Enhancement of the Inhibitory Activity of oatp Antisense Oligonucleotides by Incorporation of 2′-*O*,4′-*C*-Ethylene-Bridged Nucleic Acids (ENA) without a Loss of Subtype Selectivity

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ABSTRACT: Antisense oligonucleotides (AONs) that specifically target the genes of rat organic anion transporting polypeptide (oatp) subtypes were selected by using antisense *in vitro* selection (AIVS) and a conventional gene alignment program (GAP). When we incorporated several of our original 2′-*O*,4′-*C*-ethylene-bridged nucleic acid (ENA) residues into AONs, which were designed as gapmers containing a series of 2′-deoxynucleotides in the center, at both the 3′ and 5′ ends, the inhibitory activity of these oatp AONs was enhanced and their inhibition was mediated by RNase H cleavage. Moreover, these ENA AONs did not lose their oatp selectivity. These strategies of using AIVS and GAP to select AONs followed by incorporation of ENA residues were effective for synthesizing oatp subtype-specific AONs.

Antisense oligonucleotides (AONs)[1] can bind to the complementary sites of mRNA and inhibit translation from mRNA by an RNase H-mediated cleavage mechanism and/or blocking due to steric hindrance (*1*). They have advantages similar to the advantages of using small interfering RNAs (siRNAs) in functional genomics and gene validation studies (*1*) and have been developed as anticancer and anti-inflammation drugs (*2*−*4*). The effect of the AONs depends on their affinity for the target mRNA. However, the selection of specific and effective antisense sequences is a laborious process because the tertiary structures of mRNA are unclear and unpredictable. Although some researchers have predicted the accessible regions of AONs in mRNA with a secondary structure prediction program such as RNA mfold (*5*, *6*), this method may be unreliable because RNAs are large molecules and are thus complicated to characterize using these algorithms. To solve this problem, a typical strategy, called "gene walk", is used to select manageable numbers of AONs (from 10 to 80 oligonucleotides) from regular intervals across the targeted gene (*2*). *In vitro* screening methods, using a library of combinatorial oligonucleotides with random sequences, gel shift analysis, and mapping analysis by RNase H cleavage have been introduced (*7*−*9*). A more innovative approach, in which oligonucleotide scanning arrays are applied for selecting AONs, has also been reported (*10*).

Since natural oligodeoxynucleotides that are sensitive to nuclease are not suitable as AONs, many types of modified oligonucleotides have been developed (*1*, *11*). Oligonucleotides containing phosphorothioate linkages and oligonucleotides containing 2′-*O*-alkylnucleosides, such as 2′-*O*-methylnucleosides and 2′-*O*-methoxyethylnucleosides, in particular, are widely known and have been used as the first and second generations of AONs, respectively (*12*−*14*). Although antisense gapmers such as 2′-*O*-Me-RNA−DNA−2′-*O*-Me-RNA oligonucleotides have been widely used over the past 10 years, there is little rigorous analysis of the molecules concerned with regard to the affinity for the target, RNase H activity on the antisense−RNA hybrid, or systematic analysis of gap size (*1*, *12*). Oligonucleotides containing novel 2′-*O*,4′-*C*-methylene nucleosides (2′,4′-BNA/LNA), whose sugar puckering is fixed in the *N*-conformation, had a higher affinity for their complementary RNA than any other modified oligonucleotides (*15*−*18*). We have reported the synthesis of novel 2′-*O*,4′-*C*-ethylene nucleosides (ENA) as shown in Figure 1A (*19*, *20*). Moreover, oligonucleotides containing these ENA residues exhibited equivalent binding affinity and much greater nuclease resistance than the corresponding oligonucleotides that contained 2′,4′-BNA/LNA residues (*19*, *20*).

Organic anion transporting polypeptides (oatps) play a role in the transport of a wide spectrum of amphipathic substances such as bile acids, steroid conjugates, and eicosanoids (*21*). Although several oatps have been found in the liver, their respective functions are still unclear (*21*−*23*). To investigate the biological role of each oatp, subtype-specific inhibitors are necessary. Therefore, we attempted to use AONs as subtype-specific inhibitors for oatp1, -2, and -3, the mRNAs of which are highly homologous in sequence.

In this study, we selected AONs targeting the gene of each oatp subtype with subtype-specific sequences by using a gene alignment program (GAP) and antisense *in vitro* selection (AIVS) and compared both procedures to search for antisense

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[1] Abbreviations: AIVS, antisense *in vitro* selection; AON, antisense oligonucleotide; BNA, bridged nucleic acid; CPG, controlled pore glass; ENA, 2′-*O*,4′-*C*-ethylene-bridged nucleic acid; LNA, locked nucleic acid; GAP, gene alignment program; oatp, organic anion transporting polypeptide; siRNA, small interfering RNA. ENA is a trademark of Sankyo Lifetech Co., Ltd.

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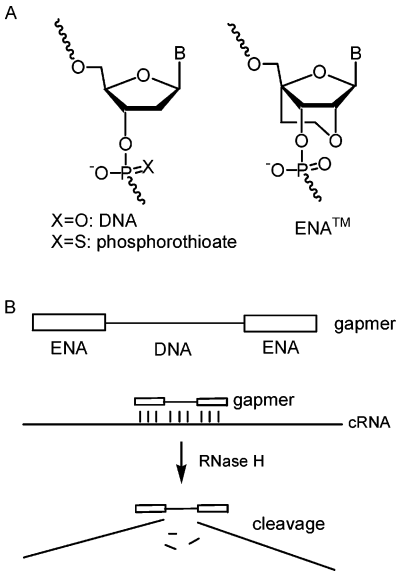

FIGURE 1: (A) Structures of DNA, phosphorothioated DNA, and ENA. (B) Design of ENA−DNA−ENA gapmers and their proposed mechanism of action mediated by RNase H.

sequences. In addition, to enhance the inhibitory activity of these oatp subtype-specific oligonucleotides, we incorporated several of our ENA residues into the AONs at both the 3′ and 5′ ends and evaluated the inhibitory activity and selectivity of these AONs for each oatp. This is the first report on the biological activity of ENA oligonucleotides as AONs.

