A link between translation of the hepatitis C virus polyprotein and polymerase function; possible consequences for hyperphosphorylation of NS5A

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Hyperphosphorylation of NS5A is thought to play a key role in controlling hepatitis C virus (HCV) RNA replication. Using a tetracycline-regulable baculovirus delivery system to introduce non-culture-adapted HCV replicons into HepG2 cells, we found that a point mutation in the active site of the viral polymerase, NS5B, led to an increase in NS5A hyperphosphorylation. Although replicon transcripts lacking elements downstream of NS5A also had altered NS5A hyperphosphorylation, this did not explain the changes resulting from polymerase inactivation. Instead, two additional findings may be related to the link between polymerase activity and NS5A hyperphosphorylation. Firstly, we found that disabling polymerase activity, either by targeted mutation of the polymerase active site or by use of a synthetic inhibitor, stimulated translation from the replicon transcript. Secondly, when the rate of translation of non-structural proteins from replicon transcripts was reduced by use of a defective encephalomyocarditis virus internal ribosome entry site, there was a substantial decrease in NS5A hyperphosphorylation, but this was not observed when non-structural protein expression was reduced by simply lowering replicon transcript levels using tetracycline. Therefore, one possibility is that the point mutation within the active site of NS5B causes an increase in NS5A hyperphosphorylation because of an increase in translation from each viral transcript. These findings represent the first demonstration that NS5A hyperphosphorylation can be modulated without use of kinase inhibitors or mutations within non-structural proteins and, as such, provide an insight into a possible means by which HCV replication is controlled during a natural infection.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus belonging to the family Flaviviridae. The virus has a 9·6 kb genome containing a single open reading frame (ORF) encoding both structural (core, E1, E2 and P7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Bartenschlager & Lohmann, 2000; Reed & Rice, 2000), which are respectively proteolytically processed as a result of host cell (Hijikata et al., 1991) and viral (Bartenschlager et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993) proteases. Flanking the ORF are 5’ and 3’ untranslated regions (UTR), both of which possess cis-acting elements necessary for virus replication (Friebe et al., 2001; Friebe & Bartenschlager, 2002; Kolykalov et al., 2000; Luo et al., 2003; Yi & Lemon, 2003), with the 5’ UTR also containing an internal ribosome entry site (RES) (Tsukiyama-Kohara et al., 1992; Wang et al., 1993).

Unusually, HCV is capable of establishing a life-long infection, irrespective of the age at which it is acquired. Long-term chronic infection can lead to clinical manifestations ranging from cryoglobulinaemia and liver fibrosis through to end-stage liver disease including cirrhosis and hepatocellular carcinoma (Poynard et al., 2003). Current combination therapy using pegylated IFN-α and ribavirin is only approximately 50 % effective and there remains a need for improved treatment regimes. One part of the virus life cycle that has potential for therapeutic intervention is replication of the viral genome. This area of research has benefited greatly from the development of the HCV replicon (Blight et al., 2000; Lohmann et al., 1999). However, establishing a level of replication within a tissue culture system to allow detection invariably results in the selection of point mutations within the coding regions of the non-structural proteins, leading to a culture-adapted phenotype (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001, 2003). Furthermore, culture adaptation is associated with attenuated virus replication in vivo (Bukh et al., 2002). Therefore, while the HCV replicon is a good model system for assessing...
the importance of viral and host cell proteins in virus replication per se, the requirement for culture-adaptation makes it less appropriate for the study of mechanisms that might determine replicative strategies adopted by the virus during a natural infection. We have previously reported the use of a baculovirus delivery system to introduce full-length and subgenomic HCV transcripts into hepatocyte-derived cell lines (McCormick et al., 2002, 2004). Advantages of this system include the facts that transduction is extremely efficient, with almost 100% of cells showing expression of viral proteins, and that the level of transcript can be controlled in the transduced cells by virtue of a tetracycline-responsive promoter. Furthermore, unlike the HCV replicon, it is also possible to achieve high levels of HCV viral transcript and protein in transduced cells without the need for robust virus replication or culture-adaptation.

