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Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus

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ABSTRACT **Chimeric genomes of poliovirus (PV) have been constructed in which the cognate internal ribosomal entry site (IRES) element was replaced by genetic elements of hepatitis C virus (HCV). Replacement of PV IRES with nt 9–332 of the genotype Ib HCV genome, a sequence comprising all but the first eight residues of the 5′ nontranslated region (5′NTR) of HCV, resulted in a lethal phenotype. Addition of 366 nt of the HCV core-encoding sequence downstream of the HCV 5′NTR yielded a viable PV/HCV chimera, which expressed a stable, small-plaque phenotype. This chimeric genome encoded a truncated HCV core protein that was fused to the N terminus of the PV polyprotein via an engineered cleavage site for PV proteinase $3C^{pro}$. Manipulation of the HCV core-encoding sequence of this viable chimera by deletion and frameshift yielded results suggesting that the 5′-proximal sequences of the HCV open reading frame were essential for viability of the chimera and that the N-terminal basic region of the HCV core protein is required for efficient replication of the chimeric virus. These data suggest that the bona fide HCV IRES includes genetic information mapping to the 5′NTR and sequences of the HCV open reading frame. PV chimeras replicating under translational control of genetic elements of HCV can serve to study HCV IRES function *in vivo* and to search for anti-HCV chemotherapeutic agents.**

Internal ribosomal entry sites (IRESs), originally discovered in the studies of translational control of picornaviruses (1–4), promote the entry of eukaryotic cellular ribosomes into mRNAs without the involvement of a 5′-terminal capping group (5).

IRES elements are highly structured RNA segments of 300–400 nt located at the 5′ nontranslated region (5′NTR) of certain viral and cellular mRNAs (5, 6). Interestingly, IRES elements of different origins do not necessarily share sequence or structural motifs, and the identification of RNA as an IRES element may be based solely on functional analyses. The IRES elements of poliovirus (PV) and hepatitis C virus (HCV) may serve as examples of such structural heterogeneity (Fig. 1).

HCV, the etiologic agent of non-A non-B hepatitis in humans, is an enveloped plus-strand RNA virus belonging to the family Flaviviridae (9–12). The 9.4-kb HCV genome encodes a single polyprotein that is processed by virus-encoded proteinases and cellular signal peptidase. Typical of flaviviruses, the core protein of the HCV virion maps to the N terminus of the polyprotein, followed by glycoproteins and nonstructural proteins (Fig. 1). The open reading frame (ORF) of the polyprotein is preceded by a nontranslated RNA segment of 332 or 341 nt depending on specific virus strains. Tsukiyama-Kohara *et al.* (13) have shown that a highly ordered sequence within the HCV 5′NTR (8) can function as an IRES, an observation that is confirmed by others (14, 15).

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PV is a nonenveloped enterovirus belonging to the family Picornaviridae. Its RNA genome is characterized by a long 5′NTR (742 nt) that contains two important cis-acting elements: the 5′ cloverleaf-like structure and the IRES element that are involved in initiation of viral RNA replication and cap-independent translation of viral mRNA, respectively (5). The PV IRES element spans roughly from nt 100 to 590, a size that is characteristic of picornaviral IRES elements. The PV genome encodes a polyprotein that is proteolytically processed by two virus-encoded proteinases (5).

The biological and pathogenic properties of PV and HCV are vastly different. PV is a highly infectious and cytolytic agent that proliferates rapidly to high titers in suitable cell cultures. The replication cycle of PV is virtually completed within 7 h. Infection of humans occurs by ingestion of the agent and is usually self-limiting. The target cells are cells of the gastrointestinal (GI) tract and can also be those of the central nervous system (CNS). GI infections cause mild symptoms, if noticeable at all, while CNS infections can progress to paralytic poliomyelitis. PV infections can be effectively controlled by vaccination with live, attenuated, or inactivated PV vaccines (5). In contrast, HCV is the causative agent of blood-borne hepatitis in humans, and the infections may progress slowly into chronic disease, cirrhosis, and even hepatocellular carcinoma (16, 17). Neither HCV vaccines nor any means of effective chemotherapy are available to control HCV infections. Replication of HCV in cell cultures to any appreciable titers has not been achieved. In fact, there is no report of successful purification of HCV for biochemical analyses from either cultured cells or tissues of infected humans or experimental animals. It is, therefore, difficult to study the biological properties of the virus and to develop HCV-specific antiviral agents that are effective in prevention and treatment of HCV-associated disease.