MATERIALS AND METHODS

Synthesis of AONs Containing 2′-O,4′-C-Ethylene Nucleosides. 2′-*O*,4′-*C*-Ethylene nucleoside 3′-*O*-phosphoramidites were synthesized according to reported methods (*19*, *20*). AONs containing 2′-*O*,4′-*C*-ethylene nucleosides were prepared by solid phase phosphoramidite chemistry using an Applied Biosystems model 392 or 394 DNA/RNA synthesizer (*19*, *20*). To attach phenyl phosphate groups at the 5′ end of AONs OA1-1−8, OA2-1−8, and OA3-1−8, phenyl 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite (*24*) was coupled to the AONs. 4,4′-Dimethoxytrityl ethylene glycol-attached controlled pore glass (CPG) was used as a polymer support (*25*). 5′-*O*-(4,4′-Dimethoxytrityl) 2′-*O*-methylnucleoside 3′-*O*-phosphoramidites were purchased from Pharmacia or Transgenomic. Other reagent solutions were purchased from Applied Biosystems. The coupling of 2′-*O*,4′-*C*-ethylene nucleoside 3′-*O*-phosphoramidites was performed under standard synthesis conditions except for a longer coupling time (15 min). To introduce phosphorothioate linkages into the AONs, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent, Aldrich) was used according to a reported method (*26*). After the coupling reaction, the CPG was treated with concentrated aqueous ammonia at 60 °C for 5 h. The crude products were purified by reverse phase HPLC [Wakosil DNA (10 mm × 250 mm) column from Wako Pure Chemical Industries, Ltd., or Chromolith (6 mm × 100 mm) column from Merck] with an acetonitrile gradient containing 0.1 M triethylammonium acetate (pH 7.0). To synthesize the oligonucleotides that did not have phenyl phosphate groups at the 5′ position, their dimethoxytrityl groups were retained for purposes of purification. After purification by reverse phase HPLC, the dimethoxytrityl group was removed by the treatment of 80% aqueous acetic acid for 20 min. The structures of the modified oligonucleotides were determined by negative ion ESI mass spectroscopy: OA1-1, calcd 5016.30, found 5016.15; OA1-2, calcd 4926.24, found 4926.06; OA1-3, calcd 5079.35, found 5079.17; OA1-4, calcd 5099.40, found 5099.37; OA1-5, calcd 4943.24, found 4943.01; OA1-6, calcd 4989.26, found 4988.98; OA1-7, calcd 4970.27, found 4969.98; OA1-8, calcd 4912.21, found 4911.91; OA1-9, calcd 4851.27, found 4850.85; OA1-10, calcd 4905.32, found 4905.52; OA1-11, calcd 4884.26, found 4883.84; OA1-12, calcd 4883.27, found 4882.96; OA2-8a, calcd 5046.31, found 5046.00; OA2-8b, calcd 5088.35, found 5088.06; OA2-8c, calcd 5130.39, found 5129.99; OA2-8d, calcd 5172.43, found 5172.25; OA2-13, calcd 4810.14, found 4809.58; OA2-13a, calcd 4992.31, found 4992.12; OA2-13b, calcd 5034.35, found 5034.24; OA2-13c, calcd 5076.39, found 5076.37; OA2-13d, calcd 5118.42, found 5118.07; OA2-14, calcd 4853.14, found 4853.24; OA3-2a, calcd 4963.24, found 4962.83; OA3-2b, calcd 5005.28, found 5005.31; OA3-2c, calcd 5047.32, found 5047.24; OA3-2d, calcd 4701.09, found 4700.85; OA3-2e, calcd 4383.89, found 4383.77; OA3-2f, calcd 5047.83, found 5047.11; OA3-2g, calcd 5047.83, found 5047.04; OA3-2h, calcd 5117.43, found 5116.30; OA3-2i, calcd 4483.99, found 4483.95; OA3-2j, calcd 4709.44, found 4708.88; OA3-15, calcd 4740.09, found 4739.72; OA3-16, calcd 4859.17, found 4858.35; OA3-17, calcd 4817.12, found 4816.38.

Injection of Xenopus Oocytes and Transport Assay. oatp2 and -3 cRNA were transcribed from plasmids pR8 and pR1, respectively, using T7 RNA polymerase according to a reported method (*23*). oatp1 cRNA was obtained according to a reported method (*22*). *Xenopus laevis* oocytes were prepared according to a procedure described previously (*23*). An aliquot of 50 nL of a solution containing 50 mM NaCl, the cRNA (100 ng/μL), and excess AON (1−900 ng/μL, approximately 1.5−1350-fold time the molar concentration of the cRNA) was injected into the *Xenopus* oocytes. In the case where the cRNA and AON were injected separately, 50 nL of the solution containing AON (1 or 10 ng/μL) was first injected into the *Xenopus* oocytes, and after incubation for 30 min at 18 °C, 50 nL of the solution containing the cRNA (100 ng/μL) was then injected into the oocytes. The oocytes were then further incubated for 3 days at 18 °C, with daily replacements of modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES (pH 7.6)], and the uptake of [^{3}H]taurocholate (11.5 μM) was assessed at room temperature in sodium-free or sodium-containing uptake buffer [100 mM choline chloride, 10 mM HEPES, 5 mM Tris, 1 mM KCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.5)].

Gene Alignment Program (GAP). As a gene alignment program, GENETYX-WIN version 4 (Software Development Co., Ltd.) was used. The GenBank accession numbers of rat oatp1−3 are L19031, U95011, and AF041105, respectively.

Antisense in Vitro Selection (AIVS) of AONs that Bind to oatp2 or -3 cRNA. Approximately 1×10^{12} RNA transcripts (5′-pppGGAAGAGAUGGCGAC[N]$_{20}$CGGUAAGCUUGGCCAC-3′) were prepared by *in vitro* transcription using an appropriate synthetic DNA template, which included a T7 RNA polymerase (T7 RNAP) promoter (*27*). The RNA

transcripts were dissolved in H_2O and purified by denaturing 10% polyacrylamide gel electrophoresis to remove unincorporated nucleotides. Approximately 100 pmol of the RNA transcripts in 100 μL of binding buffer [100 mM Tris-HCl (pH 7.2 at 37 °C), 10 mM NaCl, 160 mM KCl, 1 mM $CaCl_2$, and 13 mM $MgCl_2$] was incubated at 60 °C for 5 min and kept at 37 °C for 10 min. After an equal volume of Oligotex-dT30 Super (Roche Diagnostics) in the binding buffer was added to the solution, the mixture was incubated at 37 °C for 5 min and centrifuged at 13000*g* for 1 min. The supernatant was placed into a new tube and incubated with 100 ng of either oatp2 or -3 cRNA (ca. 0.2 pmol) at 37 °C for 30 min. After an equal volume of Oligotex-dT30 Super that was prewashed with the binding buffer was added, the mixture was incubated at 37 °C for 10 min and centrifuged at 13000*g* for 1 min. The supernatant was removed, and the oligo(dT) matrix was subsequently washed five times with 200 μL of the binding buffer. Then, the RNAs in complex with oatp2 or -3 cRNA in the oligo(dT) matrix were recovered by elution with 100 μL of H_2O. The recovered RNAs were amplified by polymerase chain reaction (RT-PCR) using primer 1 (5′-GAATTCTAATACGACTCACTATAG-GAAGAGATGGCGAC-3′) and primer 2 (5′-GTGGC-CAAGCTTACCG-3′). The resulting double-stranded DNA was used in an *in vitro* transcription reaction with the RNA population for the subsequent round of selection.

