

transformed into these strains to determine if yeast growth was affected in the absence of PKR. No retarded or stimulated growth phenotype was observed in these strains in the presence of E2.

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24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
25. RY1-1 carries two chromosomal copies of PKR at the Leu2 locus (18) and has wild-type eIF2 α .
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Replication of Subgenomic Hepatitis C Virus RNAs in a Hepatoma Cell Line

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An estimated 170 million persons worldwide are infected with hepatitis C virus (HCV), a major cause of chronic liver disease. Despite increasing knowledge of genome structure and individual viral proteins, studies on virus replication and pathogenesis have been hampered by the lack of reliable and efficient cell culture systems. A full-length consensus genome was cloned from viral RNA isolated from an infected human liver and used to construct subgenomic selectable replicons. Upon transfection into a human hepatoma cell line, these RNAs were found to replicate to high levels, permitting metabolic radiolabeling of viral RNA and proteins. This work defines the structure of HCV replicons functional in cell culture and provides the basis for a long-sought cellular system that should allow detailed molecular studies of HCV and the development of antiviral drugs.

HCV is a plus (+) strand RNA virus that causes acute and chronic liver diseases (1). Although the acute phase of infection is usually associated with mild symptoms, most patients fail to clear the virus and contract persistent infection that frequently leads to chronic liver disease, including cirrhosis and hepatocellular carcinoma. Given the high prevalence of the virus, HCV has become a focus of intensive research.

Originally cloned in 1989 (2), the viral genome is now well characterized. It has a length of ~9.6 kb and its single, long open reading frame (ORF) encodes a ~3000-amino acid polyprotein (3) (Fig. 1A). The ORF is flanked at the 5' end by a nontranslated region (NTR) that functions as an internal ribosome entry site (IRES) and at the 3' end by a highly conserved sequence essential for genome replication (4). The structural proteins are in the NH₂-terminal region of the polyprotein and the nonstructural proteins (NS) 2 to 5B in the remainder. By analogy to related +strand RNA viruses, replication occurs by means of a minus (–) strand RNA

intermediate and is catalyzed by the NS proteins forming most likely a cytoplasmic membrane-associated replicase complex.

Despite the availability of cloned infectious genomes (5), molecular studies of HCV replication and the development of antiviral drugs have been hampered by the low efficiencies of currently available cell culture systems and by the fact that the only animal model is the chimpanzee. Thus, to date, research on HCV replication has depended largely on the infection of cell lines or primary cell cultures with sera and the detection of viral replicative intermediates with the highly sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) (6).

To overcome these limitations, we established an efficient cell culture system that is based on the transfection of cloned viral consensus genome sequences. Owing to the high amount of HCV RNA, we used as starting material for cloning total liver RNA isolated from a chronically infected patient who had undergone liver transplantation (7). Using long-distance RT-PCR, we amplified the complete ORF in two overlapping fragments. Several clones of each fragment were analyzed, and an isolate-specific consensus sequence was established, which belongs to the worldwide distributed genotype 1b. The 5' and 3' NTRs were amplified separately by standard RT-PCR and were assembled with

the reconstituted consensus ORF. A 5'-flanking T7 RNA polymerase promoter and an engineered restriction site at the 3' end allowed for production of run-off RNA transcripts with authentic 5'- and 3'-terminal sequences (8). As a negative control for all transfection experiments for each parental construct, a defective genome was generated carrying an in-frame 10-amino acid deletion (Δ) encompassing the NS5B RNA polymerase active site.

We initially transfected various cell lines and primary human hepatocytes with in vitro transcripts corresponding to the cloned full-length genome or the deletion mutant. We monitored RNA replication by comparing the amounts of +strand RNA detected by RT-PCR in cells transfected with the parental or the defective genome (9). In no case was a significant difference found between the genomes, suggesting that no replication occurred.

