The 5′ untranslated region of GB virus B shows functional similarity to the internal ribosome entry site of hepatitis C virus

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Since its characterization in 1995, there has been increasing interest in the significance of GB virus B (GBV-B) due to its close phylogenetic relationship to hepatitis C virus (HCV). The genome of GBV-B is similar in length and organization to that of HCV and the two viruses share sequence similarity in their 5′ untranslated regions (5′UTR). A secondary structure model of the GBV-B 5′UTR has been proposed by comparative sequence analysis with HCV. The highly conserved secondary structure, present in HCV and the pestiviruses, is also present in the 5′UTR of GBV-B. Translation of the HCV polyprotein initiates via an internal ribosome entry site (IRES) and it is proposed that the GBV-B UTR may function in a similar manner. Dicistronic reporter constructs were made to investigate the function of the GBV-B 5′UTR. Mutational analysis and in vitro translation experiments demonstrate that GBV-B initiates translation via an IRES.

Transmission studies carried out in 1967 (Deinhardt et al., 1967) using serum from a patient (initials GB) with acute sporadic hepatitis, showed that the serum was capable of inducing hepatitis in tamarins (Saguinus species). Subsequent cross-challenge experiments showed that the agent(s) present in the GB inoculum were distinct from other known hepatotropic viruses (Holmes et al., 1973; Karayiannis et al., 1989). More recently, the genomes of two positive-stranded RNA viruses, designated GB virus A and GB virus B (GBV-A and GBV-B), have been cloned from the serum of a GB inoculum-infected animal (Simons et al., 1995a). The genome organization of these viruses, together with hepatitis G virus/GBV-C, which was cloned soon after (Linnen et al., 1996; Simons et al., 1995b), indicates that they are the most closely related viruses to hepatitis C virus (HCV) within the family Flaviviridae (Simons et al., 1995b; Muerhoff et al., 1995).

Although the original GB inoculum was of human origin, neither GBV-A nor GBV-B appear to be human pathogens (Karayiannis et al., 1997). Following experimental infection of tamarins, only GBV-B could be detected in liver samples and was associated with biochemical evidence of an acute self-limiting hepatitis (Schlauder et al., 1995).

The GBV-B genome is a positive-sense 9143 nucleotide (nt) RNA molecule, which encodes a polyprotein of 2864 amino acids (Muerhoff et al., 1995). There is significant sequence identity with HCV in specific regions, such as the putative helicase and RNA-dependent RNA polymerase of the two viruses (Simons et al., 1995b; Muerhoff et al., 1995). However, they only share 28% amino acid similarity across the entire polyprotein (Muerhoff et al., 1995).

HCV has a 5′ untranslated region (UTR) approximately 341 nt in length. This region is known to initiate translation of the HCV polyprotein via an internal ribosome entry site (IRES), which involves most of the untranslated region (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Rijnbrand et al., 1995; Reynolds et al., 1996; Honda et al., 1996a, b). As the genome of GBV-B is similar in length and organization to that of HCV, it seems likely that GBV-B may also initiate translation in a similar manner. The 5′UTR of GBV-B is 445 nt in length compared with the 341 nt length of most HCV strains. Alignment of the two sequences revealed several segments of absolute sequence identity, with two large insertions in the GBV-B sequence at positions corresponding to nt 40 and nt 102 in the HCV sequence. Excluding these insertions, the sequences share an average identity of 60%. Indeed, a model of the secondary structure of the GBV-B 5′UTR has been suggested using comparative sequence analysis with HCV (Honda et al., 1996b). This shows that the highly conserved secondary structure present in HCV and the pestiviruses may also be present in the 5′UTR of GBV-B.

In the present study, we investigated whether the IRES of GBV-B can function in a similar manner to the HCV IRES by using mono- and dicistronic reporter gene plasmid constructs. In addition, site-directed mutation analysis and in vitro
transcription/translation experiments were employed to study IRES structure–function relationships.

