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Activation and evasion of the antiviral 2′-5′ oligoadenylate synthetase/ribonuclease L pathway by hepatitis C virus mRNA

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ABSTRACT

Chronic hepatitis C virus (HCV) infections are a significant cause of morbidity and mortality worldwide. Interferon-α2b treatment, alone or in combination with ribavirin, eliminates HCV from some patients, but patients infected with HCV genotype 1 viruses are cured less frequently than patients infected with HCV genotype 2 or 3 viruses. We report that HCV mRNA was detected and destroyed by the interferon-regulated antiviral 2′-5′ oligoadenylate synthetase/ribonuclease L pathway present in cytoplasmic extracts of HeLa cells. Ribonuclease L cleaved HCV mRNA into fragments 200 to 500 bases in length. Ribonuclease L cleaved HCV mRNA predominately at UA and UU dinucleotides within loops of predicted stem-loop structures. HCV mRNAs from relatively interferon-resistant genotypes (HCV genotypes 1a and 1b) have fewer UA and UU dinucleotides than HCV mRNAs from more interferon-sensitive genotypes (HCV genotypes 2a, 2b, 3a, and 3b). HCV 2a mRNA, with 73 more UA and UU dinucleotides than HCV 1a mRNA, was cleaved by RNase L more readily than HCV 1a mRNA. In patients, HCV 1b mRNAs accumulated silent mutations preferentially at UA and UU dinucleotides during interferon therapy. These results suggest that the sensitivity of HCV infections to interferon therapy may correlate with the efficiency by which RNase L cleaves HCV mRNA.

Keywords: dsRNA; immune evasion; innate immunity; interferon; mRNA stability; RNase L; virus evolution

INTRODUCTION

The 2′-5′ oligoadenylate synthetase/ribonuclease L pathway is an important antiviral pathway present in virtually every human cell. This cytoplasmic antiviral pathway detects viral double-stranded RNA (dsRNA), leading to the activation of a latent endoribonuclease and the degradation of viral mRNA (Fig. 1; reviewed in Player & Torrence, 1998). 2′-5′ oligoadenylate synthetase (2′-5′ OAS) is the initial enzyme in the pathway. Interferons (IFN-α, IFN-β, and INF-γ) induce the expression of 2′-5′ OAS; however, the magnitude of induction of 2′-5′ OAS varies depending on the type and concentration of interferon used and on the type of cell. Three genes located on chromosome 12 are used to express small, medium, and large forms of 2′-5′ OAS (Hovnanian et al., 1998; Rebouillat & Hovanessian, 1999). Due to differential splicing, variants of the small and medium 2′-5′ OAS forms exist. The small isoforms of 2′-5′ OAS (p40/46) possess one catalytic domain and exist as tetramers. The medium isoforms of 2′-5′ OAS (p69/p71) possess two catalytic domains and exist as dimers. The large isoform of 2′-5′ OAS (p100) possesses three catalytic domains and exists as a monomer. The medium isoforms of 2′-5′ OAS are myristylated and glycosylated. The different isoforms of 2′-5′ OAS are differentially distributed within cells, but cumulatively, these enzymes are found throughout the cytoplasm and nucleus. Even though interferon increases the expression of 2′-5′ OAS, dsRNA is always a requisite cofactor for the activation of 2′-5′ OAS. In the absence of dsRNA, 2′-5′ OAS does not catalyze the synthesis of 2′-5′ oligo(A) (Fig. 1). At subsaturating concentrations (100 nM), dsRNAs from 40 bp to 110 bp in size were found to activate 2′-5′ OAS as a function of size, with larger dsRNA providing more activation than smaller dsRNA (Desai & Sen, 1997). Yet, when saturating concentrations of dsRNA were used, dsRNAs as small as 25 bp in size fully activated the medium isozyme of 2′-5′ OAS and dsRNA 15 bp long partially activated 2′-5′ OAS (Sarkar et al., 1999). RNA SELEX was used to isolate single-stranded RNA aptamers capable of activating the small isoform of 2′-5′ OAS (Hartmann et al., 1998). Finally, several small viral RNAs

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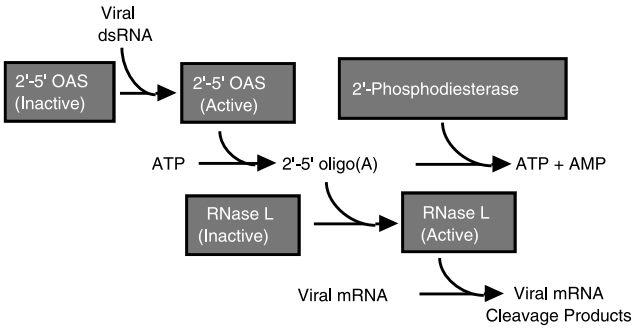

FIGURE 1. The antiviral 2′-5′ oligoadenylate synthetase/ribonuclease L pathway.

with extensive dsRNA structure have been shown to activate 2′-5′ OAS (Maitra et al., 1994; Desai et al., 1995; Sharp et al., 1999).

RNase L is encoded by one gene and is not regulated by interferon but is expressed constitutively in most cells (reviewed in Player & Torrence, 1998). RNase L is distributed throughout the cytoplasm and nucleus of cells. In the absence of 2′-5′ oligo(A), RNase L is monomeric and inactive (Fig. 1). RNase L dimerizes upon binding 2′-5′ oligo(A) (Carroll et al., 1997; Cole et al., 1997), leading to the activation of its endoribonuclease (Carroll et al., 1997; Cole et al., 1997; Dong & Silverman, 1997). Of the four ribohomopolymers [poly(A), poly(G), poly(C), and poly(U)], only poly(U) is cleaved efficiently by RNase L (Floyd-Smith et al., 1981). RNase L cleaves heteropolymeric viral mRNA preferentially at single-stranded UA and UU dinucleotides (Floyd-Smith et al., 1981; Wreschner et al., 1981b). 2′-phosphodiesterase degrades 2′-5′ oligo(A) into ATP and AMP, preventing constitutive activation of RNase L (Fig. 1).

Unlike most positive-strand RNA viruses, HCV is able to evade the host's immune response and establish chronic infections. A hypervariable region in the viral envelope glycoprotein is important in the evasion of the acquired immune response (Farci et al., 2000). HCV also evades various aspects of the innate immune response, including those associated with interferon (Korth & Katze, 2000). HCV genotype 1 infections are more frequently resistant to interferon therapy than HCV genotype 2 or 3 infections (McHutchison et al., 1998). When used alone, interferon-α2b eliminated 29% of HCV genotypes 2 and 3 infections but only 7% of HCV genotype 1 infections (McHutchison et al., 1998). When interferon was used in combination with the antiviral drug ribavirin, 28% of patients infected with HCV genotype 1 were cured of the infection and 66% of patients with HCV genotypes 2 or 3 were cured of the infection (McHutchison et al., 1998). Other studies report similar findings (Zein et al., 1996; Diamantis et al., 1998; Zylberberg et al., 2000). HCV protein NS5A binds to dsRNA-dependent protein kinase (PKR) to inhibit the antiviral effects of PKR (Gale et al., 1998; Korth & Katze, 2000). HCV E2 glycoprotein may also participate in the evasion of PKR (Taylor et al., 1999). Nonetheless, there are no genotype-specific differences in the ability of NS5A or E2 glycoproteins to inhibit PKR (Sarrazin et al., 2000). Thus, to date, there are no satisfactory explanations for the increased interferon resistance of HCV genotype 1 infections relative to HCV genotype 2 and 3 infections.

HCV mRNA possesses a 5′ nontranslated region with significant dsRNA structures (Brown et al., 1992; Rijnbrand & Lemon, 2000), a long open reading frame (ORF) encoding the viral polyprotein, and a 3′ nontranslated region with significant dsRNA structures (Kolykhalov et al., 1996; Blight & Rice, 1997). In this article, we show that HCV mRNA activates dsRNA-dependent 2′-5′ OAS leading to the activation of RNase L and the cleavage of HCV mRNA. Our data suggest that the sensitivity of HCV mRNAs to cleavage by RNase L may correlate with the sensitivity of HCV infections to interferon therapy.

RESULTS

Activation of RNase L by HCV mRNA

We initiated experiments to study the translation and replication of HCV mRNA in cell-free translation–replication reactions analogous to those used for poliovirus (Molla et al., 1991; Barton & Flanegan, 1993; Barton et al., 1995). When incubated in HeLa S10 translation–replication reactions, HCV mRNA reproducibly activated an endoribonuclease in a dose-dependent manner (Fig. 2). Increasing concentrations of HCV mRNA in the reactions lead to the appearance of ribosomal RNA fragments characteristic of RNase L activity (Fig. 2, lane 5, arrows; Wreschner et al., 1981a; Silverman et al., 1983). In a reaction with 30 nM HCV mRNA, the HCV mRNA remained intact for the 3-h period of incubation at 34 °C (Fig. 2, lane 7). In the reactions containing higher concentrations of HCV mRNA, the HCV mRNA was progressively more degraded in reactions containing more HCV mRNA (Fig. 2, lanes 8–10). The HCV mRNA was degraded into fragments from 200 to 500 bases in length (Fig. 2, lanes 8–10), consistent with 20 to 50 cleavage sites in the 9,648-base-long HCV mRNA.