The failure of these experiments might be attributable to errors in the cloned genome, low transfection efficiencies, or cytopathogenicity of HCV, which would lead to a selective loss or growth disadvantage of cells supporting virus replication. Alternatively, the cell lines used might be nonpermissive or support only low levels of viral RNA replication not detectable with our method. To overcome some of these restrictions, we constructed selectable replicons that transduced neomycin (G418) resistance only to those cells that support HCV replication. Based on recent results with flaviviruses (10) and pestiviruses (11) and on mapping of the HCV-IRES, we generated bicistronic constructs (12) (Fig.

Table 1. G418-resistant cell clones obtained after transfection of Huh-7 cells with in vitro-transcribed HCV replicon RNAs. The number of viable cell clones after subpassage of clones obtained in experiment 1 is given in the right column.

Construct	Experiment			Subpassages (exp. 1)
	1	2	3	
I ₃₇₇ neo/NS2-3'/wt	12	20	38	1
I ₃₇₇ neo/NS2-3'/ Δ	0	3	8	0
I ₃₇₇ neo/NS3-3'/wt	20	>60	40	2
I ₃₇₇ neo/NS3-3'/ Δ	2	8	18	0
I ₃₈₉ neo/NS2-3'/wt	6	20	25	1
I ₃₈₉ neo/NS2-3'/ Δ	1	10	4	1
I ₃₈₉ neo/NS3-3'/wt	30	15	17	5
I ₃₈₉ neo/NS3-3'/ Δ	1	2	6	0

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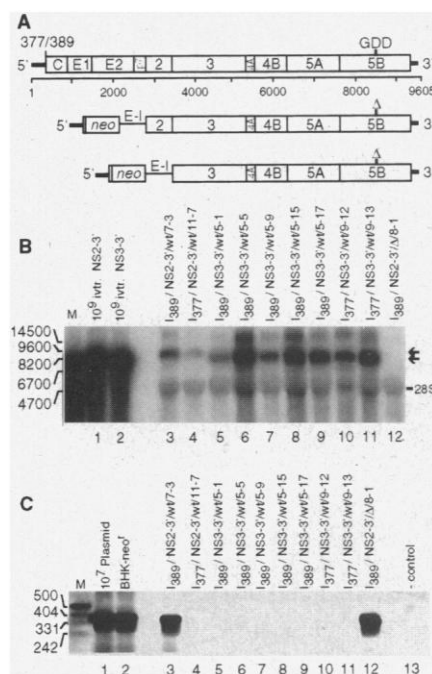
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1A). These were composed of two variants of the HCV-IRES [nucleotides (nt) 1 to 377 or 1 to 389], the neomycin phosphotransferase (*neo*) gene, the IRES of the encephalomyocarditis virus, which directs translation of HCV sequences from NS2 or NS3 up to NS5B, and the 3' NTR. Therefore, these replicons were designated I₃₇₇/NS2-3' (or I₃₇₇/NS3-3') and I₃₈₉/NS2-3' (or I₃₈₉/NS3-3'). In vitro transcripts derived from these constructs were transfected in parallel with the analogous mutants carrying the in-frame deletion of the NS5B polymerase active site (Δ constructs). Particular care was taken to remove template DNA, which might otherwise integrate into transfected cells and confer G418 resistance independent of HCV replication (13). In three separate experiments performed with the human hepatoma cell line Huh-7 (14), we observed a clear difference in the number of resistant cell clones between the parental (wild-type, wt) and the defective (Δ) replicons (Table 1) (15). Cell clones obtained in the first experiment were isolated and subpassaged. Most of the cells died during this procedure and ultimately we obtained nine clones derived from transfection with parental replicons and one clone derived from

transfections with a defective NS2-3' RNA (clone 8-1). With the exception of a reduced doubling time, no consistent difference was found between these nine cell clones and clone 8-1 or the parental Huh-7 cell line.

