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Chimeric RNase H-competent oligonucleotides directed to the HIV-1 Rev response element

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Abstract—Chimeric oligo-2′-*O*-methylribonucleotides containing centrally located patches of contiguous 2′-deoxyribonucleotides and terminating in a nuclease resistant 3′-methylphosphonate internucleotide linkage were prepared. The oligonucleotides were targeted to the 3′-side of HIV Rev response element (RRE) stem-loop IIB RNA, which is adjacent to the high affinity Rev protein binding site and is critical to virus function. Thermal denaturation experiments showed that chimeric oligonucleotides form very stable duplexes with a complementary single-stranded RNA, and gel electrophoretic mobility shift assays (EMSA) showed that they bind with high affinity and specificity to RRE stem-loop II RNA (K_D approximately 200 nM). The chimeric oligonucleotides promote RNase H-mediated hydrolysis of RRE stem-loop II RNA and have half-lives exceeding 24 h when incubated in cell culture medium containing 10% fetal calf serum. One of the chimeric oligonucleotides inhibited RRE mediated expression of chloramphenicol acetyl transferase (CAT) approximately 60% at a concentration of 300 nM in HEK 293T cells co-transfected with p-RRE/CAT and p-Rev mammalian expression vectors.
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1. Introduction

The Rev Response Element (RRE) is found in all unspliced and partially spliced HIV transcripts. Binding of HIV-encoded Rev protein to the RRE signals nuclear export of these transcripts, a process required for new virus formation.[1–3] Because the sequence of the RRE is highly conserved,[4] it has been considered an attractive target for therapeutic intervention.[5] The RRE RNA is highly structured and consists of five stem-loops. Stem-loop II, whose sequence and structure are shown in Figure 1a, contains the high-affinity binding site for Rev protein. We have shown that an antisense oligonucleotide, **2-1mp**, whose sequence is complementary to the 3′-side of stem-loop IIB (Fig. 1b), binds with high affinity to stem-loop II RNA in vitro and can competitively inhibit binding of Rev peptide.[6] This oligonucleotide, whose general structure is shown in Figure 2 (R = OCH_3), is comprised of 2′-*O*-methylribonucleotides and a nuclease resistant terminal 3′- internucleotide methylphosphonate linkage, and was shown to inhibit RRE-mediated gene expression in living cells.

Oligonucleotide **2-1mp** binds to RRE stem-loop II RNA and sterically blocks Rev interaction with this RNA. Because **2-1mp** is composed entirely of 2′-*O*-methylribonucleotides, the RRE stem-loop II RNA portion of the resulting hybrid is not expected to be a substrate for hydrolysis by RNase H. RNase H-mediated hydrolysis requires the formation of an RNA/DNA-type hybrid between the target RNA and the antisense oligonucleotide.[7] The enzyme recognizes this hybrid and cleaves only the RNA strand, leaving the antisense oligonucleotide free for another round of binding and hydrolysis.[8] The oligonucleotide can therefore potentially act in a catalytic manner to destroy its target.[9]

Oligo-2′-*O*-methylribonucleotides can be modified with deoxyribonucleotides so that their RNA hybrids are substrates for RNase H. As shown schematically in Figure 2 (R = H), the resulting 'chimeric' oligonucleotide contains an internal patch of 2′-deoxyribonucleotides flanked by 2′-*O*-methylribonucleotide 'wings'.[10–13] A short stretch of only four to six contiguous 2′-deoxyribonucleotides in a 2′-*O*-methyl sequence has been

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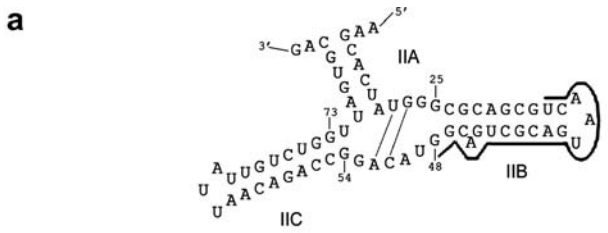