To further elucidate the nature of this endoribonucleolytic pathway, we examined whether host mRNAs exhibited the same phenomenon. We also examined whether a subgenomic HCV mRNA possessing a deletion spanning the majority of the HCV ORF was able to activate this endonucleolytic pathway. Increasing concentrations of human plakoglobin mRNA (Hu Pkg mRNA) were incubated in the HeLa S10 translation–replication reactions without effect (Fig. 3, lanes 1–5). The ribosomal RNAs within the reactions containing

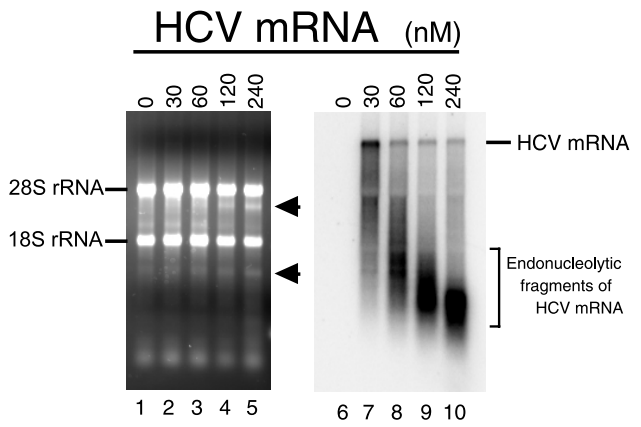

FIGURE 2. Dose-dependent activation of an endonuclease by HCV mRNA. ^{32}P-labeled HCV mRNA (30 to 240 nM as indicated) was incubated in HeLa S10 translation–replication reactions at 34 °C for 2 h (Materials and Methods, Assaying RNase L in HeLa cell extracts). Following incubation, the RNA from the reactions was fractionated by electrophoresis in 1% agarose. Left panel: Ribosomal RNA was visualized by ethidium bromide and UV light. Right panel: Radiolabeled HCV mRNA was detected by phosphorimaging of the dried gel.

Hu Pkg mRNA were unaffected (Fig. 3, lanes 1–5, top panel) and increasing amounts of Hu Pkg mRNA were detected by phosphorimaging (Fig. 3, lanes 1–5, bottom panel). In contrast, increasing concentrations of HCV P90Δ3 mRNA induced the appearance of ribosomal RNA fragments characteristic of RNase L activity (Fig. 3, lanes 8–10, top panel) coincident with the degradation of HCV P90Δ3 mRNA (Fig. 3, lanes 8–10, bottom panel). Thus, activation of this endonucleolytic degradation occurred with the viral mRNA but not with the host mRNA.

Bacteriophage T7 RNA polymerase has the capacity to initiate RNA transcription spuriously from cDNA templates containing 3′ overhangs, leading to dsRNA products (Fig. 4; Schenborn & Mierendorf, 1985). *Bsm*1 linearized HCV cDNA templates lead to the synthesis of viral RNA transcripts with authentic 3′ termini. Unfortunately, *Bsm*1 leaves a 3′ overhang on the nontemplate strand of the viral cDNA (Fig. 4A). Spurious T7 RNA polymerase transcription initiation from the 3′ overhang would result in the synthesis of RNA complementary to the HCV P90Δ3 RNA transcript (Fig. 4B). To remove the 3′ overhang from *Bsm*1-linearized cDNA templates, we used T4 DNA polymerase. T4 DNA polymerase possesses 3′-5′ exonuclease activity as well as 5′-3′ DNA polymerase activity. In the presence of four dNTP substrates, the 3′ exonuclease activity will lead to the removal of the 3′ overhang from the cDNA templates (Fig. 4A). The 5′-3′ polymerase activity and the four dNTPs prevents continued degradation of the cDNA template, leading to the conversion of the 3′ overhang into a blunt end (Fig. 4A, blunt). In the presence of dATP and dCTP, T4 DNA polymerase converts the 3′ overhang into a 5′ overhang (Fig. 4A, 5′ overhang). These modifications of the viral cDNA templates do not affect the template strand used for the synthesis of HCV P90Δ3 RNA (Fig. 4A).

Next, we determined the effect of these various cDNA termini on T7 transcription by examining ^{32}P-labeled T7 transcription products by gel electrophoresis before (Fig. 4C, lanes 1–3) and after (Fig. 4C, lanes 4–6) digestion with RNase A. HCV P90Δ3 RNA (1 μg) made from the three different *Bsm*1-linearized cDNA templates was the appropriate size and no significant contaminants were visible (Fig. 4C, lanes 1–3). The general quality of the viral RNAs, however, was different depending on the cDNA templates (Fig. 4C, lanes 1–3). HCV P90Δ3 RNA made from the 5′ overhang modified cDNA template was best as judged by the amount of full-length product (Fig. 4C, lane 3). Following the digestion of 25 μg of each RNA with RNase A, we observed a significant band of dsRNA in the T7 transcription products from the reaction containing unmodified *Bsm*1-linearized cDNA template (Fig. 4C, lane 4). A comparable band of dsRNA was not seen in the RNase A digestion products of HCV P90Δ3 RNA from cDNA templates with blunt or 5′-overhang termini (Fig. 4C, lanes 5 and 6).

Quantitation of the single-stranded and dsRNA bands by phosphorimaging allowed us to calculate the frequency of spurious transcription initiation on the cDNA template with the 3′ overhang. The dsRNA in Figure 4C, lane 4, represented 5% of the total transcription products. Therefore, T7 RNA polymerase initiated transcription from the 3′ overhang once for every 20 initiation events from the T7 promoter upstream of the HCV cDNA. Converting the 3′ overhang to a blunt or 5′ overhang eliminated $>$99% of the spurious transcription initiation. To more completely eliminate the concern that dsRNA from spurious transcription might be activating 2′-5′ OAS, we gel purified HCV P90Δ3 RNA transcripts. When the experiment described in Figure 3 was repeated using gel purified mRNAs, identical results were obtained (data not shown). As before, the host mRNA did not activate the endoribonucleolytic pathway whereas the HCV mRNA did (data not shown). These results suggest that dsRNA from spurious transcription is not responsible for the activation of 2′-5′ OAS.

Poly(I:C) is a synthetic dsRNA commonly used to activate 2′-5′ OAS, and consequently, RNase L. Therefore, we incubated poly(I:C) in HeLa S10 translation–replication reactions to determine whether the ribosomal RNA fragments observed in the previous figures were consistent with RNase L cleavage products. Poly(I:C) induced the degradation of ribosomal RNA as predicted (Fig. 5, lanes 2 and 4). HCV P90Δ3 mRNA induced the same degradation of ribosomal RNA (Fig. 5, lanes 6 and 8). In contrast, human plakoglobin mRNA did not induce the degradation of ribosomal RNA (Fig. 5, lanes 10 and 12). The band of RNA observed below

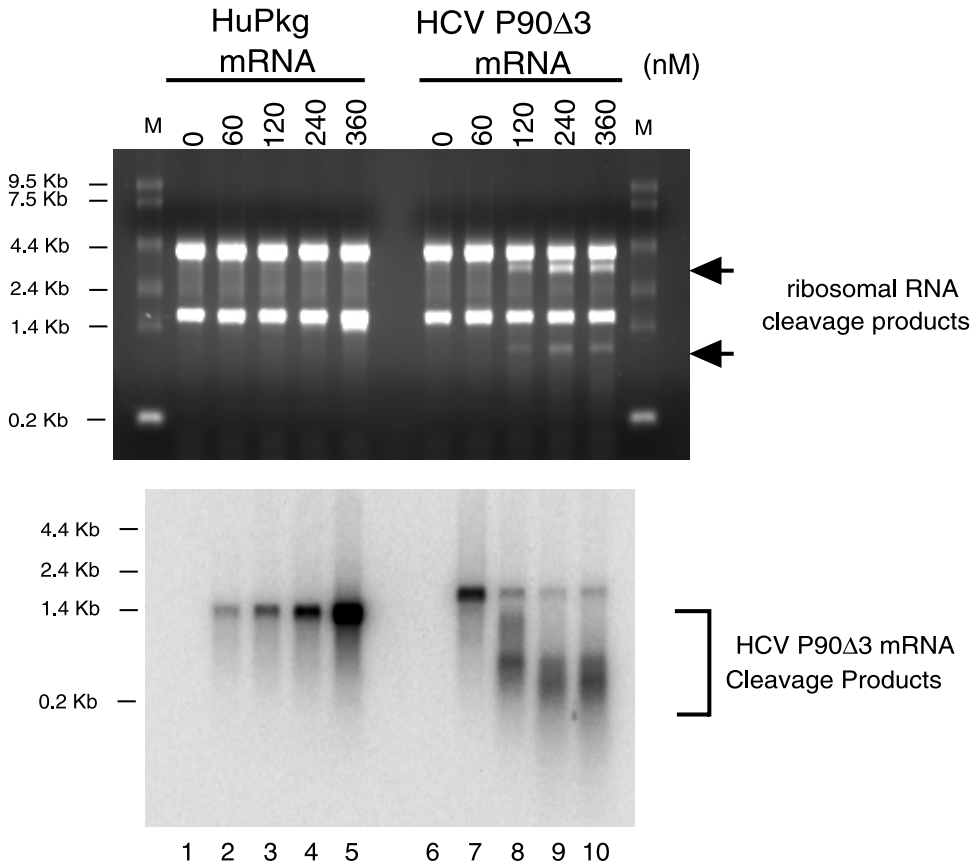

FIGURE 3. Endonuclease activation by viral mRNA but not host mRNA. Equimolar concentrations of ^{32}P-labeled human plakoglobin mRNA (lanes 1–5) and HCV P90Δ3 mRNA (lanes 6–10) were incubated in HeLa S10 translation–replication reactions as described in Figure 2. Top panel: Ribosomal RNA was detected by ethidium bromide and UV light. Bottom panel: Radiolabeled mRNAs were detected by phosphorimaging. The top and bottom panels are aligned to facilitate lane by lane comparisons.

