Determination of the Role of the Human RNase H1 in the Pharmacology of DNA-like Antisense Drugs*

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Although ribonuclease H activity has long been implicated as a molecular mechanism by which DNA-like oligonucleotides induce degradation of target RNAs, definitive proof that one or more RNase H is responsible is lacking. To date, two RNase H enzymes (H1 and H2) have been cloned and shown to be expressed in human cells and tissues. To determine the role of RNase H1 in the mechanism of action of DNA-like antisense drugs, we varied the levels of the enzyme in human cells and mouse liver and determined the correlation of those levels with the effects of a number of DNA-like antisense drugs. Our results demonstrate that in human cells RNase H1 is responsible for most of the activity of DNA-like antisense drugs. Further, we show that there are several additional previously undescribed RNases H in human cells that may participate in the effects of DNA-like antisense oligonucleotides.

RNase H hydrolyzes RNA in RNA-DNA hybrids (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2–7). Although RNases H constitutes a family of proteins of varying molecular weight, the nucleolytic activity and substrate requirements appear to be similar for the various isoforms. For example, all RNases H studied to date function as dimeric enzymes with RNase H1 from yeast, chicken, and it showed that RNase H1 is involved in mitochondrial DNA degradation (5). The human enzyme. Site-directed mutagenesis of the catalytic amino acids and the basic residues of the substrate-binding domain of human RNase H1 showed that these conserved residues are required for activity (24). Recently a novel repressor element has been reported for the enzyme (25). In contrast to human RNase H1, nothing is known about the detailed enzymological properties of human RNase H2 because the cloned and expressed enzyme is inactive.

Antisense oligonucleotides (ASOs) have proven of value in determining gene functions and may be of value as a new therapeutic class (26). Once ASOs bind via Watson-Crick hybridization to target RNAs, they may work through a variety of mechanisms of action (26–28). DNA-like ASOs are thought to work by creating a substrate for cellular RNases H after binding to target RNAs (28). Although a large body of inferential evidence supports this concept, direct proof of the mechanism is lacking, and to date compelling evidence has not been reported with regard to which of the RNases H may be responsible (27–29).

The properties of the cloned and expressed human RNase H1 have been characterized; many of the properties observed for human RNase H1 are consistent with the E. coli RNase H1 isozyme, (e.g. the cofactor requirements, substrate specificity, and binding specificity) (18, 19). In fact, the carboxy-terminal portion of human RNase H1 is highly conserved with the amino acid sequence of the E. coli enzyme. The glutamic acid and two aspartic acid residues of the catalytic site, as well as the histidine and aspartic acid residues of the proposed divalent cation-binding site of the E. coli enzyme, are conserved in human RNase H1 (20–23). In addition, the lysine residues within the highly basic α-helical substrate-binding region of E. coli RNase H1 are also conserved in the human enzyme. Site-directed mutagenesis of the catalytic amino acids and the basic residues of the substrate-binding domain of human RNase H1 showed that these conserved residues are required for activity (24). Recently a novel repressor element has been reported for the enzyme (25). In contrast to human RNase H1, nothing is known about the detailed enzymological properties of human RNase H2 because the cloned and expressed enzyme is inactive.

The abbreviations used are: ASO, antisense oligonucleotide; siRNA, small interference RNA; pfu, plaque-forming unit.

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EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Synthesis and purification of chimeric 2′-O-methoxymethyl (MOE)/deoxy phosphorothioate-modified oligonucleotides were as previously described (30, 31). Sequences of oligonucleotides and placement of 2′-O-MOE modifications are detailed below. Unmodified oligodeoxynucleotides were purchased from Invitrogen. The oligoribonucleotides were purchased from Dharmacon Research, Inc. (Boulder, CO). siRNA duplexes were formed in the solution containing 20 mM each oligoribonucleotide, 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate. Reactions were heated for 1 min at 90°C and incubated for 1 hour at 37°C after virus infection (right panel). The protein concentrations of the cell lysates were measured. The lysates were subjected to 4–20% gradient SDS-PAGE (20 μg/lane) and Western blot analysis with anti-RNase H1 (against H1 carboxyl-terminal peptides, see “Experimental Procedures”). Immunoprecipitation was performed using uninfected HeLa cell lysate with purified H1 that was covalently immobilized to agarose beads (left panel). The eluted samples were subjected to Western blot analysis with H1 Ab. C, gel renaturation assay on uninfected HeLa cell lysate (5 μg) (panel 1); samples from immunoprecipitation with H1 Ab from uninfected HeLa cell nuclear and cytosolic extracts (see “Experimental Procedures”) (panel 2); samples from immunoprecipitation with H1 Ab from the lysates of HeLa cells infected with or without H1 or control virus (panel 3).

