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Antisense Oligonucleotide Inhibition of Hepatitis C Virus Gene Expression in Transformed Hepatocytes

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Genetic and biochemical studies have provided convincing evidence that the 5′ noncoding region (5′ NCR) of hepatitis C virus (HCV) is highly conserved among viral isolates worldwide and that translation of HCV is directed by an internal ribosome entry site (IRES) located within the 5′ NCR. We have investigated inhibition of HCV gene expression using antisense oligonucleotides complementary to the 5′ NCR, translation initiation codon, and core protein coding sequences. Oligonucleotides were evaluated for activity after treatment of a human hepatocyte cell line expressing the HCV 5′ NCR, core protein coding sequences, and the majority of the envelope gene (E1). More than 50 oligonucleotides were evaluated for inhibition of HCV RNA and protein expression. Two oligonucleotides, ISIS 6095, targeted to a stem-loop structure within the 5′ NCR known to be important for IRES function, and ISIS 6547, targeted to sequences spanning the AUG used for initiation of HCV polyprotein translation, were found to be the most effective at inhibiting HCV gene expression. ISIS 6095 and 6547 caused concentration-dependent reductions in HCV RNA and protein levels, with 50% inhibitory concentrations of 0.1 to 0.2 μM. Reduction of RNA levels, and subsequently protein levels, by these phosphorothioate oligonucleotides was consistent with RNase H cleavage of RNA at the site of oligonucleotide hybridization. Chemically modified HCV antisense phosphodiester oligonucleotides were designed and evaluated for inhibition of core protein expression to identify oligonucleotides and HCV target sequences that do not require RNase H activity to inhibit expression. A uniformly modified 2′-methoxyethoxy phosphodiester antisense oligonucleotide complementary to the initiator AUG reduced HCV core protein levels as effectively as phosphorothioate oligonucleotide ISIS 6095 but without reducing HCV RNA levels. Results of our studies show that HCV gene expression is reduced by antisense oligonucleotides and demonstrate that it is feasible to design antisense oligonucleotide inhibitors of translation that do not require RNase H activation. The data demonstrate that chemically modified antisense oligonucleotides can be used as tools to identify important regulatory sequences and/or structures important for efficient translation of HCV.

Hepatitis C virus (HCV) is the major cause of transfusion-associated non-A, non-B hepatitis (4). While HCV infections are often asymptomatic, acute infections progress to a chronic state in greater than 50% of patients (9). Chronic infection can lead to liver cirrhosis, and there is an epidemiologic association of chronic HCV infection with development of hepatocellular carcinoma (42). HCV is an enveloped RNA virus and is classified as a separate genus within the *Flaviviridae* family. The HCV genome was first isolated and sequenced in 1989, and subsequently, the nucleotide sequences of isolates from many different countries have been reported (11, 15, 16, 42). The genome consists of a single strand of RNA of positive polarity approximately 9,400 nucleotides long. The structural proteins of the virus map to the 5′ end of the genome, and nonstructural proteins are encoded by the 3′ end of the genome. The genome contains a 5′ untranslated region approximately 340 nucleotides in length.

The functional importance of the 5′ noncoding region (5′ NCR) is implied by the finding that this region is highly conserved between sequenced isolates of HCV (10). The 5′ NCR of HCV forms a predicted complex secondary structure and has been shown to initiate translation at an internal AUG by a mechanism of internal ribosome entry (8, 45, 50). Deletion and mutagenesis studies have identified multiple sequence and structural motifs that are components of the HCV internal ribosome entry site (IRES) (50, 51). While the 5′ NCR and core protein coding region are well conserved, other regions of the genome exhibit sequence heterogeneity. HCV genomic variability may contribute to the establishment of viral persistence and influence response to therapy. In addition, the nucleotide sequence diversity of HCV, particularly within the envelope gene, has complicated the development of a protective vaccine.

Although the genomic characterization of HCV has resulted in the development of sensitive methods to detect HCV in blood, therapeutic options for treatment of patients remain limited. Treatment of patients with interferon alpha-2b inhibits HCV infection, but only approximately 20% of patients exhibit long-term remission (24, 26, 37). The high relapse rate associated with discontinuation of therapy and the adverse side effects of interferon treatment increase the importance of developing effective antiviral therapies for HCV infection.

Antisense oligonucleotides are useful molecular tools for probing gene function and are potentially useful therapeutic

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agents for the treatment of viral diseases (18, 19, 44, 47). Specificity of antisense oligonucleotides for complementary mRNA target sequences suggests that therapeutic use of oligonucleotides may afford advantages over nucleoside analogs (25, 39). Several studies have reported inhibition of viral gene expression in cultured cells by phosphorothioate oligonucleotides (1–3, 5, 17, 33, 53). Clinical trials to evaluate the efficacy of phosphorothioate antisense oligonucleotides against human papillomavirus, human cytomegalovirus (HCMV), or human immunodeficiency virus are in progress.

In this study, we investigated the effects of antisense oligonucleotides on expression of HCV RNA and core protein. Since a reliable cell culture system for replication of HCV is not yet available, we utilized immortalized human hepatocytes constitutively expressing the HCV 5′ NCR and core protein coding region to evaluate inhibitory effects of antisense oligonucleotides. Two phosphorothioate oligonucleotides, one complementary to sequences within the 5′ NCR and the other complementary to HCV sequences spanning the translation start codon, caused sequence-dependent reductions in HCV RNA and core protein levels. Characterization of a truncated HCV RNA product produced in cells treated with one of these phosphorothioate oligonucleotides suggests that activation of RNase H resulted in cleavage of the HCV RNA strand within the oligonucleotide-RNA duplex. Evaluation of chemically modified phosphodiester oligonucleotides possessing enhanced RNA binding affinity relative to that of phosphorothioate oligonucleotides demonstrated inhibition of HCV gene expression in cells by an RNase H-independent mechanism.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were synthesized as previously described, with only slight modifications (32). Briefly, 1-μmol-scale syntheses of oligonucleotides were performed on an Applied Biosystems 380B automated DNA synthesizer. 2′-Methoxyethoxy-modified phosphoramidites were synthesized according to published methods (38). Oligonucleotide synthesis utilized elongated coupling times to ensure nearly quantitative reactions. The 3′-terminal residue of 2′-modified oligonucleotides was not modified and was derived from the 2′-deoxy (N)-CPG resin. Oligonucleotides were deprotected by incubation in concentrated ammonia (NH_4OH) at 55°C for 12 to 16 h and recovered by ethanol precipitation. The purity of ethanol-precipitated oligonucleotides was evaluated by polyacrylamide gel electrophoresis and capillary electrophoresis.

Cells and culture conditions. Immortalization of human hepatocytes by introduction of an adenovirus vector expressing the early region of simian virus 40 has been described elsewhere (46). H8Ad17 immortalized hepatocytes were transfected with an expression vector containing the HCV type II 5′ NCR, core, and the majority of the envelope coding sequence (HCV nucleotides 1 to 1357) expressed from the immediate-early promoter of HCMV. Neomycin-selected cells, termed H8Ad17c cells, were grown in William's E medium supplemented with 2.5×10^{-5} M dexamethasone, 10^{-6} M insulin, 5 ng of epidermal growth factor per ml, 60 μg of gentamicin per ml, 400 μg of G418 per ml, and 10% fetal bovine serum.