Here we report the engineering of replicating polioviruses whose IRES element has been replaced by that of HCV. These chimeric viruses are of interest for studies of HCV and PV replication in general and of IRES-mediated translational control in particular. We provide evidence that the functional HCV IRES appears to contain a sequence of the viral ORF as well as the 5′NTR. We propose that the PV/HCV chimera may be suitable for screening anti-HCV agents that target the 5′NTR or the core protein of this hepatitis virus.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. HeLa R19 cell monolayers were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% bovine calf serum (BCS). PV 1, strain Mahoney [PV1(M)], and its derivatives were amplified in HeLa cells and titrated by plaque assay as described (18).

Abbreviations: 5′NTR, 5′ nontranslated region; HCV, hepatitis C virus; IRES, internal ribosomal entry site; ORF, open reading frame; PV, poliovirus; RT, reverse transcription.

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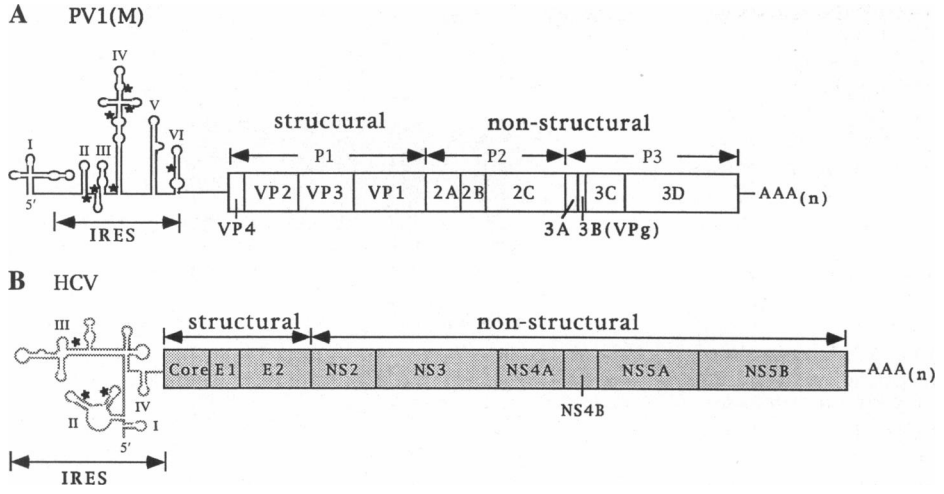

FIG. 1. Schematic diagrams of secondary structures of the 5′NTRs and gene organization of PV (*A*) and HCV (*B*). Boxes depict ORFs for viral polyproteins. Structural domains in the 5′NTRs of the viral RNAs are indicated by roman numerals and noninitiating AUGs are highlighted by stars. The 5′NTR structures of PV and HCV are modified after Pilipenko *et al.* (7) and Brown *et al.* (8), respectively.

Plasmid pT7PVM is a derivative of pT7XL, a full-length cDNA clone of PV1(M) constructed in this laboratory. Plasmid pKIV, a HCV cDNA subclone corresponding to nt 9–1772 of genotype Ib HCV genome (13), is a generous gift from A. Nomoto and M. Kohara.

DNA Manipulations. DNA cloning was accomplished by following standard procedures (19). By means of PCR-based mutagenesis, a DNA fragment encompassing the entire IRES element (nt 108–742) in pT7PVM was replaced with polylinker sequence 5′-GAA TTC GAG CTC GCT TGT TTC AA-3′ to yield plasmid pT7PVΔIRES. HCV genomic segments corresponding to the 5′NTR and the core-encoding regions were PCR amplified and inserted between the *Eco*RI and *Sac* I sites within the polylinker of pT7PVΔIRES, giving rise to a series of PV/HCV chimeric cDNA clones (Fig. 2). All the chimeric clones except pPV/HCV335 encoded an engineered PV $3C^{pro}$-specific cleavage site (LALFQ*G) in front of VP4, which ensured the release of fused HCV amino acid sequences from the PV polyprotein.

