Subgenomic Hepatitis C Virus Replicons Inducing Expression of a Secreted Enzymatic Reporter Protein

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We constructed dicistronic, subgenomic hepatitis C virus (HCV) replicons in which the sequence encoding the human immunodeficiency virus (HIV) tat protein was placed in the upstream cistron, between the HCV 5’NTR and a picornaviral 2A proteinase sequence fused to the selectable marker Neo. Stably transformed Huh7 cells expressing secreted alkaline phosphatase (SEAP) under transcriptional control of the HIV LTR promoter actively secreted SEAP following transfection with these replicon RNAs. Extracellular SEAP activity correlated closely with intracellular HCV RNA levels, as determined by Northern blotting and real-time RT-PCR analysis. These RNAs replicated efficiently despite the absence of core-protein-coding sequence downstream of the HCV IRES. The replication efficiency of replicons derived from the HCV-N strain of HCV was significantly greater than those derived from Con1 in transiently transfected cells. Using this reporter system, we have demonstrated significant differences in the response to interferon-α-2b in cell lines containing replicons derived from these two strains of HCV. © 2002 Elsevier Science (USA)

Key Words: hepatitis C virus; replication; internal ribosome entry site; core protein; replicon; interferon; tat; secreted alkaline phosphatase.

INTRODUCTION

Infection with hepatitis C virus (HCV) is an important cause of chronic liver disease throughout the world. Up to 70% of HCV infections become persistent, resulting in chronic liver disease of varying degrees of severity (Alter, 1997). Although the incidence of HCV infection has been reduced in developed countries in recent years by the screening of blood units and other public health efforts, in many areas infection remains uncontrolled. Over 170 million people are estimated to be infected with this virus worldwide. Within the United States, approximately 1.5% of the general population has serological evidence of prior infection with HCV (Alter et al., 1999). Most of these individuals remain actively infected with the virus and are thus at risk for long-term complications, including cirrhosis and hepatocellular carcinoma (Seeff, 1997). Interferon-α is widely used in developed countries for the treatment of such individuals. However, despite considerable advances in the therapy of hepatitis C, even the most effective treatment with interferon-α in combination with ribavirin fails to eliminate infection in more than 50% of those infected with the most prevalent viral genotypes (Lindsay et al., 2001; McHutchison et al., 1998). This high frequency of treatment failure points to the need for more specific, less toxic, and more active antiviral therapies for HCV.

Despite the absence of fully permissive cell cultures allowing efficient in vitro propagation of the virus, much has been learned about HCV since its discovery a little over a decade ago. Classified within the genus Hepacivirus of the family Flaviviridae, the viral genome is a single-stranded, positive-sense RNA molecule approximately 9.6 kb in length (for a review, see Major and Feinstone, 1997). The genomic RNA contains a single large open reading frame (ORF) encoding a polyprotein that is translated under the control of an internal ribosome entry site (IRES) located within the 342-nt-long 5’NTR segment. This polyprotein comprised several structural proteins (core, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) and undergoes posttranslational cleavage catalyzed by both cellular and viral-encoded proteinases (Reed and Rice, 2000). The 5’ and 3’NTRs are relatively well conserved among different HCV genotypes and contain RNA signals involved in recognition of the RNA by the viral replicase complex (Friebe et al., 2001; Kim et al., 2002, Yanagi et al., 1999; Kolykhalov et al., 2000).

Until recently, studies examining the involvement of either viral RNA elements or proteins in the replication of HCV RNA could only be carried out in chimpanzees. This technical limitation was overcome recently by demonstration of the in vitro replication competence of
subgenomic, dicistronic HCV RNAs in which most of the structural protein-coding sequence was replaced with sequence-encoding neomycin phosphotransferase (Neo) (Lohmann et al., 1999). In an effort to retain optimal translation directed by the upstream HCV IRES, the Neo sequence was fused at its 5′ end to the first 36 to 48 nt of the HCV ORF in these RNAs. At its 3′ end, the Neo sequence was fused to the IRES of encephalomyocarditis virus (EMCV), which directed the translation of downstream HCV sequence extending from either the NS2- or the NS3-coding region to the 3′ terminus of the genome (Lohmann et al., 1999). G418 selection of human hepatoma cells that were transfected with these RNAs gave rise to multiple antibiotic-resistant cell colonies containing high abundances of viral proteins and RNA (Lohmann et al., 1999). Following the original description of these replicons, cell-culture-adaptive mutations within the NS3, NS5A, and NS5B proteins were shown to significantly enhance the ability of the replicons described by Lohmann et al. to undergo autonomous amplification in cultured Huh7 cells (Krieger et al., 2001; Blight et al., 2000). In addition, replicons derived from a second genotype 1b virus, HCV-N, were shown recently to be capable of efficient replication in these cells in the absence of any additional adaptive mutations (Guo et al., 2001; Ikeda et al., 2002). These subgenomic replicons are likely to prove valuable in high-throughput screening assays that are aimed at identifying novel antiviral compounds with activity against the HCV RNA replicase, and thus they represent a substantial advance for this field.