The control ASO, ISIS 29848, is a random mixture of nucleotides at each position. The oligonucleotide sample was prepared by concurrent reaction of four amidites (A, G, C, and T) at each position of the oligonucleotides. The 2′-methoxymethyl amidites were employed at positions 1–5 and 16–20, whereas deoxyribose amidites were used at positions 6–15. The ratio of each amidite in the mixture was adjusted to ensure equivalent reactivities. The siRNA controls in transfection are either the single strand sense RNA or other RNA duplexes that were non-complementary to the target.

Antibodies—Two human RNase H1 peptides, H-CRAQVDRF-
PAARFKKFATED-OH (amino acids 46–65) corresponding to the amino-terminal region, and H-CRTSAGKEVINKEDFVALER-OH, (amino acids 231–249) corresponding to the carboxyl terminus of the full protein (GenBank™ accession number AF039652), were conjugated to diphtheria toxin with maleimidocaproyl-N-hydroxysuccinimide and used to raise polyclonal antibodies in rabbits (32). Polyclonal antibodies were also raised against the His-tagged partial human RNase H1 (amino acid 73–286) (33).

Polyclonal antibodies were further purified with protein antigen using Aminolink immobilization kits (Pierce). 200 μg of purified H1 antibodies were then directly immobilized on agarose gel by using the Seize primary immunoprecipitation kit (Pierce) to create a permanent affinity support for immunoprecipitation without the need of protein A or G beads.

Adenovirus Production—For overexpression of human RNases H1, the full-length RNase H1 cDNA coding region was amplified by PCR and cloned into the virus shuttle vector pACCMMvLPaA(-)LoxP-ssp. The insert fragments were confirmed by DNA sequencing, and then the
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Northern blot analyses of the effects of RNase H1 on the potency of cRaf triplicates. The experiment was repeated several times. Results were plotted with percentage normalized mRNA level (for normalization). The experiment was performed in G3 cDNA probe and housekeeping gene glycerol-3-phosphate dehydrogenase (G3PDH). The samples were boiled in SDS-sample buffer and then separated by SDS-PAGE using 4–20% Tris-glycine gels (Invitrogen) under reducing conditions. Molecular mass prestained markers were used to determine the protein sizes. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane and processed for immunoblotting using affinity-purified human RNases H antibodies at 0.5–1 μg/ml. The immunoreactive bands were visualized using the enhanced chemiluminescence method (Amersham Biosciences) and analyzed using PhosphorImager Storm 860 (Amersham Biosciences).

Northern Blot—Total RNA was isolated from different human cell lines using RNAeasy kits (Qiagen, Valencia, CA). The samples were boiled in SDS-sample buffer and then separated by SDS-PAGE using 4–20% Tris-glycine gels (Invitrogen) under reducing conditions. Molecular mass prestained markers were used to determine the protein sizes. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane and processed for immunoblotting using affinity-purified human RNases H antibodies at 0.5–1 μg/ml. The immunoreactive bands were visualized using the enhanced chemiluminescence method (Amersham Biosciences) and analyzed using PhosphorImager Storm 860 (Amersham Biosciences).

RNA Expression Analysis—At the indicated times following oligonucleotide treatment, total RNA was harvested from 96-well culture dishes using an RNAeasy kit and a Bio Robot 3000 (Qiagen) accord-

adenovirus (H1) was generated by the Vector Core Laboratory of the University of Michigan. The control virus (LoxP) was also generated by using the same virus shuttle vector (without any insert). This virus shares all features with the RNase H1-containing virus except for the inserted genes. The viruses were prepared by either cell lysate (titration 3–7 × 10^9 pfu/ml) or CsCl purification (titration 1.38–1.61 × 10^11 pfu/ml, 4 × 10^12 viral particles/ml).

Cell Culture and Treatment—HeLa, A549, and HepG2 cells (ATCC, Rockville, MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FCS) in 6-well, 96-well, 10- or 15-cm culture dishes. MCF7 and T24 cell (ATCC) were cultured in McCoy’s medium with 10% FCS. Mouse AML12 and HeLa cells were cultured in McCoy’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FCS) (Invitrogen) and incubated with control or H1 virus (200 pfu/cell) for 12 h before the cells were transfected with anti-cRaf ASO (ISIS13650) of different concentrations via lipofectin (see “Experimental Procedures”). The cells were harvested 24 h later, and the total RNA was prepared with RNAeasy kit (Qiagen). 5 μg of RNA/lane was subjected to 1.2% agarose/formaldehyde and further to Northern blot analysis with 32P-labeled human cRaf mRNA levels were measured with RT-PCR in which the reverse transcription and PCR amplification of cRaf mRNA were performed in 96-well format with the primer set described under “Experimental Procedures.” IC_{50} were calculated and presented under the graphs. The bars represent S.E. of the mean of 3–5 replicates of a representative experiment. B, a similar experiment with anti-PTEN ASO (ISIS116847).