Transcription and Transfection Assays. For production of RNA transcripts *in vitro*, 0.7 μg of full-length cDNA was linearized at a unique *Pvu* I site downstream of the viral genome. RNA transcripts were produced from linear cDNA by use of T7 RNA polymerase in an *in vitro* system described

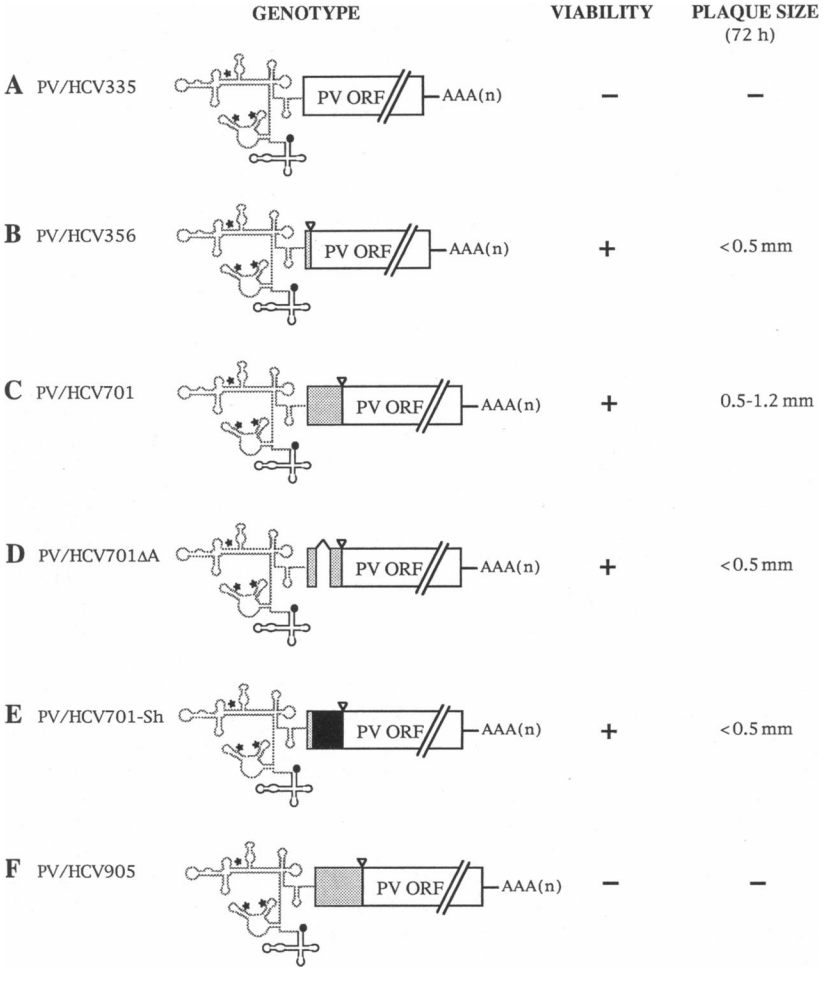

FIG. 2. Gene organization of the constructed PV/HCV chimeric genomes and growth phenotypes of the resultant chimeras. Dashed lines and shaded boxes, genomic elements originated from HCV; solid lines and open boxes, PV originated sequences; solid box, coding region for an unrelated polypeptide resulted from a frameshift in the HCV core sequence from amino acid 8 to 123; solid circles, genome-linked viral protein of PV; stars, noninitiating AUGs within the viral 5′NTRs. Triangles at the junctions between the HCV core sequences and the PV ORF indicate an engineered $3C^{pro}$ cleavage site.

previously (20). HeLa R19 monolayers cultured in 35-mm dishes were transfected as described (21) and grown in 2 ml of DMEM containing 2% BCS at 37°C for up to 4 days.

Characterization of Viral Growth Phenotype. The plaque phenotypes of wild-type (wt) PV and the PV/HCV chimeras were characterized by plaque assay on HeLa R19 cell monolayers seeded on six-well plates (22). After a 3-day incubation at 37°C, the plaques were developed by staining with 1% crystal violet. One-step growth kinetics of the wt or the chimeric PV were measured as described (21).

RNA Isolation, Reverse Transcription (RT)-PCR, and Sequencing. A 35-mm dish of HeLa R19 cells, infected at a multiplicity of infection of 10 with wt PV or its derivatives, was harvested in 200 μl of lysis buffer and total RNA was isolated as described (21). Thirteen micrograms of the isolated total RNA was amplified by RT-PCR using 5′NTR- or 2A-specific primers in a reaction mixture described previously (21). The resulting DNA fragments were subjected to sequence analyses.