In this article, we describe a useful refinement of these subgenomic replicons that simplifies detection of HCV RNA replication in both transiently transfected cells and established cell clones selected under antibiotic pressure. By modifying the upstream cistron so that it expresses the tat protein of human immunodeficiency virus (HIV) in addition to the Neo resistance marker, we developed replicon RNAs that are capable of signaling their presence and abundance in cells by the secretion of placental alkaline phosphatase (SEAP), expressed under transcriptional control of the HIV LTR. This system is unique in that it permits the autonomous replication of the viral RNA to be monitored in intact cells by an enzymatic assay of SEAP activity in the media bathing the cells. Using these novel reporter replicons, we show the effect of interferon-α on the replication of RNAs derived from two different strains of HCV in stably transformed cell cultures.

RESULTS

Tat-SEAP enzyme reporter system

The HIV tat protein is a potent transcriptional transactivator of its LTR promoter element. Unlike most known eukaryotic transcriptional transactivators, tat functions via an interaction with an RNA structure, the transactivating the transcription of functional mRNAs from sequences placed under control of the HIV LTR promoter. We have taken advantage of the small size of the tat protein, and the manner in which it functionally regulates the LTR promoter, to develop a system in which a replication-competent, subgenomic HCV RNA expressing tat induces the expression of SEAP placed under transcriptional control of the LTR in stably transformed liver cells.

pEt2AN is an expression plasmid in which the HIV tat-coding sequence is fused to sequence encoding the FMDV 2A proteinase and the positive, selectable marker neomycin phosphotransferase (Neo) (Fig. 1A). The small FMDV 2A polypeptide sequence possesses autocatalytic activity (Ryan and Drew, 1994), resulting in the scission of the peptide backbone at its C-terminus and the release of Neo. The translation of this mini-polyprotein is driven by the EMCV IRES sequence located just upstream of the protein-coding sequence (Fig. 1A), while transcription is directed by a composite CMV/T7 promoter. We used this plasmid to determine the level of SEAP expressed by stably transformed Huh7 cells (selected for blasticidin resistance) in which the SEAP sequence had been integrated under transcriptional control of the HIV LTR. SEAP activity was measured in the supernatant culture medium before and after transfection of the cells with pEt2AN. Results obtained with one clonally isolated cell line, En5-3, are shown in Fig. 1B.

This cell line produced a minimal basal level of SEAP activity, while transfection of the cells with pEt2AN DNA led to an approximately 100-fold increase in the secretion of SEAP into the medium in response to tat expression (Fig. 1B). The secretion of SEAP from En5-3 cells began to increase between 24 and 48 h after DNA transfection and reached maximal levels at 72 to 96 h. In contrast, the transfection of En5-3 cells with RNA transcribed in vitro from pEt2AN led to an immediate increase in SEAP activity that was maximal when first assayed at 24 h posttransfection and subsequently decreased over time, reaching background levels 72 h later (Fig. 1C). Since the cell culture medium bathing these transfected cells was replaced at 24 h intervals in these experiments (see Materials and Methods), the SEAP activity measured at each time point reflected the amount of the reporter protein secreted into the medium over the preceding 24-h period. The delay in SEAP secretion following DNA vs RNA transfection is likely to represent the time required for RNA transcription to occur, while the rapid decline of SEAP following RNA transfection reflects degradation of the transfected RNA and...
the tat protein translated from it. These encouraging results suggested that the expression of tat from a replicating subgenomic HCV RNA could provide a simple and useful approach to monitoring the presence and abundance of replicon RNA in En5-3 cells.

Subgenomic HCV replicons expressing tat

To test this hypothesis, we constructed a plasmid with a transcriptional unit containing a dicistronic, subgenomic HCV replicon similar to that reported originally by Lohmann et al. (1999), but in which the 5′ cistron encodes the tat-2A-Neo mini-polyprotein present in pEt2AN (Fig. 1), fused in-frame downstream of the N-terminal 14 amino acid residues of the HCV core protein sequence (Fig. 2, Btat2ANeo). The second cistron in this replicon contained the NS3-5B segment of the Con1 HCV sequence placed under the translational control of the EMCV IRES, as in the original HCV replicons (Lohmann et al., 1999). Because the effect of the fusion of the N-terminal segment of the core protein on tat function was uncertain, we also constructed a variant in which the 5′ cistron contained no HCV protein-coding sequence, and in which HCV IRES-directed translation initiated at the tat-coding sequence (Fig. 2, Btat2ANeo). The results of these experiments confirmed the activity of the FMDV 2A proteinase within the mini-polyprotein, as protein species migrating with the mobilities expected for the unprocessed tat2ANeo and the tat2ANeo precursor proteins and the fully processed Neo protein were evident in SDS–PAGE gels of the translation products from Btat2ANeo (Fig. 3A, Lanes 2 and 3). The tat2A cleavage product was not observed due to its small size. The results also suggested that the absence of the core-protein-coding sequence in Btat2ANeo RNA (Fig. 3A, compare Lane 3 with Lane 2). In contrast, the quantity of NS3 produced from the downstream cistron was relatively increased in lysates programmed with