Antisense Drug Activity—IC_{50} were calculated and presented under the graphs. The bars represent S.E. of the mean of the triplicates. The experiment was repeated several times.

HeLa cells were split into 6000 cells/well in 96-well plates and then infected with H1 or control (LoxP) viruses (200 pfu/cell). 12 h later, the cells were transfected with the anti-cRaf (A) ASO (ISIS 13650) at different concentrations. The cells were harvested 24 h later. cRaf mRNA levels were measured with RT-PCR in which the reverse transcription and PCR amplification of cRaf mRNA were performed in 96-well format with the primer set described under “Experimental Procedures.” IC_{50} were calculated and presented under the graphs. The bars represent S.E. of the mean of 3–5 replicates of a representative experiment. B, a similar experiment with anti-PTEN ASO (ISIS116847). C, with anti-c-Jun NH_{2}-terminal kinase 2 ASO (ISIS101759). D, a similar experiment in A549 cells.

Northern blot analyses of the effects of RNase H1 on the potency of the cRaf ASO in HeLa cells. The cells were split into 10^6 cells/10-cm plate and incubated with control or H1 virus (200 pfu/cell) for 12 h before the cells were transfected with anti-cRaf ASO (ISIS13650) of different concentrations via lipofectin (see “Experimental Procedures”). The cells were harvested 24 h later, and the total RNA was prepared with RNAeasy kit (Qiagen). 5 μg of RNA/lane was subjected to 1.2% agarose/formaldehyde and further to Northern blot analysis with 32P-labeled human cRaf cDNA probe and housekeeping gene glycerol-3-phosphate dehydrogenase (G3PDH) (for normalization). The experiment was performed in triplicate. Results were plotted with percentage normalized mRNA level versus ASO concentration. The bars represent S.E. of the mean of the triplicates. The experiment was repeated several times.

Fig. 2. Effects of RNase H1 overexpression on the potency of DNA-like ASOs. HeLa cells were split into 10 6 cells/10-cm plate and then infected with H1 or control (LoxP) viruses (200 pfu/cell). 12 h later, the cells were transfected with the anti-cRaf (A) ASO (ISIS 13650) at different concentrations. The cells were harvested 24 h later. cRaf mRNA levels were measured with RT-PCR in which the reverse transcription and PCR amplification of cRaf mRNA were performed in 96-well format with the primer set described under “Experimental Procedures.” IC_{50} were calculated and presented under the graphs. The bars represent S.E. of the mean of 3–5 replicates of a representative experiment.
ing to the manufacturer’s protocol. The RNA concentration was measured with the ribogreen RNA quantitation reagent (Molecular Probes, Eugene, OR). Gene expression was analyzed using quantitative RT/PCR as described elsewhere (35). Total RNA was analyzed in a final volume of 50 μl containing 200 nM gene-specific PCR primers, 0.2 mM of each dNTP, 75 nM fluorescently labeled oligonucleotide probe, 1/100 RT/PCR buffer, 5 mM MgCl₂, 2 units of platinum TaqDNA polymerase (Invitrogen), and 8 units of ribonuclease inhibitor. Reverse transcription was performed for 30 min at 48°C followed by PCR: 40 thermal cycles of 30 s at 94°C and 1 min at 60°C, using an ABI Prism 7700 sequence detector (Foster City, CA).

The following primer/probe sets were used. Human c Raf kinase (GenBank™ accession number X03484): forward primer, AGCTTGGAGAGCAGATCGCAA; reverse primer, AAAGCTTGACTATTTGTAGGAGATGT; and probe, AGATGCCGTGTTTGTAGGCTTCACG. Human PTEN phosphatase (GenBank™ accession number U92436): forward primer, AATGGCTAAGTGAAGATGACCATCAT; reverse primer, TGCACATATCATTACACCAGTTCGT; and probe, TTGCAGCATATTCACTGTAAAGCTGGAAAG. Human Jnk2 protein kinase (GenBank™ accession number U35003.1): forward primer, CGCTGGCCTCAGACACAGA; reverse primer, CTAACCTATCATCGACAGCCTTCA; and probe, AGCAGTCTTGATGCCTCGACGGG. Human RNase H1 (GenBank™ accession number AF039652): forward primer, GGTTTCCTGCTGCCAGATTTAA; reverse primer, GGCTTGCAGATTTCCTGACAA; and probe, TTTGCCACAGAGGATGAGGCCTGG. Mouse Jnk1 protein kinase (GenBank™ accession number BU611812.1): forward primer, CAACGTCTGGTATGATCCTTCAGA; reverse primer, GTGCTCCCTCTCATCTAACTGCTT; and probe, AAGCCCCACACCACAAAGATCCCGX.