***In Vitro* Translation and *In Vivo* Labeling.** *In vitro* translation was performed in a HeLa cell extract (23) supplemented with either PV1(M) RNA or PV/HCV chimeric RNAs at 34°C for up to 7 h. To label viral polypeptides *in vivo*, 35-mm dishes of HeLa cells were inoculated with 300 μl of lysates derived from cells transfected with PV1(M) or PV/HCV RNAs and incubated for 3.5 h (wt PV) or 20 h (PV/HCV chimeras) at 37°C. The infected cells were then labeled with Tran^{35}S-label (ICN) according to a previous report (24).

RESULTS

Construction of Chimeric PV/HCV Genomes. The 5′NTR of HCV contains several noninitiating AUGs and stem–loop structures, a property that is also found in IRES elements of picornaviruses. To test whether the HCV genome indeed encodes a functional IRES element in a replicating RNA genome *in vivo*, we constructed a total of six PV/HCV chimeric genomes (Fig. 2) in which the PV IRES was exchanged with HCV genetic segments. In chimera PV/HCV335 (Fig. 2*A*), a segment (nt 108–742) encoding the PV IRES element was replaced with nt 9–332 of the 5′NTR of genotype Ib HCV (13). PV/HCV356, -701, and -905 contained sequences of the HCV ORF encoding 8, 123, and 191 amino acids of the HCV core protein, respectively, in addition to HCV 5′NTR (Fig. 2 *B*, *C*, and *F*). PV/HCV701ΔA was a derivative of PV/HCV701 that harbored an internal deletion (amino acids 41–107) in the HCV core sequence (Fig. 2*D*). PV/HCV701-Sh was a frameshift mutant created from PV/HCV701 by inserting 2 nt downstream of the 7th codon and deleting 2 nt downstream of the 123rd codon of the HCV core-encoding sequence. In all the chimeras, the HCV 5′NTR was preceded by the 5′ cloverleaf of the PV genome to ensure proper replication of the chimeric genome (5). A 3C^{pro} cleavage site was introduced at the junction between the HCV core sequences and the PV polyprotein. It was anticipated that cleavage at this site would readily liberate a free N terminus of VP4 as described (25).

Translation of the Chimeric RNA *In Vitro*. All chimeric cDNAs were transcribed into full-length viral RNAs, and the ability of the transcript RNAs to direct protein synthesis was tested by *in vitro* translation in a HeLa cell extract (23). As shown in Fig. 3, all of the PV/HCV chimeric RNAs were competent to produce PV-specific polypeptides, an observation demonstrating the integrity of the chimeric constructs. Several points are noteworthy. First, the HCV 5′NTR (nt 9–335) in PV/HCV335 RNA was able to replace the PV IRES *in vitro*, a result suggesting that this RNA segment is capable of directing ribosomes into the chimeric RNA (13–15) in spite of the presence of the 5′-terminal uncapped cloverleaf. Nevertheless, PV/HCV335 was a poor template under standardized conditions of translation (lane 3). A quantitative analysis

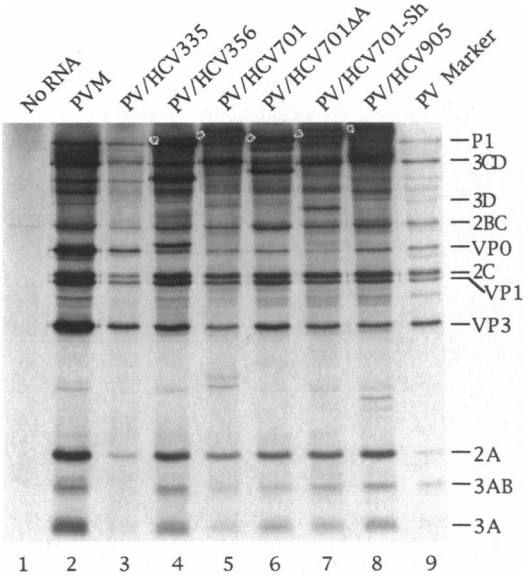

FIG. 3. Protein profiles of translation products generated from the PV/HCV chimeric RNAs in a HeLa cell-derived *in vitro* translation system. Protein bands corresponding to the PV-encoded polypeptides are indicated. Unprocessed Δcore-P1 precursors are denoted by asterisks.

of this effect relative to translations of other chimeric RNAs, however, was not carried out. Second, the translational efficiency significantly improved when HCV-specific sequences downstream of nt 335 were inserted into the uncapped RNAs (lanes 4–8). These sequences encoded portions of the HCV core protein (Fig. 2). Third, despite the presence of a favorable 3C^{pro}-specific cleavage site between the truncated core and the PV polyprotein, processing at the Δcore*P1 junction was very slow (Fig. 3, lanes 4–8).