The main criteria for functional replicons are the formation of viral RNAs of correct size and the absence of (integrated) plasmid DNA that could confer G418 resistance. To detect HCV RNAs in these cells, we isolated total RNAs and analyzed a sample on Northern blots using a +strand-specific RNA probe (Fig. 1B). With the exception of clone 8-1, homogeneous RNAs of correct lengths (\sim 8640 nt for the NS2-3' and \sim 7970 nt for the NS3-3' replicon) were detected, suggesting that functional replicons conferred the G418 resistance. Although the amount of HCV RNA detected in these clones was variable during passages, the highest amount was consistently obtained with cell clones 5-5, 5-15, and 9-13 (lanes 6, 8, and 11). To exclude the possibility that resistance was due to plasmid DNA integrated into the host cell genome and transcribed under control of a cellular promoter, we analyzed DNA of each clone by *neo*-specific PCR (Fig. 1C). With the exception of cell clones 7-3 and 8-1 (lanes

Fig. 1. Structure of the HCV subgenomic replicons and detection of viral RNA replication in transfected Huh-7 cells. (A) (Top) Schematic of the HCV genome, indicating the location of cleavage products within the polyprotein and the 5' and 3' NTRs (thick lines). The positions of the 3' borders of the HCV-IRES selected for construction and the "GDD active site" of the NS5B RNA polymerase are shown above. Numbers below the genome refer to the nucleotide positions of our consensus isolate. The structures of the selectable replicons composed of the 5' HCV-IRES, the *neo* gene, the EMCV-IRES (E-I), and HCV sequences from NS2 or NS3 up to the authentic 3' end are given below. Δ indicates the position of the 10-amino acid deletion in the NS5B polymerase (amino acids 2732 to 2741 of the polyprotein). (B) Detection of +strand HCV RNA in subpassaged Huh-7 cell clones. Total RNA was isolated from the cells (20), and 10 μ g of RNA corresponding to 5×10^5 cells was analyzed by denaturing agarose gel electrophoresis. Replicon RNA was detected by Northern blot with a radiolabeled RNA probe complementary to the *neo* gene and the HCV-IRES. In vitro transcripts (10^9) (ivtr.) corresponding to the parental I₃₈₉-replicons were analyzed in parallel (lanes 1 and 2). Arrows point to HCV RNAs. Lane M, positions of RNA size markers (in nucleotides); the position of the 28S ribosomal RNA is indicated on the right. The RNA marker fragments contain HCV sequences and therefore hybridize with the RNA probe. (C) Absence of integrated replicon DNA in most selected cell clones. DNA was isolated from Huh-7 clones with nucleospin columns (Macherey-Nagel, Düren, Germany) and subsequently treated with ribonuclease A for 1 hour. After phenol-chloroform extraction and ethanol precipitation, 1 μ g of DNA corresponding to 4×10^4 to 8×10^4 cells was analyzed by PCR with *neo*-specific primers (5'-TCAAGACCGACCTGTCCGGTGCCC-3' and 5'-CTTGAGCCTGGCGAACAGTTCGGC-3'). Amplified fragments were analyzed by Southern (DNA) blot with a digoxigenin-labeled DNA probe corresponding to the *neo* gene. As a positive control, PCR was performed with 10^7 plasmid molecules or 1 μ g of DNA from a BHK cell line stably transfected with the *neo* gene (lanes 1 and 2), and as a negative control PCR was performed without a DNA template (lane 13). Lane M, molecular size markers (in base pairs).



3 and 12), no *neo*-DNA was detected, confirming that G418 resistance of most clones was conferred by HCV RNA replicons. Irrespective of these results, generation of HCV RNAs of the correct size from integrated plasmid DNAs is highly unlikely, because the plasmid used for in vitro transcription contained neither a eukaryotic promoter nor a polyadenylation signal. Therefore, in the case of clone 7-3, resistance most likely was mediated both by the replicon and integrated *neo*