18S rRNA in lanes 10 and 12 of Figure 5 corresponds to human plakoglobin mRNA (Fig. 5, lanes 10 and 12).

These experiments demonstrated that: (1) HCV mRNA (and HCVP90Δ3 mRNA) activated an endoribonuclease, (2) the endoribonuclease cleaved HCV mRNA and ribosomal RNA, and (3) the fragments of ribosomal RNA corresponded in size to those obtained in reactions containing poly(I:C), a synthetic dsRNA known to activate 2′-5′ OAS and RNase L.

Activation of 2′-5′ OAS by HCV mRNA

Because RNase L requires 2′-5′ oligo(A) to activate its endoribonuclease, we assayed for the synthesis of 2′-5′ oligo(A) in HeLa S10 translation–replication reactions. 2′-5′ OAS typically makes 2′-5′ oligo(A) dimers, trimers, and tetramers with smaller amounts of larger oligomers (Fig. 6A). The 5′-terminal triphosphate of 2′-5′ oligo(A) can be removed with alkaline phosphatase, yielding 2′-5′ oligo(A) "cores." We used [α-^{32}P]ATP as a substrate for the synthesis of radiolabeled 2′-5′ oligo(A). Radiolabeled 2′-5′ oligo(A) was detected by electrophoresis of samples in 20% polyacrylamide 7 M urea and phosphorimaging (Fig. 6B; Miele et al., 1991). In reactions without dsRNA, no oligomers of ATP were detected (Fig. 6B, lane 1). When double-stranded poly(I:C) was incubated in the reactions, considerable amounts of radiolabeled 2′-5′ oligo(A) were made (Fig. 6B, lane 3; Nilsen et al., 1981, 1982; Miele et al., 1991). Phosphatase treatment of poly(I:C) reaction products before electrophoresis converted the tri-dimer 2′-5′ oligo(A) into a dimer core (Fig. 6B, lane 4; Miele et al., 1991). Likewise, phosphatase treatment converted the tri-trimer and tri-tetramer forms of 2′-5′ oligo(A) into trimer core and tetramer core, respectively (Fig. 6B, lane 4; Miele et al., 1991). HCV P90Δ3 mRNA activated 2′-5′ OAS, leading to the synthesis of tri-dimer, tri-trimer, and tri-tetramer forms of 2′-5′ oligo(A) (Fig. 6B, lane 5). The tri-dimer, tri-trimer, and tri-tetramer forms from the HCV P90Δ3 reactions were converted to dimer, trimer, and tetramer cores upon digestion with alkaline phosphatase (Fig. 6B, lane 6). Human plakoglobin mRNA did not activate 2′-5′ OAS activity in these reactions (Fig. 6B, lanes 7–8). Thus, as shown for the

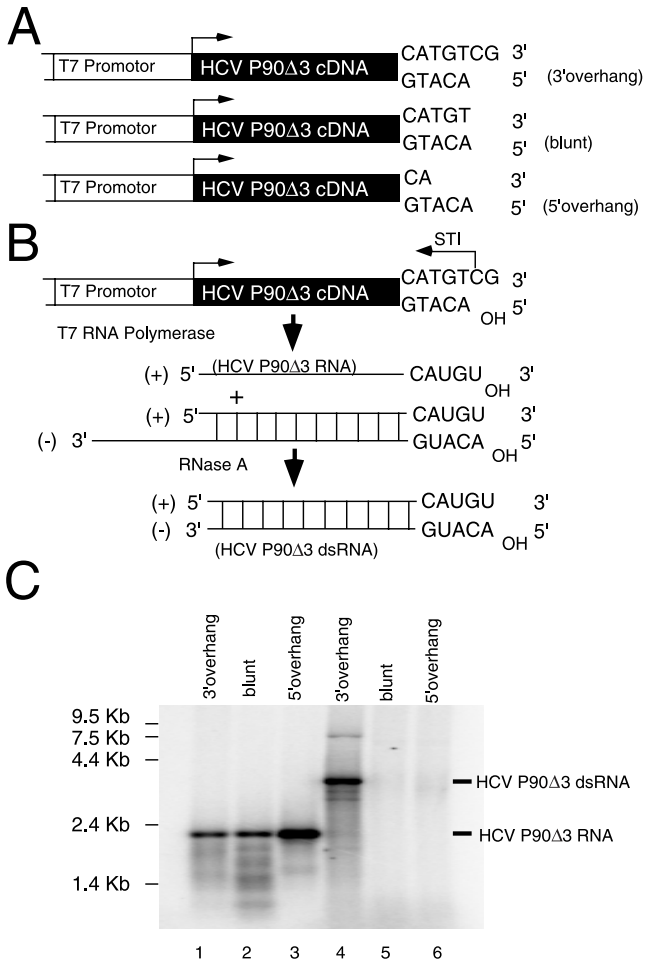

FIGURE 4. Modification of cDNA templates and T7 transcription of HCV P90Δ3 RNA. To make HCV T7 RNA transcripts with authentic 3′ termini, HCV cDNAs were linearized with *Bsm*1 immediately downstream of the viral cDNA and used as templates for runoff transcription. **A:** cDNA templates. *Bsm*1 digestion created a 3′ overhang in the HCV P90Δ3 cDNA (unmodified). Bacteriophage T4 DNA polymerase was used as described in Materials and Methods to convert the 3′ overhang of *Bsm*I-digested cDNA into blunt and 5′ overhangs. These modifications only affected the nontemplate (top) strand of the cDNA. **B:** Spurious transcription initiation (STI). Bacteriophage T7 RNA polymerase initiates transcription spuriously from cDNAs with 3′ overhangs (Schenborn & Mierendorf, 1985). Transcription from both the T7 promoter and from the 3′ overhang in the *Bsm*1-linearized cDNA leads to the synthesis of both (+) strand and (−) strand transcripts of HCV P90Δ3 RNA. Double-stranded forms of HCV P90Δ3 RNA are resistant to digestion with RNase A. **C:** HCV P90Δ3 RNA transcripts. ^{32}P-labeled transcripts of HCV P90Δ3 RNA before (lanes 1–3) and after (lanes 4–6) digestion with RNase A. HCV P90Δ3 RNA from unmodified cDNA (lanes 1 and 4), blunt modified cDNA (lanes 2 and 5), and 5′ overhang modified cDNA (lanes 3 and 6). Lanes 1–3: 1 μg of HCVP90Δ3 RNA per lane. Twenty-five micrograms of HCV P90Δ3 RNA transcription products from each cDNA template were digested with 10 ng/mL RNase A in 0.3 M NaCl for 30 min at 37 °C. Lanes 4–6: The RNase A-digested products were precipitated with ethanol and fractionated on the gel. The mobility of double-stranded HCV P90Δ3 RNA and single-stranded HCV P90Δ3 RNA is indicated.

activation of RNase L (Figs. 3 and 5), HCV mRNA specifically activated 2′-5′ OAS under conditions in which host mRNA did not (Fig. 6B).

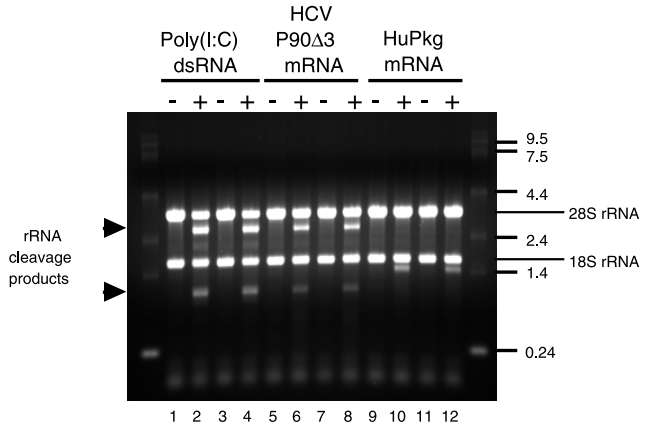

FIGURE 5. HCV P90Δ3 mRNA-activated endonuclease and dsRNA-activated endonuclease (RNase L) produced identical rRNA cleavage products. Poly(I:C) (50 μg/mL), HCV P90Δ3 mRNA (360 nM), and human plakoglobin mRNA (360 nM) were incubated in HeLa S10 translation–replication reactions at 34 °C for 30 min. RNA from the reactions was fractionated by electrophoresis in 1% agarose and detected by ethidium bromide and UV light. RNA from reactions containing poly(I:C) (lanes 2 and 4), HCV P90Δ3 mRNA (lanes 6 and 8), and human plakoglobin mRNA (lanes 10 and 12). Cellular RNA from mock reactions without exogenous RNA (lanes 1, 3, 5, 7, 9, and 11).