Replication of the PV/HCV Chimeras. To test whether chimera PV/HCV335 could produce viable virus, RNA transcripts from this construct were transfected into HeLa cells. It was found that this chimeric genome failed to produce any viable virus upon transfection (Fig. 4*A*). Moreover, inoculation of HeLa cells with lysates derived from PV/HCV335 transfected cells failed to reveal any viral-specific protein synthesis (Fig. 4*B*, lane 2). These results indicate that replacement of the PV IRES with nt 9–332 of the HCV 5′NTR resulted in a defect in viral replication.

We considered it possible that the lethal phenotype of PV/HCV335 RNA was due to the lack of an efficiently functioning IRES *in vivo*. Based on the results of *in vitro* translation, we tested whether sequences of the HCV ORF could enhance internal ribosome entry into the HCV 5′NTR *in vivo* to yield viable virus. Accordingly, we transfected cells with chimeric RNAs PV/HCV356 and -701 that contained either 24 or 369 nt of the HCV ORF between HCV 5′NTR and PV ORF. The inserted HCV ORF sequences in these two constructs encode the N-terminal 8 and 123 amino acids of the HCV core protein, respectively. It was observed that RNA transcripts from both constructs were able to generate viable chimeric viruses in transfected cells (Figs. 4 and 5). The viability of these chimeras suggests that an efficient HCV IRES element contains not only the sequences from the 5′NTR but also sequences of the adjacent HCV ORF. Interestingly, upon transfection of HeLa cells, PV/HCV701 RNA produces ≈100 times more infectious virus (henceforth referred to as W1-PV/HCV701) than PV/HCV356 RNA (Fig. 5*A*). In addition, the plaques produced by W1-PV/HCV701 were larger than those produced by W1-PV/HCV356 (data not shown).

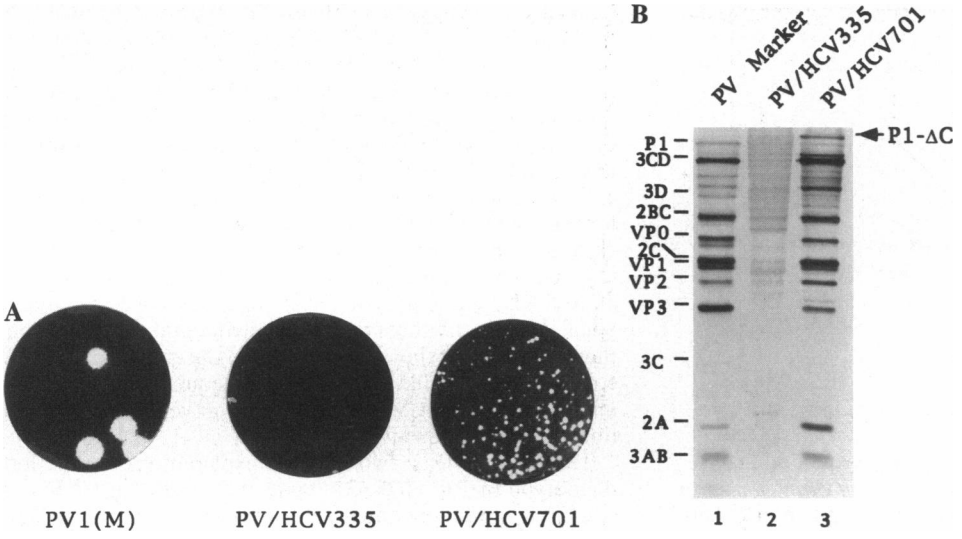

FIG. 4. Properties of wt PV and chimeric PV/HCV constructs. (*A*) Plaque phenotypes of PV1(M) and chimeras W1-PV/HCV335 and -701 on HeLa cell monolayers. Infected cells were inoculated with cell lysates derived from transfections with the corresponding RNA. After incubation at 37°C for 72 h, viral plaques were developed by 1% crystal violet staining. (*B*) Translation of wt PV and chimeras PV/HCV335 and -701 in HeLa cells. Unprocessed HCV ΔCore-PV P1 fusion protein is indicated. Positions of PV-encoded polypeptides are denoted on the left.