After six rounds in total, to recover the RNA bound to oatp2 or -3 cRNA, a Micron YM-3 centrifugal filter device (Millipore) was used instead of the method using Oligotex-dT30 Super. After incubation of the mixture of the RNA and oatp2 or -3 cRNA at 37 °C for 30 min, the mixture was passed through the filter device and spun at 13000*g* and 20 °C for 20 min, producing ca. 10 μL of retentate. The binding buffer was added to the retentate, and the solution was centrifuged at 13000*g* and 20 °C for 20 min. This treatment was repeated two more times, and the recovered RNA was amplified by RT-PCR. The eighth generation of RNA transcripts was cloned using a TOPO-TA cloning kit (Invitrogen) and sequenced with an ABI PRISM apparatus (model 3700, Perkin-Elmer).

RESULTS AND DISCUSSION

Selection of Subtype-Specific Antisense Sequences Targeting oatp Genes by GAP. When the open reading frame sequences of oatp1−3 genes were aligned, more than 86% of the nucleotides were identical (*21*, *23*). To search for specific antisense sequences, we used a gene alignment program, GAP, as a conventional procedure to select eight regions (I−VIII) that had a relatively low level of homology among these three genes and designed AONs having relatively short lengths of 15 nucleotides [OA1-1−8, OA2-1−8, and OA3-1−8 (Table 1)]. Relatively short AONs were used because longer AONs might show a greater propensity to tolerate mismatches and bind to nontarget sequences. In the comparison of the sequences of each oatp subtype-specific AON, as there were several residues that were unique to each, high specificity of these AONs was anticipated (Table 1).

To enhance their inhibitory activity, three or four ENA residues were incorporated into the AONs near the 3′ and 5′ ends (Table 1). It was hoped that these AONs would form duplexes with the target oatp cRNA, which would then be a substrate for RNase H, as the AONs were designed as gapmers containing a series of 2′-deoxynucleotides in the center (Figure 1B). To facilitate the purification of the AONs, phenyl phosphate groups were attached at the 5′ end of the AONs to increase their hydrophobicity for good separation of the desired products on reverse phase HPLC. In addition, to stabilize the AONs against 3′-exonucleases, 2-hydroxyethyl phosphate groups were attached at the 3′ end of the AONs as reported previously (*25*).

We evaluated the inhibitory activity of these oatp AONs on the transporting activity of oatp by co-injecting each oatp cRNA and the AON into *Xenopus* oocytes. If the AONs led to oatp cRNA degradation by RNase H-mediated cleavage and/or blocked the translation of oatp cRNA due to steric hindrance, the oatp would not function as a transporter of [^{3}H]taurocholate in the oocytes.

Table 1: Sequences of AONs Targeting oatp[a]

Name[b]	Region	Sequence (5′-3′)	Mismatches against oatp1, -2 or -3[c]
OA1-1	I	AAATTGGGCCAGCAA	no, 8, 6
OA2-1		TCAAAGGGCCAATGG	8, no, 6
OA3-1		CTATTGGGCCAAAGA	6, 6, no
OA1-2	II	CAACTTTGCCATCTT	no, 5, 6
OA2-2		TCTCTTTGGCATTTT	5, no, 4
OA3-2		CCTCCTTGTCATTGT	6, 4, no
OA1-3	III	TATGAGGAATATTGC	no, 5, 6
OA2-3		CATAAGGAAGACTAC	5, no, 5
OA3-3		TATGAGAAGGACCAC	6, 5, no
OA1-4	IV	AAGTGACAAAGAAAA	no, 9, 6
OA2-4		TAGCTAAGGAAAGAC	9, no, 6
OA3-4		AAGTGCAAGAAATAC	6, 6, no
OA1-5	V	CAGCAACTGCTGCAT	no, 6, 6
OA2-5		CTGCCACTGGAAAAT	6, no, 4
OA3-5		CGGCAACTGGGAAGT	6, 4, no
OA1-6	VI	TGTGCCTGAAACTGT	no, 5, 4
OA2-6		GACGTCTGAAGCTGT	5, no, 5
OA3-6		TGCGCCTGAAGTTAT	4, 5, no
OA1-7	VII	GCTATAGGCAACCCC	no, 6, 4
OA2-7		GCAGCCGGCAATCCA	6, no, 5
OA3-7		GCTGCAGGCAACACT	4, 5, no
OA1-8	VIII	CCTTCTCTCCGAGCA	no, 6, 6
OA2-8		CCTTCAAGGTGAGCT	6, no, 4
OA3-8		TCTTCACTGTGATCT	5, 4, no

[a] 2-Hydroxyethyl phosphate groups were attached at the 3′ end of all AONs. Phenyl phosphate groups were attached at the 5′ end of AONs OA1-1−8, OA2-1−8, and OA3-1−8. ENA residues are colored red. Matched sequences between two of three genes are shaded. [b] AONs are coded as follows: OA(number of oatp subtype)-(number of region). [c] The number of mismatches of each AON against each oatp gene is given.

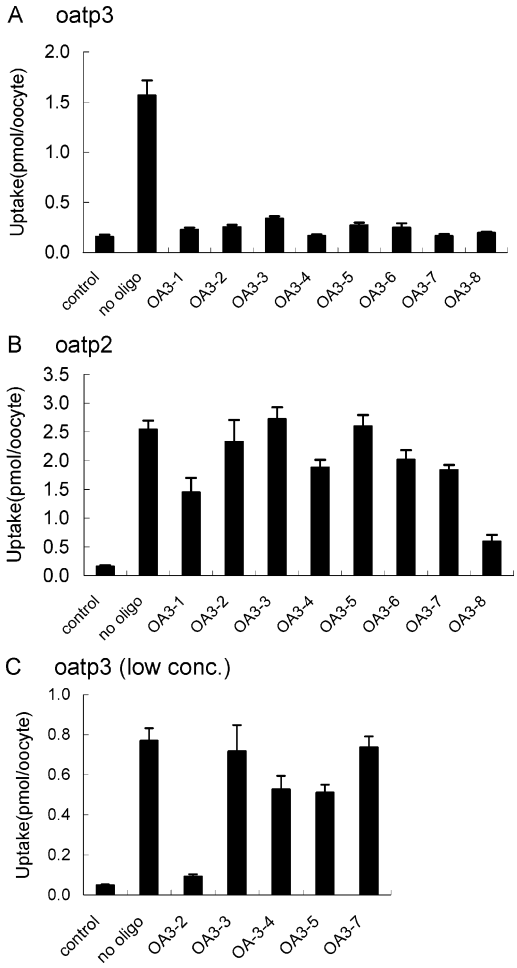

FIGURE 2: (A) Effects of ENA AONs OA3-1−8 on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 900 ng/μL ENA AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error. As a control, 50 nL of water was injected into the oocytes instead of the cRNA and ENA AON. (B) Effects of OA3-1−8 on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 900 ng/μL ENA AON. (C) Effects of a low concentration of OA3-2−5 and OA3-7 on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 9 ng/μL ENA AON.