Animal Experiments—Eight-week-old female Balb/c mice were purchased from Jackson Laboratory. Mice were treated with various doses of ASO (ISIS 22023, anti-mouse Fas) in saline (Invitrogen) or with

![Figure 3](image1.png) Overexpression of human RNase H1 enhances ASO activity in mouse cell lines. A, overexpression of human RNase H1 in mouse AML12 and HeLa cell lines. Adenoviral infection and Western blot analyses were performed as described in Fig. 1B and under “Experimental Procedures.” B, overexpression of human RNase H1 increases anti-mouse c-Jun NH₂-terminal kinase 1 ASO potency. Experimental procedures were as described for Fig. 2 except for the increase in virus dosage (400 pfu/cell).
saline alone in 200 µl by intraperitoneal injection before treatment with the RNase H1-containing adenovirus (6 × 10^9 pfu or 1.7 × 10^11 viral particles in 200 µl of phosphate-buffered saline) or the control LoxP virus (6 × 10^9 pfu or 1.5 × 10^11 viral particles in 200 µl of phosphate-buffered saline) by intravenous injection, according to the indicated schedules. Total RNA was extracted from mouse liver using RNAeasy kits (Qiagen). RNase protection assays (RPA) were performed according to the manufacturer’s instructions (BD Biosciences). RPA template mApo-3 and a custom template (BD Biosciences) were used as probes. 20 µg of proteins of cell lysate were used for gel renaturation assay to test RNase H1 activity.

Statistics—To determine the IC50 values displayed in many figures, the curves were fit with a curve-fitting program in the linear dose range and the IC50 interpolated. To evaluate the statistical significance of treatment effects, pairwise comparisons of each point on each curve between treatment and control were performed using two-sided Student’s t test. For every point in the RNase H1-treated samples, the p value compared with control was less than 10^–41.

RESULTS

Overexpression of Human RNase H1—For overexpression of human RNase H1, a strain of adenovirus containing the insert shown in Fig. 1A was developed. Fig. 1B (right panel) shows that the full-length enzyme was overexpressed in HeLa and A549 cells. Peak expressions were observed 36–48 h after infection. In addition, the enzyme could be overexpressed in T24, MCF7, HepG2, and H293 cells (data not shown). Fig. 1B
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(left panel) shows a Western blot of the immunoprecipitated lysate from untreated HeLa cells; the right panel demonstrates that the full-length human RNase H1 virally encoded enzyme was overexpressed and comigrated with the enzyme from uninfected cells.

To determine whether the overexpressed RNase H1 was active, we employed the gel renaturation assay. As previously reported, human RNase H1 can be renatured and was active in this assay (Fig. 1C). Human RNase H1 activity was present in both the cytosolic and nuclear fractions of uninfected HeLa cells. To confirm that the activity was indeed human RNase H1, the enzyme was immunoprecipitated from HeLa cells and then subjected to the gel renaturation assay (Fig. 1C, panel 2), again showing that the enzyme was present in both the cytosolic and nuclear fractions. Overexpression of the full-length human RNase H1 resulted in increased activity in the gel renaturation assay (Fig. 1C, panel 3).

Overexpression of Human RNase H1 Increases the Potency of DNA-like ASOs—To evaluate the effect of overexpression of the RNases H on the potency of DNA-like ASOs, HeLa and A549 cells were infected with either the control (Lox P) or RNase H1 virus or RNase H1-containing adenovirus and then subjected to the gel renaturation assay (Fig. 1C, panel 2), again showing that the enzyme was present in both the cytosolic and nuclear fractions. Overexpression of the full-length human RNase H1 resulted in increased activity in the gel renaturation assay (Fig. 1C, panel 3).

Reduction of Human RNase H1 Reduces the Potency of DNA-like ASOs—To complement the overexpression experiments, we have reduced RNase H1 levels and evaluated the effects of reduced enzyme levels on the potencies of DNA-like ASOs. Potent selective DNA-like ASOs and siRNA were identified for both enzymes by cellular screening as previously described (26, 42).