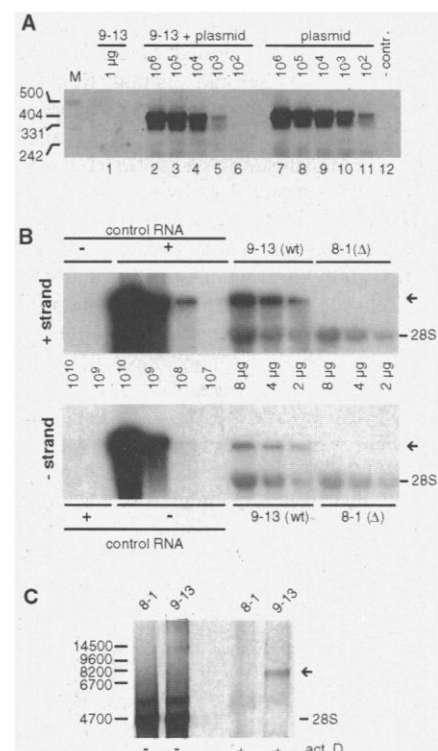


Fig. 2. Characterization of cell clone 9-13, which harbors the NS3-3' replicon RNA. (A) Exclusion of *neo* DNA was done by PCR and Southern blot as in Fig. 1C with 1 μ g of DNA (lane 1). Sensitivity was determined by using 10^6 to 10^2 plasmid molecules (I₃₇₇/NS3-3'/wt) either directly for PCR (lanes 7 to 11) or after addition of 1 μ g of 9-13 DNA (lanes 2 to 6). As a negative control, PCR was performed without DNA (lane 12). (B) Quantification of HCV + and -strand RNA. Eight, 4, or 2 μ g of total RNA isolated from cell clones 9-13 and 8-1 were analyzed by Northern blot in parallel with analogous in vitro transcripts of given polarity (control RNA). + Strand RNA (upper panel) was detected with an RNA probe complementary to the *neo* gene and the HCV IRES, and -strand RNA (lower panel) was detected with an RNA probe complementary to the NS3 sequence. Arrows indicate the positions of replicon RNAs. (C) Replication of HCV RNA is resistant to actinomycin D. About 5×10^5 cells of clones 9-13 and 8-1 were incubated with 100 μ Ci of [³H]uridine for 16 hours in the absence (-) or presence (+) of actinomycin D (act. D, 4 μ g/ml). After labeling, total RNA was prepared and analyzed by denaturing agarose gel electrophoresis. Only one-tenth of total RNA is shown in the first two lanes. Radiolabeled RNAs were visualized with a BAS-2500 Bio-Imager (Fuji).

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DNA sequences, whereas resistance of cell clone 8-1 was conferred exclusively by integrated plasmid DNA.

To confirm that G418 resistance was mediated by autonomously replicating HCV RNAs, we chose cell clone 9-13 for further analysis, because it contained high amounts of HCV RNA, and used clone 8-1 throughout as a negative control. To rule out the presence of *neo*-DNA in clone 9-13 with high stringency, we performed a PCR assay that allowed detection of <0.02 *neo* copies per cell (Fig. 2A). Even with this level of sensitivity, no plasmid DNA was found. To calculate the amounts of HCV + and -strand RNAs in these cells, we analyzed serial dilutions of total RNA on Northern blots using strand-specific radiolabeled RNA probes (Fig. 2B). About 10^8 +strand RNA copies per microgram of total RNA were detected; this corresponds to 1000 to 5000 molecules per cell. A 5- to 10-fold lower amount of -strand RNA was detected, consistent with the notion that -strand RNA is the replicative intermediate serving as template for synthesis of excess +strand molecules. Because this reaction is carried out by the NS5B RNA-dependent RNA polymerase, generation of HCV RNAs should be resistant to actinomycin D, an antibiotic that selectively inhibits RNA synthe-

sis from DNA but not RNA templates. To test this hypothesis, we incubated cells with [3 H]uridine in the presence of actinomycin D and analyzed the radiolabeled RNAs (Fig. 2C). In agreement with the inhibitor profile of the NS5B polymerase (16), replication of HCV RNA was not affected by actinomycin D, whereas synthesis of cellular RNAs was blocked.