The basis for the study reported here was the observation that a single point mutation blocking the activity of the viral polymerase, NS5B, caused an increase in the hyper-phosphorylation of NS5A in the context of a non-culture-adapted replicon. This was of interest because some of the more potent culture-adapted mutations tend to cluster within a region of the viral non-structural protein NS5A (Blight et al., 2000; Krieger et al., 2001), leading to speculation that this protein might be critically involved in controlling replication. Moreover, results from more recent studies suggest that the differential phosphorylation of this protein is important, and indicate that the hyper-phosphorylated form of NS5A is inhibitory to replication (Appel et al., 2005; Evans et al., 2004; Neddermann et al., 2004). It was also unexpected because previous studies had suggested that neither the coding nor the non-coding regions downstream of NS5A played a significant role in modulating hyperphosphorylation (Asabe et al., 1997; Koch & Bartenschlager, 1999; Neddermann et al., 1999). Our investigation has shown that, in the non-culture-adapted replicon con1, NS5A hyperphosphorylation is influenced by elements in the HCV genome downstream of the NS5A coding region. In addition, it has also revealed the importance of translational activity from the replicon transcript in determining levels of hyperphosphorylation.

METHODS

Cells and viruses. Mammalian cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 1 x non-essential amino acids and antibiotics. Sf9 cells were maintained in TC100 with 10% FCS and antibiotics and used to isolate, amplify and titrate baculovirus clones using standard procedures. Recombinant virus was generated using the Bac-to-Bac system (Invitrogen) according to the manufacturer’s recommendations. Concentrated virus stocks were obtained by clarification of the virus supernatant using a 0.45 µm filter, centrifugation (26 000 r.p.m. in a Sorvall AH-629 rotor for 1 h at 4 °C) and resuspension in PBS. For transduction and DNA transfection experiments, cells were seeded 20–24 h in advance at a cell density of 2·0 x 10^5 cells cm^-2. Unless otherwise stated, cells were then incubated with 1 x 10^7 p.f.u. ml^-1 of both BACtTA and a replicon-containing baculovirus construct for 4 h and then allowed to recover for 20 h in tetracycline-free medium before harvesting.

DNA constructs. We have recently shown that the commercial Bac-to-Bac system (Invitrogen) allows more rapid generation of recombinant baculoviruses for mammalian expression and dramatically reduces the occurrence of point mutations within clones (unpublished data). For this reason, all HCV-containing baculovirus constructs used in this study were derived from a modified pFastBac transfer vector. Firstly, an oligonucleotide pair (FASTBat1, FASTBat2; sequences available on request) was cloned into SnuBjAvrI-cl-cut pFastBac1, generating pFB(Xbal-HindIII). Replicon-containing mammalian expression cassettes were then transferred into pFB (Xbal-HindIII) by sequential cloning of the Xbal–HindIII and HindIII–HindIII fragments from pBACrep5.1neo(451k) and pBACrepGNDneo (McCormick et al., 2004), generating pFBrep5.1neo and pFBrepcon1GNDneo, respectively. Other full-length replicon constructs, pFBrepcon1neo and pFBrep5.1GNDneo, were produced by exchange of Sfi–Sfi fragments between pFK-I389neo/NS3–3’/con1 (Lohmann et al., 1999) and pFBrep5.1neo and between pFK-I389neo/NS3–3’/5.1 (Krieger et al., 2001) and pFBrepcon1GNDneo, respectively.

To generate replicon-construclks lacking part of the 3’ end of the genome, the Sfi–Sfi fragment of pFK-I389neo/NS3–3’/con1 was introduced into Sfi-cut pBACrep5.1neo(T7/NotI) (McCormick et al., 2004), generating pFBrepcon1neo(T7/NotI). The primer pairs NS9con1 and NS9A5e(5’)-con1 were used in PCRs with pFBrepcon1neo or pFBrepcon1GNDneo as template to generate DNA fragments which were cloned into Xhol/NotI-cut pBACrepcon1neo(5’/NotI). The HindIII–HindIII fragment from the resultant vectors were subsequently transferred to HindIII-cut pFBrepcon1neo, generating pFBrepcon1neo(D583’U), pFBrepcon1neo(A3’U) and pFBrepcon1GNDneo(A3’U).