The N-Terminal Basic Region of HCV Core Protein Promotes Replication of the PV/HCV Chimera. The efficient replication of W1-PV/HCV701 relative to W1-PV/HCV356 can be attributed either to the involvement of the truncated HCV core protein or to core-encoding RNA sequences. To differentiate between these two possibilities, we modified the core-encoding region in the chimeric cDNA by a partial deletion (Fig. 2, PV/HCV701ΔA) or a frameshift mutation (Fig. 2, PV/HCV701-Sh). Plaque assays revealed that PV/HCV701ΔA and -Sh produced viable viruses with a minute plaque phenotype similar to that of W1-PV/HCV356 (data not shown). The virus titers from cells transfected with PV/HCV701ΔA or -Sh RNAs were 10- to 100-fold lower than of that with the parental PV/HCV701 RNA (Fig. 5*A*). In addition, immunoblotting using a monoclonal antibody against the 2C protein of PV indicated that W1-PV/HCV356, -701ΔA, and -701-Sh were less efficient in viral proliferation compared with that of W1-PV/HCV701 (Fig. 5*B*). PV/HCV701ΔA bears a 66-amino acid deletion in the N-terminal basic region of the truncated core protein, and PV/HCV701-Sh contains a frameshift that retains only the first 7 amino acids of the core protein (see *Materials and Methods* for specific nucleotide changes). Hence, we suggest that the integrity of the N-terminal basic region (amino acids 1–123) of the HCV core protein is a major determinant for efficient replication of the PV/HCV chimera.

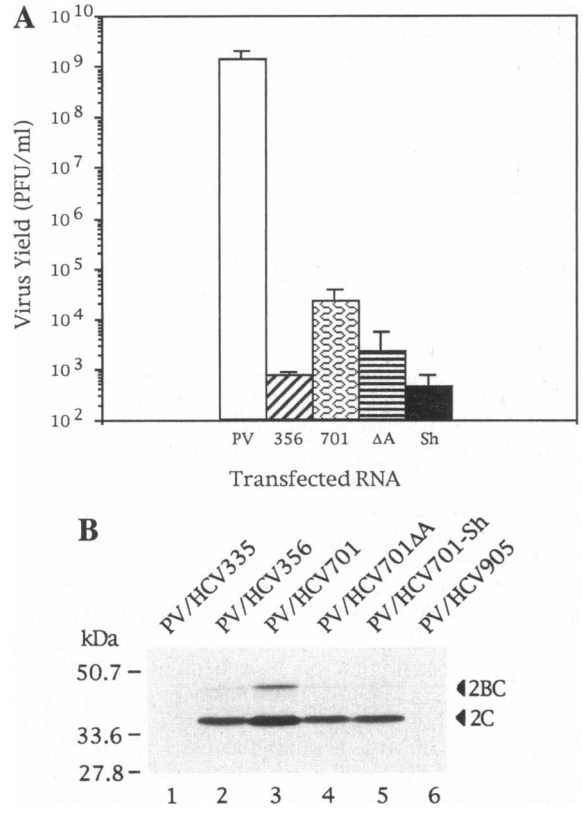

FIG. 5. (*A*) Comparison of virus yields from cells transfected with wt PV RNA or PV/HCV chimeric RNAs. HeLa R19 plates (35 mm) were transfected with 10 μg of RNA transcripts of the wt PV and the chimeric constructs. Three days after transfection, the cells were lysed and the virus titer in each cell lysate was determined by plaque assays. Each column represents average virus titer from three independent transfection assays. Note that transfection of cells with RNA transcripts from PV/HCV335 and PV/HCV905 did not yield any viable virus. PFU, plaque-forming units. (*B*) Detection of viral specific protein synthesis by immunoblotting with a monoclonal antibody against the 2C protein of PV. HeLa cells were transfected with RNA transcripts from the corresponding chimeric constructs and harvested 3 days after transfection. Each lysate was blind passaged through HeLa cells for three cycles. Cell lysates from the third passage were subjected to SDS/12.5% PAGE and detected by Western blotting with 2C-specific monoclonal antibody 33.7.

PV/HCV905, a chimeric genome that contains HCV sequences for the 5′NTR and the entire core protein, did not yield any viable virus in transfected cells. However, *in vitro* translation and the processing pattern of PV/HCV905 RNA transcripts were similar to that of PV/HCV701 (Fig. 3, lane 8). Replication of either plus- or minus-strand viral RNA in cells transfected with PV/HCV905, as examined by RT-PCR, was undetectable (data not shown), an observation suggesting that the construct had a defect in RNA replication.