b	2-1mp	5'	mr-CpCpGpUpCpApGpCpGpUpCpApUpUpGpA 3'
	6d-1mp	5'	mr-CpCpGpUpCpApGpCpGpUpCpApUpUpGpA 3'
	6d/3P-1mp	5'	mr-CpCpGpUpCpApGpC*pGpU*pC*pApUpUpGpA 3'
	8d-1mp	5'	mr-CpCpGpUpCpApGpCpGpUpCpApUpUpGpA 3'
	8d/4P-1mp	5'	mr-CpCpGpUpC*pApGpC*pGpU*pC*pApUpUpGpA 3'
	c2RNA	3'	r-GpGpCpApGpUpCpGpCpApGpUpApApCpU 5'
	6dr-1mp	5'	mr-CpCpGpUpCpNpNpNpNpNpNpApUpUpGpA 3'

Figure 1. (a) RRE stem-loop II RNA structure predicted by M-fold.[43] The U:A and G:C base pairs of stem IID[2,44] are indicated by connecting lines, but are not predicted by M-fold. The purine-rich bubble between stems IIB and IIC is the initial high-affinity binding site of the Rev protein.[45,46] The oligonucleotide binding site is shown as a black line along the 3′ side of stem-loop IIB. (b) Sequences of chimeric oligonucleotides and **c2RNA**. The notations '**mr**-' and '**r**-' denote 2′-*O*-methyl and 2′-ribose sugars, respectively. 2′-Deoxyribonucleotides are in bold and 2′-deoxyribo-5-propynylpyrimidines are marked with an asterisk. Methylphosphonate linkages are denoted by '**p**' and N is A, G, C or T.

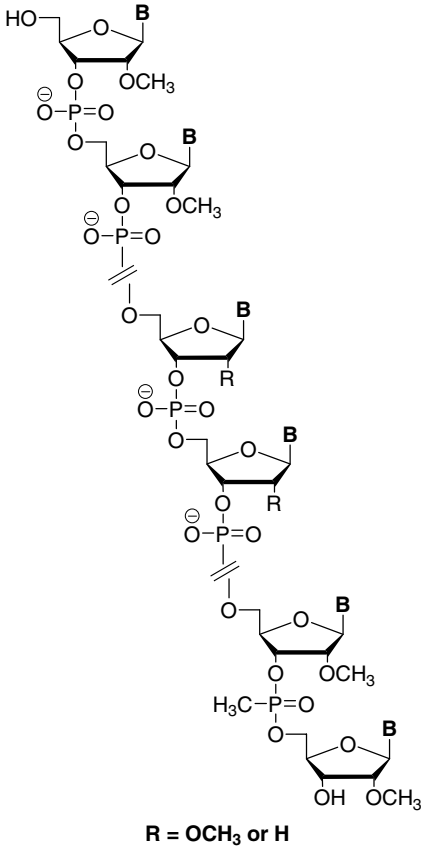


Figure 2. General structure of antisense oligonucleotide. The chimeric oligonucleotides contain 2′-deoxyribonucleotides (R = H) in the central region of the oligonucleotide.

reported to be sufficient for RNase H-mediated target hydrolysis.[11,14] This chimeric strategy, which was originally described by Giles and Tidd,[15–17] potentially enables one to design antisense oligonucleotides that are resistant to nuclease degradation; have high binding affinity and specificity for their designated target; and are able to degrade their target in a catalytic manner, properties that are desirable for creating an effective antisense oligonucleotide.

In the study described here, we examine the properties of a series of nuclease resistant chimeric oligo-2′-*O*-methylribo/deoxyribonucleotides whose sequences are identical to that of **2-1mp**. We show that these chimeric oligonucleotides bind to the RRE stem-loop II RNA with high affinity and specificity; direct RNase H-mediated cleavage of their RRE stem-loop II target; and that one of these oligonucleotides effectively inhibits RRE mediated gene expression in HEK 293T cells in culture.