Several monocistronic or dicistronic reporter gene constructs were made as shown in Fig. 1(a). These were employed to determine whether the GBV-B 5' UTR contains an IRES, and to compare it to the IRES of HCV. All plasmids were constructed in Bluescript II KS transcription vectors (Stratagene). The HCV sequences (nt 341 to 855) were derived from a British patient infected with genotype 1b virus and were subcloned as described by Reynolds et al. (1995).

GBV-B sequences were obtained by RT–PCR of liver extracts from GBV-B-infected tamarins (Choo et al., 1989). Dicistronic expression cassettes consisted of two reporter genes in series, which were transcribed from the T7 promoter. The upstream gene, chloramphenicol acetyltransferase (CAT) (pSV2-CAT; Gorman et al., 1982), was translated in a cap-dependent manner and acted as an internal control for basal levels of protein translation. The downstream reporter gene, either HCV core/ΔE1 or secreted alkaline phosphatase (SEAP) (Clontech), was out of frame with the upstream gene and under the translational control of either the HCV IRES or the GBV-B 5' UTR. Plasmid pKCUS4 carries the CAT gene and the HCV IRES (nt -341 to +15) cloned immediately adjacent to the AUG of SEAP. pKCUC1 is a similar dicistronic vector but with SEAP replaced by core/ΔE1 of HCV. pKCGBS is also similar to pKCUS4, but has the 5' UTR from GBV-B (nt -445 to +21) cloned as a SalI–AatII fragment upstream of the SEAP gene. This cloning strategy resulted in an AatII site being introduced between the IRES and the start codon of the SEAP gene.

All reporter plasmids were transcribed using Ribonuclease T7 large scale RNA production kit (Promega). RNA transcripts were used to programme nuclease-treated rabbit reticulocyte lysate (Promega) in vitro translation reactions (Pelham & Jackson, 1976) as described by Grace et al. (1991). In addition, the extent to which these processes are affected by altering potassium ion concentration was also studied. Potassium chloride was added to the rabbit reticulocyte lysate to create added potassium chloride concentrations ranging from 0 to 100 mM. The commercial rabbit reticulocyte lysate is supplemented with potassium ions to 79 mM in the final translation reaction (Promega).

Analysis of HCV dicistronic plasmids, pKCUC1 and pKCUS4 (Fig. 1b), demonstrated that translation of the upstream reporter gene, CAT, was reduced as the concentration of potassium chloride increased. However, the reporter genes under translational control of the HCV IRES (core/ΔE1 and SEAP) were not inhibited by high potassium levels. This result is consistent with the observation that

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**Fig. 1.** (a) Schematic diagram of the plasmid constructs used in this study. (b) Autoradiogram of a 10% SDS–polyacrylamide gel of the polypeptides synthesized by in vitro translation of RNA transcripts (1 µg per lane) from HCV dicistronic reporter constructs. (c) Autoradiogram of a 10% SDS–polyacrylamide gel of the polypeptides synthesized by in vitro translation of RNA transcripts (1 µg per lane) from GBV-B dicistronic reporter constructs. Reactions were carried out in varying concentrations of potassium ions, from 0 to 100 mM added potassium chloride. The positions of SEAP (61 kDa), CAT (24 kDa) and Core/E1 (31 kDa) reporter proteins are indicated. All autoradiograms were scanned using a Hewlett Packard ScanJet ADF with Hewlett Packard DeskScan II software.
Table 1. Mutations introduced in the HCV IRES as reported by Wang et al. (1994) and Reynolds et al. (1995) and corresponding mutations made in the GBV-B 5' UTR, designated mut1–mut8