Treatment of H8Ad17c human hepatocytes with oligonucleotides. Six-well dishes were seeded at a density of 5×10^5 cells per well. The cells were rinsed once with Optimem (GIBCO-BRL) and treated for 4 h with oligonucleotide in Optimem in the presence of 5-μg/ml DOTMA-DOPE solution (Lipofectin) (7). Following treatment, the cells were rinsed once and refed with growth medium. Total RNA or protein was harvested 18 to 20 h after treatment with oligonucleotide.

Northern (RNA) analyses. Total RNA was isolated by using RNAzol B (Tel-Test, Inc.) according to the protocol suggested by the manufacturer. Blots were prepared from formaldehyde-agarose gels and probed with a ^{32}P-radiolabeled 1.4-kb *Hin*dIII-*Bam*HI fragment of HCV DNA or a fragment of human glycerol-3-phosphate dehydrogenase (G3PDH) (Clonetech). The blots were hybridized at 68°C in Rapid Hyb (Amersham). The blots were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and then washed for 30 min at 60°C. Oligonucleotide probes were labeled at the 5′ end by using [γ-^{32}P]ATP and polynucleotide kinase. Northern blot hybridizations were performed at 45°C, and the membranes were washed at room temperature. HCV and G3PDH RNAs were visualized and quantitated with a PhosphorImager (Molecular Dynamics).

Western blot (immunoblot) analyses of HCV core proteins. Cell extracts were prepared in 1× Laemmli sample buffer (34). Samples were boiled for 5 min, and cell debris was removed by centrifugation. The proteins were electrophoresed on SDS–16% polyacrylamide gels and electrotransferred to a polyvinylidene difluoride membrane in carbonate buffer (Novex) at 200 mA for 2.0 h. After being blocked in phosphate-buffered saline containing 2% normal goat serum and 0.3% Tween 20, the blots were probed simultaneously with a polyclonal antibody (2243, 1:1,500) from an HCV-positive patient and a monoclonal antibody to G3PDH (RGM2, 1:50,000; Advanced Immunochemical). Following incubation with the primary antibody, the blots were rinsed and incubated with ^{125}I-radiolabeled goat anti-human antibody (1:3,000) for detection of HCV proteins and with ^{125}I-radiolabeled goat anti-mouse antibody (1:5,000) for detection of G3PDH (ICN). Immunoreactive proteins were visualized and quantitated with a PhosphorImager.

HCV RNA mapping. The 3′ ends of RNA molecules were mapped by RNA ligase-mediated amplification of cDNA ends (35). Total RNA was isolated from cells treated with ISIS 6547 by the RNAzol B method. An oligonucleotide (oligonucleotide 1, 5′-AATGCTAAACCGGAATTCCGGATACAT-3′) containing an *Eco*RI site was ligated to total RNA with T4 RNA ligase (New England Biolabs). Free oligonucleotides were removed by Sephadex G-50 column filtration. cDNA synthesis was primed with a second oligonucleotide (oligonucleotide 2) complementary to the ligated oligonucleotide. First-strand reverse transcription was carried out by using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). PCR amplification was performed according to the manufacturer's suggested protocol (InVitrogen PCR optimizer kit) using *Taq* DNA polymerase. The reaction mixtures contained oligonucleotide 2, complementary to the ligated oligonucleotide, and an HCV gene-specific oligonucleotide (oligonucleotide 3, 5′-TGTCGTGCAGCCTCGAGGACC-3′) designed to contain an *Xho*I site. An initial cycle (5 min at 94°C) was followed by 35 cycles of the PCR (1 min at 94°C, 2 min at 55°C, 3 min at 72°C) and then 7 min at 72°C. The amplified products were separated by agarose gel electrophoresis. DNA fragments were electrophoresed onto NA 45 paper (Schleicher & Schuell), eluted, and cloned into plasmid Bluescript KS(+) (Stratagene) digested with *Eco*RI and *Xho*I. Sequence analysis was performed by using Sequenase 2.0 according to the suggestions of the manufacturers (U.S. Biochemicals and Amersham).

Evaluation of oligonucleotide-RNA duplex stability. Absorbance-versus-temperature curves of duplexes were measured as previously described (40). The melting temperatures (T_ms) were determined in buffer containing 100 mM Na^+, 10 mM phosphate, and 0.1 mM EDTA (pH 7.0). Reported parameters are averages of at least three experiments.

RESULTS

Effects of phosphorothioate oligonucleotides on HCV RNA levels in transformed hepatocytes. Although the HCV genome exhibits nucleotide sequence diversity, the characterization of well-conserved blocks of nucleotide sequences located in the 5′ NCR and core protein coding region has provided rational target sequences for antisense oligonucleotides. Antisense phosphorothioate oligonucleotides that were complementary to the HCV 5′ NCR, translation start codon (nucleotides 342 to 344), or core protein coding sequence were designed. The oligonucleotides were 20 bases long and were designed to hybridize to HCV RNA at 10-nucleotide intervals. The sequences and target sites of 23 of the oligonucleotides evaluated for activity in this study are shown in Table 1.

Antisense oligonucleotides were first evaluated for effects on HCV RNA levels. To date, a dependable and reproducible cell culture assay system for replication of HCV has not been developed. Therefore, we utilized an immortalized human hepatocyte cell line expressing a portion of the HCV genome (Fig. 1). H8Ad17c cells constitutively express the HCV 5′ NCR, core protein coding region, and the majority of the envelope gene (E1) from the HCMV immediate-early promoter. Cells were treated with oligonucleotides in the presence of the cationic liposome complex DOTMA-DOPE, which has been shown to increase uptake of phosphorothioate oligonucleotides (7). Reductions in HCV RNA levels were assayed by Northern analysis as described in Materials and Methods. We observed two major HCV transcripts in H8Ad17c cells, one approximately 1.6 kb in length and a smaller transcript presumably resulting from premature termination of transcription (Fig. 2A). Northern blots were stripped and hybridized with a probe specific for G3PDH to normalize RNA levels. The antisense oligonucleotides were evaluated at a dose of 170 nM.

TABLE 1. Effects of antisense phosphorothiote oligonucleotides on HCV RNA levels in H8Ad17c cells

ISIS no.	HCV nucleotide no.	Oligonucleotide sequence (5′→3′)	RNA level (% control ± SD)[a]
6481	100–119	TCCTGGAGGCTGCACGACAC	73 ± 5
6483	110–129	GGAGGGGGGGTCCTGGAGGC	100 ± 3
6097	120–139	GGCTCTCCCGGGAGGGGGGG	37 ± 12
6533	130–149	AGACCACTATGGCTCTCCCG	53 ± 14
6494	140–159	CCGGTTCCGCAGACCACTAT	89 ± 23
6552	170–179	CCGGTCGTCCTGGCAATTCC	68 ± 11
6497	180–199	AAGAAAGGACCCGGTCGTCC	89 ± 15
6556	190–209	GGGTTGATCCAAGAAAGGAC	120 ± 10
6509	240–259	ACTCGGCTAGCAGTCTCGCG	62 ± 13
6095	260–279	GCCTTTCGCGACCCAACACT	35 ± 14
6538	270–289	GTACCACAAGGCCTTTCGCG	38 ± 17
6510	280–299	CTATCAGGCAGTACCACAAG	39 ± 13
6511	300–319	CCGGGGCACTCGCAAGCACC	94 ± 31
6546	310–329	ACGAGACCTCCCGGGGCACT	59 ± 21
6512	320–339	TGCACGGTCTACGAGACCTC	47 ± 9
6547	330–349	GTGCTCATGGTGCACGGTCT	37 ± 13
6517	340–359	TTTAGGATTCGTGCTCATGG	67 ± 17
6548	350–369	TTCTTTGAGGTTTAGGATTC	95 ± 24
6522	360–379	CGTTTGGTTTTTCTTTGAGG	69 ± 5
6527	371–390	GGTTGGTGTTACGTTTGGTT	92 ± 7
9651	Randomized	ACGCCCGTGTCATGCGTTCT	119 ± 12
6961	Randomized	TACGTTTCTATGTCGATGGG	121 ± 17
8167	Randomized	GCTTGGAACCGCTGGAGATC	165 ± 26