Design of oatp3-Specific ENA AONs by GAP. When we evaluated the inhibitory activity of eight oatp3 AONs, OA3-1−8 (Table 1), all inhibited the function of oatp3 by more than 85% (Figure 2A). To evaluate the subtype specificity of these oatp3 AONs and select the highly oatp3-specific AONs, oatp2-mediated transport activity was measured in the presence of oatp3 AONs at a relatively high concentration that was 1350-fold times the molar concentration of the oatp2 cRNA. Almost all of them, OA3-2−5 and OA3-7, did not show any inhibitory activity against oatp2-mediated transport, although OA3-1 and OA3-8 inhibited the oatp2 activity by more than 40% (Figure 2B). This was possibly due to their unfavorable binding to the target sites and/or other accessible sites with partially complementarities followed by recruiting RNase H because of the use of the high concentration of the AONs with large numbers of mismatches. Among oatp3-specific AONs, OA3-2−5 and

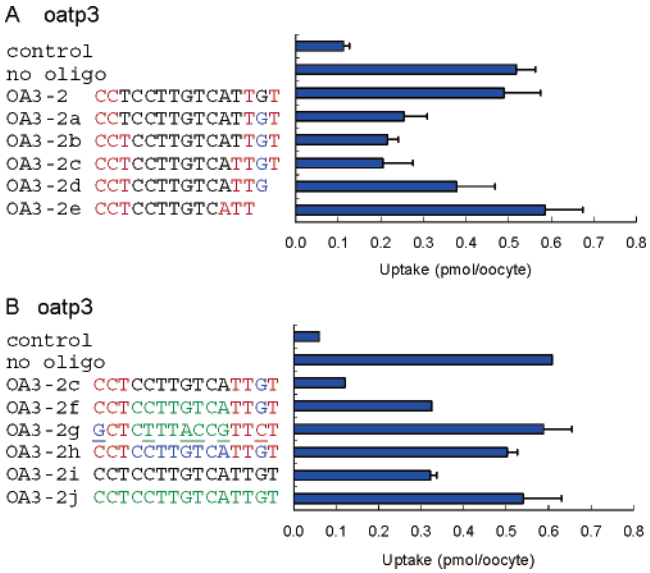

FIGURE 3: (A) Effect of modifying ENA AON OA3-2 by adding ENA residues on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 0.9 ng/μL ENA AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error. As a control, 50 nL of water was injected into the oocytes instead of the cRNA and ENA AON. (B) Derivatives of ENA AON OA3-2c targeting oatp3 and their effects on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 1 ng/μL AON. ENA residues, 2′-*O*-methylnucleoside units, and phosphorothioate linkages are colored red, blue, and green, respectively. The C of the ENA residue represents a 5-methylcytosine base. Mismatched sequences are underlined. 2-Hydroxyethyl phosphate groups were attached at the 3′ end of all AONs. Phenyl phosphate groups were attached at the 5′ end of AONs OA3-2a−2e.

OA3-7, OA3-2 exhibited the highest, by more than 90%, inhibitory activity even at a low concentration that was 100-fold lower than that used for the experiment depicted in panels A and B of Figure 2 (Figure 2C).

More ENA residues were incorporated into OA3-2 to synthesize OA3-2a to OA3-2c. When their AONs were evaluated at a much lower concentration that was 10-fold lower than that used for the experiment depicted in Figure 2C, we found that the inhibitory activities of OA3-2a−2c were increased in comparison to that of the parent AON OA3-2 (Figure 3A). The AONs that were shorter than OA3-2, OA3-2d and OA3-2e, decreased their activity more than the longer AONs, such as OA3-2a−2c, probably due to their weaker affinity for oatp3 cRNA.

When the inhibitory activity of OA3-2c was compared with those of an ENA AON partially modified with phosphorothioate linkages between the DNA residues, OA3-2f, a natural DNA AON, OA3-2i, and a fully phosphorothioate-modified DNA AON, OA3-2j, OA3-2c exhibited the most potent activity with >90% inhibitory activity as shown in Figure 3B. The AONs with ENA residues, OA3-2c and -2f, showed higher activity than the AONs without ENA residues, OA3-2i and -2j, respectively. Furthermore, the AONs with phosphodiester linkages, OA3-2c and -2i, showed higher activity than the AONs with phosphorothioate linkages, OA3-2f and -2j, respectively. These results demonstrated that the antisense efficiency of these AONs depended on their

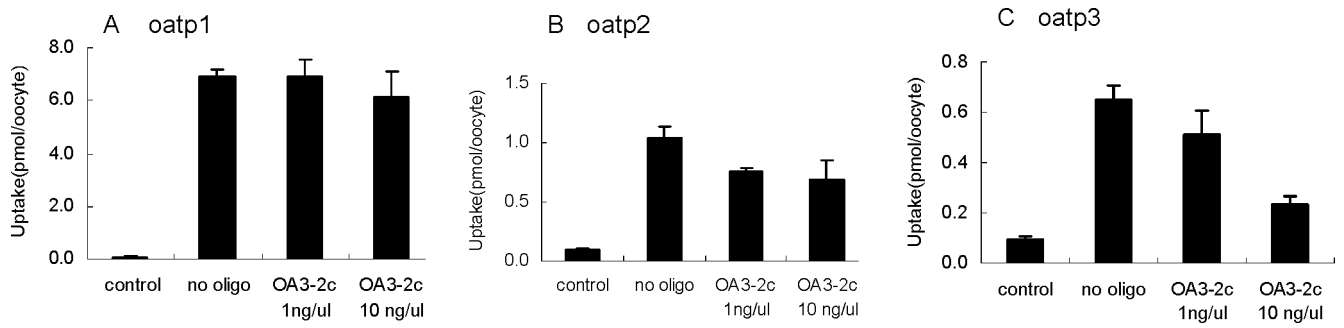

FIGURE 4: Effects of ENA AON OA3-2c targeting oatp3 on the uptake of taurocholate in (A) oatp1, (B) oatp2, and (C) oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of 1 or 10 ng/μL OA3-2c. After the cells had been incubated for 30 min at 18 °C, 50 nL of 100 ng/μL oatp1, oatp2, or oatp3 cRNA was then injected into the oocytes. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error. As a control, 50 nL of water was injected into the oocytes instead of the cRNA and the ENA AON.

affinity for the target molecule, which has already been reported in the literature (*11*, *19*, *20*). In this assay, nuclease resistance of the AONs may not affect the results due to more rapid degradation of cRNA than of the AONs, since the natural DNA AON, OA3-2i, had moderate activity.