To generate the encephalomyocarditis virus (EMCV) defective replicon constructs, the RsplI(polished)–BsrGI fragment from pFK-I389neo/NS3–3’/5.1 was first transferred into LITMUS38B (NEB), generating pLRM-EMCVwt. Mutagenesis was performed using the GeneEditor mutagenesis kit (Promega) in combination with mutagenic oligonucleotide EMCV1(del31). Sequencing was used to identify mutated clones, but also revealed that a small stretch of nucleotides between the end of the neo ORF and the start of the EMCV IRES was consistently deleted (nucleotides 1243–1252). Such clones were deemed acceptable because this region is not expected to affect either neo expression or EMCV IRES activity. A clone containing the mutated EMCV IRES was then restricted with KpnI and StuI and a KpnI–Xhol(polished) fragment from pFK-I389neo/NS3–3’/con1 was cloned into the vector. Finally, the Pmel–Mld fragment from this latter vector was removed and cloned into Pmel/Mld-cut pFBrepcon1neo and pFBrepcon1GNDneo, generating pFBrepcon1neo(ED) and pFBrepcon1GNDneo(ED), respectively.

Northern blot analysis. RNA was harvested from cells using Trizol (Invitrogen), electrophoresed through a MOPS/formaldehyde gel and transferred to BrightStar Nylon Plus membrane (Ambion) using standard procedures. Biotinylated probes and markers were generated using Biotin-Chem-Link reagent (Roche). Hybridization was performed overnight at 42 °C in UltraHyb (Ambion) and bound probe was detected using the BrightStar detection kit (Ambion).

Western blot analysis. Cells were lysed in RIPA (50 mM Tris/HCl, pH 8-6, 150 mM NaCl, 1% (v/v) NP-40, 0-5% w/v sodium deoxycholate, 0-1% SDS) supplemented with 2x Complete protease inhibitor cocktail (Roche), 1 mM Na3VO4 and 1 mM NaF and the protein concentration of samples was determined using BCA reagent (Pierce). Samples containing equal amounts of protein (typically 5–10 µg per well) were separated by SDS-PAGE and transferred...
to PVDF membrane (Millipore). Membranes were blocked with 5% (w/v) low-fat dried milk, 0.1% Tween 20 (Merck) in Tris-buffered saline and incubated with a 1:10000 dilution of either sheep anti-NS3, anti-NS5A or anti-NS5B serum (raised against a His-tagged NS5B fusion protein expressed in E. coli) or a 1:5000 dilution of a rabbit anti-neomycin phosphotransferase (NPT) polyclonal antibody (Upstate). Bound antibody was detected with an appropriate HRP-conjugated secondary antibody (Sigma) in conjunction with ECL reagent (Amersham Pharmacia Biotech) and light emissions were captured by a Luminescent Image Analyser LAS-1000 (Fujifilm) and analysed using Advanced Image Data Analyser version 2.0 software (Raytek Scientific).

RESULTS

Differences in NS5A hyperphosphorylation between HCV replicons with point mutations in NS5B

We have previously developed a baculovirus system for the delivery and controlled expression of HCV genomes (full-length and replicon) in hepatocyte-derived cell lines (McCormick et al., 2004). Using this system we expressed a series of different bicistronic replicon transcripts, including a non-culture-adapted HCV replicon (con1) and a culture-adapted HCV replicon (5.1), either with a functional polymerase (FBrepcon1neo and FBrep5.1neo) or with a point mutation within the active site of NS5B that abolished polymerase activity (FBrepcon1\textsuperscript{GND}neo and FBrep5.1\textsuperscript{GND}neo) (Fig. 1a). Expression of NPT, NS3 and NS5B was confirmed by Western blot (Fig. 1b), demonstrating that proteins from both cistrons were expressed and processed appropriately. Recent work has highlighted the importance of NS5A hyperphosphorylation in controlling HCV replication, so it was of interest to examine hyperphosphorylation of NS5A following transduction of HepG2 cells with the different constructs. Consistent with previous reports, hyperphosphorylation of NS5A (p58 formation) was found to be reduced in cells transduced with the culture-adapted replicon constructs (FBrep5.1neo and FBrep5.1\textsuperscript{GND}neo) compared with the non-culture-adapted constructs (FBrepcon1neo and FBrepcon1\textsuperscript{GND}neo) (Fig. 1c). Unexpectedly,