To identify the most potent ASOs and siRNAs to inhibit human RNase H1, we screened candidates designed to bind to multiple sites in the RNAs. The most potent ASO (ISIS 194178) was located at nucleotides 36–40 on the intracellular concentrations of target RNAs evaluated. Fig. 2A–C shows that the potencies of ASOs designed to bind to human c-Raf, PTEN, or c-Jun NH2-terminal kinase 2 were significantly increased by overexpression of human RNase H1 in HeLa cells. Fig. 2D shows that similar results were observed in A549 cells. Fig. 2E shows that similar results were observed whether RT-PCR or Northern blot analyses were performed. The IC50 values for each ASO under each condition are shown under the graphs.

Because DNA-like ASOs are frequently used in vivo and are being evaluated in multiple clinical trials in humans (26), we extended our observations to include in vivo experiments. To do this we first demonstrated that human RNase H1 could be overexpressed in mouse cells (Fig. 3A). As was observed in human cells, overexpression of human RNase H1 increased the potency of a DNA-like ASO designed to bind to mouse c-Jun NH2-terminal kinase 1 RNA (Fig. 3B).

To determine whether overexpression of human RNase H1 in mouse liver increased the potency of DNA-like ASOs, we evaluated the effects of a well characterized mouse Fas ASO (41). Groups of mice were treated with the control and human RNase H1-containing adenovirus as described under “Experimental Procedures.” Fig. 4A shows that human RNase H1 was significantly overexpressed in the liver of the animals that were infected with the adenoviruses containing the insert. The human RNase H1 expressed in mouse liver was active in the gel renaturation assay. Moreover, the degree of overexpression was reasonably consistent. Fig. 4B shows that the ASO caused the selective reduction of Fas RNA in mouse liver. To evaluate the effects of overexpression of RNase H1 on the potency of the Fas ASO, the bands were quantitated and normalized to glyceraldehyde-3-phosphate dehydrogenase. Those data were then used to construct the dose response curves shown in Fig. 4C. The effects of overexpression of human RNase H1 were further confirmed by immunostaining of Fas protein with a Fas antibody (data not shown). Under the conditions employed, there was no evidence of significant liver toxicity and no histological differences among the saline, the LoxP control, and human RNase H1 virus-treated livers (data not shown). Furthermore, the concentrations of Fas ASO in the liver of mice treated with different doses of ASO were similar in mice treated with control virus or RNase H1-containing virus (Group 20 mg/kg: control, 29.0 ± 1.9; RNase H1, 31.1 ± 2.3. Group 30 mg/kg: control, 37.5 ± 5; RNase H1, 40.9 ± 4.1. Group 40 mg/kg: control, 50.1 ± 7.8; RNase H1, 48.5 ± 3.0 µg of parent ASO/g liver). Thus, the effects observed were neither secondary to hepatotoxicity nor due to changes in uptake of the Fas ASO into liver as a result of adenovirus infection.

The effects of various concentrations of each of the optimized inhibitors were then evaluated (Fig. 5). Both the ASO and siRNA inhibitors resulted in potent dose-dependent selective loss of RNase H1 RNA in both HeLa and A549 cells (Fig. 5A). Both the 1.3- and the 5-kb bands thought to be preprocessed human RNase H1 RNA were reduced. Further, the RNase H1 activity in both cell lines was reduced as shown in the gel renaturation assay (Fig. 5B). There were no effects on RNase H2 levels (data not shown). The duration of effect for both the ASO and siRNAs was greater than 48 h (data not shown).

Fig. 6 shows that reduction in the levels of human RNase H1 reduced the potency of the ASO targeting c-Raf RNA in HeLa cells. Increasing concentrations of the siRNA to human RNase H1 resulted in a comparable reduction in the potency of the c-Raf ASO. Further, there is a clear correlation between the reduction of c-Raf RNA by the ASO and the cellular level of human RNase H1 ($R^2 = 0.91$ or 0.69; $p < 0.01$) (Fig. 6, A and B).
Note that an extrapolation of the dose response curves for the c-Raf ASO would not demonstrate zero antisense activity when there was no human RNase H1 mRNA.

Fig. 7 shows that an ASO versus human RNase H1 reduced the potency of the c-Raf ASO in HeLa and A549 cells. These results were entirely comparable with the effects of siRNAs to RNase H1.

There Are Multiple RNase H-like Activities in Human Cells—Two related questions are prompted by the RNase H1 inhibition experiments. First, if RNase H1 is required for the activity of DNA-like ASO, why was the c-Raf ASO active when cellular RNase H1 was reduced by more than 90%? Second, if only RNase H1 is involved in the activities of DNA-like ASOs, why do the RNase H1 inhibition experiments generate curves that do not extrapolate to zero activity at zero RNase H1?