PV/HCV701 Produces a Stable Chimeric Virus. W1-PV/HCV701 displayed a small plaque phenotype on HeLa cell monolayers (Fig. 4*A*), an observation implying that the replacement of PV IRES with that of an unrelated virus caused a defect in viral replication. The analysis of virus growth kinetics revealed that W1-PV/HCV701 grew to ≈10% of the wt PV level in a single replication cycle (Fig. 6). Interestingly, eclipse of the viral infectivity, an indication of the early events of uptake and virus uncoating, was delayed by 2 h. After seven passages through cultured cells, the viral plaques remained small, and the inserted HCV sequences as well as the 2A-encoding region of the chimera remained unchanged, an observation suggesting that this chimera is genetically stable in cell culture.

DISCUSSION

IRES elements, a class of highly ordered RNA segments located in the 5′NTR of certain viral and cellular mRNAs,

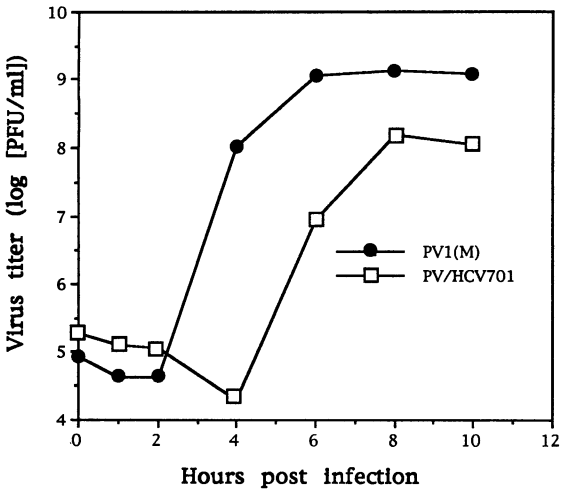

FIG. 6. One-step growth kinetics of PV1(M) and W1-PV/HCV701 in HeLa R19 cells grown at 37°C. PFU, plaque-forming units.

allow entry of the translational machinery into mRNAs without recognition of a 5′-end cap structure (1–4, 22, 26, 27). Certain RNA viruses have developed this mode of translation to evade or even abrogate cap-dependent translation of host cellular mRNAs.

A large body of evidence supports the original observation by Tsukiyama-Kohara *et al.* (13) that RNA sequences in the 5′NTR can function as an IRES (14, 15). These studies have been carried out with monocistronic and dicistronic mRNAs in which reporter genes are expressed under the translational control of the HCV 5′NTR sequence. The properties of the PV/HCV chimeras described here extend these observations and allow additional conclusions to be drawn.

First, the HCV RNA segments in the genomes of viable PV/HCV chimeras are preceded by the noncapped, highly structured PV cloverleaf (24, 28, 29). These features are incompatible with the mechanism of cap recognition and scanning known to function in the translational initiation of the majority of eukaryotic mRNAs. The replacement of the PV IRES by genetic segments of the HCV genome leading to the generation of replicating PV/HCV chimeras proves the existence of a functional HCV IRES *in vivo.*

Second, translation of PV/HCV335 RNA in a HeLa cell-free extract was less efficient than those of PV/HCV356 or -701 RNAs. While the difference in *in vitro* translation may not be extraordinary, several attempts to generate viable virus from PV/HCV335 RNA have failed. Remarkably, when a short sequence (21 nt) specifying the first several amino acids of the HCV core protein was inserted downstream of the HCV 5′NTR of PV/HCV335, replication-competent virus (W1-PV/HCV356) emerged upon transfection, displaying a minute plaque phenotype. There are two possibilities to explain this phenomenon. The introduction of HCV coding sequences could either improve the IRES-driven translation or it could stimulate viral RNA synthesis. Currently, we believe it is more likely that the HCV coding sequences contribute to the HCV IRES function because of the observed effect of the HCV ORF sequences on translation (Fig. 3).