![Fig. 1. Polymerase-defective replicon transcripts have altered hyperphosphorylation of NS5A. (a) Schematic of baculovirus constructs containing either the non-culture-adapted bicistronic HCV replicon (con1) or a culture-adapted replicon (5.1) (differences indicated by *), with or without a point mutation in NS5B that disables polymerase activity (indicated by †). Also shown are the tetracycline-responsive promoter (P\textsubscript{tet}) and hepatitis delta ribozyme (H\textsubscript{D}V), which allow production of polI-derived replicon transcripts with appropriate 5’ and 3’ ends. (b) Representative Western blots for NPT, NS3 and NS5B in HepG2 cell lysates mock-transduced (lane 1) or co-transduced with BACtTA and either FBrepcon1neo (lane 2), FBrepcon1\textsuperscript{GND}neo (3), FBrep5.1neo (4) or FBrep5.1\textsuperscript{GND}neo (5). (c) Representative Western blots for NS5A expression using the cell lysates from (b) (top panel) or from transduced Huh7 cells (lower panel) and maintaining the same lane order. The percentage of NS5A hyperphosphorylated was determined by densitometry and is depicted graphically. The data from HepG2 cells are from five separate experiments (means ± SEM); the Huh7 cell data result from a single experiment.](http://vir.sgmjournals.org)
the level of NS5A hyperphosphorylation was increased in HepG2 cells transduced with the non-culture-adapted replicon containing a non-functional NS5B polymerase (FBrepcon1\(^{GND}\)neo) compared with cells transduced with the related construct containing a functional polymerase (FBrepcon1neo). In contrast, disabling polymerase activity in the 5.1 replicon background had little or no effect. Immunofluorescence was also used to examine the cellular localization of NS5A in the four constructs. While there were differences in the localization pattern of NS5A between the 5.1- and con1-based constructs, an observation that has been reported when comparing other replicon constructs (Moradpour et al., 2004), no such difference was seen when comparing FBrepcon1neo and FBrepcon1\(^{GND}\)neo (data not shown). Therefore, differences in NS5A hyperphosphorylation expressed from the con1 and con1\(^{GND}\) transcripts do not appear to be due to gross changes in cellular distribution of NS5A, although the effects of subtle changes in distribution cannot be discounted.

While HepG2 cells are efficiently transduced with baculovirus, they do not support replication of con1-based replicons. For this reason, expression of NS5A from the same four constructs was examined in Huh7 cells, which are capable of supporting replicon replication. Although this proved more difficult because of the much lower levels of replicon transcript production by baculovirus in these cells, it was clear that, as in HepG2 cells, a greater proportion of NS5A was hyperphosphorylated in FBrepcon1\(^{GND}\)neo-transduced cells compared with FBrepcon1neo-transduced cells. In addition, there was little or no difference in the extent of NS5A hyperphosphorylation in FBrep5.1neo- and FBrep5.1\(^{GND}\)neo-transduced cells, although it was higher than that seen in FBrepcon1-transduced Huh7 cells, in contrast to the results seen in HepG2 cells. However, as the differential effects of inactivation of polymerase on the hyperphosphorylation of NS5A in con1 and 5.1 genomic backgrounds are the same as in HepG2 cells, all subsequent work was done in this cell line because of the limited levels of expression achievable using Huh7 cells.

**Viral elements downstream from NS5A affect hyperphosphorylation**

Our observation that a point mutation in NS5B which disabled polymerase activity also increased NS5A hyperphosphorylation conflicted with previous reports suggesting that elements downstream from NS5A had little impact on hyperphosphorylation (Asabe et al., 1997; Koch & Bartschenschlager, 1999; Neddermann et al., 1999). In order to address this issue, con1 and con1\(^{GND}\) replicons with a series of deletions from the 3' end were transferred into baculovirus constructs. These included a con1 replicon lacking both NS5B and the 3' UTR [FBrepcon1neo(Δ5B3'U)], a con1 replicon missing just the 3'UTR [FBrepcon1neo(Δ3'U)] and a con1\(^{GND}\) replicon missing the 3' UTR [FBrepcon1\(^{GND}\)neo(Δ3'U)] (Fig. 2a). Northern analysis of HepG2 cells transduced with these and the original FBrepcon1neo and FBrepcon1\(^{GND}\)neo constructs demonstrated