For analysis, the viral proteins were radiolabeled with 35 S-methionine and 35 S-cysteine and isolated by immunoprecipitation. All HCV proteins were detected, and they corresponded in size to the proteins observed after transient expression of the same replicon construct in naïve Huh-7 cells (Fig. 3A). Immunostaining with NS3- and NS5A-specific antisera revealed that NS3 and NS5A localized almost exclusively to the cytoplasm, although there was a small amount of NS5A staining in the nucleus (Fig. 3B). This predominant cytoplasmic localization of viral antigens provides strong evidence that HCV RNA replicates in the cytoplasm, as is the case for most RNA viruses.

Our data define the structures of selectable HCV RNAs replicating autonomously and to high levels in a human hepatoma cell line. The fact that the structural proteins and NS2 are not required for replication emphasizes the close evolutionary relationship be-

tween HCV and the animal pathogenic pestiviruses, for which analogous RNAs have been described (11). Although replication of these RNAs has not been quantified yet at a per cell basis, pestiviruses appear to replicate more efficiently, yielding titers of $\sim 1.5 \times 10^4$ genomes per cell in the acute phase of infection (17). The ~ 10 -fold lower value we found with the HCV replicons might reflect intrinsically lower HCV replication or the fact that the cell clones we established resemble a persistent and not an acute infection.

The low number of cell clones obtained may indicate that only a few cells in the culture support HCV RNA replication, or that a level of replication required for G418 resistance was reached in only a few cells. Alternatively, high-level replication may reflect an adaptation of the replicon to the host cell. As such adaptation would require one or several mutations, formation of an adapted replicon would be rare. However, this possibility is unlikely for two reasons: first, sequence analysis of several replicons recloned from two different cell clones did not reveal consistent mutations (18); second, upon serial passage of the replicons in naïve Huh-7 cells, we did not observe a significant increase of the number of colonies (19). Thus, the low efficiency most likely is attributable to particular host cell conditions or factors present in only a few cells.

The replicons described in this study may allow a detailed analysis of HCV replication, pathogenesis, and evolution in cell culture. In principle, viral RNAs can be generated in unlimited quantities, and the viral genome can be manipulated for genetic analyses of HCV functions that are essential for replication. Functional replicons also provide a cell-based assay system for the evaluation of antiviral drugs.