[a] H8Ad17c cells were treated with oligonucleotides at a final concentration of 170 nM. RNA levels were detected by Northern blot hybridization analysis and quantitated with a PhosphorImager. HCV RNA levels are normalized to G3PDH RNA levels and are expressed relative to the value for control, untreated H8Ad17c cells. Values are the means of a minimum of two experiments. Control, noncomplementary oligonucleotides 9651 and 6961 have the same base compositions as oligonucleotides 6547 and 6517, respectively.

The majority of antisense oligonucleotides evaluated inhibited HCV RNA expression, although the level of inhibition varied between oligonucleotides (Table 1). While the levels of both HCV transcripts were reduced following oligonucleotide treatment, only the 1.6-kb RNA transcript was used to quantitate inhibitory activity of oligonucleotides. Treatment of H8Ad17c cells with oligonucleotide 6097, 6095, 6538, 6510, or 6547 at a final concentration of 170 nM resulted in a 60 to 70% reduction in HCV RNA levels. Two oligonucleotides, one complementary to the 5′ NCR (6095) and one targeting sequences spanning the translation start codon (6547), showed the most potent inhibitory activity. Treatment of H8Ad17c cells with control noncomplementary phosphorothioate oligonucleotides (9651, 6961, and 8167) did not reduce HCV RNA expression.

The effects of oligonucleotides 6095 and 6547 on HCV RNA and protein levels were evaluated at escalating doses, with a maximal dose of 200 nM (Fig. 2). In the HCV RNA assay, dose-response experiments demonstrated at least a 50% reduction in HCV RNA levels at a dose of 100 nM for antisense oligonucleotides 6095 and 6547 (Fig. 2B). Two respective control oligonucleotides with base-matched compositions but noncomplementary to HCV did not reduce HCV RNA (Fig. 2A and B). The results of Western blot analysis of effects on HCV core protein levels were consistent with results of Northern blot analyses and demonstrated dose-dependent reductions in HCV core protein after treatment of cells with oligonucleotide 6095 or 6547 (Fig. 2C and D).

Treatment of H8Ad17c cells with oligonucleotide 6095 or 6547 resulted in a significant reduction in HCV RNA levels. The activities of oligomers complementary to sequences spanning the nucleotide 260 or 330 region of HCV RNA were investigated in an effort to identify oligonucleotides with greater potency than 6095 and 6547. An experiment was performed to determine activities of oligonucleotides designed to hybridize to HCV RNA at 2-base intervals. Effects of oligonucleotides complementary to the nucleotide 260 or 330 region on HCV RNA levels are shown in Table 2. Oligonucleotides complementary to HCV sequences between nucleotides 322 and 340 showed variable levels of activity. Oligonucleotides 10491, 6547, and 10486 showed the greatest effects on HCV RNA after treatment of H8Ad17c cells at a final concentration of 170 nM. All of the oligonucleotides complementary to the nucleotide 260 region reduced HCV RNA to similar levels, suggesting that this region of the HCV RNA encompassing nucleotides 254 to 270 may represent a therapeutically useful target for antisense oligonucleotides.

Effects of truncated or mismatched oligonucleotides on HCV RNA levels. Oligonucleotides 6095 and 6547 were further characterized by shortening the oligonucleotides or by introducing base mismatches. Derivative oligonucleotides of the 6095 sequence that were shortened from the 5′ end, the 3′ end, or both were synthesized. Truncation to 18, 17, or 15 nucleotides reduced HCV RNA levels by as much as 40% relative to that of oligonucleotide 6095 (Table 3). Greater effects on potency were observed after introduction of nucleotide substitutions. A single-base substitution was tolerated, causing a 40% reduction in activity. Two-base mismatches had variable effects on activity, depending on the location of the mismatches. Oligonucleotide 10967, containing base substitutions at the 5′ and

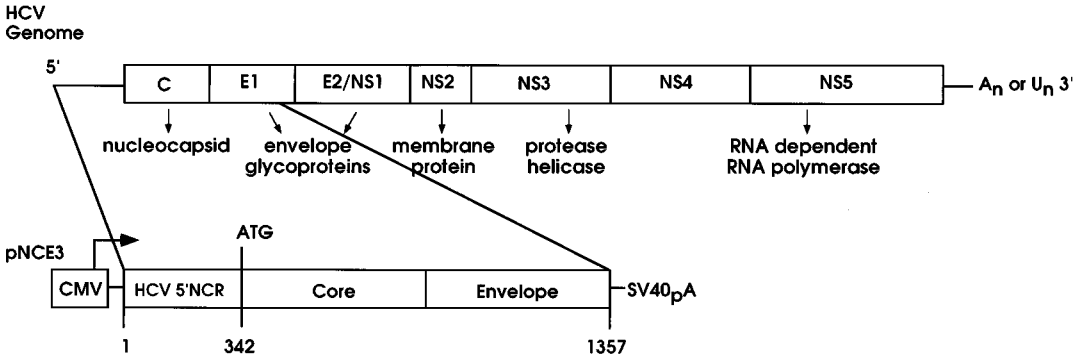

FIG. 1. Schematic representation of the HCV genome and protein coding domains. The open reading frame is shown as an open box. Immortalized human hepatocytes were transfected with plasmid pNCE3. H8Ad17c cells were selected for neomycin resistance and express the HCV 5′ NCR, core, and the majority of envelope sequences from the HCMV immediate-early promoter.