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**Fig. 2.** Regions downstream from the NS5A coding region can influence hyperphosphorylation. (a) Schematic of baculovirus constructs containing HCV replicons with deletions at the 3' end. (b) Northern blot using total RNA from HepG2 cells mock-transduced (lanes 1 and 5) or co-transduced with BACTA and either FBrepcon1neo(Δ5B3'U) (lanes 2 and 6), FBrepcon1neo(Δ3'U) (3), FBrepcon1neo (4), FBrepcon1\(^{GND}\)neo(-Δ3'U) (7) or FBrepcon1\(^{GND}\)neo (8). The blot was first probed for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript (lower panel) and then stripped and reprobed for the replicon transcript (upper panel; probe encompassed nucleotides 254–7935 of pFK-I389 neo/NS3-3'9/5.1). (c) Western blot for NS3, NS5A and NS5B using cell lysates derived from the same experiment in (b) and with the order of the lanes maintained. (d) Graphical representation of four separate experiments where the level of hyperphosphorylated NS5A was determined by densitometry (means ± SEM).
Inhibition of polymerase activity increases translational activity from the replicon transcript

Although deletion of the 3′ end of the HCV replicon had demonstrated that NS5B could modulate NS5A hyperphosphorylation, it was not obvious why polymerase activity per se should reduce the formation of p58. In an attempt to ascertain whether polymerase activity could influence NS5A hyperphosphorylation, HepG2 cells were transduced with FBrepcon1neo(A3′U) or FBrepcon1GNDneo and then maintained in medium either containing no supplements or supplemented with either SB-750330-0Z (an NS5B polymerase inhibitor) (Dhanak et al., 2002) or vehicle alone (DMSO). In contrast to the increase in NS5A hyperphosphorylation that was observed by mutating the active site of NS5B (Fig. 1b), Western analysis revealed that SB-750330-0Z did not increase the formation of p58 in FBrepcon1neo-transduced cells (Fig. 3a). However, several other changes were observed in the presence of the drug. Firstly, an accumulation of what appeared to be an NS5A5B precursor was observed, given that it was detectable by both NS5A and NS5B antiserum and migrated on SDS-PAGE with an apparent molecular mass equivalent to NS5A and NS5B combined (~120 kDa) (Fig. 3b). Generation of this precursor was also far more apparent in FBrepcon1neo-transduced cells compared with FBrepcon1GNDneo-transduced cells (data not shown). Secondly, the presence of the inhibitor but not DMSO alone increased expression of NPT, NS3 and NS5A in FBrepcon1neo- but not FBrepcon1GND neo-transduced cells (Fig. 3a, c; Table 1). This appeared to be as a result of increased translational activity from both IRES elements within the HCV replicon, as Northern analysis (Fig. 3d) demonstrated that neither DMSO nor the polymerase inhibitor affected replicon transcript levels. These data are consistent with the notion that functional polymerase activity suppresses translational activity from both the EMCV and HCV IRES elements. However, intriguingly, the data indicated that, whereas genetic elimination of polymerase function resulted in an increase in NS5A hyperphosphorylation, pharmacological inhibition of the polymerase had no such effect.

Translational activity from the HCV replicon determines the level of NS5A hyperphosphorylation

As these two methods of polymerase inactivation are mechanistically distinct, this suggested that there was a complex relationship between translation and NS5A hyperphosphorylation. It seemed unlikely that the enhanced hyperphosphorylation exhibited by FBrepcon1GNDneo might simply reflect a link between hyperphosphorylation and the abundance of the polyprotein. However, to test this hypothesis and to gain some insight into the regulation of hyperphosphorylation, we used two approaches to regulate the levels of protein translation. The first approach involved modulating the transcript levels by altering the concentration of tetracycline: HepG2 cells were transduced with either FBrepcon1neo or FBrepcon1GNDneo and then maintained for 16 h in varying concentrations of tetracycline. As expected, increasing concentrations of tetracycline resulted in reduced levels of non-structural protein expression, as determined by Western analysis of NS5A (Fig. 4). In cells transduced with FBrepcon1neo, reduction of viral protein expression had a negligible effect on the percentage of total NS5A that was hyperphosphorylated. The situation was slightly different in FBrepcon1GNDneo-transduced cells in that reduction of non-structural protein expression caused a modest increase in overall levels of hyperphosphorylated NS5A. However, the level of hyperphosphorylated NS5A in FBrepcon1neo- and FBrepcon1GNDneo-transduced cells did not coincide over the range of expression levels examined. Therefore we conclude that differences in the overall level of non-structural protein within the cell (as determined by varying replicon transcript levels) cannot account for the phenomenon of increased p58 formation in FBrepcon1GNDneo-transduced cells.