FIG. 1. Enzyme reporter system. (A) Organization of pEt2AN. Solid square, the CMV promoter region; solid arrow, T7 promoter; a thick line, EMCV IRES; open box, open reading frame encoding polyprotein tat-2A-Neo. (B) SEAP expression following pEt2AN DNA transfection into En5-3 cells (●). The expression of tat from this plasmid is dependent on the CMV promoter. Note that SEAP activity is reported in arbitrary units. DNA was transfected using Fugene reagent. SEAP expression from En5-3 cells without DNA transfection was also shown (■). (C) SEAP expression following electroporation of En5-3 cells with RNA transcribed in vitro from pEt2AN (●). SEAP expression from En5-3 cells without RNA transfection was also shown (■).

FIG. 2. HCV replicons inducing enzymatic reporter protein

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Btat2ANeo RNA compared to BΔCtat2ANeo, suggesting that the reduction in the activity of the HCV IRES in the former RNA may have a complementary, beneficial effect on the downstream EMCV IRES. This suggests that there may be intercistronic competition for translation factors between the HCV and EMCV IRES elements in these replicon RNAs, as noted previously with other dicistronic RNAs (Whetter et al., 1994b).

We next assessed the activities of tat proteins expressed from the upstream cistron in the BΔCtat2ANeo and Btat2ANeo replicons (Fig. 2) in transient transfections of these replicon RNAs in En5-3 cells. SEAP activity was monitored in the supernatant media at 72 h post-transfection, in the absence of Neo selection. The results of these experiments indicated that the tat protein was significantly less active when expressed as a fusion protein with the N-terminal 14 amino acid segment of core (Fig. 3B, compare BΔCtat2ANeo, BΔCtat2ANeo(SI), and Btat2ANeo, Btat2ANeo(SI), and Btat2ANeo(RG) RNAs). Although the tat proteins expressed from these RNAs also have a C-terminal fusion with the FMDV 2A proteinase, this C-terminal fusion does not abrogate the transactivating activity of tat, as evidenced in the experiments shown in Figs. 1B and 1C. Replication of the RNAs did not contribute to the expression of SEAP in the transient transfection experiment shown in Fig. 3B, as the amount of SEAP induced by transfection of an NS5B deletion mutant, Btat2ANeo(ΔGDD), was only slightly less than that induced by its parent, Btat2ANeo. Similarly, the cell-culture-adaptive NS5A S2205I and NS5B R2889G mutations (Fig. 2) engineered into these RNAs had no effect on the level of SEAP expression under these conditions (Fig. 3B).

Stable cell lines expressing SEAP under control of replicon-mediated tat expression

Efforts to select stable, G418-resistant colonies following transfection of En5-3 cells with Btat2ANeo or BΔCtat2ANeo were unsuccessful. These results are consistent with the very low frequency of colony formation with the unmodified Con1 NS3-5B sequence, as reported by Lohmann and others (Lohmann et al., 1999; Blight et al., 2000; Ikeda et al., 2002). However, it was possible to select G418-resistant En5-3 clones following transfection of the modified Btat2ANeo containing the adaptive S2205I mutation and BΔCtat2ANeo RNAs containing the adaptive S2205I and R2889G mutations in NS5A and NS5B (Fig. 2), respectively. The efficiency of colony formation was substantially lower with these replicons, even with the adaptive mutations, than what has been reported in the literature (Lohmann et al., 2001; Blight et al., 2000) or what we have observed previously (Ikeda et al., 2002) with dicistronic, subgenomic HCV replicons. This may reflect the use of the clonal, blasticidin-resistant En5-3 cell line rather than the parental Huh7 cells.
Moreover, the number of colonies selected with Btat2ANeo(SI) RNA was approximately 10-fold lower than with Btat2ANeo, suggesting that the absence of the short, ΔC core protein-coding sequence in Btat2ANeo(SI) decreases the efficiency of colony selection. This could be due to the lower level of Neo expressed from this RNA (Fig. 3), or potentially to other effects on replication of the subgenomic RNA.

Because replicons containing the genotype 1b, HCV-N sequence have proven to be substantially superior to Con1 replicons in their ability to induce the selection of G418-resistant Huh7 cell clones (Ikeda et al., 2002; Guo et al., 2001), we constructed a parallel series of replicons containing the tat2ANeo sequence in the upstream cistron with the downstream cistron, NS3-NS5B sequence derived from HCV-N: Ntat2ANeo, Ntat2ANeo(SI), and Ntat2ANeo(RG) (Fig. 2). Transfection with each of these RNAs led to the selection of stable, G418-resistant colonies. The number of G418-resistant colonies selected with Ntat2ANeo(RG) was at least 100-fold higher than with Btat2ANeo(SI). Overall, the efficiency of colony selection observed with replicon RNAs that lacked any core-protein-coding sequence (Fig. 2) could be ordered as follows, from high to low: Ntat2ANeo(SI), Ntat2ANeo(RG), Ntat2ANeo, Btat2ANeo(SI). This is consistent with our previous observations with subgenomic HCV replicons expressing only Neo from the upstream cistron (Ikeda et al., 2002).