RNase L cleavage sites in HCV mRNA

Because RNase L reportedly cleaves heteropolymeric RNAs preferentially at single-stranded UA and UU dinucleotides (Wreschner et al., 1981b), we sought to determine whether HCV mRNA in HeLa S10 translation–replication reactions was cleaved preferentially at particular sites. Primer extension was used to identify cleavage sites in HCV mRNA (Fig. 7). HCV mRNA (Fig. 7A, lane 1) was degraded (by RNase L) in HeLa S10 translation–replication reactions (Fig. 7A, lane 3). Each HCV mRNA molecule was cleaved one or more times as evidenced by the disappearance of intact HCV mRNA (Fig. 7A, lane 3). A control template for primer extension was prepared from an unincubated HeLa S10 translation–replication reaction containing HCV mRNA (Fig. 7A, lane 2). A 5′ ^{32}P-labeled primer complementary to HCV nt 9348 to 9377 (the 3′ terminus of the HCV ORF) identified four RNase L cut sites over 300 bases of HCV mRNA sequence (Fig. 7B, lane 3). Intact HCV mRNA did not yield primer extension products at these sites (Fig. 7B, lane 2). A DNA sequencing ladder was generated using the same primer as that used for primer extension (Fig. 7B–E). The precise RNase L cleavage sites in HCV mRNA were identified by examining the size of the primer extension products alongside DNA sequencing ladders (Fig. 7C–E). Predicted RNA stem-loop structures were identified at each RNase L cut site using the RNA Fold algorithm of Michael Zucker (Mathews et al., 1999; Fig. 7C–E). For the purposes of analysis, we defined a cut site as one or more cleavages within a common loop of a predicted stem-loop structure. Multiple cleavages were detected at cut

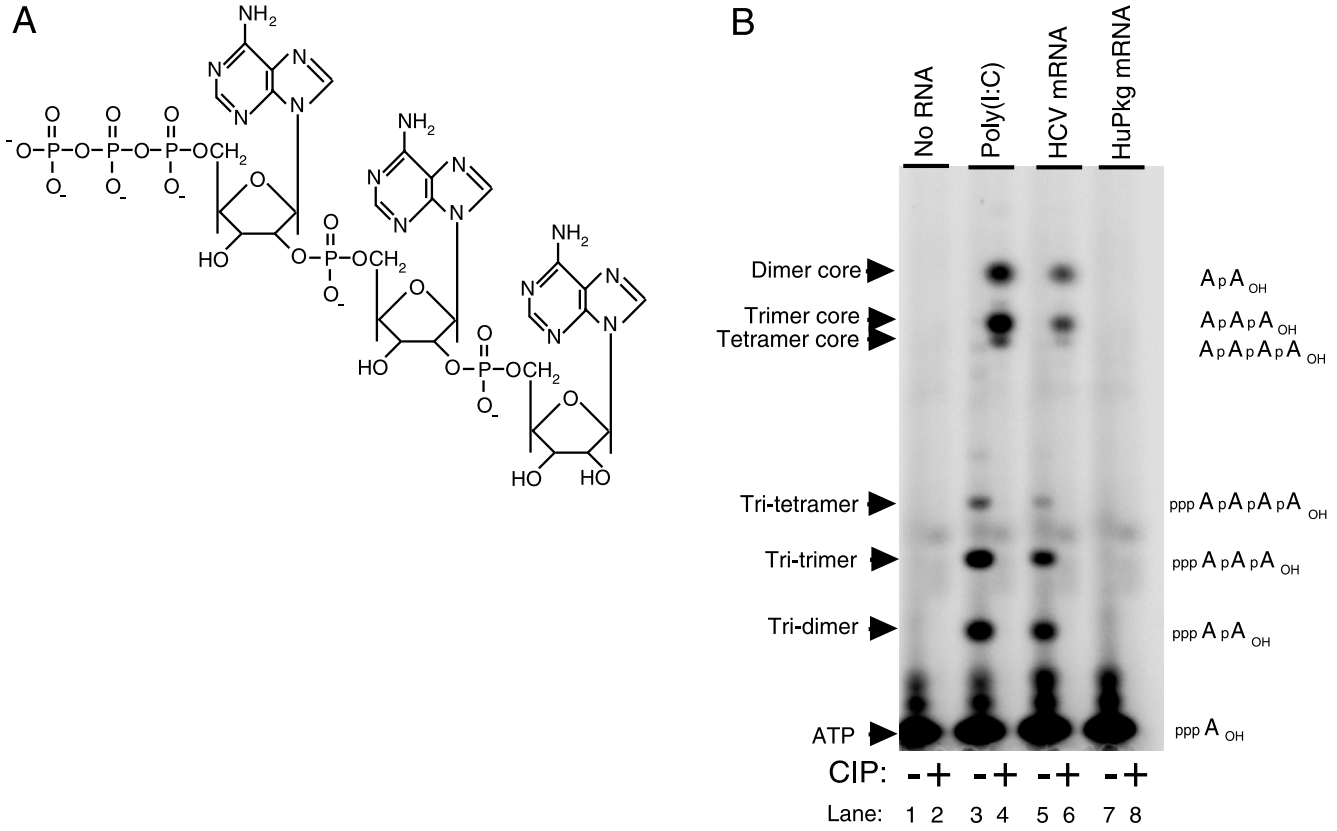

FIGURE 6. Activation of 2′-5′ oligoadenylate synthetase by HCV P90Δ3 mRNA. **A:** 2′-5′ oligoadenylate tri-trimer. **B:** Synthesis of radiolabeled 2′-5′ oligoadenylate. Poly(I:C) (50 μg/mL), HCV P90Δ3 mRNA (360 nM), and human plakoglobin mRNA (360 nM) were incubated in reactions containing HeLa cell extract and α-^{32}P[ATP] as described in Materials and Methods (Assaying 2′-5′ OAS). A portion of each reaction was left untreated, and a second portion was digested with calf intestinal phosphatase. Reaction products were separated by electrophoresis in 7 M urea 20% polyacrylamide. Mock reaction without RNA (lanes 1 and 2). Reactions containing poly(I:C) (lanes 3–4), HCV P90Δ3 mRNA (lanes 5–6), and human plakoglobin mRNA (lanes 7–8). Radiolabel within the gel was detected by phosphorimaging. The mobilities of specific molecules are indicated.

sites #1, #3, and #4 (Fig. 7C–E). A UA dinucleotide was present in the single-stranded loop at each cut site. Most cleavages were detected following UA and UU dinucleotides, although other dinucleotides immediately proximal to UA dinucleotides were also cleaved (Fig. 7C,D). Using other primers, we found UA dinucleotides were present at almost every RNase L cleavage site identified in the HCV 1a ORF (data not shown).

Because the primer was end labeled, the amount of primer extension product at each cut site is a function of the RNase L cleavage frequency at each site. Yet, because many HCV mRNA templates were cleaved more than once, cleavage sites downstream from other cleavage sites may exhibit less signal than those proximal to the primer. The amount of primer extension product at each cut site was determined by phosphorimaging. Cut site #4 had the greatest amount of primer extension product, followed by cut site #1, cut site #3, and cut site #2 (Fig. 7B). Indeed, cut site #4 had 10 times more primer extension product than cut site #2 (Fig. 7B). Unlike cut sites #1, 3, and 4, cut site #2 appeared to be in an unfavorable structure for RNase L cleavage (Fig. 7D). Cut sites #1 and #4, the most frequently cleaved sites, exhibited larger loop structures than that at cut site #3. These results are consistent with the prediction that HCV mRNA would be cleaved by RNase L at single-stranded UA and UU dinucleotides.

Intriguingly, HCV mRNA is naturally deficient in UA and UU dinucleotides. The 9,033-nt-long HCV genotype 1a ORF has a 21.2% U and 19.9% A base composition. Based on this base composition, one could predict a UA dinucleotide every 23.7 nt [1/(0.212 $\times$ 0.199) = 23.7]. Over the 9,033-nt-long ORF, one would predict 381 UA dinucleotides and 405 UU dinucleotides. In reality, the HCV genotype 1a ORF has only 291 UA dinucleotides (90 fewer than predicted by the base composition) and 369 UU dinucleotides (36 fewer than predicted by the base composition). This deficit of 126 UA and UU dinucleotides is reflected in the codon bias of the HCV genotype 1a ORF (Table 1). Leucine is encoded by six codons (UUA, CUA, UUG, CUU, CUC, and CUG) and there are 305 leucines encoded within the HCV genotype 1a ORF. If the six codons were used