Although the AON, OA3-2g, which had mismatched sequences, had no inhibitory activity, OA3-2f, which had matched sequences, showed moderate activity by approximately 50% inhibition. It was shown that these ENA AONs discriminated against the oatp cRNAs based on Watson−Crick base pairs and that RNase H recognized matched base pairs of a heteroduplex of cRNA and ENA AONs.

The 2′-*O*-methylnucleoside-modified AON OA3-2h, which does not undergo RNase H cleavage (*28*), exhibited weak inhibitory activity of ca. 20% in comparison to that of the AON OA3-2c, which undergoes RNase H cleavage, suggesting that the main mechanism of action of these gapmer-designed AONs was by an RNase H-mediated cleavage reaction.

Since in the assay that we described, the oatp cRNA and AON were mixed in a test tube and then injected into *Xenopus* oocytes, this treatment may have facilitated the binding of the AONs to cRNA and enhanced the inhibitory activity of the AONs. To investigate this possibility and the subtype selectivity of each AON, the oatp cRNA and AON were separately injected into the oocytes. Although the AON OA3-2c slightly inhibited oatp2-mediated transport in a dose-independent manner, possibly due to nonhybridization effects, the AON OA3-2c had a high and selective activity for oatp3 without inhibiting the function of oatp1 (Figure 4). This selectivity of the AON OA3-2c would be sufficient to clarify the function of oatp3 in rat intestine, because expression of oatp3 was observed despite the lack of expression of oatp2 in this organ (*23*). In this case, by using GAP, we could successfully obtain oatp3-specific ENA AON OA3-2c.

Design of oatp1- or oatp2-Specific ENA AONs by GAP. Using the same GAP as that used for the sequence selection of the oatp3 AONs, AONs targeting oatp1, OA1-1−8, were designed and synthesized (Table 1). Although, overall, the activities of the synthesized AONs were low, those of OA1-6 and OA1-8 were relatively high (data not shown), and thus, they were selected as lead AONs. ENA residues were then incorporated into these lead AONs to synthesize new AONs. However, the activity of these oligonucleotides did not improve dramatically (data not shown).

To prepare more potent AONs, we selected other AONs, OA1-9−12, by using a different GAP. These oatp1 AONs had unique sequences and were not complementary to oatp2 or -3. However, the targeted sequences of oatp2 and -3 that they carried were similar (see the Supporting Information). When OA1-9−12 were synthesized and evaluated, OA1-9−11 showed high inhibitory activity against oatp1 even at a low concentration (see the Supporting Information); in addition, OA1-9 and -10 had good selectivity for oatp2 (see the Supporting Information). These results suggested that the GAP strategy was helpful for finding specific sequences.

ENA AONs OA2-1−8, with sequences complementary to oatp2 as determined by using the same GAP as that for oatp3 AONs, were synthesized and evaluated (Table 1). All inhibited the function of oatp2 by more than 90% at a high concentration (Figure 5A). However, OA2-1, OA2-2, and OA2-7 inhibited on oatp3-mediated transport by more than 50% at a high concentration (Figure 5B). In particular, we found that OA2-8 was effective on oatp2-mediated transport at a concentration relatively lower than that used for panels A and B of Figure 5 to select the AON with the highest activity (Figure 5C). Although we selected OA2-8 as a lead compound and incorporated several ENA residues into OA2-8, the activity of these oligonucleotides, OA2-8a−8d, did not improve as we expected, possibly due to their maximum inhibitory activity (Figure 5D). To search for more effective antisense sequences for oatp3, we switched from using the GAP procedure to antisense *in vitro* selection, focusing on antisense binding sites in the tertiary cRNA structure.

Search for Antisense Sequences by AIVS. To locate regions on oatp cRNA that are accessible to antisense molecules for binding, we developed a method of antisense *in vitro* selection, AIVS, using oatp2 or -3 cRNA and a pool of numerous RNA transcripts that were prepared by *in vitro* selection using appropriate DNA templates synthesized as antisense molecules (Figure 6A). To select ENA AONs using this AIVS, we chose a pool of RNA transcripts rather than DNA transcripts as antisense molecules bound to oatp cRNA, because we have found that the duplex structures of our ENA oligonucleotides and target RNA molecules are close to those of natural RNA−RNA duplexes (*20*). The RNA transcripts were incubated with oatp cRNA under intracellular-like conditions in a buffer containing a low concentration of NaCl (10 mM), a high concentration of KCl (160 mM), and metal ions such as Ca^{2+} and Mg^{2+} (*29*). To avoid nonspecific

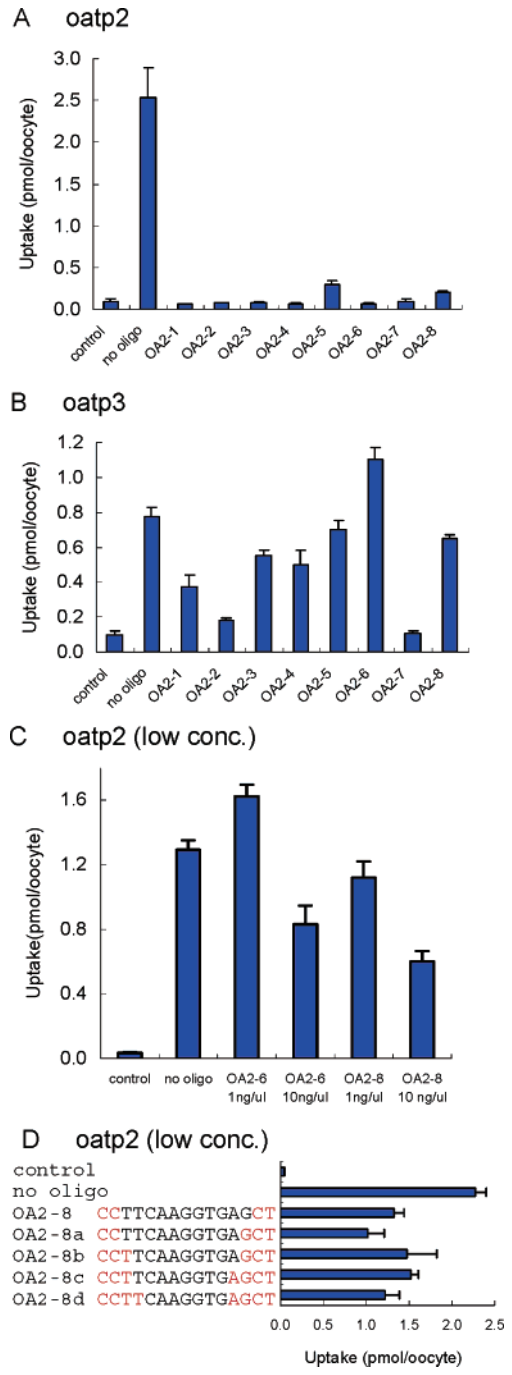

FIGURE 5: (A) Effects of ENA AONs OA2-1−8 on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 900 ng/μL ENA AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error. As a control, 50 nL of water was injected into the oocytes instead of the cRNA and ENA AON. (B) Effects of OA2-1−8 on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 900 ng/μL ENA AON. (C) Effects of a low concentration of OA2-6 and -8 on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 1 or 10 ng/μL ENA AON. (D) Effects of adding further ENA residues to the AON, OA2-8, on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 10 ng/μL ENA AON. ENA residues are colored red. The C of the ENA residue represents a 5-methylcytosine base. 2-Hydroxyethyl phosphate groups were attached at the 3′ end of all AONs.