Replicon RNA was readily detected by Northern analysis of G418-resistant cell lines selected following transfection with BΔCtat2ANeo(SI), Btat2ANeo(SI), and Ntat2ANeo(RG) (Fig. 4A). The abundance of the viral RNA was significantly greater in the BΔCtat2ANeo(SI) cell line selected for testing than in cell lines supporting replication of Btat2ANeo(SI) and Ntat2ANeo(RG). While the total abundance of the replicon RNAs (see Materials and Methods) increased in each of the cell lines studied over a 120-h period following passage of the cells (Fig. 4A), quantitative real-time RT-PCR assays showed a trend toward a reduction in the intracellular abundance of the replicon RNA relative to the abundance of GAPDH mRNA as the cells approached confluence at 120 h (Fig. 4B). This is similar to the reduction in intracellular abundance of replicon RNAs reported recently by Pietschmann et al. (2001). Once confluent, the intracellular abundance of the replicon RNAs appeared to be similar in all three cell lines studied. These results confirm that there is no requirement for core-protein-coding sequence for replication of these dicistronic, subgenomic viral RNAs.

We also examined the cell lines shown in Fig. 4 for viral protein expression as well as secretion of SEAP. NS5A antigen was readily detected within the cytoplasm in each cell line (Figs. 5A, 5C, and 5D), while no NS5A antigen was detectable in normal En5-3 cells stained in parallel (Fig. 5B). The abundance of the viral protein was significantly greater in cells containing BΔCtat2ANeo(SI) (Fig. 5A) than Btat2ANeo(SI) (Fig. 5C) or Ntat2ANeo(RG) (Fig. 5D), consistent with the greater abundance of replicon RNA detected in the former by Northern analysis (Fig. 4A). In contrast, the SEAP activities expressed by

![FIG. 3.](image-url) (A) Product of in vitro translation reactions programmed with the indicated RNAs. (*) The expected positions of the major protein products anticipated to be produced from the dicistronic RNAs. (B) SEAP activity present in tissue-culture media 72 h following transient transfection with synthetic RNAs transcribed from the indicated plasmids.
these cell lines showed a very different relationship to the abundance of the replicon RNA. Each of the cell lines secreted increased amounts of SEAP that were detectable above the low background activity present in En5-3 media (Fig. 6A). However, the level of SEAP activity expressed by the BΔCtat2A(SI) cell line was minimally above background and much lower than that secreted by the Btat2ANeo(SI) or Ntat2ANeo(RG) cell lines, despite a higher abundance of viral RNA and viral proteins in the former. Sequencing of cDNA amplified by RT-PCR from the replicon RNAs present in the BΔCtat2A(SI) cells did not identify any mutations within the upstream, ΔCtat2ANeo cistron (data not shown), ruling out adventitious mutations as a potential cause for the minimal level of SEAP expressed by these cells. The Btat2ANeo(SI) and Ntat2ANeo(RG) cell lines demonstrated robust secretion of the reporter protein, reaching levels at least 100-fold above background after 5 days in culture (Fig. 6A). These results are consistent with the results of the transient transfections presented above (Fig. 3B) and serve to confirm that the fusion of tat to the N-terminal segment of the core protein sharply diminishes its ability to functionally transactivate the HIV LTR.

In the experiment shown in Fig. 6A, it is important to note that the media was completely replaced at 24 h intervals and that the cells were thoroughly washed before being refed with fresh media. Thus, the results shown represent the quantity of SEAP secreted by the Btat2ANeo(SI) and Ntat2ANeo(RG) cells during successive 24 h periods. The secretion of SEAP correlated closely with the abundance of replicon RNA in the Btat2ANeo(SI) and Ntat2ANeo(RG) cells as determined by densitometry of Northern blots (Fig. 6B, \( R^2 = 0.983 \) and 0.939 by linear regression analysis, respectively). In aggregate, these results demonstrate that the expression of tat from subgenomic HCV RNAs that are replicating in En5-3 cells effectively signals the secretion of SEAP, thereby providing an easily measurable and accurate marker of viral RNA replication that does not require lysis or destruction of the cell monolayer.

**Impact of cell-culture-adaptive mutations on the replication of tat-expressing HCV replicons in transient transfection assays**

Further studies of these replicons focused on those with no core protein sequence fused to tat, since the fusion with the core sequence effectively inactivated the transactivating function of tat. To determine whether the activation of SEAP expression in En5-3 cells by tat was sufficiently sensitive for detection of the replication of subgenomic RNAs in transient transfection assays, replicon RNAs were transfected into En5-3 cells using elec-
troporation, and the cells were followed for a period of 20
days in the absence of G418 selection. Included in this
experiment were the Btat2ANeo and Ntat2ANeo repli-
cons and mutants containing cell-culture-adaptive muta-
tions that were derived from them, as shown schemati-
cally in Fig. 2B. The supernatant media bathing the trans-
formed cells was removed and replaced with fresh media
at 24-h intervals, as in the experiment shown in Fig. 6A,
and the cells were collected by trypsinization and pas-
saged into fresh culture vessels at 7 and 14 days. The
levels of SEAP activity present in the media that was
removed from cells transfected with the replicon RNAs
based on the Btat2ANeo (Con1) sequence (Fig. 2) are
shown in Fig. 7A, while Fig. 7B shows SEAP activities in
media collected from cells transfected with replicons
derived from the HCV-N sequence.