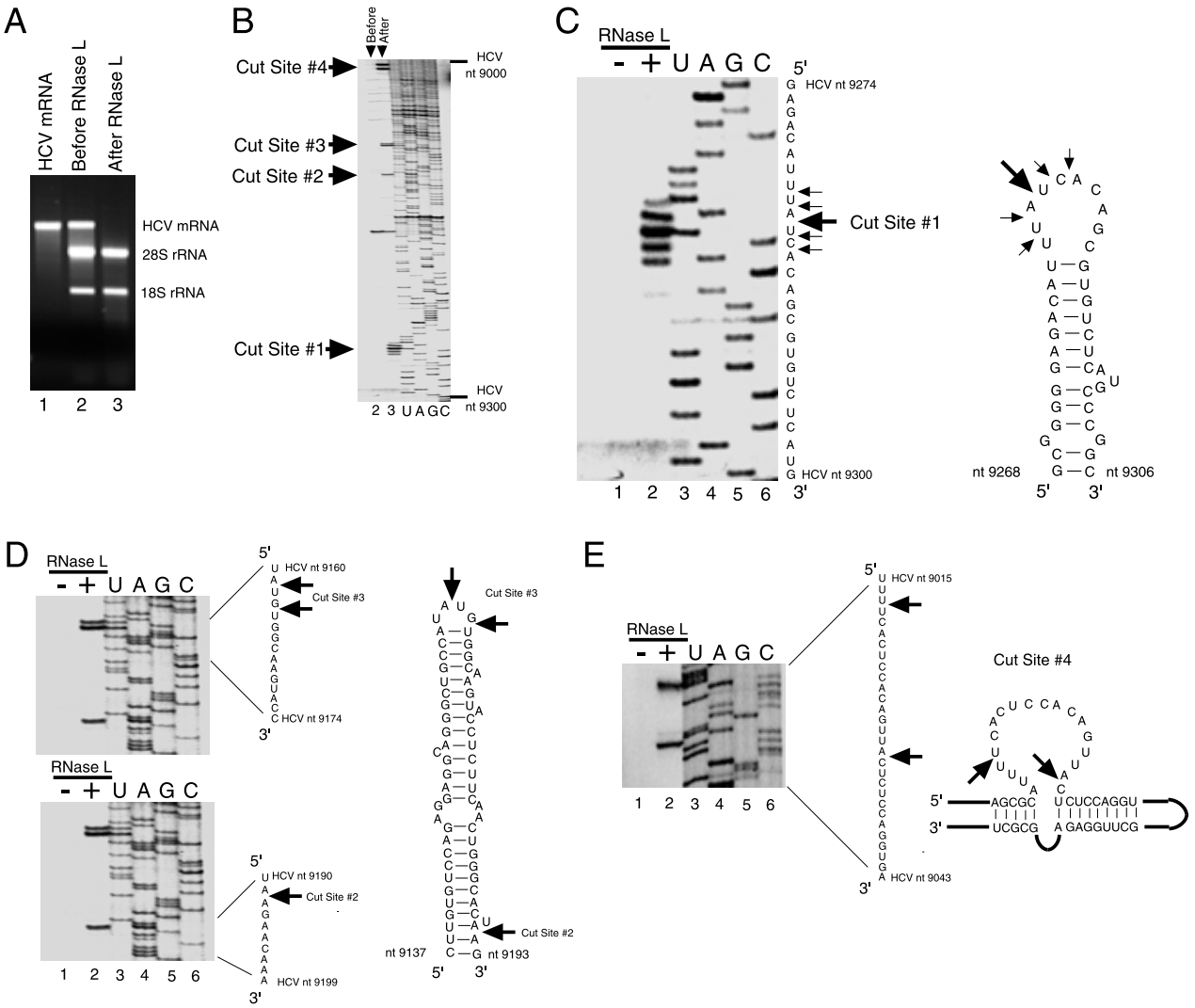

FIGURE 7. Identification of RNase L cleavage sites in HCV mRNA. **A:** HCV mRNA before and after digestion with RNase L. HCV mRNA (240 nM) was added to HeLa S10 translation–replication reactions as indicated in Materials and Methods. One reaction was maintained on ice whereas the second was incubated at 34 °C for 10 min to activate RNase L. The RNAs from both reactions were prepared by phenol:chloroform extraction, ethanol precipitation, and solubilization in water. A portion of HCV mRNA alone (lane 1), RNA from the unincubated reaction (lane 2), and RNA from the incubated reaction (lane 3) were fractionated by electrophoresis in 1% agarose. RNA was detected by ethidium bromide and UV light. **B:** Primer extension. RNAs corresponding to those in lanes 2 and 3 of **A** were used as templates for primer extension as described in Materials and Methods (Primer extension). A 5′ ^{32}P-labeled primer complementary to HCV nt 9348 to 9377 was annealed to the RNA templates and extended using Superscript reverse transcriptase. The primer extension products were separated by electrophoresis in a 7 M urea 6% polyacrylamide gel and detected by phosphorimaging. **C–E:** RNase L Cleavage sites. Primer extension products were fractionated alongside a DNA sequencing ladder to identify specific cleavage sites.

equally, each codon would be used approximately 50 times. The UUA leucine codon, which possesses both a UU and a UA dinucleotide, is used only 9 times in the HCV genotype 1a ORF. The CUA codon encoding leucine is used only 21 times, the UUG codon encoding leucine is used only 38 times, and the CUU codon encoding leucine is used 52 times. The two other codons encoding leucine, CUC and CUG, are used 87 and 98 times, respectively. The codon bias for valine is also noteworthy; GUA and GUU are underrepresented (used 60 times) relative to GUC and GUG (used 181 times). These observations suggest that HCV genotype 1a mRNA has evolved to avoid UA and UU dinucleotides. The reduced frequency of these dinucleotides in HCV mRNA could be due to codon selection (Nakamura et al., 2000) or RNase L selection. HCV genotype 1b, 2a, 2b, 3a, and 3b mRNAs also have fewer UA and UU dinucleotides than predicted by the base composition (Table 2). Furthermore, relatively interferon-resistant genotypes (1a and 1b) have fewer UA and UU dinucleotides than relatively interferon-sensitive genotypes (2a, 2b, 3a, and 3b; Table 2). Based on these obser-

TABLE 1. HCV genotype 1a codon usage.

UUU phe 31	UCU ser 28	UAU tyr 29	UGU cys 32
UUC phe 56	UCC ser 70	UAC tyr 69	UGC cys 71
UUA leu 9	UCA ser 27	UAA OCH	UGA OPA
UUG leu 38	UCG ser 25	UAG AMB	UGG trp 71
CUU leu 52	CCU pro 55	CAU his 29	CGU arg 15
CUC leu 87	CCC pro 83	CAC his 38	CGC arg 38
CUA leu 21	CCA pro 35	CAA gln 36	CGA arg 13
CUG leu 98	CCG pro 34	CAG gln 52	CGG arg 34
AUU ile 24	ACU thr 43	AAU asn 25	AGU ser 15
AUC ile 74	ACC thr 90	AAC asn 61	AGC ser 49
AUA ile 33	ACA thr 33	AAA lys 30	AGA arg 26
AUG met 56	ACG thr 50	AAG lys 63	AGG arg 55
GUU val 35	GCU ala 55	GAU asp 33	GGU gly 42
GUC val 83	GCC ala 113	GAC asp 86	GGC gly 104
GUA val 25	GCA ala 45	GAA glu 32	GGA gly 35
GUG val 98	GCG ala 64	GAG glu 84	GGG gly 74

vations, we predicted that HCV mRNA from relatively interferon-sensitive genotypes would be more readily cleaved by RNase L than HCV mRNA from more interferon-resistant genotypes.

Comparison of HCV 1a and HCV 2a mRNAs

To test whether HCV mRNAs from interferon-sensitive and interferon-resistant genotypes were differentially sensitive to 2′-5′ OAS and RNase L, we compared HCV 1a mRNA with HCV 2a mRNA (Figs. 8 and 9). HCV 1a mRNA, a relatively interferon-resistant genotype, has 73 fewer UA and UU dinucleotides than HCV 2a mRNA, a relatively interferon-sensitive genotype (Table 2). HCV 1a and HCV 2a mRNAs activated equal amounts of 2′-5′ oligoadenylate synthetase in a dose-dependent manner (Fig. 8). This result was not unexpected, as the dsRNA structures in the 5′ and 3′ NTRs of HCV 1a and 2a mRNAs are very highly conserved (Yanagi et al., 1999) and it appears that these dsRNA structures are responsible for the activation of 2′-5′ oligoadenylate synthetase (Figs. 3, 5, and 6). At 15 nM and below, HCV mRNA did not activate 2′-5′ oligoadenylate synthetase or RNase L (Figs. 8 and 9). Above 15 nM, dsRNA elements of HCV mRNA activated 2′-5′ oligoadenylate synthetase and RNase L (Figs. 8 and 9). Even though HCV 1a and 2a mRNAs activated 2′-5′ OAS equally, HCV 2a mRNA was more readily degraded by RNase L (Fig. 9). Thus, as predicted by the analysis of UA and UU dinucleotide frequency, HCV 2a mRNA was more sensitive to cleavage by RNase L than HCV 1a mRNA. From these results, we suggest that variation in RNase L cleavage sites in HCV mRNAs is the molecular basis for the interferon sensitivity or resistance of HCV infections. The number of potential RNase L cleavage sites in the ORF of HCV mRNAs varies significantly between genotypes as well as between different isolates within a genotype (Table 2). For instance, an isolate of HCV 3a mRNA possesses 203 more UA and UU dinucleotides than an isolate of HCV 1b mRNA (Table 2, HCV 3a mRNA compared with HCV 1b mRNA from patient 2). Variation within genotypes is also substantial as HCV 1b mRNA from one patient had 85 more UA and UU dinucleotides than the HCV 1b mRNA from another patient (Table 2, HCV 1b patients 2 and 3). In general, relatively interferon-sensitive HCV genotypes have more potential RNase L

TABLE 2. Potential RNase L cleavage sites in HCV mRNA.[a]

Virus[b]	UA dinucleotides[c]	UU dinucleotides[c]	UA and UU dinucleotides		
			Observed[c]	Predicted[d]	Missing[e]
HCV 1a	291	369	660	786	126
HCV 1b	300	363	663	801	138
HCV 2a	313	420	733	838	106
HCV 2b	345	447	792	898	106
HCV 3a	376	446	822	924	102
HCV 3b	350	419	769	886	117
HCV 1b (Patient 1)[f]	292	348	640		
HCV 1b (Patient 2)[f]	269	350	619		
HCV 1b (Patient 3)[f]	314	390	704		

[a] Single-stranded UA and UU dinucleotides represent preferential cleavage sites for RNase L. The number of UA and UU dinucleotides in the ORFs of HCV mRNA are fewer than predicted.
[b] Nucleotide sequences: HCV genotype 1a, (Kolykhalov et al., 1997); HCV genotype 1b, D90208 ; HCV genotype 2a, AF177036, (Yanagi et al., 1999); HCV genotype 2b, D01221; HCV genotype 3a, D28917; HCV genotype 3b, 26556; HCV genotype 1b patient 1, D50483; HCV genotype 1b patient 2, D50485; HCV genotype 1b patient 3, D50484.
[c] Number of UA and/or UU dinucleotides within the ORF of each virus.
[d] Number of UA and UU dinucleotides predicted in the ORF of each virus based on the base composition.
[e] Difference in number of UA and UU dinucleotides predicted and those observed.
[f] Interferon nonresponders described in Enomoto et al. (1995).