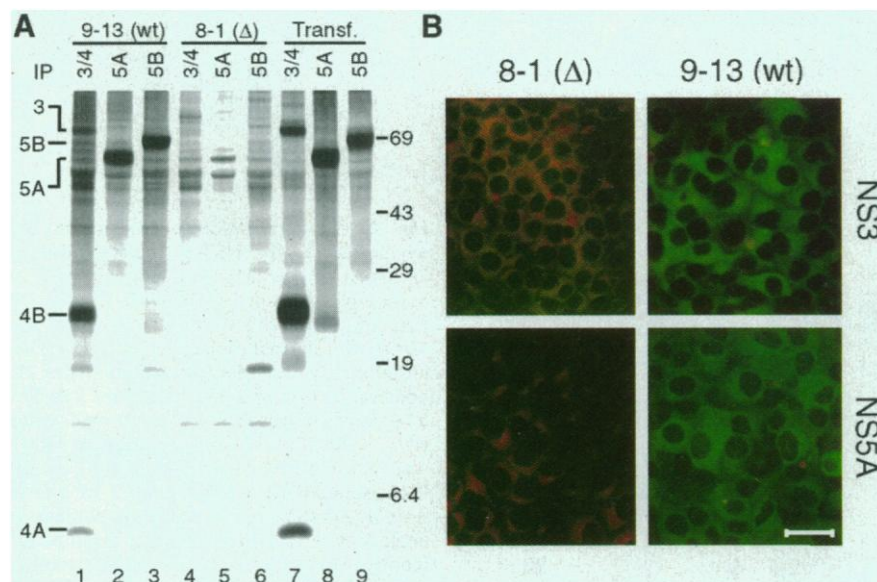


Fig. 3. Detection of HCV antigens in cell clone 9-13. (A) 9-13 (wt) and 8-1 (Δ) cells were incubated with protein labeling mixture (NEN Life Science) for 16 hours, and HCV proteins were isolated from cell lysates by immunoprecipitation (IP) under nondenaturing conditions (21) with antisera specified above the lanes. Immunocomplexes were analyzed by Tricine SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. To obtain authentic size markers, the homologous replicon construct ($I_{377}/NS3-3'/wt$) was transiently expressed with the vaccinia virus T7-hybrid system (22) in Huh-7 cells and processed in parallel (lanes 7 to 9). Positions of HCV-proteins are given on the left, and molecular size standards (kilodaltons) on the right. The NS3/4-specific antiserum preferentially reacts with NS4A and NS4B. (B) Subcellular localization of HCV antigens as determined by immunofluorescence. Twenty-four hours after seeding on cover slips, 9-13 (wt) and 8-1 (Δ) cells were fixed with methanol-acetone, incubated with polyclonal NS3- or NS5A-specific antisera, and bound antibody was detected with a fluorescein isothiocyanate-conjugated antibody to rabbit immunoglobulin G. Cells were counterstained with Evans blue. Bar, 25 μ m.

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7. Total RNA was isolated from explanted liver (~ 100

- mg) (20), and 1 μ g was used for reverse transcription with primers A6103 (GCTATCAGCCGGTTCATCACTGC) or A9413 (CAGGATGGCCTATTGGCCGTGAG) and the Expand Reverse Transcriptase System (Boehringer Mannheim, Germany). PCR was performed with the Expand Long Template System (Boehringer Mannheim) in buffer containing 2% dimethylsulfoxide. After 1 hour at 42°C, one-eighth of the mixture was used for the first PCR with primers A6103 and S59 (TGTCTCACCAGCAAGCGTCTAG) or A9413 and S4542 (GATGAGCTCGCCGCAAGCTGTCC). After 40 cycles, one-tenth was used for the second PCR with primers S59 and A4919 (AGCA-CAGCCCGCTCATAGCACTCG) or S4542 and A9386 (TTAGTCCCCGTTTCATCGGTGG). After 30 cycles, the PCR products were purified by preparative agarose gel electrophoresis, and eluted fragments were ligated into vector pCR2.1 (Invitrogen) or pBSK II (Stratagene). Four clones of each fragment were analyzed and a consensus sequence was established. To resolve ambiguities, we amplified shorter PCR fragments covering the corresponding region and sequenced multiple clones. The 3' NTR was obtained by conventional PCR with an antisense primer covering the last 24 nt of the genome (4). The authentic 5' NTR downstream of the T7 promoter was generated by PCR with an oligonucleotide corresponding to a truncated T7 promoter (TAATACGACTCACTATAG) and the first 88 nt of HCV and a plasmid carrying one of the 5' fragments of the genome. The complete genome was assembled from subgenomic fragments carrying the least numbers of nonconsensus nucleotide changes and inserted into a modified pBR322 vector. Nonconsensus changes were removed by site-directed mutagenesis. To generate run-off transcripts with an authentic 3' end, we modified the 3' NTR of our isolate (terminating with TGT) to match the sequence of genotype 3 [clone WS; A. A. Kolykhalov, S. M. Feinstone, C. M. Rice, *J. Virol.* **70**, 3363 (1996)] terminating with AGT, which allowed us to introduce a recognition sequence for the restriction enzyme Sca I (AGTACT) at the end of the 3' NTR. A guanine was replaced with an adenine nucleotide at position 8180 of the genome to remove an internal Sca I site. After assembly of the full-length genome with appropriate 5' and 3' NTRs, the complete HCV sequence [European Molecular Biology Laboratory (EMBL) accession number AJ238799] was verified.
8. Plasmid DNA was linearized with Sca I and used for in vitro transcription reactions containing 80 mM Hepes (pH 7.5), 12.5 mM MgCl₂, 2 mM spermidine, 40 mM dithiothreitol, 2 mM of each nucleoside triphosphate, RNasin (1 U/ml), DNA template (50 μ g/ml), and T7 RNA polymerase (~2 U/ μ l). To increase the yields, after 2 hours at 37°C an extra 1 U of T7 RNA polymerase was added per microliter, and the reaction was incubated for an additional 2 hours. DNA was removed by extraction with acid phenol [W. Kedzierski and J. C. Porter, *BioTechniques* **10**, 210 (1991)] and treatment with 2 U of deoxyribonuclease (DNase) per microgram of DNA for 60 min at 37°C. RNA was purified and analyzed by denaturing agarose gel electrophoresis.