The transfection of any of these replicon RNAs into
En5-3 cells resulted in a high initial level of SEAP ex-
pression that was present in the culture media as early
as 12 h after electroporation (Figs. 7A and 7B). This early,
high level of SEAP secretion persisted for approximately
3 days and was due to translation of the transfected input
RNA, as in the experiment shown in Fig. 3B. This high
initial SEAP level was also observed with replication-
defective mutants containing a deletion in the NS5B
sequence involving the GDD polymerase motif (ΔGDD
mutants) (Figs. 7A and 7B). The SEAP activity secreted
into the media of cells transfected with Btat2ANeo(ΔGDD)
and Ntat2ANeo(ΔGDD) began to decrease by Day 4 and
reached baseline values similar to those observed with
normal En5-3 cells by 8 days after electroporation (Figs.
7A and 7B). In contrast, other, replication-competent
RNAs, particularly those derived from the HCV-N se-
quence, demonstrated increased levels of SEAP expres-
sion at later time points that were significantly above the
En5-3 cell background and thus indicative of replication
of the transfected RNA.

In experiments with replicon RNAs derived from the
Con1 sequence, significant increases in SEAP activity
above that observed with the Btat2ANeo(ΔGDD) mutant
were seen only in cells transfected with Btat2ANeo(SI).
There was no apparent difference in the levels of
SEAP expressed by cells transfected with the Btat2ANeo
and Btat2ANeo(RG) replicons. Cells transfected with
Btat2ANeo(SI) demonstrated a low level but sustained
increase in SEAP activity above background beginning
about 10 days after transfection (Fig. 7A). However, the
secretion of SEAP was modest in magnitude, and never
more than several-fold above background. In sharp con-
trast, the HCV-N based replicons were remarkably more
potent in terms of their abilities to elicit sustained in-
creases in SEAP expression (Fig. 7B). Levels of SEAP
secretion up to 100-fold above background were ob-
served with Ntat2ANeo(SI) and Ntat2ANeo(RG), as well
as Ntat2ANeo(SIΔi5A). This latter replicon contains both
the S2205I substitution in NS5A and the deletion of a
natural four amino acid insertion that is present in the
NS5A sequence of HCV-N (Fig. 2B). This natural insertion
in NS5A, which was present in cDNA cloned from human
serum (Beard et al., 1999), has been shown to contribute
substantially to the replication capacity of replicons containing the wild-type HCV-N sequence in Huh7 cells (Ikeda et al., 2002). The results shown in Fig. 7 are consistent with those we have published recently (Ikeda et al., 2002) concerning the relative abilities of subgenomic RNAs containing the Con1 and HCV-N NS3-NS5B sequences (with or without cell-culture-adaptive mutations in NS5A and NS5B) to transduce the selection of G418-resistant cell clones. These results also provide independent confirmation of the ability of the S2205I and R2889G mutations to enhance the replication capacity of subgenomic, genotype 1b RNAs in cultured cells (Blight et al., 2000; Krieger et al., 2001; Ikeda et al., 2002).

We also examined transiently transfected cells for expression of NS5A antigen at 12 and 19 days after electroporation. These studies demonstrated that the proportion of cells containing a detectable abundance of NS5A was significantly greater following transfection with Ntat2ANeo(RG) and Nrat2ANeo(SI) than Ntat2ANeo or Btat2ANeo(SI) (Fig. 8, compare E, F, G, and H, with A, B, C, and D, respectively). Thus, these results parallel closely the results of the SEAP assays shown in Fig. 7. Interestingly, the intensity of staining of individual positive cells appeared similar with each of the replicon RNAs, suggesting that the level of SEAP expression may correlate with the proportion of cells in which replicon amplification is occurring, rather than the intracellular abundance of the replicon under these conditions. As this experiment was carried out in the absence of G418 selection, it is uncertain whether those cells that did not stain positively for NS5A antigen contained levels of the viral protein that were below the threshold of detection or, alternatively, none at all.

Interferon suppression of HCV RNA replication

To demonstrate the utility of the tat-expressing HCV replicons, we assessed the ability of recombinant interferon α-2b to suppress the replication of Btat2ANeo(SI) and Ntat2ANeo(RG) in stable, G418-resistant cell clones. Recently seeded cell cultures were fed with media containing various concentrations of recombinant interferon α-2b ranging from 0 to 100 units/ml. The medium was subsequently removed completely at 24 h intervals, and the cells were washed thoroughly and refed with fresh interferon-containing media. Results are shown in Fig. 9 and demonstrate dose-dependent inhibition of SEAP secretion in both cell lines. As shown, cells cultured in the absence of interferon, or at the lowest concentration of interferon, showed an increasing level of SEAP secretion over successive 24 h intervals, consistent with the growth of the cells. At the highest concentration of interferon tested (100 units/ml), this trend was reversed and SEAP expression declined over time in the absence of demonstrable cellular cytotoxicity. Independent quantitative RT-PCR assays for HCV RNA demonstrated that the decline in SEAP secretion was closely matched by similar decreases in the intracellular abundance of RNA (compare Fig. 9 and Fig. 10). The decline in intracellular RNA preceded the decreases in SEAP secretion by approximately 24 h, most likely reflecting the kinetic delay in tat signaling of SEAP secretion.