Whereas inactivation of the polymerase causes an increase in translation without changing replicon transcript levels (Fig. 3c, d), the changes in NPT and non-structural protein expression following addition of tetracycline simply reflect alterations in replicon transcript levels. This raised the alternative possibility that the amount of non-structural protein translated from each replicon transcript might influence NS5A hyperphosphorylation. We tested this by utilizing a second approach in which we reduced the translational activity of the EMCV IRES by mutating a tetraloop
sequence within the EMCV IRES of the con1 and con1GND replicon from GCGA to GTTA. This change has been shown previously to reduce IRES activity by approximately 80% (Robertson et al., 1999).

HepG2 cells were transduced with baculovirus constructs containing these two replicons [FBrepcon1neo(ED) and FBrepcon1GNDneo(ED)] or with the wild-type FBrepcon1neo and FBrepcon1GNDneo and the cell lysates were subjected to Western analysis (Fig. 5). As expected, expression of NS3, NS5A and NS5B was much lower in cells containing replicons with the mutated EMCV IRES compared with replicons with a wild-type IRES (Fig. 5b). However, in contrast to reducing HCV non-structural protein expression by changing replicon transcript levels, where little effect was seen on NS5A hyperphosphorylation, a decrease in HCV non-structural protein expression as a result of reduced EMCV IRES activity suppressed p58 formation (Fig. 5a). Furthermore, unlike FBrepcon1neo- and FBrepcon1GNDneotransduced cells, there seemed to be no difference in the extent to which NS5A was hyperphosphorylated between FBrepcon1neo(ED)- and FBrepcon1GNDneo(ED)-transduced cells, nor was there any noticeable difference in overall levels of NPT and non-structural protein expression. Another difference between cells containing replicons with a functional or attenuated EMCV IRES was that, in the latter case, NPT expression was increased, perhaps indicating the existence of competition between the two IRESs within the replicon. Further evidence that all these differences in expression and phosphorylation of proteins from the

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**Table 1.** Summary of NPT and NS3 expression after pharmacological inhibition of polymerase activity

Values shown represent percentage of expression compared with mock-treated cells from four separate experiments (means ± SEM).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Treatment</th>
<th>NPT (%)</th>
<th>NS3 (%)</th>
</tr>
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<tbody>
<tr>
<td>FBrepcon1neo</td>
<td>1 % DMSO</td>
<td>115 ± 7</td>
<td>114 ± 8</td>
</tr>
<tr>
<td></td>
<td>4 μM SB-750330-0Z</td>
<td>159 ± 10</td>
<td>194 ± 15</td>
</tr>
<tr>
<td>FBrepcon1GNDneo</td>
<td>1 % DMSO</td>
<td>102 ± 1</td>
<td>88 ± 4</td>
</tr>
<tr>
<td></td>
<td>4 μM SB-750330-0Z</td>
<td>104 ± 4</td>
<td>87 ± 4</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Increased translational activity from the HCV replicon upon pharmacological inhibition of polymerase activity. (a) Representative Western blot showing hyperphosphorylation of NS5A in HepG2 cells mock-transduced (lane 1) or co-transduced with BACtTA and either FBrepcon1neo (lanes 2–4) or FBrepcon1GNDneo (lanes 5–7). After transduction, cells were maintained in medium lacking additional supplements (lanes 2 and 5) or supplemented with either 1 % DMSO (lanes 3 and 6) or with 1 % DMSO and 4 μM SB-750330-0Z (a polymerase inhibitor) (lanes 4 and 7). The extent of NS5A hyperphosphorylation was assessed by densitometry and the results of three separate experiments are shown graphically (means ± SEM). (b) Western blots for NS5A and NS5B from the same cell lysates (lanes 1–4) described in (a) but indicating the location of the NS5A5B precursor (arrow) and of the processed NS5A and NS5B proteins (asterisk). (c) Western analysis showing the presence of NPT and NS3 in cell lysates from (a). (d) Total cellular RNA derived from the experimental groups described in (a) was also subject to Northern blot analysis using both a probe for the replicon (an NS5B coding region encompassing nucleotides 6266–7100 of pFK-I389neo/NS3-3’/5.1) and a control GAPDH probe.
replicon ORFs were due to translational activity was provided by Northern analysis, which showed that the amount of replicon transcript was comparable between cells transduced with BACtTA and either FBrepcon1neo or FBrepcon1^GND^neo (lanes 2–7) which were maintained in tetracycline-free medium (lanes 1 and 2) or medium supplemented with 0·02, 0·04, 0·06, 0·08 and 0·10 μg tetracycline ml⁻¹ (lanes 3–7, respectively). The graph summarizes the results for both FBrepcon1neo- and FBrepcon1^GND^neo-transduced cells from two separate experiments where the extent of NS5A hyperphosphorylation was determined by densitometry.