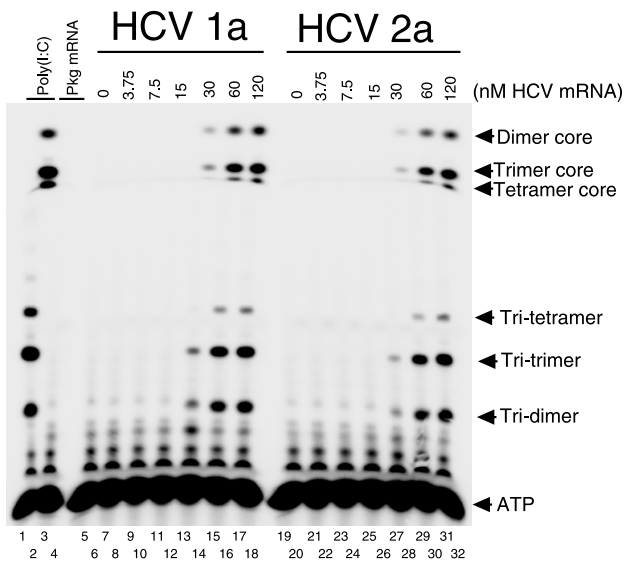

FIGURE 8. Dose-dependent activation of dsRNA-dependent 2′-5′ oligoadenylate synthetase by HCV 1a and HCV 2a mRNAs. RNAs were incubated in reactions containing [α-^{32}P]ATP and 2′-5′ oligoadenylate synthetase (Materials and Methods, Assaying 2′-5′ OAS). Poly(I:C) (50 μg/mL), a synthetic dsRNA, was used as a positive control (lanes 1 and 2). Human plakoglobin mRNA (120 nM) was used as a negative control (lanes 3 and 4). HCV 1a (lanes 5–18) and HCV 2a (lanes 19–30) mRNAs were incubated in the reactions at the indicated concentrations. A portion of the products from each reaction was treated with calf intestinal phosphatase (even numbered lanes) and a second portion was untreated (odd numbered lanes). Radiolabeled products were fractionated by electrophoresis in 20% polyacrylamide 7 M urea TBE gels and detected by phosphorimaging. Radiolabeled products are identified on the right side of the figure.

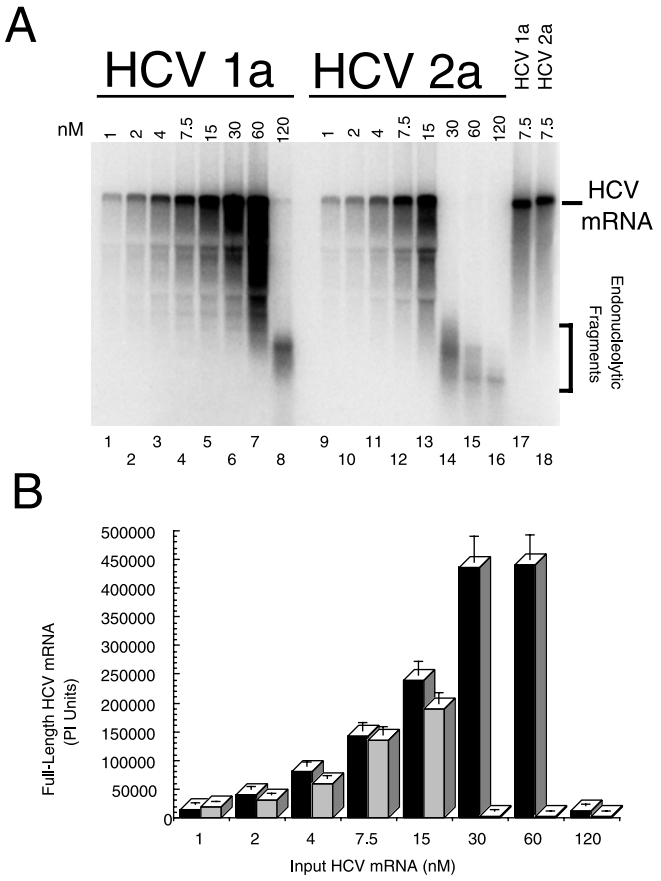

FIGURE 9. Dose-dependent activation of the antiviral 2′-5′ oligoadenylate synthetase/RNase L pathway by HCV 1a and HCV 2a mRNAs. ^{32}P-labeled HCV1a and 2a mRNAs were transcribed from cDNA clones and incubated for 2 h at 34 °C at the indicated concentrations in HeLa S10 translation–replication reactions. **A:** RNA from the reactions was separated by electrophoresis in 1% agarose and radiolabeled HCV mRNA was detected by phosphorimaging. Lanes 1–8: HCV 1a mRNA. Lanes 9–16: HCV 2a mRNA. Lanes 17 and 18: HCV 1a mRNA and 2a mRNA, respectively, before incubation in HeLa S10 translation-replication reactions. **B:** Full-length HCV 1a mRNA (black bar) and 2a mRNA (light bar) was quantified by phosphorimaging and plotted versus concentration of HCV mRNA incubated in the reaction.

cleavage sites in their mRNA than relatively interferon-resistant genotypes (Table 2).

Evolution of UA and UU dinucleotides in vivo

To determine whether RNase L cleavage sites are important in vivo, we reanalyzed the data of Enomoto et al. (1995) to see if interferon treatment selected for mutations at UA and UU dinucleotides (Fig. 10). Each of the patients examined was infected with a HCV quasispecies possessing reduced numbers of UA and UU dinucleotides (Table 2). Patient 2 was infected by a quasispecies with especially reduced numbers of UA and UU dinucleotides (Table 2). Interferon treatment resulted in significant evolution in the HCV quasispecies (Fig. 10). Ninety-four silent mutations accumulated in the HCV quasispecies during 4 weeks of interferon therapy, eliminating 14 UA dinucleotides and 9 UU dinucleotides (Fig. 10, Patient 1). The 94 silent mutations were not randomly distributed throughout the viral ORF but localized in particular regions (Fig. 10, Patient 1). Similar results were found for two additional interferon-nonresponder patients treated for 17 and 26 weeks, respectively; however, the regions of HCV mRNA with mutations varied from patient to patient (Fig. 10, Patients 2 and 3). HCV mRNA from the patient treated with interferon for 17 weeks accumulated 130 silent mutations that eliminated 9 UA dinucleotides and 28 UU dinucleotides (Fig. 10, Patient 2). HCV mRNA from the patient treated with interferon for 26 weeks accumulated 134 silent mutations that eliminated 22 UA dinucleotides and 19 UU dinucleotides (Fig. 10, Patient 3). Many of the silent mutations that accumulated during interferon therapy also created new UA and UU dinucleotides (data not shown). Thus, it appears that potential RNase L cleavage sites in the quasispecies of HCV mRNA were dramatically remodeled during interferon therapy, although not quantitatively removed from HCV mRNA.

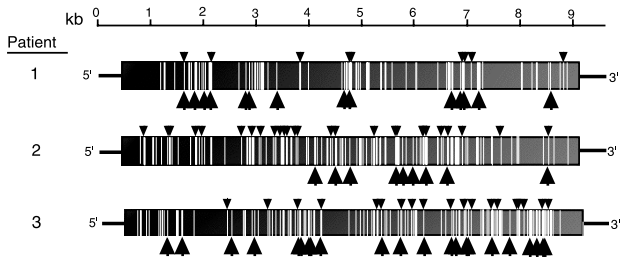

FIGURE 10. Evolution of UA and UU dinucleotides in HCV mRNA during interferon therapy. The quasispecies of HCV1b mRNA from three interferon nonresponder patients was determined before and after interferon therapy (Enomoto et al., 1995). The HCV mRNA quasispecies was determined after 4 weeks of interferon therapy in patient 1 (nucleotide sequences D50483 and D50480), 17 weeks of interferon therapy in patient 2 (nucleotide sequences D50485 and D50481), and 26 weeks of interferon therapy in patient 3 (D50484 and D50482). Location of silent mutations is indicated by vertical white lines. Location of silent mutations that eliminated a UA dinucleotide are indicated by large arrow. Location of silent mutations that eliminated a UU dinucleotide are indicated by small arrow.