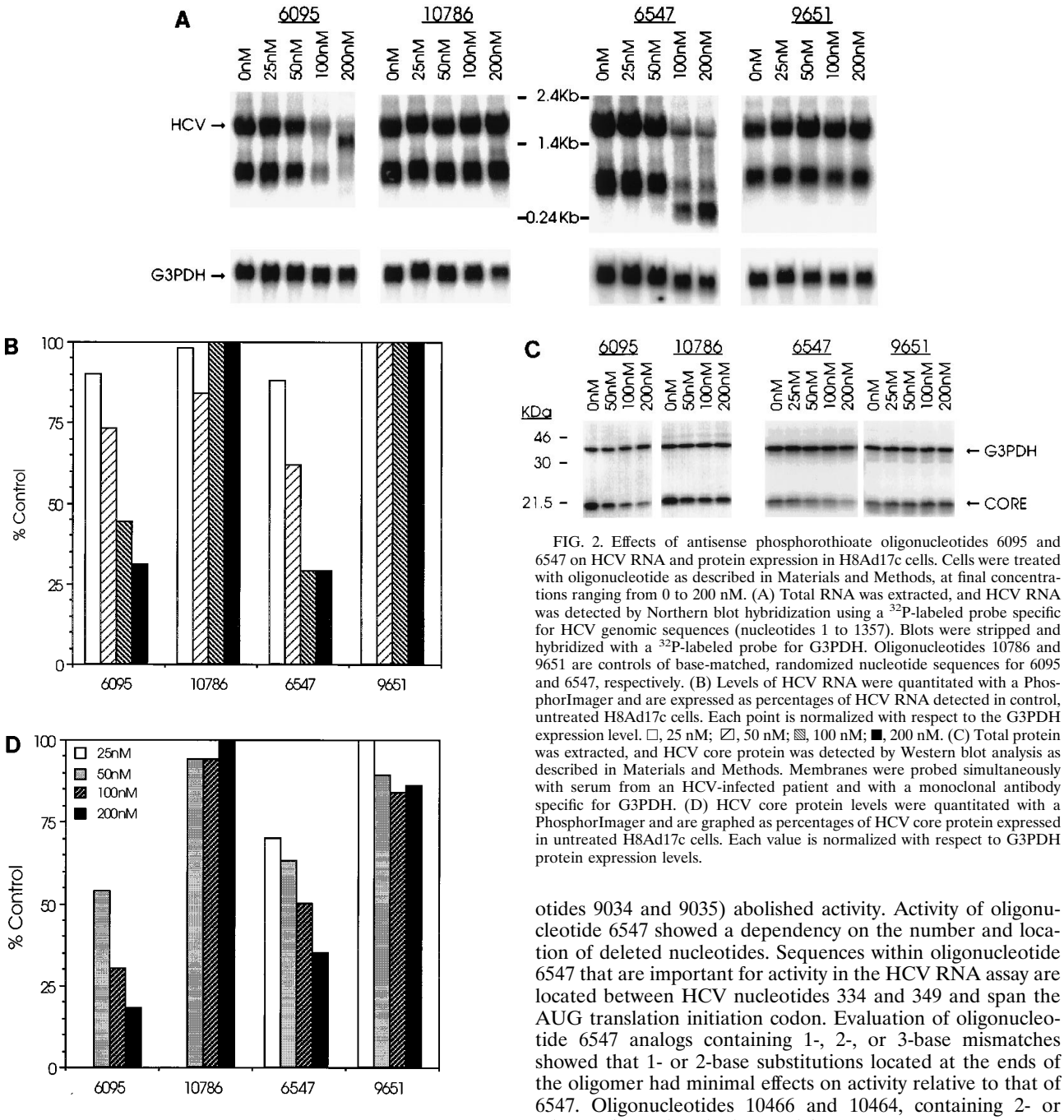

FIG. 2. Effects of antisense phosphorothioate oligonucleotides 6095 and 6547 on HCV RNA and protein expression in H8Ad17c cells. Cells were treated with oligonucleotide as described in Materials and Methods, at final concentrations ranging from 0 to 200 nM. (A) Total RNA was extracted, and HCV RNA was detected by Northern blot hybridization using a ^{32}P-labeled probe specific for HCV genomic sequences (nucleotides 1 to 1357). Blots were stripped and hybridized with a ^{32}P-labeled probe for G3PDH. Oligonucleotides 10786 and 9651 are controls of base-matched, randomized nucleotide sequences for 6095 and 6547, respectively. (B) Levels of HCV RNA were quantitated with a PhosphorImager and are expressed as percentages of HCV RNA detected in control, untreated H8Ad17c cells. Each point is normalized with respect to the G3PDH expression level. □, 25 nM; ▨, 50 nM; ▧, 100 nM; ■, 200 nM. (C) Total protein was extracted, and HCV core protein was detected by Western blot analysis as described in Materials and Methods. Membranes were probed simultaneously with serum from an HCV-infected patient and with a monoclonal antibody specific for G3PDH. (D) HCV core protein levels were quantitated with a PhosphorImager and are graphed as percentages of HCV core protein expressed in untreated H8Ad17c cells. Each value is normalized with respect to G3PDH protein expression levels.

3′ ends, reduced HCV RNA to levels observed after treatment with oligonucleotide 10964, containing a single-base mismatch, or 10552, containing a deletion of nucleotides at the 5′ and 3′ ends. Two-base mismatches placed in internal sequences (oligonucleotide 10961) or 3-base mismatches (oligonucleotide 10963) resulted in a complete loss of activity consistent with the predicted decrease in hybridization affinity previously demonstrated for oligonucleotides containing internal mismatched bases (28, 29).

Deletion of oligonucleotide 6547 from the 5′ end gradually reduced activity in the HCV RNA assay. In contrast, oligonucleotide 6547 sequence analogs with as many as 4 bases deleted from the 3′ end were effective at reducing HCV RNA levels. Deletion of 10 nucleotides from the 5′ or 3′ end (oligonucleotides 9034 and 9035) abolished activity. Activity of oligonucleotide 6547 showed a dependency on the number and location of deleted nucleotides. Sequences within oligonucleotide 6547 that are important for activity in the HCV RNA assay are located between HCV nucleotides 334 and 349 and span the AUG translation initiation codon. Evaluation of oligonucleotide 6547 analogs containing 1-, 2-, or 3-base mismatches showed that 1- or 2-base substitutions located at the ends of the oligomer had minimal effects on activity relative to that of 6547. Oligonucleotides 10466 and 10464, containing 2- or 3-base mismatches, had no activity.

Characterization of a truncated HCV RNA product induced by oligonucleotide 6547. Treatment of H8Ad17c cells with oligonucleotide 6547 resulted in a concentration-dependent loss of the major HCV transcript and in the appearance of an HCV RNA product of approximately 0.3 to 0.4 kb (Fig. 2A). The kinetics of the smaller transcript were investigated following treatment with oligonucleotide 6547. Total RNA was harvested at various times following treatment and analyzed by Northern blot hybridization. The small RNA detected by an HCV probe for nucleotides 1 to 1357 (probe 1-1357) was apparent as early as 2 h after treatment of cells with oligonucleotide 6547 (Fig. 3A). The level of the small RNA gradually increases with time as the level of the major transcript decreases (Fig. 3B). The origin of the small HCV RNA was

TABLE 2. Effects of phosphorothioate oligonucleotides complementary to HCV 260 and 330 regions on HCV RNA levels

Oligonucleotide group and ISIS no.	HCV nucleotide no.	% Decrease in HCV RNA[a]
HCV 260 region		
10498	254–273	83
10497	256–275	84
10496	258–277	84
6095	260–279	84
10495	262–281	85
10494	264–283	84
10493	266–285	89
10492	268–287	81
9553	270–289	78
HCV 330 region		
10491	322–341	74
10490	324–343	41
10489	326–345	65
9690	328–347	54
6547	330–349	73
10488	332–351	52
10487	334–353	52
10486	336–355	70
10485	338–357	47
6517	340–359	28

[a] Reductions in HCV RNA levels were determined by Northern analysis as described in Materials and Methods. H8Ad17c cells were treated with an oligonucleotide dose of 170 nM.

investigated by Northern blot hybridization with ^{32}P-labeled DNA fragments and oligonucleotide probes specific for regions of the HCV RNA expressed in H8Ad17c cells (Fig. 4A). The truncated RNA hybridizes with a probe specific for the entire HCV sequence expressed in H8Ad17c cells, while a probe for HCV sequences from nucleotides 709 to 1357 did not detect the truncated RNA. These results indicated that the truncated RNA originated from the 5′ half of HCV RNA present in H8Ad17c cells (Fig. 4B).