**DISCUSSION**

A growing body of evidence points to hyperphosphorylation of NS5A as being important in controlling HCV RNA replication. Mutations that are associated with increased replicative fitness of HCV replicons in tissue culture often lead to a reduced level of NS5A hyperphosphorylation (Appel *et al.*, 2005; Evans *et al.*, 2004; Neddermann *et al.*, 2004). Perhaps more convincing is the finding that kinase inhibitors that block or reduce NS5A hyperphosphorylation also facilitate replication of a non-culture-adapted replicon (Neddermann *et al.*, 2004). Indeed, a speculative model has been proposed in which hyperphosphorylation disrupts binding of NS5A to human vesicle-associated membrane protein-associated protein A (hVAP-A), a cellular protein considered necessary for efficient replicon replication (Evans *et al.*, 2004). However, if hyperphosphorylation of NS5A does have a regulatory role in virus replication, this also implies it is a dynamic process in which the levels of hyperphosphorylation are in turn altered in response to one or more stimuli that affect HCV replication. To date, no evidence has been put forward to suggest how hyperphosphorylation might be modulated. Instead, studies have been devoted to identifying both the cellular kinases involved in NS5A phosphorylation (Coito *et al.*, 2004; Reed *et al.*, 1997) and the components of the viral genome necessary, when expressed either in cis or in trans, to facilitate hyperphosphorylation (Asabe *et al.*, 1997; Koch & Bartenschlager, 1999; Neddermann *et al.*, 1999). Indeed, it is difficult to utilize replicon-containing cell lines to examine how hyperphosphorylation might be modulated, since culture-adapted mutations are likely to affect the dynamics of hyperphosphorylation. We have developed a baculovirus delivery system which enables efficient delivery of full-length and subgenomic replicon transcripts into HepG2 cells (McCormick *et al.*, 2002, 2004), thus by-passing the need for culture-adaptation. It was therefore of interest to observe that, in cells containing a non-culture-adapted HCV replicon (con1), the presence of a point mutation in NS5B that disabled polymerase activity modulated the degree of NS5A hyperphosphorylation. Subsequent attempts to determine how polymerase activity could affect hyperphosphorylation led us to three conclusions: firstly, that RNA elements downstream of the NS5A coding region, and possibly expression of NS5B as well, influence NS5A hyperphosphorylation (Fig. 2); secondly, that polymerase activity suppresses translation from both the HCV and EMCV IRES within the HCV replicon (Fig. 4); and finally, and more importantly, we also found that reducing the translational activity of the EMCV IRES reduces hyperphosphorylation of NS5A in a non-culture-adapted replicon background. This is not due to a change in overall levels of non-structural protein present within the cell, as reducing the amount of replicon transcript within each cell did not have the same effect. To our knowledge, this represents the first evidence that modulation of NS5A hyperphosphorylation might be controlled in part by factors that influence translational activity from the HCV genome.