DISCUSSION

In this article, we demonstrate that HCV mRNA was detected and destroyed by the antiviral 2′-5′ OAS/RNase L pathway present in HeLa cell extracts. HCV 2a mRNA was detected and destroyed by 2′-5′ OAS and RNase L more readily than HCV 1a mRNA. HCV genotype 2 and 3 mRNAs exhibit more potential RNase L cleavage sites than HCV genotype 1 mRNAs and HCV genotype 2 and 3 infections respond more favorably to interferon than HCV genotype 1 infections (McHutchison et al., 1998). These results are consistent with the conclusion that the sensitivity of HCV mRNA to cleavage by RNase L correlates with the sensitivity of HCV infections to interferon therapy. We also report that the evolution of UA and UU dinucleotides in the quasispecies of HCV mRNA during interferon therapy is consistent with selection by RNase L.

Activation of 2′-5′ OAS by HCV mRNA

dsRNA molecules as small as 15 bp in length can partially activate 2′-5′ OAS (Sarkar et al., 1999). Our results suggest that HCV mRNA possesses one or more dsRNA structures capable of activating 2′-5′ OAS. The 5′ and 3′ nontranslated regions of HCV mRNA exhibit significant amounts of dsRNA structure. The 5′ nontranslated region of HCV mRNA encodes a highly structured internal ribosome entry site (Wang et al., 1993, 1994; Rijnbrand & Lemon, 2000). The 3′ nontranslated region of HCV mRNA possesses various dsRNA stem-loops around a central poly(U)-poly(U/C) domain (Kolykhalov et al., 1996; Blight & Rice, 1997). Furthermore, there is some complementarity between the very 5′ and 3′ termini of HCV mRNA (Kolykhalov et al., 1996). Host mRNAs, by and large, do not possess comparable amounts of dsRNA structure within their 5′ and 3′ nontranslated regions nor within their coding regions. Because human plakoglobin mRNA did not activate 2′-5′ OAS under conditions in which HCV P90Δ3 mRNA did, we suggest that 2′-5′ OAS is able to distinguish between host and HCV mRNAs by recognizing (binding) dsRNA structure(s) in the 5′ and/or 3′ nontranslated regions of HCV mRNA.

In our reactions, HCV mRNA exhibited a threshold concentration ~20 nM for the activation of 2′-5′ OAS and RNase L (Figs. 8 and 9). Below this concentration, HCV mRNA was stable and translated efficiently in our reactions. Above this concentration, the HCV mRNA was rapidly cleaved by RNase L. Twenty nanomolar HCV mRNA corresponds to approximately 27,000 mRNA molecules per infected hepatocyte (assuming a cytoplasmic volume of 3,000 μM^3). Within infected hepatocytes, HCV mRNA concentrations may increase gradually until they reach threshold concentrations capable of activating 2′-5′ OAS and RNase L. Furthermore, because 2′-5′ OAS expression is dramatically increased by interferon, concentrations of HCV mRNA may be regulated by the magnitude of the immune response. As interferon and 2′-5′ OAS concentrations in liver tissue rise, HCV mRNA concentrations may be reduced, down-regulating viral gene expression and replication. Regulating HCV gene expression and replication inversely with the magnitude of the immune response may provide HCV infections a mechanism for avoiding excessive provocation of the acquired immune response. Avoiding excessive provocation of the acquired immune response may contribute to the ability of HCV infections to establish persistent infection. Thus, rather than completely avoiding this antiviral pathway, HCV mRNA may intentionally activate 2′-5′ OAS and RNase L as a means of regulating antigen expression inversely with the magnitude of the immune response.

Cleavage of HCV mRNA by RNase L

Because HCV 1a mRNA was degraded into fragments 200 to 500 nt in length (Fig. 2), we predict that HCV 1a mRNA (9,648 nt long) possesses 20 to 50 RNase L cleavage sites interspersed throughout the viral mRNA (i.e., sites that can be cleaved by RNase L efficiently). HCV mRNA was cleaved by RNase L at single-stranded UA and UU dinucleotides (Fig. 7). Nonetheless, HCV mRNA is naturally deficient in UA and UU dinucleotides, preferred sequences for RNase L cleavage (Table 2). Furthermore, many of the UA and UU dinucleotides within the dsRNA portion of stem-loop structures cannot be cleaved by RNase L. In the 300-base-long region of HCV mRNA examined in Figure 7, there are 9 UA dinucleotides and 14 UU dinucleotides. RNase L cleavage was only detected at 4 out of 9 UA dinucleotides and 3 out of 14 UU dinucleotides (see

Fig. 7). Furthermore, the frequency of cleavage at different sites was more than 10-fold different. These results support the conclusion that sequence and structure influence the efficiency by which RNase L cleaves particular sites. The reduced frequency of UA and UU dinucleotides in the ORF of HCV mRNA could be due to codon selection, RNase L selection, or both. Codon selection against UA and UU dinucleotides may occur due to reduced concentrations of particular tRNAs in human cells (Nakamura et al., 2000). A comprehensive analysis of RNase L cleavage sites and UA/UU dinucleotide distribution in HCV mRNA will help distinguish between codon selection and RNase L selection as the root of decreased UA and UU dinucleotide frequencies.

Mechanism for interferon resistance

We hypothesize that the sensitivity of HCV infections to interferon therapy is directly related to the sensitivity of HCV mRNA to cleavage by RNase L. UA and UU dinucleotides within the ORF of HCV mRNA constitute the most likely RNase L cleavage sites. Relatively interferon-resistant genotypes of HCV have significantly fewer UA and UU dinucleotides within their ORFs than more interferon-sensitive genotypes (Table 2). Because only one cleavage of HCV mRNA is required to "kill" the viral mRNA, we suggest that under physiologic conditions, RNase L activity is tightly regulated by 2′-phosphodiesterase and RNase L inhibitor (Benoit De Coignac et al., 1998; Martinand et al., 1998) such that only limited activation of RNase L occurs. The concept of highly regulated and localized activation of RNase L is supported in the literature (Li et al., 1998; Player & Torrence, 1998). Under conditions of tightly regulated RNase L activity, some HCV mRNA molecules with fewer RNase L cleavage sites may avoid RNase L cleavage altogether whereas HCV mRNAs with more RNase L cleavage sites are each cleaved at least once. This is not to say that most HCV mRNA molecules are resistant to cleavage by RNase L. As shown in this article, HCV 1a mRNA, a relatively interferon-resistant genotype, can be cleaved robustly by RNase L. Furthermore, serum HCV RNA levels drop dramatically during interferon therapy regardless of genotype (Keeffe et al., 1999). Nonetheless, only a small number of HCV mRNAs need to avoid cleavage to prevent the eradication of an infection by interferon treatment. If a small number of HCV mRNAs persist during interferon treatment, the magnitude of the infection will increase following the withdrawal of therapy, as occurs in a significant number a patients who relapse (Davis et al., 1998; Camma & Craxi, 2000).

In addition to the theoretical paucity of RNase L cleavage sites within the HCV ORF, HCV mRNA possesses a poly(U) sequence within its 3′-nontranslated region (Kolykhalov et al., 1996). Because poly(U) is a substrate for RNase L (Floyd-Smith et al., 1981), the poly(U) sequence within the 3′-nontranslated region of HCV mRNA might function as a decoy sequence, luring activated RNase L away from the viral ORF. Although cleavage of the poly(U) sequence within the 3′-nontranslated region by RNase L would prevent the replication of that particular HCV RNA molecule, it would not preclude the continued translation of the 3′-truncated HCV mRNA. Such a strategy could facilitate the continued expression of HCV proteins in the context of activated RNase L. Unfortunately, we have not been able to determine whether RNase L cleaves the poly(U) tract of HCV mRNA in our reactions because reverse transcriptase fails to traverse the poly(U) tract of HCV mRNA efficiently in our primer extension experiments (data not shown).

Because of the high error rate of the RNA-dependent RNA polymerase involved in HCV RNA replication, HCV infections are composed of populations of genetically related variants known as a quasispecies (Enomoto & Sato, 1995; Forns et al., 1999; Cabot et al., 2000; Farci et al., 2000). Numerous investigators have examined the evolution of HCV during interferon treatment, yet none have detected specific nucleotide or amino acid changes associated with interferon resistance (Sakuma et al., 1996, 1999; Sarrazin et al., 1999, 2000; De Mitri et al., 2000). Mutations at wobble positions of codons with UA, UU, or other dinucleotide RNase L cleavage sites could allow specific variants within the HCV quasispecies to evade RNase L cleavage without altering the amino acid sequence of the viral proteins. Because wobble position codons throughout the HCV ORF can be altered in this fashion, each unique HCV variant in each individual patient may exhibit differential RNase L susceptibility, and therefore, differential interferon sensitivity. We found that the HCV mRNA quasispecies evolved rapidly during interferon therapy, especially at UA and UU dinucleotides representing potential RNase L cleavage sites (Fig. 10). The frequency, location, and structural presentation of UA and UU dinucleotides in the ORFs of HCV mRNA are quite variable between genotypes due to the 30 to 34% sequence variation between genotypes. Indeed, the frequency, location, and structural presentation of UA and UU dinucleotides in the ORFs of HCV mRNA are quite variable within genotypes (Fig. 10; Table 2; data not shown). Remodeling of potential RNase L cleavage sites in the HCV mRNA quasispecies is likely ongoing throughout chronic infections due to silent mutations by the RNA-dependent RNA polymerase and intermittent selection by RNase L cleavage.