Third, the addition of an RNA segment encoding the N-terminal 123 amino acids of the core protein to the HCV 5′NTR yielded a chimera (W1-PV/HCV701) with remarkably improved growth capability. However, RNA sequences of the HCV ORF *per se* were not sufficient to generate PV derivatives with growth properties matching that of W1-PV/HCV701, as shown by deletion or frameshift mutations of the core-encoding sequences (W1-PV/HCV701ΔA and -Sh, respectively). In fact, viral titers obtained from cells transfected with PV/HCV356, -ΔA, or -Sh RNAs were approximately 2 orders of magnitude lower than that with PV/HCV701 RNA. We speculate that the HCV 5′NTR together with a short RNA sequence immediately downstream of the authentic AUG constitutes the bona fide HCV IRES, a unique property that was recently also observed by Reynolds *et al.* (30) using dicistronic mRNAs. In addition, expression of the N-terminal basic sequence of the HCV core protein appears to significantly stimulate replication of the chimera, possibly by optimizing the HCV IRES function *in vivo* (see below). It could be argued that the diminished replication of W1-PV/HCV356, -ΔA, and -Sh compared to W1-PV/HCV701 may result from altered RNA structures due to changes in the RNA sequences. Such altered structures could be detrimental to replication of these chimeras or they may destabilize the chimeric RNAs. We consider this less likely because the sequences introduced in mutant W1-PV/HCV701-Sh resulted in very small modifications of the RNA sequence.

There are at least two possible explanations for the lethal phenotype of PV/HCV335. First, the 5′NTR of HCV is too weak for cap-independent translation to allow for sufficient production of viral proteins *in vivo* (Fig. 3). Second, the genotype of PV/HCV335, but not the genotypes of other PV/HCV chimeras, interferes with genome replication. We cannot rigorously eliminate this latter possibility. However, previous studies involving extensive sequence changes between the IRES and the authentic AUG initiating the PV polyprotein translation did not abrogate genome replication (31).

We have shown that PV/HCV335 lacking HCV ORF sequences has some, albeit weak, template activity for *in vitro* translations in HeLa cell extracts (Fig. 3). This observation confirms the results published by Tsukiyama-Kohara *et al.* (13) and Wang *et al.* (14) who used monocistronic and dicistronic mRNAs. Internal ribosomal entry, therefore, can be promoted by the HCV 5′NTR without addition of the HCV ORF sequences. However, it appears that initiation of translation by the HCV 5′NTR alone is quite inefficient (ref. 30; Fig. 3).

It has been proposed that the PV IRES is essential for genome replication (32). The HCV genetic segments used in this study share little, if any, sequence or structural homology with the PV IRES element. Based on the replication kinetics of W1-PV/HCV701 (Fig. 6), we suggest that RNA sequences within the PV IRES are dispensable for PV proliferation. This is also in agreement with our previous studies of a PV chimera that replicates under translational control of the IRES element from encephalomyocarditis virus, a picornavirus distantly related to PV (33).

The mechanism of IRES function is poorly understood. Apart from cis-acting signals mapping to different parts of the highly structured RNA segments, virus-encoded and host cell-specific trans-acting factors have been identified (5). For picornavirus IRES elements, the trans-acting factors include La autoantigen (34), polypyrimidine track binding protein (p57/PTB) (35, 36, 40), and, in the case of PV, the viral proteinase $2A^{pro}$ (37). Available evidence suggests that picornavirus IRESs are active in translation only in the presence of the RNA binding protein p57/PTB (36). The HCV core protein is a basic protein with numerous activities including RNA binding and ribosome binding (ref. 38 and references therein). It is intriguing to speculate that the truncated version of the HCV core protein in W1-PV/HCV701 may function as a translational activator. On the other hand, PV/HCV905, the construct that encoded a full-length core protein, expressed a lethal phenotype. It is noteworthy that a hydrophobic sequence mapping near the C terminus of the core protein may function as a signal sequence for the E1 protein of HCV (38). We have shown previously that introduction of a signal sequence into ORFs of dicistronic PV genomes completely inhibits viral replication (21). It is possible, therefore, that PV/HCV905 is nonviable because of a block of viral replication caused by the putative signal sequence at the C terminus of the core protein.

Seven passages of W1-PV/HCV701 through cultured cells did not change its phenotype, and no sequence alterations were observed in the inserted HCV sequence or PV 2A-encoding region. Thus, mutational events that may lead to a possible adaptation of the chimera for more efficient internal initiation of translation did not occur. In contrast, PV containing mutated PV IRES sequences yielded second site mutations either in the modified IRES or in 2A-encoding sequences (39).

It has not been possible to develop an efficient tissue culture or experimental animal system for the study of proliferation and pathogenicity of HCV. W1-PV/HCV701, a PV chimera containing essential genetic elements of HCV IRES and a large portion of the HCV core-encoding sequence, may be suitable for analyzing a function(s) of these HCV elements *in vivo* and for assaying compounds for their potential in anti-HCV chemotherapy.

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