interaction between the RNA transcript and oatp cRNA and to select strong binding sequences, we carried out the selection at 37 °C, which is higher than that in the case of the antisense evaluation in *Xenopus* oocytes. The mixture was passed through an oligo(dT) matrix to collect the complexes of the RNA transcripts and oatp cRNA. The RNA transcripts in complex with oatp cRNA were reverse-transcribed, producing single-stranded DNA. This DNA fragment was amplified by PCR using T7 promoter-containing primers, and large amounts of the selected RNA were produced by transcribing with T7 RNA polymerase. This process was repeated five more times. In the seventh and eighth rounds, due to the possibility of a large number of A-rich clones binding to the oligo(dT) matrix, we switched methods from using the oligo(dT) matrix to using a centrifugal filtration device, which ensured that unbound RNAs were passed through a filter. The bound RNAs were recovered, amplified by RT-PCR, and transcribed with T7 RNA polymerase as described above. The eighth generation of RNA transcripts was cloned, and 20 RNA clones were sequenced. Although some of the cloned RNA had relatively high occurrence frequencies and sequences almost fully complementary to the oatp2 or -3 cRNA (Figure 6B), the other RNA clones had low frequency, maybe due to their weak binding to the large target RNA molecules. Their cloned sequences were completely different from those selected by GAP as shown in Table 1.

To compare between the activities of the AONs selected by GAP and AIVS, those with a fully complementary sequence based on the selected sequences were synthesized (Figure 6B) and their inhibitory activity was evaluated in *Xenopus* oocytes as described above (Figure 6C,D). The selected AON for oatp2, OA2-13, showed better inhibitory activity than the control oligonucleotide, OA2-14, which was the nonselected sequence segment next to that of OA2-13 (Figure 6B,C). However, both OA2-13 and OA2-14 inhibited the oatp2-mediated transport. This region, including the AONs, OA2-13, and OA2-14, might be suitable for the design of AONs. Moreover, for the oatp3 AONs, the regions of antisense sequences highly complementary to oatp3 cRNA were chosen to construct OA3-15 and -17. The AON OA3-15 had a high inhibitory activity of more than 80% compared with that of the control AON, OA3-16, which had a nonselected sequence. The inhibitory activity of AON OA3-17 (approximately 30% inhibition) was weaker than that of AON OA3-15. However, the activity of the AON without the ENA residues, OA3-17, was higher than those of the ENA AONs which were selected by GAP as shown in Figure 3C (OA3-3−5 and OA3-7), even though these ENA AONs contained three or four ENA residues. These results indicate that AIVS is a useful method for searching for desired AONs.

Since we have already obtained effective oatp3 AONs such as OA3-2c, but not oatp2 AONs, oatp2 AON OA2-13 was the next focus. On the basis of the sequence of the AON OA2-13, new AONs (OA2-13a−13d), which were constructed by incorporating several ENA residues, were synthesized and evaluated (Figure 7A). Among these compounds, OA2-13b−13d exhibited excellent inhibitory activity that was much higher than that of the parent OA2-13 (Figure 7A). These AONs, OA2-13b−13d, inhibited oatp2 transport activity by more than 90%, and their activities were greater