Is it now possible to account for the original observation that the non-culture-adapted replicon differed in NS5A hyperphosphorylation depending on whether or not NS5B was inactivated by a point mutation within the active polymerase site? Our results clearly show that removal of elements downstream of the NS5A coding region alter
hyperphosphorylation. Therefore, it is possible that the difference in hyperphosphorylation between con1 and con1\textsuperscript{GND} is a result of direct structural changes within NS5B that affect its association with other viral non-structural and cellular proteins, rather than a lack of polymerase function. However, the mutation is present within the active site of NS5B and so is unlikely to be directly accessible to other non-structural and/or cellular proteins, although it is pertinent to note that the level of NS5B protein was reduced relative to the other non-structural proteins when inactivated by the GND mutation, suggesting decreased stability of the non-functional protein. Two further hypotheses may explain the data. The first is based on the result from experiments with the 3’ deletion constructs (Fig. 2) which are consistent with, but not proof of, a model where NS5B serves to suppress NS5A hyperphosphorylation. In this case, if NS5B stability is decreased by the GND point mutation, as already mentioned, suppression of NS5A hyperphosphorylation might be relieved. The second explanation is based on the positive correlation that exists between the activity of the EMCV IRES and the level of NS5A hyperphosphorylation. If functional polymerase activity suppresses translation from the replicon transcript, as is observed, this in turn would be expected to reduce NS5A hyperphosphorylation. In support of this hypothesis was the finding that, in the context of a defective EMCV IRES, the con1 and con1\textsuperscript{GND} transcripts displayed similar translational activity and had a reduced but comparable level of NS5A hyperphosphorylation. In other words, under conditions where there was no difference in translational activity from the non-culture-adapted replicon with or without a functional polymerase, the level of NS5A hyperphosphorylation was the same. This was not seen when FBrepcon1neo-transduced cells were treated with the polymerase inhibitor, despite an increase in translation from the two IRESs within the replicon transcript. However, this observation has to be viewed with some caution, as a significant build-up of the NS5A5B precursor also occurred in the presence of inhibitor, suggesting that the inhibitor has global effects on NS5B in addition to its effect on polymerase activity which may in turn alter hyperphosphorylation of NS5A.

Another finding of the study was that replicon-derived translated products increased when a point mutation was introduced into NS5B that disabled polymerase activity or when a polymerase inhibitor was present. The reasons for this are currently being investigated but may be the result of a number of possibilities. Firstly, we have previously shown that the presence of a polymerase-competent but not a polymerase-defective replicon in HepG2 cells triggers an interferon response (McCormick et al., 2004), which may in turn modulate translational activity from the replicon transcript through phosphorylation of eIF2\textalpha (Goodbourn et al., 2000). Alternatively, it may be that viral transcripts are recruited into membrane-bound replication complexes as a result of polymerase activity, which then decreases their availability to cellular translational machinery. Indeed, a
dependence on polymerase activity for recruitment to such complexes has already been observed with poliovirus (Teterina et al., 2001). Finally, competition may occur between the translating ribosomes moving in the 5′ to 3′ direction and the replication complex moving in the 3′ to 5′ direction on the same viral transcript. Such clashes are normally thought to be avoided in positive-strand viral RNA replication, as they have been shown to suppress polymerase activity and therefore would affect viral fitness (Barton et al., 1999). Nonetheless, it is possible that, either in HCV replication generally or in the system that is employed in this study, the switch from translation of the viral transcript to use of the transcript for RNA-dependent RNA polymerase-catalysed production of negative strand is ‘leaky’. Under such circumstances both replication and translation might be suppressed.

One of the most interesting findings of this study was that the introduction of a defective EMCV IRES into the HCV replicon resulted in a significant reduction in the hyperphosphorylation of NS5A. It is difficult at present to speculate on the mechanism whereby the machinery responsible for the hyperphosphorylation of NS5A can respond to the rate at which protein translation is initiated on HCV-related transcripts. However, the ability of the virus to respond to transcript utilization in this way may be very important for the regulation of virus replication and progeny production, given the well-established inverse correlation between hyperphosphorylation of NS5A and replicon replication (Appel et al., 2005; Blight et al., 2000; Evans et al., 2004; Neddermann et al., 2004). It is conceivable that such a mechanism might provide the virus with the means to respond to the intracellular environment so that replication could be modulated in response, for example, to the immune status of the host. Such a facility may help explain the almost unique ability of HCV routinely to initiate lifelong chronic infections.

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