We observed several additional higher molecular mass (50–70-kD bands) as well as several lower molecular mass bands from cell homogenates in the gel renaturation assay. These bands were observed in a variety of cells when the renaturation assay was performed under standard conditions (10 mM Mg$^{2+}$). The level of activity and the number of extra bands varied from cell type to cell type and from cell preparation to cell preparation (Fig. 8A). When the assay was performed in the presence of 0.5 mM Mn$^{2+}$, the extra RNase H activity bands were more apparent and at least one higher molecular mass activity band was observed in all cell lines studied (Fig. 8B). This is a representative experiment that has been repeated more than five times.

Fig. 9, A and B, shows that reduction of RNase H1 with either an ASO or siRNA in both HeLa and A549 cells reduced the RNase H1 band of activity and had no effect on the higher molecular mass bands of RNase H activity. In Fig. 9C, we performed quantitative immunoprecipitation of RNase H1 in cell lysates. The supernatant after immunoprecipitation of

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**Fig. 8.** Several RNases H are present in human cells. Cell lysates were prepared in RIPA lysis buffer from human HeLa, A549, T24, MCF7, and HepG2 cells as described under “Experimental Procedures.” 20 μg of protein from each lysate were used in gel renaturation assay (see “Experimental Procedures”). Lanes 1–2, HeLa cell lysates; lanes 3–4, A549 lysates; lanes 5–6, T24; lane 7, MCF7; lane 8, HepG2 lysate. The lysates from lanes 2, 4, and 6 were prepared with the lysis buffer without phosphatase inhibitors. A, gel renaturation assay in the presence of Mg$^{2+}$. B, gel renaturation in the presence of Mn$^{2+}$. This is a representative experiment that has been repeated more than five times.

**Fig. 9.** Several RNases H are present in human cells, and they are not RNase H1. A, gel renaturation assay in the presence of Mg$^{2+}$ of HeLa cell lysates prepared as described under “Experimental Procedures.” Prior to preparation of the lysates, the HeLa cells were treated with either a control ASO or the RNase H1 ASO (ISIS194178) at the concentration indicated. This is a representative experiment repeated more than three times. B, gel renaturation assay in the presence of Mn$^{2+}$ of A549 cell lysates. Cells were treated with a control or the siRNA for RNase H1 as indicated. C, Western blot analysis of RNase H1 from HeLa cell lysates. Cell lysates were prepared as previously described. These were subjected to immunoprecipitation with the purified polyclonal antibodies to human RNase H1. The supernatant was separated from the protein A beads by centrifugation. All samples were then subjected to SDS-PAGE and probed with the purified human RNase H1 antibody. D, gel renaturation assay of the HeLa cell lysates after immunoprecipitation. Experimental procedures are as described earlier.
RNase H1 contained no detectable RNase H1. Further, in the gel renaturation assay (Fig. 9D) there was no RNase H1 activity in the immunoprecipitation supernatant. Nevertheless, in the immunoprecipitation supernatant, several of the novel RNase H activity bands remained (Fig. 9D). The same results are produced from similar experiments with anti-H2 antibody immunoprecipitation (data not shown).

These results show that there are several previously unidentified RNase H activities in human cells that are not RNase H1 or H2 yet are active in a gel renaturation assay. Neither inhibition at the RNA level with ASOs or siRNAs nor precipitation with RNase H1 or H2 antibodies affected the level of activities of the unidentified RNases H.

DISCUSSION

Although it has been assumed that DNA-like ASOs cause target RNA reduction by binding to the target RNA and creating a DNA-RNA duplex that serves as a substrate for RNase H, definitive proof that this mechanism is responsible for the observed effects in mammalian cells and animals is lacking (27, 28). In cell-free systems, the addition of E. coli RNase H or human RNase H1 to DNA-RNA duplexes results in degradation of the target RNA (18, 43, 44). The ability of DNA-like ASOs to cause a reduction in target RNAs in cells has been demonstrated many times as well (28). Moreover, changes in the structure of a DNA-like ASO that resulted in loss of the ability of the duplex to serve as a substrate for RNase H in cell-free systems have been reported to result in a loss of target RNA reduction in cells treated with the modified ASO (28, 45). Additionally, Giles et al. (29) used reverse ligation PCR to identify cleavage products from bcr-abl mRNA in cells treated with a DNA-like ASO. Nevertheless, none of these studies directly demonstrates that DNA-like ASOs reduce target RNA by activating RNase H nor has the specific RNase H that is responsible been identified. Because knock-outs of human RNase H1 are lethal (5, 14), we have chosen to overexpress human RNase H1 and to employ ASO and siRNA reduction of RNase H1 as complementary approaches to determine whether RNase H1 is required for target RNA reduction by DNA-like ASOs.