 9. Purified in vitro transcripts corresponding to the parental or the inactivated HCV genome were used for transfection of human hepatoma cell lines and primary human hepatocytes. Cell lines were maintained in a medium as described [B. J. Yoo *et al.*, *J. Virol.* **69**, 32 (1995)] and passaged once a week. Total RNA was prepared from transfected cells, and serial dilutions were used for RT-PCR amplification of the 5' NTR or an NS5B sequence covering the 10-amino acid deletion. This allowed discrimination between the parental and the inactivated genome carrying the in-frame deletion. We monitored RNA replication by comparing the amounts of HCV RNA found in cells transfected with the wild-type or the inactivated genome. Input RNA was detected for up to three passages, with similar amounts seen for both genomes.
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 18. As will be reported elsewhere (V. Lohmann and R. Bartenschlager, in preparation), we recloned HCV replicons from 1 μ g of total RNA by RT-PCR using primers S59 and A9413 (7). For amplification of 5' and 3' NTRs, we used an RNA ligation approach before PCR. Among 10 sequenced replicons, no converging mutations were found. Each replicon contained 6 to 12 amino acid substitutions scattered throughout the HCV ORF. The NTRs were highly conserved, and only sporadic nucleotide changes were observed.
 19. HCV RNA contained in total RNA of cell clones 5-15 and 9-13 was quantified by Northern blot, and 20 μ g of total RNA were used for transfection (15). An equivalent number of in vitro-transcribed replicon molecules was supplemented with total RNA from naïve Huh-7 cells to the same concentration and transfected in parallel. Cotransfection of a construct directing the expression of firefly luciferase was used to correct for transfection efficiency. No significant difference in the number of G418-resistant colonies was found between total RNA isolated from the two cell clones and the in vitro RNA mixture.
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 23. We thank R. Devos and H. Schaller for critical reading of the manuscript and stimulating discussions; P. Hahn, K. Rispeter, and P. Hilgert for technical assistance; B. Moss for vaccinia virus vTF7-3; M. Billeter for plasmid encoding T7 RNA polymerase; and M. J. Reddehase for continuous support and critical reading of the manuscript. Supported by grants from Roche Products, the German Ministry for Research and Technology (01 KI 9653/9), and the German Research Society (Ba 1505/1-2).

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Positive Selection of Natural Autoreactive B Cells

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Lymphocyte development is critically influenced by self-antigens. T cells are subject to both positive and negative selection, depending on their degree of self-reactivity. Although B cells are subject to negative selection, it has been difficult to test whether self-antigen plays any positive role in B cell development. A murine model system of naturally generated autoreactive B cells with a germ line gene-encoded specificity for the Thy-1 (CD90) glycoprotein was developed, in which the presence of self-antigen promotes B cell accumulation and serum autoantibody secretion. Thus, B cells can be subject to positive selection, generated, and maintained on the basis of their autoreactivity.

Although it is widely accepted that B cells with self-reactivity are deleted or rendered functionally inactive (I), autoantibodies can

be found in the serum of healthy animals, referred to as "natural autoantibodies," in an apparent paradox to the clonal tolerance theory (2, 3). In contrast with disease-associated hypermutated immunoglobulin G (IgG) antibodies, these natural autoantibodies are predominantly IgM, encoded by mostly unmutated germ line variable (V) region genes, and are independent of T cell help for secretion. Natural autoantibody constitutes a large fraction of serum Ig, and the B cells that produce natural autoantibodies frequently express CD5, a phenotype rare in spleen, but more common in the peritoneal cavity of mice (4,

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