HCV replicons will be useful for further characterization of interactions between HCV RNA and the antiviral 2′-5′ oligoadenylate synthetase/RNase L pathway in vivo (Lohmann et al., 2001; Pietschmann et al., 2001). It will be important to determine the effect of variable numbers of UA and UU dinucleotides in HCV RNA on the magnitude of viral gene expression and the mag-

nitude of RNA replication. Most of all, it will be important to determine in vivo the effect of variable numbers of UA and UU dinucleotides in HCV RNA on the sensitivity of HCV infections to interferon.

MATERIALS AND METHODS

cDNAs

A plasmid (p90/HCVFLlong pU) with cDNA encoding infectious HCV genotype 1a RNA was kindly provided by Dr. Charlie Rice, Rockefeller University, New York (Kolykhalov et al., 1997). A plasmid (pJ6CF) with cDNA encoding infectious HCV genotype 2a RNA was kindly provided by Jens Bukh, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Hepatitis Viruses Section (Yanagi et al., 1999). A plasmid (pT7Hum-Pkg.myc) with cDNA encoding human plakoglobin mRNA was kindly provided by Dr. Mike Klymkowsky, University of Colorado, Boulder (Merriam et al., 1997). Plasmid HCV p90Δ3 was constructed by deleting HCV nt 403 to 7920 from p90/HCVFLlong pU with the restriction endonuclease *Aat*II. HCV p90Δ3 clones were grown in the presence of tetracycline and identified by analysis with restriction enzymes.

mRNA transcripts

Plasmids were linearized with restriction enzymes to allow for runoff transcription of mRNAs. Plasmids p90/HCVFLlong pU and HCV p90Δ3 were linearized with *Mlu*1 unless otherwise indicated. Bacteriophage T4 DNA polymerase (New England Biolabs, Beverly, Massachusetts) was used to modify the termini of *Bsm*1-linearized plasmids, converting 3′ overhangs into blunt or 5′ overhangs (Sambrook & Maniatis, 1989). To convert 3′ overhangs into blunt termini, 20 μg of *Bsm*1-linearized cDNA was incubated for 15 min at 20 °C in a 20-μL reaction containing 20 U of T4 DNA polymerase and 1 mM of each dNTP as described by the manufacturer (New England Biolabs, Beverly, Massachusetts). To convert a 3′ overhang into a 5′ overhang, 20 μg of *Bsm*1-linearized cDNA were incubated for 15 min at 4 °C in a 20-μL reaction containing 20 U of T4 DNA polymerase and 2 mM dATP and 2 mM dCTP as described by the manufacturer (New England Biolabs). Following incubation, the reactions were heated at 75 °C for 10 min, phenol:chloroform:isoamyl alcohol extracted, and ethanol precipitated. Plasmid pJ6CF was linearized with *Mlu*I. Plasmid pT7Hum-Pkg.myc was linearized with *Bam*H1. Linearized plasmid was incubated in reactions containing bacteriophage T7 RNA polymerase and ribonucleotide triphosphates as described by the manufacturers (Epicentre, Madison, Wisconsin). Products of the transcription reactions were purified by precipitation in 2.5 M ammonium acetate, pH 7.0, and washed with ethanol. RNAs were solubilized in water and quantified by OD_{260nm}. To make ^{32}P-labeled mRNAs, α-^{32}P[CTP] was added to the T7 transcription reactions.

Gel purification of mRNA

mRNA (50 μg) was loaded into a large preparative well and separated by electrophoresis in a 2% agarose (SeaPlaque Low Melting Temperature GTG; BioWhittaker Molecular Applications, Rockland, Maine) gel containing TAE buffer (40 mM Tris-acetate, 1 mM EDTA). A marker lane was removed from the gel, stained with ethidium bromide, and the mobility of mRNA determined using UV light. A band of agarose containing the mRNA was excised, weighed, and processed using a QIAquick gel extraction kit (Qiagen, Valencia, California). mRNA recovered from the gel was ethanol precipitated, solubilized in water, and quantitated by OD_{260nm}.

Assaying RNase L in HeLa cell extracts

Cytoplasmic HeLa cell extracts [S10 and translation initiation factors (IFs)] were prepared as previously described (Barton et al., 1996). HCV and human plakoglobin mRNAs were incubated at the indicated concentrations in reactions containing 50% by volume HeLa S10 extract, 20% by volume HeLa cell IFs, and 10% by volume 10× reaction mix (10 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 600 mM KCH_3CO_2, 300 mM creatine phosphate, 4 mg/mL creatine kinase, 155 mM HEPES-KOH, pH 7.4). Poly(I:C) (Sigma, St. Louis, Missouri), a synthetic dsRNA, was used as a positive control to activate 2′-5′ OAS. Canine microsomal membranes (Promega, Madison, Wisconsin) were added to reactions containing full-length HCV mRNA to facilitate HCV polyprotein processing. Reactions were incubated at 34 °C for the indicated periods of time.

Following incubation, reactions were solubilized in SDS sample buffer [0.5% sodium dodecyl sulfate (Sigma), 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl]. RNA from the reactions was purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. RNA was solubilized in water and separated by electrophoresis in 1% agarose gels containing MOPS-formaldehyde buffer and 0.1 μg/mL ethidium bromide. Portions of RNA were also used as template in primer extension reactions as described below. RNA within 1% agarose gels was visualized using UV light. Radiolabeled mRNAs within 1% agarose gels were detected by phosphorimaging of the dried gels.

Assaying 2′-5′ OAS

α-^{32}P[ATP] was used as a substrate to synthesize radiolabeled 2′-5′ oligo(A). Addition of α-^{32}P[ATP] to complete HeLa S10 translation–replication reactions, such as those used to assay RNase L above, led to the accumulation of only small amounts of radiolabeled 2′-5′ oligo(A) in response to dsRNA (data not shown). When S10 was omitted from the reactions, significant amounts of radiolabeled 2′-5′ oligo(A) accumulated in response to dsRNA. Furthermore, treatment of HeLa cells with 50 U of interferon-γ for 18 h before the preparation of cell extracts increased the amount of 2′-5′ OAS in HeLa cell IFs. Therefore, we assayed for the synthesis of 2′-5′ oligo(A) in 20-μL reactions containing 50% by volume 1× S10 buffer (40 mM HEPES-KOH, pH 7.4, 120 mM KCH_3CO_2, 5.5 mM $Mg(CH_3CO_2)_2$, 10 mM KCl, 6 mM DTT), 20% by volume HeLa cell IFs from interferon-γ treated cells, 10% by volume 10× reaction mix, and 10 μCi α-^{32}P[ATP] (≥800 Ci per mmol, Amersham, Arlington Heights, Illinois). RNAs were incubated in the reactions as indicated in the figure legends (Figs. 6 and 8). Reactions were incubated at 30 °C for 10 min. Reactions were terminated by heating at

100 °C for 10 min. Denatured proteins were removed from the samples by centrifugation at 13,000 × *g* for 10 min. A portion of the supernatant (4 μL) was mixed with calf intestinal phosphatase (1 μL of 1 U/μL) and incubated at 37 °C for 15 min. Samples (untreated and phosphatase treated) were mixed with equal volumes of 2× urea sample buffer (18 M urea, 8.9 mM Tris base, and 8.9 mM boric acid, pH 8.3, 0.2 mM EDTA, 20% (w/v) sucrose, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol) and fractionated by electrophoresis (500 V for 16 h) in 7 M urea 20% polyacrylamide TBE gels as previously described (Miele et al., 1991). Radiolabel in the gel was detected by phosphorimaging.

Primer extension

RNA templates were prepared as described above (Materials and Methods, Assaying RNase L in HeLa cell extracts). A primer complementary to HCV nucleotides 9348 to 9377 was end labeled in polynucleotide kinase reactions (20 μL) containing 20 pmol primer, 60 μCi γ-^{32}P[ATP] (3,000 Ci/mmol; Amersham), and 20 U polynucleotide kinase (New England Biolabs). The reactions were incubated at 37 °C for 10 min. Approximately one half of the radiolabel was transferred to the primer. Polynucleotide kinase was inactivated by incubating the reactions at 90 °C for 2 min. The reactions were diluted 1:10 with water such that the primer was at a concentration of 0.1 pmol/μL. 5′-end-labeled primer (0.1 pmol; ~300,000 cpm) and template (4 μg total RNA) were mixed in reactions (18 μL) containing 1 mM each dNTP, 10 mM DTT and 1× SuperScript II 1st strand buffer (Gibco-BRL, Long Island, New York). Primers and templates were annealed by incubating the reactions at 58 °C for 20 min followed by 10 min at room temperature. RNasin (1 μL at 10 U/μL; Gibco-BRL) and SuperScript II reverse transcriptase (1 μL of 200 U/μL; Gibco-BRL) were added to the reactions before incubation at 50 °C for 30 min. Stop solution (10 μL; USB Sequenase Kit, Cleveland, Ohio) was added to terminate the reactions. A DNA sequencing ladder was generated using unlabeled primer, p90/HCVFLlong pU DNA template, α-^{35}S[dATP], and Sequenase (USB) as described by the manufacturer. Samples were heated ≥75 °C for 5 min before being fractionated by electrophoresis (50 °C constant temperature) in a 7 M urea 6% polyacrylamide TBE gel. Gels were fixed with 15% methanol/5% acetic acid and dried. Radiolabel was detected by phosphorimaging.

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