DNA-like ASOs Cause RNA Reduction by Serving as Substrates for Human RNase H1—For the first time, in this report we provide direct evidence demonstrating that DNA-like ASOs work via an RNase H mechanism. We show that overexpression of human RNase H1 increases the potency of several ASOs against several target RNAs in several human cell lines (Fig. 2). We also demonstrate that overexpression of human RNase H1 in mouse cells and mouse liver increases the potency of DNA-like ASOs (Figs. 3 and 4). More compellingly, reduction of RNase H1 results in a loss of potency for ASO to RNA target in several human cell lines (Figs. 6 and 7). Thus, both overexpression and reduction of RNase H1 demonstrate that RNase H1 is involved in the effects of DNA-like ASOs. This is the first direct demonstration of the role of an RNase H in the activity of a DNA-like ASO. The conclusions differ from a previous publication: ten Asbroek et al. (46) concluded that RNase H2 plays an essential role in the activity of DNA-like ASOs based on indirect evidence. In essence, they inferred that RNase H2 must be involved because it is more prevalent and differs in subcellular localization from RNase H1. Obviously, direct evidence based on selective alteration of the levels of both enzymes provides a more compelling answer.

Other RNases H May Play a Role in the Activity of DNA-like ASOs—The failure of siRNA or ASO inhibition of RNase H1 to eradicate ASO activity suggests that although RNase H1 plays a dominant role other RNases H may be involved, particularly if RNase H1 is reduced. We have identified several RNases H that are not immunoprecipitated by antibodies to RNase H1 or H2 (Figs. 8 and 9). These enzymes are not reduced by ASOs or siRNAs to RNase H1. They are active in the gel renaturation assay, suggesting that they are active as monomers and can be renatured. Some of them are more active in the presence of Mn2+ than Mg2+, and their activities are quite variable from cell line to cell line and within cell lines. These data suggest that these enzymes differ from the cloned mammalian RNase H and that they are regulated differently from RNase H1, because the levels of these enzymes vary far less than the novel activities we have observed. They may even be chimeric proteins with polymerase or other enzymatic activities fused to a peptide with RNase H activity. We are in the process of isolating and characterizing the enzymes.

Implications for Antisense Therapeutics—The demonstration that human RNase H1 plays a dominant role in the activities of DNA-like ASOs suggests that additional studies that explore the substrate preferences, enzymology, and regulatory processes for RNase H1 should support improved design of antisense agents. The demonstration that increases in RNase H1 activity correlated with increases in potency suggests that recruitment of RNase H1 to the ASO-RNA duplex and/or cleavage of the RNA by the enzyme is limiting for ASO activity. Any strategy that would improve these processes should improve ASO potency. Medicinal chemical approaches that optimize the structures of chimeric ASOs, the placement of DNA-like domains relative to preferred sites of cleavage, or enhance recruitment of the enzyme are in progress. It will also be important to better understand the novel RNases H and begin to design ASOs optimized for them.