A second Northern blot hybridization analysis was performed to more closely determine the origin of the truncated RNA. Total RNA prepared from untreated H8Ad17c cells and cells treated with oligonucleotide 6547 was transferred to nylon membranes and hybridized with a panel of probes (oligonucleotides and DNA fragments) specific for sequences at the 5′ end of the HCV transcript. The truncated RNA was detected by probes 1-709 and 160-190 but not with probes for sequences from nucleotides 430 to 461 or 329 to 709 (Fig. 4C). On the basis of the estimated size of the truncated transcript and results of hybridization analyses, the truncated RNA appeared to represent HCV sequences from approximately nucleotides 1 to 330, or 5′ to the position where oligonucleotide 6547 hybridized.

One mechanism by which antisense oligonucleotides are believed to cause a reduction in the levels of complementary target RNAs is through activation of RNase H cleavage of the RNA component of the oligonucleotide-RNA duplex (19, 21, 40). The 3′ end of the truncated RNA transcript was more precisely determined by using RNA ligase-mediated amplification of cDNA ends (35). Total RNA was prepared from H8Ad17c cells treated with oligonucleotide 6547. An oligonucleotide dose of 300 nM was used to ensure production of a high percentage of truncated HCV RNA relative to the larger HCV transcripts expressed in H8Ad17c cells. RNA ligation of oligonucleotide to the 3′ ends of total RNA, reverse transcription, and cDNA amplification of the truncated RNA species are described in Materials and Methods. Seven clones were analyzed, and sequence data revealed that, while variability in the exact 3′-end sequence was apparent, all were located within the region targeted for hybridization by oligonucleotide 6547 (Fig. 5). Sequence composition can influence RNase H activity, but the enzyme is not known to be sequence specific (40). Three of the clones also contained 1 to 3 nucleotides that were not consistent with the HCV RNA sequence or oligonucleotides used in amplification reactions. The origin of these nucleotides is unknown, but such nucleotide additions may have occurred during amplification or cloning of cDNAs. The results suggest that the truncated HCV RNA transcript(s) represents almost the entire 5′ untranslated region of HCV extending as far 3′ as nucleotide 340. A truncated RNA with these characteristics is expected after RNase H cleavage of the HCV RNA at the position where oligonucleotide 6547 hybridizes.

Inhibition of HCV core protein expression by uniformly modified oligonucleotides. Previous studies have suggested that, while RNase H is found in the nucleus and the cytoplasm, the enzyme is localized mainly to the nucleus (12, 21). Replication of HCV in the cytoplasm of infected cells may require a mechanism of action which is not dependent on RNase H cleavage of duplexed RNA. Oligonucleotides with uniform substitutions at the 2′ position of the ribose do not serve as substrates for RNase H (31, 32). Chemical modification of antisense oligonucleotides at the 2′ position has been shown to increase duplex stability and, depending on the modification, can enhance resistance of the oligonucleotide to exo- and en-

TABLE 3. Effects of oligonucleotide 6095 and 6547 sequence analogs on HCV RNA levels

ISIS no.	Sequence[a]	Relative % reduction of HCV RNA[b]
6095 series		
6095	GCCTTTCGCGACCCAACACT	100
10483	--CTTTCGCGACCCAACACT	71
10482	---TTTCGCGACCCAACACT	60
10481	-----TCGCGACCCAACACT	81
10479	GCCTTTCGCGACCCAAC---	100
10478	GCCTTTCGCGACCCA-----	88
10552	-CCTTTCGCGACCCAACAC-	80
10551	--CTTTCGCGACCCAACA--	56
10964	GCCTTTCGCGACCC**T**ACACT	60
10967	**T**CCTTTCGCGACCCAACAC**G**	62
10963	**T**CCTTTCGC**T**ACCCAACAC**G**	0
10961	GCCT**A**TCGCGACCC**T**ACACT	0
6547 series		
6547	GTGCTCATGGTGCACGGTCT	100
10471	--GCTCATGGTGCACGGTCT	76
10470	---CTCATGGTGCACGGTCT	60
9038	-----CATGGTGCACGGTCT	40
9034	----------TGCACGGTCT	9
9559	GTGCTCATGGTGCACGGT--	81
9557	GTGCTCATGGTGCACGG---	92
9558	GTGCTCATGGTGCACG----	88
9036	GTGCTCATGGTGCAC-----	53
9035	GTGCTCATGG----------	0
10969	**C**TGCTCATGGTGCACGGTCT	90
10970	**T**TGCTCATGGTGCACGGTC**G**	88
10466	GT**C**CTCATGGT**G**ACGGTCT	0
10464	**C**TGCT**G**ATGG**A**GCACGGTCT	0

[a] Hyphens, deleted bases; boldface underlined bases, sequence changes with respect to 6095 or 6547 sequence.

[b] % inhibition of oligonucleotide (170 nM)/% inhibition of oligonucleotide 6095 or 6547) × 100.

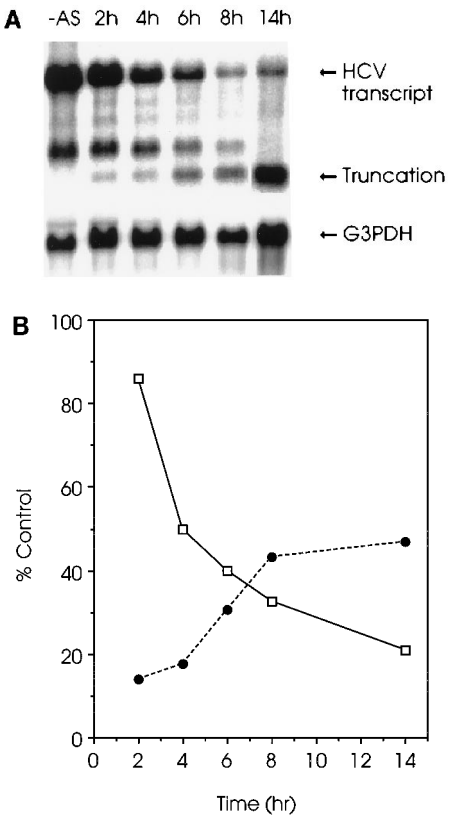

FIG. 3. Kinetic analysis of HCV RNA inhibition by oligonucleotide 6547 and appearance of truncated HCV RNA species. (A) Total RNA was extracted from H8Ad17c cells at the indicated times after treatment with oligonucleotide 6547 at a final concentration of 400 nM. Northern blot analysis was performed as described in Materials and Methods. (B) The decrease in the major HCV transcript and concomitant appearance of the truncated HCV RNA transcript were quantitated with a PhosphorImager. G3PDH levels were also quantitated to normalize the amount of RNA loaded for each sample.

donucleolytic degradation (23, 40, 43). Modification of phosphodiester oligonucleotides at the 2′ position with methoxyethoxy increases nuclease resistance to a level similar to that seen with a phosphorothioate linkage and increases hybridization affinity over that of an unmodified phosphodiester oligonucleotide (22). Modifications at the 2′ position have also been shown to enhance resistance to endonucleolytic degradation (23).

Hybridization of HCV 2′-methoxyethoxy phosphodiester antisense oligonucleotides to synthetic cRNA target was evaluated. Results of the hybridization analysis are shown in Table 4. 2′-Methoxyethoxy modification, combined with a phosphodiester backbone, significantly enhances duplex stability, as demonstrated by higher T_m values than those obtained for phosphorothioate oligonucleotides.