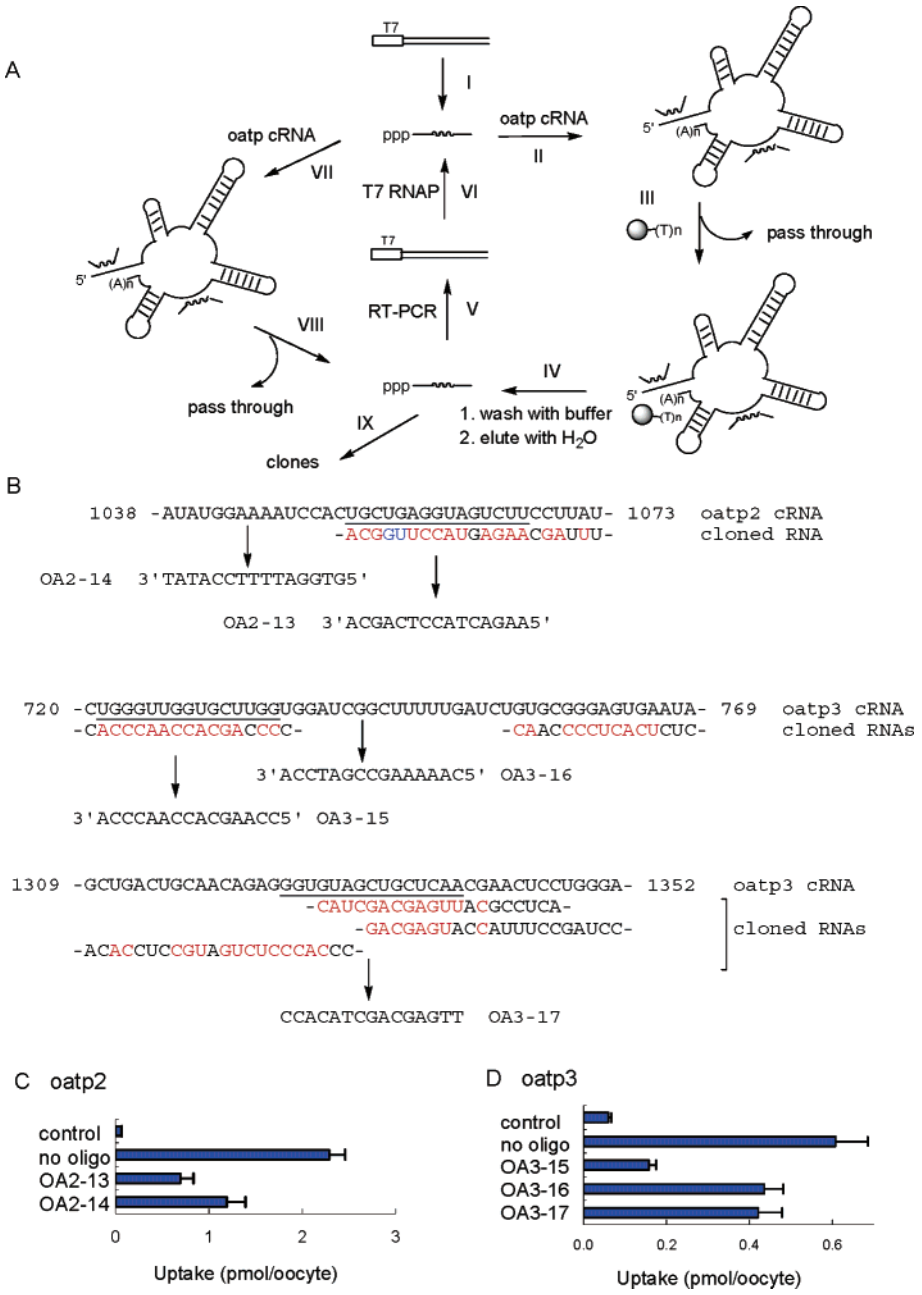

FIGURE 6: (A) AIVS of AONs targeting oatp2 or -3 from a population of random RNA fragments. (I) RNA fragments were prepared by *in vitro* transcription from a mixture of ca. 1×10^{12} double-stranded DNA templates using T7 RNA polymerase (T7 RNAP). Each template comprised a 20-nucleotide domain with a different sequence that was flanked by primer binding sequences, of which one was for the promoter of T7 RNAP (T7). (II) Purified RNA products were dissolved in the binding buffer and mixed with oatp2 or -3 cRNA. (III) An oligo(dT) matrix was added to the mixture. (IV) Unbound RNAs were removed by washing the matrix with the binding buffer. Bound RNA was eluted with H_2O. (V) Recovered RNAs were amplified by RT-PCR. (VI) The resulting double-stranded DNAs were transcribed to generate the subsequent population of RNA fragments. (VII) After the sixth round, step VII was performed. Purified RNA products were dissolved in the binding buffer and mixed with oatp2 cRNA. (VIII) The mixture was passed through a Micron YM-3 centrifugal filtration device, and RNAs bound to oatp2 or -3 cRNA were recovered on the filter. (IX) Recovered RNAs were amplified by RT-PCR, and double-stranded DNA from the final round of selection was cloned for further analysis. (B) Nucleotide sequences of oatp2 and -3 cRNA and the RNA transcripts isolated by AIVS. Matched sequences between cRNA and cloned RNAs are colored red. Sequences for wobble base pairs between cRNA and cloned RNAs are colored blue. The designed AON for oatp2 was named OA2-13. As a control, OA2-14 synthesized on the basis of the sequence segment next to that for OA2-13 was selected. The designed AONs for oatp3 were named OA2-15 and -17. As a control, OA2-16 synthesized on the basis of the sequence segment next to that for AO2-15 was selected. Phenyl phosphate groups and 2-hydroxyethyl phosphate groups were attached at the 5′ and 3′ ends of these oligonucleotides, respectively. (C) Effects of AONs OA2-13 and -14 targeting oatp2 on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 100 ng/μL AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error. (D) Effects of AONs OA3-15−17 targeting oatp3 on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp3 cRNA and 100 ng/μL AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. Uptake values represent means ± the standard error.

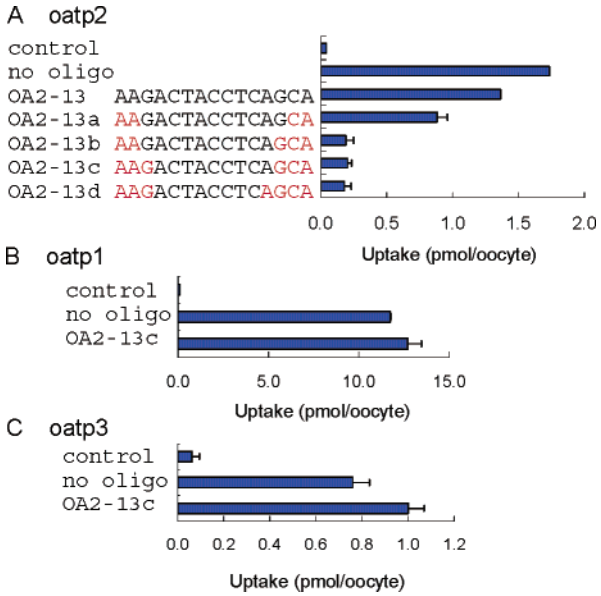

FIGURE 7: (A) Effects of adding further ENA residues to AON OA2-13 on the uptake of taurocholate in oatp2 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp2 cRNA and 10 ng/μL ENA AON. After the cells had been cultured for 3 days, the uptake of taurocholate was assessed as described in Materials and Methods. ENA residues are colored red. The C of the ENA residue represents a 5-methylcytosine base. 2-Hydroxyethyl phosphate groups were attached at the 3′ end of all AONs. (B) Effects of ENA AON, OA2-13c, on the uptake of taurocholate in oatp1 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 100 ng/μL oatp1 cRNA and 10 ng/μL ENA AON. (C) Effects of ENA AON OA2-13c on the uptake of taurocholate in oatp3 cRNA-injected oocytes. Oocytes were injected with 50 nL of a solution containing 10 ng/μL oatp3 cRNA and 100 ng/μL ENA AON.

than those of OA2-8 and OA2-8a−8d (maximum of 50% inhibition), which were selected by the GAP procedure as shown in Figure 5. In particular, OA2-13c exhibited highly specific inhibitory activity; that is, it did not inhibit the function of oatp1 (Figure 7B). Moreover, OA2-13c did not inhibit the function of oatp3 (Figure 7C).

Comparison between GAP and AIVS Methods in Selecting Effective and Subtype-Specific Antisense Sequences. Many researchers have tried to find both effective and specific antisense sequences using various methods (*5*−*10*). Single-strand regions of target mRNA were thought to be accessible regions for AONs (*10*). The best way to obtain effective antisense sequences is to know the structure of the target RNA. However, it is very hard to predict the tertiary structures of the huge mRNA molecules by computer analysis (*5*, *6*) or even by experimental procedures, such as with chemical probes and enzymatic digestion (*30*). In this report, we used two procedures, a gene alignment program (GAP) and antisense *in vitro* selection (AIVS), to obtain effective and subtype-specific antisense sequences targeting each oatp subtype. Using GAP, alignment of the oatp1−3 genes revealed sequences with a relatively low level of homology among the three genes. There were eight such regions which were selected (Table 1). In the case of the oatp3 AONs, OA3-1−8, almost all could be distinguished from oatp2 cRNA due to good mismatch discrimination (Figure 2A,B). In particular, OA3-2 had the most effective antisense sequence at a low concentration (Figure 2C). For the oatp1 and oatp2 AONs, when the inhibitory activity of OA1-1−8 and OA2-1−8 was evaluated, the selectivity for the target oatp was relatively high, but the inhibitory activity against each target was not very high (Figure 5). To prepare more potent oatp1 AONs, we selected oatp1-specific sequences that were unlikely to bind to oatp2 or oatp3 genes by redoing the alignment (see the Supporting Information). As a result, the obtained AONs, OA1-9 and -10, were found to show a high and specific inhibitory activity (see the Supporting Information). These results indicated that the AONs with sequences determined by a rational sequence alignment procedure were specific but not always effective as the tertiary structure of the target RNA is not taken into consideration.