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REFERENCES

1. Stein, H., and Hansen, P. (1969) Science 166, 393–395
2. Iyayi, M., and Kondo, K. (1991) Nucleic Acids Res. 19, 4443–4449
3. Iyayi, M., McKelvin, D., Chatterjee, S. K., and Crouch, R. J. (1991) Mol. Gen. Genet. 227, 438–445
4. Kanaya, S., and Iyayi, M. (1992) J. Biol. Chem. 267, 10184–10192
5. Busen, W. (1980) J. Biol. Chem. 255, 9434–9443
6. Rong, Y. W., and Carl, P. L. (1990) Biochemistry 29, 383–389
7. Crouch, R. J., and Walder, J. A. (1993) Biochimie (Paris) 75, 6472–6479
8. Crouch, R. J., and Dirksen, M. L. (1982) in Cold Spring Harbor Symp. Quant. Biol., Vol. 5, pp. 115–125, John Wiley and Sons, Inc., New York
9. Eder, P. S., and Walder, J. A. (1991) J. Biol. Chem. 266, 6472–6479
10. Frank, P., Albert, S., Cazenave, C., and Toulme, J. J. (1994) Nucleic Acids Res. 22, 5247–5254
11. Wu, H., Lima, W. F., and Croke, S. T. (1998) Antisense Nucleic Acid Drug Dev. 8, 53–61
12. Busen, W., Peters, J. H., and Hansen, P. (1977) Eur. J. Biochem. 74, 203–208
13. Turchi, J. J., Huang, L., Andrade, R. S., Kim, Y., and Bambara, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8903–8907
14. Cerritelli, S. M., Frolova, E. G., Feng, C., Grinberg, A., Love, P. E., and Crouch, R. J. (2003) Mol. Cell. 11, 807–815
15. Frank, P., Braunshofer-Reiter, C., Wintersberger, U., Grimm, R., and Busen, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12872–12877
16. Frank, P., Braunshofer-Reiter, C., Poellt, A., and Holzmann, K. (1998) Biochim. Biophys. Acta 1379, 1407–1412
17. Cerritelli, S. M., and Crouch, R. J. (1998) Genomics 53, 300–307
18. Wu, H., Lima, W. F., and Croke, S. T. (1999) J. Biol. Chem. 274, 28270–28278
19. Lima, W. F., and Croke, S. T. (1997) Biochemistry 36, 390–398
20. Kanaya, S., Katsuda-Nakai, C., and Ikebara, M. (1991) J. Biol. Chem. 266, 11621–11627
21. Nakamura, H., Oda, Y., Iwai, S., Inoue, H., Ohtsuka, E., Kanaya, S., Kimura, S., Katsuda, C., Katayanagi, K., Morikawa, K., Miyashiro, H., and Ikebara, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11535–11539
22. Katayanagi, K., Miyagawa, M., Matsushita, M., Ishikawa, M., Kanaya, S., Ikebara, M., Matsuoka, T., and Morikawa, K. (1992) Nature 347, 306–309
23. Yang, W., Hendrickson, W. A., Crouch, R. J., and Satow, Y. (1999) Science 282, 1398–1405
24. Landt, O., Grunert, H., and Hanh, U. (1990) Gene 96, 125–128
25. Lima, W., Wu, H., Nichols, J. G., Manalili, S. M., Drader, J. J., Hofstadler, S. A., and Croke, S. T. (2003) J. Biol. Chem. 278, 14906–14912
26. Crooke, S. T. (2003) in Burger’s Medicinal Chemistry, (Abraham, D. J., ed) 6th Ed., Vol. 5, pp. 115–166, John Wiley and Sons, Inc., New York
27. Crooke, S. T. (1999) Biochim. Biophys. Acta 1489, 30–42
28. Crooke, S. T. (2001) in Antisense Technology: Principles, Strategies, and Applications (Crooke, S. T., ed) pp. 1–28, Marcel Dekker, Inc., New York
29. Giles, R. V., Spiller, D. G., and Tidd, D. M. (1995) Antisense Res. Dev. 5, 23–31
30. McKay, R., Miraqulis, L., Cammins, L., Owens, S., Sasmor, H., and Dean, N. M. (1999) J. Biol. Chem. 274, 1715–1722
31. Baker, B. F., Lot, S. S., Condon, T. P., Cheng-Flournoy, S., Lesnik, E. A., Sasmor, H. M., and Bennett, C. F. (1997) J. Biol. Chem. 272, 11994–12000
32. Harlow, E., and Lane, D. (1988) Antibodies: a Laboratory Manual, pp. 53–158, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York
33. Wu, H., Lima, W. F., and Crooke, S. T. (2001) J. Biol. Chem. 276, 23547–23553
34. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
35. Winer, J., Kwang, C., Jung, S., Shackel, I., and Williams, P. M. (1999) Anal. Biochem. 270, 41–49
36. Monia, B. P. (1997) Anti-cancer Drug Design 12, 327–339
37. Geary, R. S., Leeds, J. M., Fitchett, J., Burckin, T., Truong, L., Spainhour, C., Creek, M., and Levin, A. A. (1997) Drug Metab. Dispos. 25, 1272–1281
38. Bost, F., McKay, R., Dean, N., and Mercola, D. (1997) J. Biol. Chem. 272, 33422–33429
39. Bost, F., McKay, R., Bost, M., Potapova, O., Dean, N., and Mercola, D. (1999) Mol. Cell. Biol. 19, 1938–1949
40. Butler, M., Popoff, I. J., Gaarde, W. A., Witchell, D., Murray, S. F., Dean, N. M., Bhanot, S., and Monia, B. P. (2002) Diabetes 51, 1028–1034
41. Zhang, Z., Cook, J., Nickel, J., Yu, R., Stecker, K., Myers, K., and Dean, N. M. (2000) Nature Biotechnol. 18, 862–867
42. Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003) J. Biol. Chem. 278, 7108–7118
43. Crooke, S. T., Lemonidis, K., Neilson, L., Griffey, R., Lesnik, E. A., and Monia, B. P. (1995) Biochem. J. 312, 599–609
44. Lima, W. F., Mohan, V., and Crooke, S. (1997) J. Biol. Chem. 272, 18191–18199
45. Chiang, M.-Y., Chan, H., Zounes, M. A., Freier, S. M., Lima, W. F., and Bennett, C. F. (1991) J. Biol. Chem. 266, 18162–18171
46. ten Asbroek, A. L., van Groenigen, M., Mosj, M., and Baas, F. (2002) Eur. J. Biochem. 299, 583–592