Surprisingly, the Nrat2ANeo(RG) replicon (Fig. 9B) was approximately 10-fold more resistant to interferon than the Btat2ANeo(SI) replicon (Fig. 9A). This relative interferon resistance was reflected also in differences in the degree of suppression of the intracellular abundance of

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**FIG. 7.** SEAP activity following transient transfection of En5-3 cells with (A) Btat2Aneo and (B) Ntat2Aneo with various mutations. Wt(C), SI (■), RG (▲), dGDD (×), N-Δ5AS1 (*). Arrow indicates trypsinization and passage of cells.
HCV RNA following interferon treatment of these cells (compare the decrease in Btat2ANeo(SI) RNA abundance at different interferon concentrations in Fig. 10A with the decreases in Ntat2ANeo(RG) RNA abundance shown in Fig. 10B). A similar level of interferon resistance was observed in separate experiments with an independently selected, G418-resistant clone supporting the replication of the Ntat2ANeo(RG) replicon (data not shown), suggesting that the resistance observed in Figs. 9B and 10B was not an idiosyncratic feature of the particular cell clone tested. Studies are currently in progress to determine the molecular basis of this difference in the response of the two replicons to interferon α-2b.

DISCUSSION

We have described here a novel enzymatic reporter system that permits the detection and quantitation of HCV RNA replication in intact cell monolayers. The system is based on the expression of the tat transactivator protein by replicating subgenomic RNA replicons, and the subsequent induction of SEAP synthesis in En5-3 cells that contain the SEAP gene under transcriptional control of the HIV LTR promoter. SEAP is secreted efficiently into the medium bathing these cells, where it is readily quantified as an accurate marker of viral RNA abundance. We adapted both Con-1 and HCV-N replicons for use in this system and have shown that the induction of SEAP is a useful measure of the replicon RNA abundance in stable, G418-resistant cell lines (Fig. 6), as well as in cells that have been transiently transfected by these RNAs (Fig. 7). Parallel measurements of RNA abundance and SEAP expression in two separate stable cell lines demonstrated a remarkable degree of correlation (Fig. 6B), providing strong validation of the system.

We have utilized this system to document the inhibition of HCV-N and Con-1 HCV RNA replication in En5-3 cells following treatment with recombinant interferon α-2b (Figs. 9 and 10). We found Ntat2ANeo(RG) to be about 10-fold less sensitive to interferon than Btat2ANeo(SI). These results differ from those reported recently by Guo et al. (2001), who found comparable interferon sensitivities with simple subgenomic dicistronic replicons constructed from these two viral sequences. We are currently investigating the molecular basis of the difference we observed in the interferon responsiveness of these replicons. Using the tat-expressing replicons, we have also been able to demonstrate the inhibition of viral RNA replication by prototype antiviral compounds that have activity against the viral NS3 proteinase or NS5B RNA-dependent RNA polymerase (Minkyung, Yi, and Stanley M. Lemon, unpublished data). Thus, we believe that this unique and simple system for monitoring viral RNA replication is likely to prove useful in future antiviral drug discovery efforts.

Because measurements of SEAP are technically simpler and considerably less expensive than quantitative RT-PCR assays for viral RNA, this system is likely to prove advantageous for high throughput screening for compounds with antiviral activity. An additional technical advantage over HCV replicons that express luciferase or most other conventional reporter proteins is that SEAP activity is measured in supernatant culture fluids and does not require the lysis of cells. This permits serial measurements of the kinetics of RNA amplification in single cultures of cells (Fig. 7). One potential drawback of this system is that suppression of SEAP activity by candidate antiviral compounds could result from inhibition of the activity of either the 2A protease or the tat, or even (as with other published dicistronic HCV replicons) the EMCV IRES. To address this issue, we established a stably transformed cell line that constitutively expresses the tat2ANeo polyprotein under the translational control of the EMCV IRES (data not shown). This cell line (Et2AN) was established by transfection of pEt2AN DNA (Fig. 1) into En5-3 cells, followed by selection with G418. In contrast to the results shown in Fig. 9, where interferon α-2b suppressed the secretion of SEAP from the replicon-bearing cell lines, there was no suppression of the secretion of SEAP by the Et2AN cell line at comparable concentrations of interferon (data not shown). This indicates that the effect of interferon-α on SEAP secretion from the replicon cell line was due to specific suppression of the replication of HCV RNA, and not the fortuitous suppression of 2A, tat, or EMCV IRES activity. It also demonstrates the absence of nonspecific toxicity at the concentrations of interferon tested and is consistent with the suppression of HCV RNA abundance in these cells shown in Fig. 10.