Inhibition of HCV core protein expression by antisense oligonucleotides containing a uniform methoxyethoxy modification at the 2′ position was investigated. 2′-Methoxyethoxy phosphodiester analogs of oligonucleotides 6095 and 6547 and a third oligonucleotide (6517 analog) complementary to the translation start codon and core protein coding sequences were evaluated for effects on HCV core protein levels. Oligonucleotides 11154 and 11153, 2′-methoxyethoxy phosphodiester analogs of oligonucleotides 6095 and 6547, respectively, did not affect core protein levels in H8Ad17c cells (data not shown). However, cells treated with oligonucleotide 11155, a 2′-methoxyethoxy phosphodiester analog of oligonucleotide 6517, showed a concentration-dependent reduction in HCV core protein expression (Fig. 6A). The activity of oligonucleotide 11155 was comparable to that of 6095 and enhanced relative to that of the phosphorothioate sequence analog 6517 shown in Table 1. A 2′-methoxyethoxy-modified control oligonucleotide of base-matched, randomized sequence (11179) did not inhibit HCV core protein expression (Fig. 6B). Treatment of H8Ad17c cells with oligonucleotide 11155 did not reduce HCV RNA levels, indicating that the observed inhibition of core protein synthesis did not depend on activation of RNase H (Fig. 6C).

DISCUSSION

The experiments whose results are presented here demonstrate inhibition of HCV gene expression in transformed hepatocytes by antisense oligonucleotides. Phosphorothioate oligonucleotides were utilized in our initial studies since the phosphorothioate linkage confers enhanced nuclease stability compared with that of unmodified, phosphodiester oligonucleotides (13, 23, 30, 52). Inhibition of HCV gene expression was examined by designing phosphorothioate antisense oligonucleotides complementary to the HCV 5′ NCR, the start codon for translation or the core protein coding region.

An immortalized human hepatocyte cell line constitutively expressing HCV RNA and core protein was utilized to evaluate inhibitory effects of antisense oligonucleotides. Reductions in HCV RNA or core protein levels are significant, since the HCMV immediate-early promoter yields HCV RNA and core protein expression levels that are likely to be considerably greater than those found in HCV-infected patients. One possible shortcoming of our studies is that HCV-specific transcripts are likely to be capped following RNA polymerase II nuclear transcription and processing. It is not known whether HCV RNA contains a 5′ cap structure similar to that of mRNAs of flaviviruses. Results from in vitro studies suggest a cap-independent mechanism of translation (45). Although a cap structure may be present at the 5′ end of HCV RNA expressed in H8Ad17c cells, translation is likely to occur by a mechanism of internal ribosome entry since extensive secondary structure within the 5′ NCR and the presence of multiple AUG codons upstream of the initiator AUG will interfere with ribosome scanning (27). Capped cellular mRNAs that use an internal ribosome binding mechanism for translation have been identified. The capped mRNAs for immunoglobulin heavy-chain-binding protein (BiP) and the *Antennapedia* gene of *Drosophila melanogaster* contain functional IRES elements (36, 41).

H8Ad17c cells were treated with phosphorothioate antisense oligonucleotides, and concentration-dependent reductions in HCV RNA and protein expression were demonstrated. Inhibition of expression was dependent on the sequence of the oligonucleotide in that activity of oligonucleotides varied and noncomplementary oligonucleotides did not reduce HCV RNA or core protein levels. We identified two oligonucleotides that specifically reduced HCV RNA levels, with an approximate 50% effective concentration of 100 to 200 nM. Our findings are similar to those reported by Alt et al. in which inhibitory activity of antisense oligonucleotides was evaluated by cotransfection of HepG2 cells with oligonucleotides and a DNA plasmid containing HCV sequences fused to a luciferase reporter gene (3). Oligonucleotides complementary to HCV sequences from nucleotides 264 to 282 and 326 to 348 were identified as potent inhibitors of luciferase gene expression. Our studies differ from those of others in that oligonucleotides

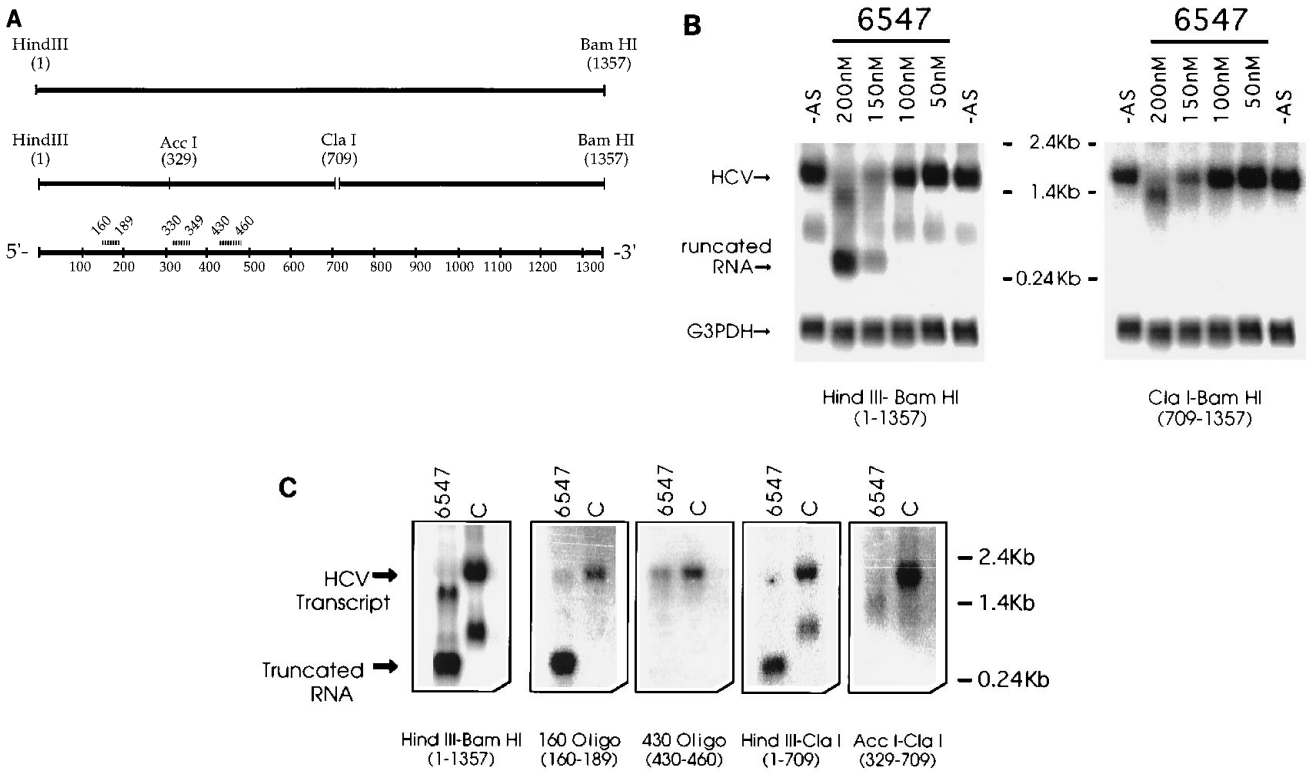

FIG. 4. Hybridization analysis of the truncated HCV RNA species. (A) Schematic representation of HCV DNA fragments and oligonucleotides used in Northern blot hybridization analyses of HCV RNAs. Restriction endonuclease sites denote the origin of DNA fragments. Oligonucleotide probes (hatched boxes) are indicated. (B) Total RNA was prepared from H8Ad17c cells 20 h following treatment with oligonucleotide 6547. Duplicate Northern blots were hybridized with either a probe specific for HCV sequences from nucleotides 1 to 1357 or a probe specific for HCV sequences from nucleotides 709 to 1357. Both membranes were stripped and hybridized with a probe specific for G3PDH. (C) Total RNA was extracted from untreated H8Ad17c cells (lanes C) and from cells treated with oligonucleotide 6547. Duplicate Northern blots were hybridized with HCV DNA probes complementary to HCV 5′ NCR sequences and core protein coding sequences. Hybridization conditions for oligonucleotide probes are described in Materials and Methods. HCV and G3PDH transcripts were visualized with a PhosphorImager.

were evaluated for activity by using an immortalized human hepatocyte cell line expressing HCV sequences rather than an in vitro translation assay or transient-transfection assay (3, 48). Our findings demonstrate that cotransfection of cells with antisense oligonucleotide and target RNA is not required to achieve specific inhibition of HCV gene expression.