<table>
<thead>
<tr>
<th>Mutations in HCV 5' UTR</th>
<th>Mutations in GBV-B 5' UTR</th>
<th>Mutation no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut2 and mut3 (as below)</td>
<td>Mut2 and mut3 (as below)</td>
<td>1</td>
</tr>
<tr>
<td>12tGUCC12s → AGAA</td>
<td>12tGUCC12s → AGAA</td>
<td>2</td>
</tr>
<tr>
<td>31tGGAC32s → UUCU</td>
<td>31tGGAC32s → UUCU</td>
<td>3</td>
</tr>
<tr>
<td>AUG311 → AUU</td>
<td>AUG311 → AUU</td>
<td>4</td>
</tr>
<tr>
<td>AUG311 → CUG</td>
<td>AUG311 → CUG</td>
<td>5</td>
</tr>
<tr>
<td>AUG311 → AAG</td>
<td>AUG311 → AAG</td>
<td>6</td>
</tr>
<tr>
<td>AUG311 → GAG</td>
<td>AUG311 → GAG</td>
<td>7</td>
</tr>
<tr>
<td>AUG311 → GCG</td>
<td>AUG311 → GCG</td>
<td>8</td>
</tr>
</tbody>
</table>

potassium ion concentration has a differential effect on cap-dependent and cap-independent, or IRES driven, translation (Jackson, 1991).

RNA transcripts were generated from the GBV-B dicistronic SEAP plasmid (pKCGBS) and were translated in vitro using rabbit reticulocyte lysate (Fig. 1c). Both CAT and SEAP were produced from the GBV-B transcripts. The fact that SEAP protein was produced indicated that translation had occurred via internal ribosome entry in the GBV-B 5' UTR, since SEAP is out of frame with the upstream reporter gene, CAT, and is not expressed from transcripts lacking GBV-B sequences in the intercistronic space. Increasing the concentration of potassium ions reduced CAT expression but appeared to have less of an effect upon translation of SEAP, which was downstream of the GBV-B 5' UTR. Levels of SEAP expression appeared to be similar to those seen when translation was under the control of the HCV IRES. These observations indicate that the 5' UTR of GBV-B contains an IRES, as predicted from the secondary structure of the RNA.

There are several reports in the literature describing site-directed mutagenesis of the HCV IRES. These studies have allowed identification of regions important for IRES function and confirmed AUG311 as the authentic initiation codon. Mutations described by Reynolds et al. (1995) and Wang et al. (1994) affecting either the initiating AUG or the polypyrimidine tract II (Py II) domain of the HCV IRES were repeated in the corresponding regions of the GBV-B 5' UTR. The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate dicistronic expression vectors carrying the GBV-B 5' UTR with the mutations shown in Table 1 (Wang et al., 1994; Reynolds et al., 1995). The mutations were designated mut1–mut8. The effect of each mutation on the translational efficiency of the IRES was determined by in vitro translation (Fig. 2a) and subsequent densiometric measurement of the autoradiogram using an Epson GT9000 scanner and Phoretix 1D gel analysis v4.0 software (Fig. 2b). The effect of each mutation was also determined by measuring SEAP activity in a cell-based transfection assay (Fig. 2c).

The transfection assays were carried out in BT7-H cells (Whetter et al., 1994) infected with recombinant vaccinia virus vTF7-3, expressing T7 RNA polymerase (Fuerst et al., 1986), and performed as described by Reynolds et al. (1995). After 17 h incubation the medium was removed from the cells and...
SEAP levels were measured using the Great Escape SEAP detection kit (Clontech). Luminescence measurements were made using a Wallac Microbeta plate counter. Data were reproducible and there was fair correlation between the results, whether the effects of the mutations were analysed either by in vitro translation or by measuring reporter gene (SEAP) activity in the cell-based assay.