In developing these replicons, we have shown that none of the viral core protein-coding sequence is required for replication of HCV RNA. There has been considerable controversy over the role of this sequence in viral translation since Reynolds et al. (1996) first suggested that the 5’ proximal 33 nt of the core sequence were an integral part of the viral IRES and required for efficient cap-independent translation. Recently, however, Rijnbrand et al. (2001) demonstrated that the requirement is not for any specific sequence, but rather for a lack of secondary RNA structure within the core-coding sequence immediately downstream of the initiator AUG. This is consistent with prior work by Honda et al. (1996) that indicated that stable RNA structure within the vicinity of the AUG is very detrimental to IRES-directed translation. Because of concerns that the 5’ proximal core-coding sequence might be required for optimal activity of the HCV IRES, the original dicistronic, subgenomic HCV replicons that were constructed by Lohmann et al. (1999) contained RNA encoding 12 or 16 amino acids of the core protein fused in-frame to the Neo gene in the upstream cistron. We found that replicons in which the tat
sequence was fused directly to the HCV IRES had reduced translation of the upstream tat2ANeo minipolyprotein (Fig. 2A), but were nonetheless capable of replication and the transduction of G418-resistant cell lines (Figs. 4 and 5). These results demonstrate that none of the core-coding sequence is required for viral RNA replication. Other subgenomic HCV replicons have recently been described in which all core protein sequence had been removed, but in these replicons translation of the upstream cistron was driven by a picornaviral IRES and the HCV 5’NTR sequence functioned only in template recognition by the RNA replicase complex (Kim et al., 2002).

**MATERIALS AND METHODS**

**Cells**

En5-3 cells are a clonal cell line derived from Huh7 cells by stable transformation with the plasmid pLTR-SEAP (see below). These cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal calf serum, 2 μg/ml blasticidin (Invitrogen), penicillin, and streptomycin. Following transfection with replicon RNAs, cells supporting replicon (Invitrogen) digested with 

**Plasmids**

The plasmid pLTR-SEAP was generated as follows. pcDNA6/V5-His (Invitrogen) was digested with BgIII-BamHI to remove the CMV promoter. The vector was then self-ligated, digested with EcoRV-NotI, and re-ligated to a DNA fragment encoding SEAP under transcriptional control of the HIV LTR that was amplified from pBCHIVSEAP (a generous gift of Dr. Bryan Cullen, Duke University, Durham, NC) using the oligonucleotide primer pairs; 5'-CTAGCTAGCGCTGAGACCTGGAAAAACATGGGAG-3’ and 5’-ATAAGAATGCGGCCGCTTAAGCCGGGTACGTGC-3’. The resulting plasmid was transfected into Huh7 cells using a nonliposomal transfection reagent (FUGENE, Boehringer Mannheim), and stably resistant cells were selected in the presence of blasticidin (Invitrogen) digested with BgIII and NotI. These fragments were ligated to pcDNA6/V5-His (Invitrogen) digested with HindIII and NotI to generate pEt2AN.

To construct the replicon plasmid pΔCtat2Aneo, the genotype 1a infectious clone, pCV-H77c (generously provided by Dr. Robert Purcell, National Institutes of Health, Bethesda, MD), was digested with SphI and the small fragment was re-ligated. A single T-to-A nucleotide change was engineered in this plasmid at nt 444 of the HCV sequence using QuickChange (Stratagene) mutagenesis, generating a novel HpaI site at this position. This resulting plasmid was digested with HpaI and XbaI to generate a DNA fragment representing the HCV 1a 5’NTR and immediately downstream sequence encoding the first 14 amino acids of the HCV polyprotein. A second DNA fragment representing the tat, 2A, and partial neo sequence was excised from pEt2AN by digestion with Stul and SphI. Finally, the plasmid pBNeo/wt (Fig. 1), containing the sequence of the I_{iso}neo/NS3-3’ replicon of Lohmann et al. (generously provided by Dr. Michael Murray of the Schering-Plough Research Institute), was digested with SphI and XbaI to generate a fragment representing the C-terminal neo sequence, EMCV IRES, and downstream elements of the HCV replicon. These three fragments were ligated to generate pBΔCtat2Aneo (Fig. 1), which contains the 5’NTR and downstream 42 nt of core-coding sequence of the H77 strain of HCV (genotype 1a) and the NS3-5B and 3’NTR sequence of the Con1 strain of HCV (genotype 1b). The plasmid pBtat2Aneo was generated by QuickChange mutagenesis of pBΔCtat2Aneo, with deletion of the 42 nucleotides of core-coding sequence and fusion of the tat sequence directly downstream of 5’NTR of HCV. pNtat2Aneo was constructed by exchanging the large BsrGI-XbaI fragment of pBtat2Aneo with the analogous HCV sequence derived from the plasmid pHCV-N, resulting in replacement of most of the NS3-NS5B and 3’NTR sequence. A similar strategy was employed for the construction of variants of these replicon plasmids containing various cell-culture-adaptive mutations or a deletion of the GDD motif in the NS5B protein, as described by Ikeda et al. (2002).

