translation of cellular proteins (GAPDH, β-actin) or cell growth after transduction of HeLa 5′*tk* cells with retroviral vectors expressing ribozymes compared with untransduced or control Rz transduced reporter cells (Fig. 5).

Discussion

Ribozymes are useful tools in inhibiting RNA expression from both viral and cellular genes (14). Hairpin ribozymes (10) as well as hammerhead ribozymes (15–17) targeting highly conserved regions of the 5′-end of the 9.5-kb HCV RNA molecule have been shown to significantly inhibit HCV core protein expression. In this study, we used a reverse functional genomics approach to discover potent ribozymes that specifically interfere with essential factors controlling viral replication (specifically IRES co-factors). These ribozymes would expand the repertoire of inhibitors of HCV replication that can be used in gene therapy strategies. In addition, the selected ribozymes helped to identify their corresponding cellular gene targets, which might represent suitable candidates for the development of antiviral drugs.

The basic premise of our approach is to have a stable reporter system for the selection of functional ribozymes from a random ribozyme library. In brief, we have constructed a bicistronic reporter with an SV40 promoter driving the expression of hygromycin B phosphotransferase (cap-dependent translation) and HCV IRES-mediated translation of the herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) (cap-independent translation). On application of the purine nucleoside analogue ganciclovir (GCV) ([9-(1,3-dihydroxy-2-propoxy)methyl] guanine), HSV-*tk* is able to monophosphorylate GCV, which is then further metabolized to its triphosphate form by endogenous cellular kinases (18). Triphosphorylated GCV is a toxic metabolite and competes with guanine triphosphate for incorporation into the growing DNA chain during S phase, leading to subsequent cell death (19). In our approach, HSV-*tk* translated by the HCV 5′-UTR serves as a negative selectable marker. On transfection into HeLa cells, the expression of *tk* confers sensitivity to GCV, leading to cell death on exposure to the drug. In contrast, cells with particularly active ribozymes inhibiting the expression of genes involved in IRES-mediated translation of HSV-*tk*, GCV-uptake and metabolism, as well as molecules involved in the pathway of GCV-induced cell damage, are selected through their concomitant resistance to GCV. Active ribozyme can be rescued from these cells, and partial sequence information of gene candidates can be obtained via the sequence of the ribozyme binding arms. After identification of a particular ribozyme targeting the gamma subunit of human eukaryotic initiation factor 2B (eIF2Bγ), we cloned the entire gene based on the sequence of the ribozyme binding domains. Interestingly, a second

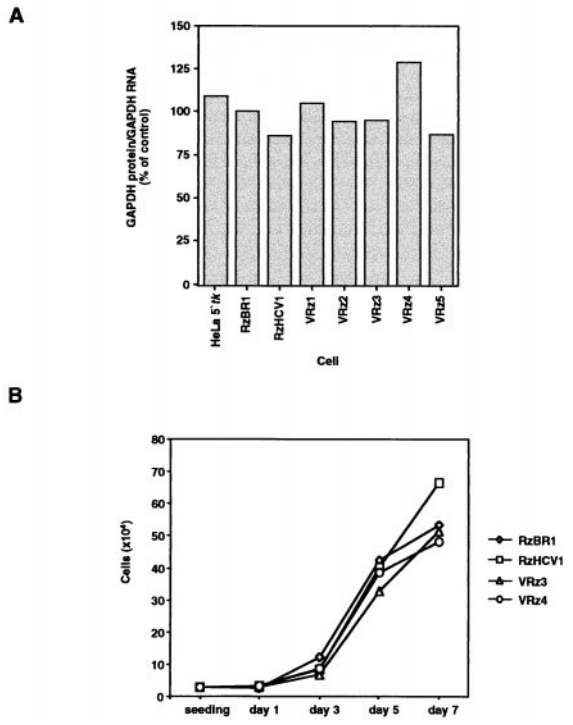


Fig. 5. Ribozyme effect on cap-dependent translation and cell growth. (*A*) Cap-dependent translation of cellular proteins was analyzed in hygromycin B- and ganciclovir-resistant reporter cells derived from transduction with ribozyme vectors. GAPDH protein/GAPDH RNA is shown as an example of cap-dependent translation in cells stably expressing ribozymes against eIF2Bγ. (*B*) Hygromycin- and ganciclovir-resistant reporter cells transduced with ribozyme vectors were analyzed for growth characteristics under relative serum starvation (0.5% FCS). Cells were seeded at 0.3×10^4cells/cm^2 into 6-well plates and were counted on the following days as indicated.

ribozyme selected independently apparently targets the gamma subunit of human eukaryotic initiation factor 2 (eIF2γ). The involvement of RzHCV1 and RzHCV2 and their gene targets in conferring HCV IRES-mediated ganciclovir resistance is further supported by the following observations: (*i*) RzHCV1 and RzHCV2 persisted through several rounds of selection; (*ii*) these Rzs individually were able to reproducibly confer a GCV-resistant phenotype whereas their disabled counterparts failed to do so; (*iii*) ribozymes directed against additional sites within the eIF2Bγ and eIF2γ mRNA induced the same GCV-resistant phenotype; and (*iv*) RNA analysis, Western blotting, and functional assays demonstrated a decrease in IRES-mediated translation of HCV core protein whereas cap-dependent translation was not impaired.

Both eIF2 and eIF2B are translation initiation factors that bind to EMCV IRES with high affinity (20). Recently, the involvement of eIF2 in HCV IRES-mediated translation has been further characterized (6). eIF2 delivers Met-tRNA^{Met} to the ribosome as a ternary initiation complex with GTP and is subsequently released as an inactive complex with GDP (21). eIF2B is the guanine nucleotide-exchange factor responsible for recycling eIF2 to its active GTP-bound state, a key regulatory step in eukaryotic protein synthesis (22). However, the specific roles of the γ subunits of both factors are not clear. Based on this selection system using a randomized ribozyme library, we show that internal initiation mediated by HCV IRES can be substantially inhibited by partial depletion of eIF2γ and eIF2Bγ whereas general protein translation and cell growth remain unaffected. This indicates a crucial, previously not recognized role of eIF2γ and eIF2Bγ as functionally important cofactors of HCV IRES-mediated translation. However, we observed only a moderate (20%) decrease in eIF2Bγ and eIF2γ mRNA levels in the GCV-resistant colonies derived from transduction with the corresponding functional ribozyme vectors (data not shown), suggesting that higher degrees of inhibition may result in cellular toxicity or impaired survival.

Our data also support previous findings that absolute conservation of the base-pairing in the ribozyme binding sequences might not be required for optimal activity (23) because both RzHCV1 and RzHCV2 have one or more mismatches with their presumed target sequences. Despite the requirement for the GUC triplet to cleave a target RNA (24), ribozyme *in vitro* selection data have indicated that absolute conservation of the base-pairing in helices I and II is not required, and single mismatches in the binding domains (as in case of RzHCV1 targeting human eIF2Bγ mRNA) might therefore be acceptable to still maintain (or even favor) substrate cleavage (23). This observation may increase the challenge of applying this powerful approach for functional gene discovery in general. However, as in our case, additional genomics information (e.g., expected functional category or expression patterns of the candidate genes) will help to narrow down target genes for further validation. The identification of two proteins in the same functional pathway with independently selected ribozymes suggests that the randomized ribozyme vector library may be an effective strategy to elucidate distinct metabolic pathways involved in other functional selection systems. Furthermore, eIF2γ and eIF2Bγ as well as additional cellular factors to be identified and characterized by this approach might serve as potential targets to specifically inhibit HCV translation and replication.

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