Mut2 (CUCC to AGAA) between nt 242 and 245 altered the sequence of Py II found at the base of domain III and has the potential to destroy base-pairing and secondary structure in this region. No in vitro protein translation or in vivo SEAP activity could be detected from the GBV-B IRES when this mutation was present. These results from both in vitro and in vivo experiments suggest that the RNA structure in this region is important for GBV-B IRES function, in a similar way to the equivalent region of the HCV IRES. Nucleotides 242–245 are predicted to base-pair with nt 424–427 (Honda et al., 1996b). Disruption of this base-pairing might account for the observed decrease in translational efficiency of the mutated RNA. Therefore, a second mutation was studied, mut3 (GGAG to AAGA), between nt 424 and nt 427. These base substitutions should also cause disruption of the base-pairing interaction predicted in Py II found at the base of domain III. As expected, the in vitro and in vivo translational efficiency of the GBV-B IRES was greatly reduced and again indicates the importance of the RNA secondary structure in this region. However, when both compensatory mut2 and mut3 were introduced together in the same construct (mut1), the function of the IRES was partially restored, even though the primary sequence of the two regions had been altered. This suggests that the secondary structure of the RNA is more important than the primary sequence during ribosome binding.

It has been observed that the HCV IRES can accommodate certain mutations at initiation codon AUG\textsubscript{446} with very little effect on the efficiency of translation (Reynolds et al., 1995). Similar mutations were applied to the GBV-B IRES and their relative effects upon translation from a dicistronic expression cassette were observed. Mutations in initiating codon AUG\textsubscript{446} demonstrated that changes AUG → AUU (mut4) and AUG → CUG (mut5) could be tolerated, although translation occurred at a reduced level. Mutation of the authentic initiation codon to AAG, GAG or GCG (mut6, mut7 and mut8) completely abolished translation and no SEAP activity was detected in the cell-based assay. Thus, site-directed mutagenesis of the GBV-B 5′UTR confirms that AUG\textsubscript{446} is the authentic initiating codon of polyprotein synthesis.

Introduction of mutations similar to those shown to affect HCV IRES function (Wang et al., 1994; Reynolds et al., 1995) had a similar effect on GBV-B IRES function. It was noted that mut4 (AUG → AUU) was better tolerated in vitro than in vivo. In comparison with the other mutations, particularly mut1 (Py II double mutation) and mut5 (AUG → CUG), the relative activity of mut4 was reversed between the in vitro and in vivo assays. These experiments have been repeated on several occasions and yielded identical results; the reason for this observation is not clear.

The results presented here confirm that there is an IRES in the GBV-B genome capable of conferring internal initiation of translation of a downstream heterologous reporter cistron as predicted from the model of the secondary structure of the RNA (Honda et al., 1996b). The function of the GBV-B IRES was investigated experimentally in comparative studies with constructs containing the HCV IRES. Expression levels of the reporter gene under IRES control were similar in both the HCV and the GBV-B dicistronic plasmid constructs, suggesting a similar level of activity of the GBV-B and HCV IRES.

In vitro translation studies of transcripts containing the GBV-B 5′UTR under conditions of varying potassium chloride concentration revealed that the expression of the downstream reporter was more tolerant to increasing potassium ion concentrations. The tolerance to high potassium ion levels was similar to that seen when RNA transcripts containing the HCV 5′UTR, which is known to contain an IRES, replaced that of GBV-B. In both cases translation of the upstream cistron was greatly reduced as potassium levels increased. Potassium ion concentration is known to have a differential effect on cap-dependent and cap-independent, or IRES driven, translation (Jackson, 1991). Consequently, these results support the view that the 5′UTR of GBV-B contains an IRES.

Site-directed mutagenesis of the GBV-B 5′UTR confirms that AUG\textsubscript{446} is the initiating codon of polyprotein synthesis. Introduction of mutations similar to those shown to affect HCV IRES function (Wang et al., 1994; Reynolds et al., 1995) had the same effect on GBV-B IRES function. These results indicate that the secondary structure of the RNA is more important than the primary sequence during ribosome binding (Wang et al., 1994; Reynolds et al., 1995).

In conclusion, the above observations confirm that the 5′UTR of GBV-B contains an IRES and that this IRES functions in a similar manner to that of HCV. In view of this, GBV-B may be of value as a potential surrogate virus for evaluation of potential anti-HCV compounds.

References


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