Another procedure for finding effective AONs, namely, AIVS using oatp2 and -3 cRNA and a pool of random RNA fragments, was also used. This technique has been developed for finding new aptamers and ribozymes and is classified as a combinatorial approach using potential oligonucleotide libraries (*31*, *32*). We applied this method to selection of antisense sequences of oatp2 and -3 cRNA (Figure 6A). With this strategy, although the tertiary structure of the target cRNA is taken into consideration, visualization of this tertiary structure is not necessary. Eventually, the sequences of the accessible regions of cRNA can be deduced by comparing the antisense sequences from the pool of random RNA fragments. As a combinatorial strategy similar to the *in vitro* selection described above, mapping of the accessible regions using RNase H cleavage has been reported (*8*, *9*). Since this method is carried out under conditions that are optimal for RNase H reaction in the presence of a high concentration of sodium salts, the tertiary structure of the RNA under these reaction conditions might differ from that under intracellular-like conditions under which a high concentration of potassium salts, a low concentration of sodium salts, and some metal ions are present (*29*). Following selection of oatp2 AONs by AIVS under intracellular-like conditions, antisense sequences were observed from the cloned RNA, and the oatp2 AON OA2-13 showed inhibitory activity that was higher than those of OA2-1−8, whose sequences were selected by GAP (Figures 5 and 6). Although we eventually constructed only oatp2-specific AON OA2-13c, selected by AIVS as shown in Figure 7, oatp3-specific AONs also could be similarly obtained on the basis of selected sequences such as OA3-15. These results indicate that AIVS based on the tertiary structure of the target RNA might be useful for the selection of AONs. To our knowledge, this is the first time that a large RNA molecule has been used for the *in vitro* selection of antisense sequences.

Incorporation of ENA Residues for Increased Inhibitory Activity into AONs Selected by GAP and AIVS. Finding effective AONs that function at low concentrations is important for minimizing nonspecific inhibition, which can sometimes present contradictory results. For this reason, ENA oligonucleotides, which have high affinity for their complementary RNA ($\Delta T_m = 3-5$ °C/modification), were expected to enhance the antisense efficiency of the AONs (*19*, *20*). The addition of such ENA residues into AONs, such as OA3-2 and OA2-13, enhanced their antisense efficiency as shown in Figures 3A and 7A, respectively. When six ENA residues were incorporated into 15mer AONs OA3-2c and OA2-13c, the maximum inhibitory activity was obtained. It is known that oligonucleotides that contain ENA residues do not undergo RNase H cleavage as is the case for 2′,4′-

BNA/LNA oligonucleotides (*33*, *34*). In this study, the mechanism of action of the gapmer-designed AONs might mainly be by RNase H-mediated cleavage based on the results shown in Figure 3. The RNase H cleavage occurred by recognition of the heteroduplex of the target RNA bound to the DNA gap region of the AONs that were longer than eight DNA residues (Figure 3B). These results confirmed the previous results in which seven base pairs of DNA residues were sufficient for RNase H cleavage to occur in cells (*35*). In particular, we found that OA3-2c gave good results (>80% inhibition) at a very low concentration that was only ca. 1.5-fold equivalent to that of the oatp3 cRNA (Figure 3A). Furthermore, OA3-2c exhibited good selectivity, discriminating between oatp1 and oatp2, which were undesired targeted genes (Figure 4). Similar selectivity was also observed for OA2-13c (Figure 7). These results indicate that the finally obtained ENA AONs, OA2-13c and OA3-2c, did not induce RNase-H-mediated cleavage at the regions of partial complementarity of the AONs in the nontarget oatp cRNA, even though these ENA AONs made a helix with the nontarget cRNA.

In the case of ribozymes and aptamers, it is known that modification after *in vitro* selection does not always improve the selection of candidates (*36*, *37*). However, the combination of AIVS followed by ENA modification successfully selected effective ENA AONs, probably due to the structural properties of ENA, which are similar to those of natural RNA (*20*). Some other modified nucleic acids such as 2′,4′-BNA/LNA, PNA, morpholino, and phosphoramidate (3′-NP DNA), which are capable of hybridizing to mRNA with increased thermal stabilities (*1*), might also be suitable for using in combination with the AIVS method to search for antisense molecules.

From these results, it was clearly demonstrated that the incorporation of ENA residues into AONs increased their antisense activity without any loss of selectivity. In drug discovery, when one searches for inhibitors and activators, a severe problem is designing small molecules that can specifically distinguish among subtypes of enzymes that have a high level of homology such as protein kinases and phosphatases (*2*, *38*, *39*). AONs, and in particular ENA AONs, might resolve this kind of problem.

In Vivo Application of oatp ENA AONs. AONs are widely applied *in vivo* to elucidate the mechanism of target genes related to diseases such as cancer, inflammation, and diabetes (*13*, *40*−*43*). Our final goal is to clarify the function of each rat oatp *in vivo*. To do this, functional analysis using AONs as inhibitors could be one of the best strategies. It has been reported that rat oatp2 mRNA is expressed in the brain, retina, and liver and that rat oatp3 mRNA is found in the retina, liver, and kidney (*23*). On the other hand, rat oatp1 mRNA is expressed in the liver, kidney, brain, skeletal muscle, and colon (*21*, *23*). Specifically, all three oatps are expressed in rat liver. Thus, a subtype-specific inhibitor would be required for the functional analysis of oatps. It is known that AONs are distributed mainly to the liver and kidney, less so to skeletal muscles, and not at all to the brain (*44*, *45*). For the analysis of oatps in the liver and kidney, subtype-specific oatp ENA AONs, such as OA1-9, OA2-13c, and OA3-2c, which were designed and constructed in this study, might be useful *in vivo*. These *in vivo* studies using ENA AONs are now in progress.

SUPPORTING INFORMATION AVAILABLE

Table of the sequences of AONs targeting oatp and a graph showing the effects of OA1-9−12 on the uptake of taurocholate. This material is available free of charge via the Internet at http://pubs.acs.org.

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