Analogs of oligonucleotides 6095 and 6547 containing internal base mismatches showed reduced potency consistent with the lower T_ms predicted for these oligonucleotide-RNA duplexes (28, 29). Evaluation of oligonucleotide 6095 and 6547 analogs containing nucleotide deletions revealed potential differences in their respective HCV target regions. Truncation of oligonucleotide 6095 from the 5′ or the 3′ end to a 15-mer did not greatly reduce potency. Oligonucleotide 6095 and truncated analogs 10478 and 10481 all target a stem-loop structure located within the HCV 5′ NCR at nucleotides 253 to 282 (Fig. 7). The functional importance of this stem-loop structure and sequences located upstream has been demonstrated by others using deletion mutagenesis (51). Deletion of oligonucleotide 6547 from the 5′ end significantly reduced inhibitory activity, while deletion of as many as 4 nucleotides from the 3′ end only slightly affected activity. Deletion analysis of oligonucleotide

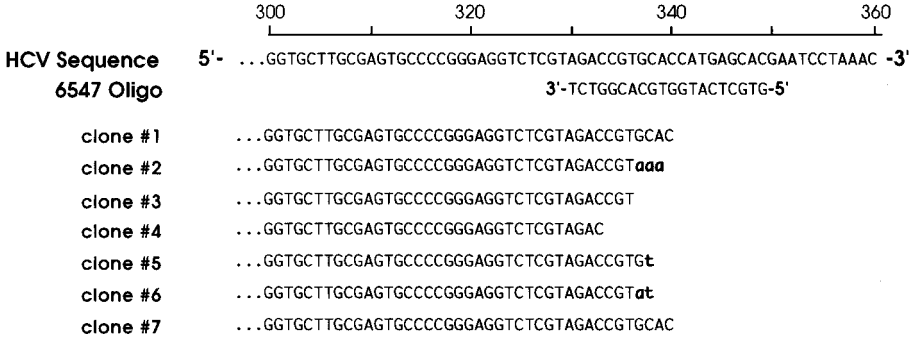

FIG. 5. Sequence comparisons among different cDNA clones representing the truncated HCV RNA. Total RNA was extracted from H8Ad17c cells following treatment with oligonucleotide 6547. RNA ligase-mediated amplification of cDNA ends was done as described in Materials and Methods. The sequences of seven clones were determined. The position of the 3′ end of each clone is shown relative to the HCV genomic sequence and site of oligonucleotide 6547 hybridization.

TABLE 4. Hybridization affinities of HCV antisense oligonucleotides

HCV coordinates	T_m (°C)[a]		
	DNA P═S	DNA P═O	2′-Methoxyethoxy–P═O
260–279	58.6	67.4	>90
330–349	53.5	64.5	>90
340–359	50.8	57.9	83.8

[a] Denaturation temperatures (T_ms) were measured versus length-matched RNA complement in 100 mM Na^+–10 mM phosphate–0.1 mM EDTA (pH 7.0) at a 4 μM strand concentration. Hybridization was evaluated spectrophotometrically, and reported values are the means of three experiments.

6547 suggests that sequences complementary to the HCV translational start codon and core protein coding region are important for inhibitory activity.

Antisense oligonucleotides exert inhibitory effects through a variety of mechanisms. Inhibition of RNA splicing, inhibition of translation of mRNA, and degradation of RNA by RNase H have been reported previously (1, 14, 33). Reduction of HCV RNA levels by oligonucleotides 6095 and 6547 is likely caused by RNase H cleavage of the RNA strand of the oligonucleotide-RNA duplex. Analogs of oligonucleotides 6095 and 6547 uniformly modified at the 2′ position and unable to serve as substrates for RNase H did not affect HCV RNA levels. Further evidence for an RNase H mechanism of action was provided by molecular characterization of the truncated HCV RNA observed after treatment of cells with oligonucleotide 6547. The 3′ end of the truncated RNA was shown to map within the site of oligonucleotide 6547 hybridization to HCV RNA. Although not extensively characterized, oligonucleotide 6095 also produced a truncated RNA product consistent with RNase H. In this case, the stable product mapped to the 3′ side of the oligonucleotide 6095 hybridization site, suggesting that there may be sequences between nucleotides 260 and 330 which confer stability to the RNA. The stability of the truncated RNA after cleavage may be due to extensive secondary structure in this region (representing almost the entire 5′ NCR) and to inhibition of enzymatic activity of nonspecific RNases by phosphorothioate oligonucleotides (20). The advantages of an RNase H-mediated termination of HCV gene expression are twofold: inhibition of both translation and viral replication through destruction of the HCV genome. However, because HCV replicates in the cytoplasm of infected cells, inhibition strategies may require application of oligonucleotides containing chemical modifications that specifically enhance RNA binding affinity.

The replicative cycle of HCV takes place in the cytoplasm of infected cells, in which RNase H levels have been reported to be reduced relative to those of the nucleus (12, 21). Several uniformly 2′-methoxyethoxy-modified phosphodiester oligonucleotides were evaluated to identify an RNase H-independent inhibitory compound. The 2′-methoxyethoxy modification confers stability to snake venom phosphodiesterase that is comparable to that of a phosphorothioate oligonucleotide (22). 2′-Methoxyethoxy phosphodiester analogs of oligonucleotides 6095, 6547, and 6517 showed dramatically increased hybridiza-