**RNA transcription and transfection**

RNA was synthesized with T7 MEGAScript reagents (Ambion), after linearizing plasmids with XbaI. Following
treatment with RNase-free Dnase to remove template DNA and precipitation of the RNA with lithium chloride, the RNA was transfected into En5-3 cells. Transfection was done by electroporation, as described previously. Briefly, 10 µg RNA was mixed with 5 × 10⁶ cells suspended in 500 µl phosphate-buffered saline, in a cuvette with a gap width of 0.2 cm (Bio-Rad). Electroporation was with two pulses of current delivered by the Gene Pulser II electroporation device (Bio-Rad), set at 1.5 kV, 25 µF, and maximum resistance.

**In vitro translation**

In vitro transcribed RNA, prepared as described above, was used to program *in vitro* translation reactions in rabbit reticulocyte lysate (Promega). About 1 µg of each RNA, 2 µl [³⁵S]methionine (1000 Ci/mmol at 10 mCi/ml), and 1 µl of an amino acid mixture lacking methionine were included in each 50 µl reaction mixture. Translation was carried out at 30°C for 90 min. Translation products were separated by SDS–PAGE followed by autoradiography or PhosphorImager (Molecular Dynamics) analysis.

**Northern analysis for HCV RNA**

We seeded replicon-bearing cells into six-well plates at a density of 2 × 10⁵ cells/well and harvested the RNA from individual wells at daily intervals. Total cellular RNAs were extracted with TRizol reagent (Gibco-BRL) and quantified by spectrophotometry at 260 nm. One-half of the total RNA extracted from each well was loaded onto a denaturing agarose-formaldehyde gel, subjected to electrophoresis, and transferred to positively charged Hybond-N+ nylon membranes (Amersham-Pharmacia Biotech) using reagents provided with the NorthernMax Kit (Ambion). RNAs were immobilized on the membranes by UV-crosslinking. The membrane was hybridized with a ³²P-labeled antisense riboprobe complementary to the 3’-end of NS5B sequence (HCV nt 8990–9275), and the hybridized probe was detected by exposure to X-ray film.

**FIG. 8.** Immunofluorescence analysis of NS5A antigen 12 days and 19 days following transfection of En5-3 cells with the replicon RNAs indicated. Cells were cultured in the absence of G-418.
Indirect immunofluorescence analysis

Cells were grown on chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol/acetone (1:1 V/V) for 10 min at room temperature. A 1:10 dilution of a primary, murine monoclonal antibody to NS5A (MAB7022P, ME Biotechnology Services) was prepared in PBS containing 3% bovine serum albumin and incubated with the fixed cells for 1 h at room temperature. Following additional washes with PBS, specific antibody binding was detected with a goat anti-mouse IgG FITC-conjugated secondary antibody (Sigma) diluted 1:70. Cells were washed with PBS, counterstained with DAPI, and mounted in Vectashield mounting medium (Vector Laboratories) prior to examination by a Zeiss AxioPlan2 fluorescence microscope.

Alkaline phosphatase assay

SEAP activity was measured in 20-μl aliquots of the supernatant culture fluids using the Phospha-Light Chemiluminescent Reporter Assay (Tropix), and the man-
ufacturer’s suggested protocol reduced one-third in scale. The luminescent signal was read using a TD-20/20 Luminometer (Turner Designs, Inc.). In most time-course experiments, the culture medium was replaced every 24 h. Thus, the SEAP activity measured in these fluids reflected the daily production of SEAP by the cells.

Real-time quantitative RT-PCR analysis of HCV RNA

Quantitative RT-PCR assays were carried out using TaqMan chemistry on a PRISM 7700 instrument (ABI). For detection and quantitation of HCV RNA, we used primers complementary to the 5′NTR region of HCV (Takeuchi et al., 1999), with in vitro transcribed HCV RNA included in the assays as a standard. Results were normalized to the estimated total RNA content of the sample, as determined by the abundance of cellular GAPDH mRNA detected in a similar real-time RT-PCR assay using reagents provided with TaqMan GAPDH Control Reagents (Human) (Applied Biosystems).

Sequence analysis of cDNA from replicating HCV RNAs

HCV RNA was extracted from cells, converted to cDNA, and amplified by PCR as described previously (Ikeda et al., 2002). First-strand cDNA synthesis was carried out with Superscript II reverse transcriptase (Gibco-BRL), and PFU-Turbo DNA polymerase (Stratagene) was used for PCR amplification of the DNA. The amplified DNAs were subjected to direct sequencing using an ABI 9600 automatic DNA sequencer.

Interferon treatment of cell cultures

Selected replicon-bearing cell lines were seeded into 12-well plates. The media was replaced 24 h later with fresh, G418-free media containing various concentrations of recombinant interferon α-2b ranging from 0 to 100 units/ml. The medium was subsequently completely removed every 24 h; the cells washed and refed with fresh interferon-containing media. SEAP activity was measured in the media removed from the cells as described above.

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