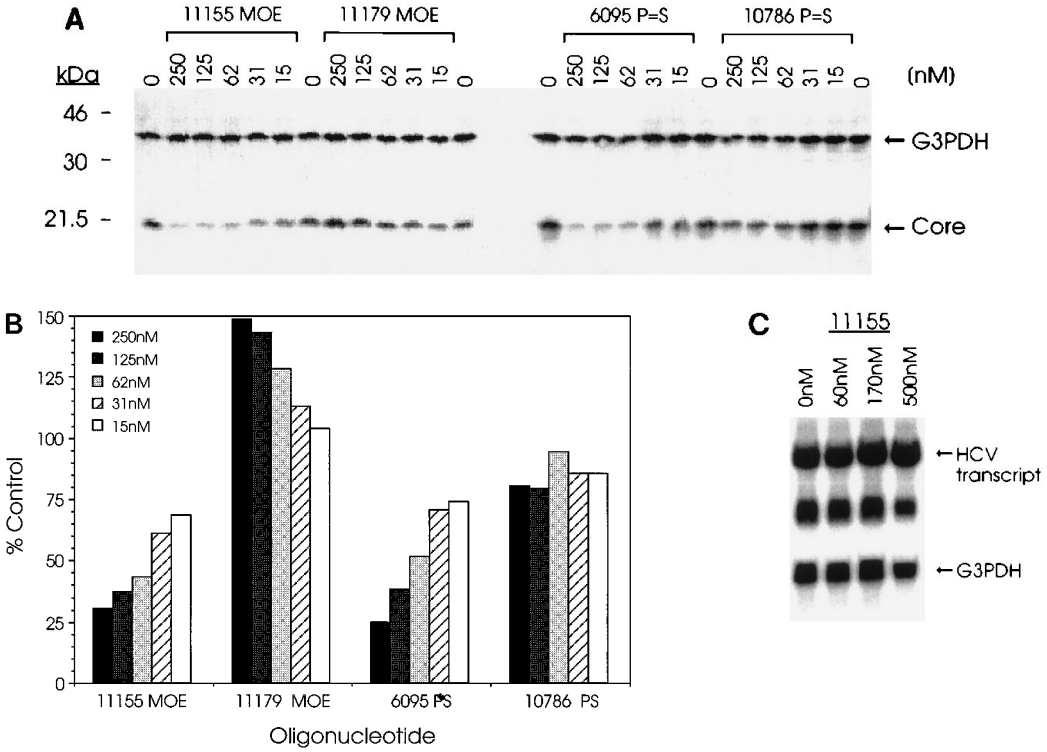

FIG. 6. Dose response of inhibition of core protein expression by 2′-methoxyethoxy phosphodiester-modified oligonucleotide 11155. (A) Immunoblots of total protein prepared from H8Ad17c cells treated with 2′-methoxyethoxy phosphodiester (MOE) and 2′-deoxyphosphorothioate (P═S) oligonucleotides. Core protein was detected by using polyclonal serum taken from an HCV-infected patient and ^{125}I-labeled secondary antibody. Noncomplementary oligonucleotides 11179 and 10786 containing randomized nucleotide sequences were evaluated as controls. Randomized oligonucleotides 11179 and 10786 are identical in nucleotide composition to antisense oligonucleotides 11155 and 6095, respectively. (B) Reductions in HCV core protein levels were quantitated by using a PhosphorImager. Values were normalized for G3PDH protein levels and were graphed as percentages of core protein expression in control, untreated H8Ad17c cells. (C) Northern blot analysis of HCV RNA levels in H8Ad17c cells treated with 2′-methoxyethoxy–P═O antisense oligonucleotide 11155.

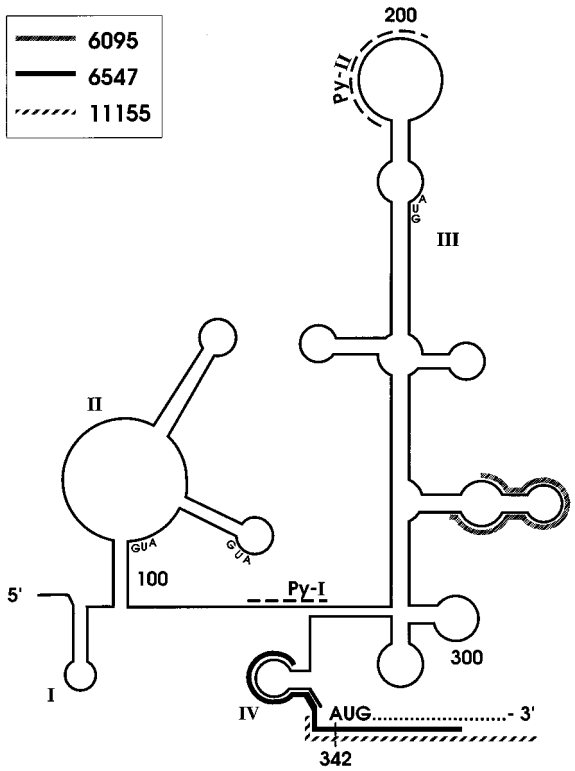

FIG. 7. Secondary structure predicted for the HCV 5′ NCR. The structure was originally reported by Brown et al. (8). The locations of internal AUG sequences and polypyrimidine tracts are noted. The start codon used for initiation of translation is located at nucleotide 342. Oligonucleotides 6095, 6547, and 11155 are complementary to regions of the HCV genome shown with double solid lines. There is a 10-base overlap in the region where oligonucleotides 6547 and 11155 are predicted to hybridize to the HCV genome.

tion affinity relative to that of unmodified phosphodiester and phosphorothioate oligonucleotides. Despite favorable in vitro biophysical characteristics, 2′-methoxyethoxy phosphodiester analogs of oligonucleotides 6095 and 6547 did not inhibit core protein expression, suggesting that activation of RNase H may be required for inhibition by oligonucleotides complementary to these sites. RNase H cleavage at the site of oligonucleotide 6095 or 6547 hybridization would result in the loss of sequences (and RNA structures) essential for HCV IRES function. Alternatively, secondary and tertiary structures present in these regions may sterically hinder hybridization of chemically modified oligonucleotides. An RNA pseudoknot structure located upstream of the initiator AUG near the site of oligonucleotide 6547 hybridization has been postulated (49). 2′-Methoxyethoxy-modified oligonucleotide 11155 (complementary to HCV nucleotides 340 to 359) was a potent inhibitor of core protein expression and did not affect HCV RNA levels, suggesting inhibition of HCV translation. Oligonucleotide 11155 is an attractive compound since it contains a phosphodiester linkage combined with a 2′ ribo modification. It is likely to exhibit fewer nonspecific effects at high doses than a phosphorothioate oligonucleotide (44).

The RNase H-independent inhibitory activity of oligonucleotide 11155 suggests that the initiator AUG is involved in HCV IRES function. Hybridization of antisense oligonucleotide 11155 to HCV RNA may interfere with formation of the putative RNA pseudoknot and/or specific RNA-protein interactions required for efficient IRES function. However, to date, a cellular factor(s) binding to HCV RNA in the vicinity of the AUG and core protein coding region has not been identified. While an analysis of polysome-associated HCV RNA was not performed in this study, oligonucleotides uniformly modified at the 2′ position with methoxyethoxy have been shown to interfere with the process of translation. Treatment of cells with a 2′-methoxyethoxy-modified antisense oligonucleotide complementary to human ICAM-1 mRNA did not affect ICAM mRNA stability or transport but reduced levels of ICAM-1 mRNA associated with polysomes (6).

Antisense oligonucleotides have proven useful tools for probing HCV IRES function. We identified several antisense oligonucleotides which specifically inhibit HCV gene expression. The most potent compounds are complementary to HCV sequences located in the 5′ NCR and core protein coding region which are functionally important for efficient cap-independent translation. Two active oligonucleotides, 6547 and 11155, target the initiator AUG and core protein coding sequence (Fig. 7). Inhibitory activity observed with oligonucleotide 11155 provides evidence that the initiator AUG and core region are important components of the HCV IRES.

Antisense oligonucleotides represent a promising new class of therapeutic agents. The mechanisms by which oligonucleotides are known to inhibit gene expression are distinct from the actions of therapeutic agents, such as interferon and nucleoside analogs, currently used for treatment of viral infections. Evaluation of in vitro and in vivo activities of antisense oligonucleotides, with an emphasis on nuclease-resistant, chemically modified compounds such as those described here, will advance the rational design and eventual development of antisense oligonucleotides as novel therapeutic agents for HCV infections.

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