2. Results and discussion

2.1. Chimeric oligo-2′-deoxy-/2′-*O*-methylribonucleotides

The chimeric antisense oligonucleotides are targeted to the 3′-side of the stem-loop IIB region of RRE stem-loop II RNA as shown in Figure 1a. This region is immediately adjacent to the Rev high affinity binding site, which is located between stem-loops IIB and IIC. These oligonucleotides, whose sequences are shown in Figure 1b, are derived from oligo-2′-*O*-methylribonucleotide **2-1mp**.[6] Oligonucleotides **6d-1mp** and **8d-1mp** contain patches of six and eight deoxyribonucleotides, respectively, flanked by 2′-*O*-methylribonucleotides. Each oligonucleotide terminates at its 3′-end with a nuclease resistant methylphosphonate internucleotide linkage. Chimeric oligonucleotides **6d/3P-1mp** and **8d/4P-1mp** were also prepared in which the deoxyribopyrimidine nucleotides were replaced with deoxyribo-5-propynylpyrimidine nucleotides. The 5-propynyl modification has been shown to increase the stability of oligonucleotide/RNA duplexes, particularly when contiguous 5-propynylpyrimidines are present. This increased stability is attributed to increased π-stacking

interactions between the bases in the duplex, and to conformational preorganization of the oligonucleotide, both of which contribute to a decreased entropy cost for binding to the RNA target.[18,19] Oligodeoxyribonucleotides that contain 5-propynylpyrimidines form hybrids with RNA that are cleaved by RNase H and have been shown to inhibit gene expression in cell culture.[20,21]

2.2. Thermal stability of chimeric oligonucleotide/RNA duplexes

Ultraviolet thermal denaturation experiments were used to assess the stability of hybrids formed between the chimeric oligonucleotides and the single-stranded RNA target **c2RNA**, whose sequence corresponds to the oligonucleotide binding site in RRE stem-loop II RNA. The experiments were carried out in buffer containing 0.1 M sodium chloride. As shown in Table 1 the hybrid formed between **2-1mp**, whose backbone consists entirely of 2′-*O*-methylribonucleotides, and **c2RNA** has a very high T_m, 74 °C. Introduction of six deoxyribonucleotides reduces the T_m of the **6d-1mp/c2RNA** duplex by 10 °C. The T_m of the **8d-1mp/c2RNA** duplex, which contains eight deoxyribonucleotides, is reduced an additional 4 °C. The T_m of the latter duplex is comparable to the T_m, 59 °C, of the duplex formed between **c2RNA** and its complementary all phosphodiester oligodeoxyribonucleotide.[6]

Table 1. Melting temperatures of **oligonucleotide/c2RNA** duplexes

Oligonucleotide	T_m [a] (°C)
2-1mp	74
6d-1mp	64
6d/3P-1mp	70
8d-1mp	60
8d/4P-1mp	69

[a] Melting experiments were carried out in buffer containing 50 mM MOPS (3-(*N*-morpholino)propane sulfonic acid), pH 7.0, and 100 mM sodium chloride at a concentration of 1 μM per strand.

The high T_m of the **2-1mp/c2RNA** hybrid is attributed to the C3′-endo sugar pucker adopted by the 2′-*O*-methylribonucleotides of **2-1mp**. As a consequence, the oligonucleotide is preorganized in an A-type conformation that resembles the final conformation of the hybrid. This preorganization reduces the entropic penalty for hybrid formation, thus leading to greater duplex stability.[22] The reduced stability of the **6d-1mp/c2RNA** and **8d-1mp/c2RNA** duplexes results from the increased conformational flexibility of the deoxyribonucleotide patch whose sugar residues exist in a dynamic equilibrium between the C2′-endo and C3′-endo sugar pucker. This increased flexibility causes an increased loss of entropy upon hybrid formation, thus reducing the stability of the duplex.[23]

Replacement of the deoxypyrimidine residues of **6d-1mp** and **8d-1mp** with 2′-deoxy-5-propynylpyrimidines increases the T_ms of duplexes formed between **c2RNA** and **6d/3P-1mp** and **8d/4P-1mp** by 6 °C and 9 °C, respectively. The 5-propynyl group shifts the sugar pucker equilibrium of the deoxynucleotides towards the C3′-endo conformation,[19,24] and increases stacking interactions with neighboring bases. The increase in stability provided by the 5-propynyl groups averages 2.0–2.3 °C per modification, a value that agrees with the reported 2–3 °C per modification increase in T_m for single, centrally-located propynyl modifications in DNA/RNA hybrids.[18]

The circular dichroism spectra of the hybrids formed between the chimeric oligonucleotides and **c2RNA** are all very similar as shown in Figure 3. The strong positive

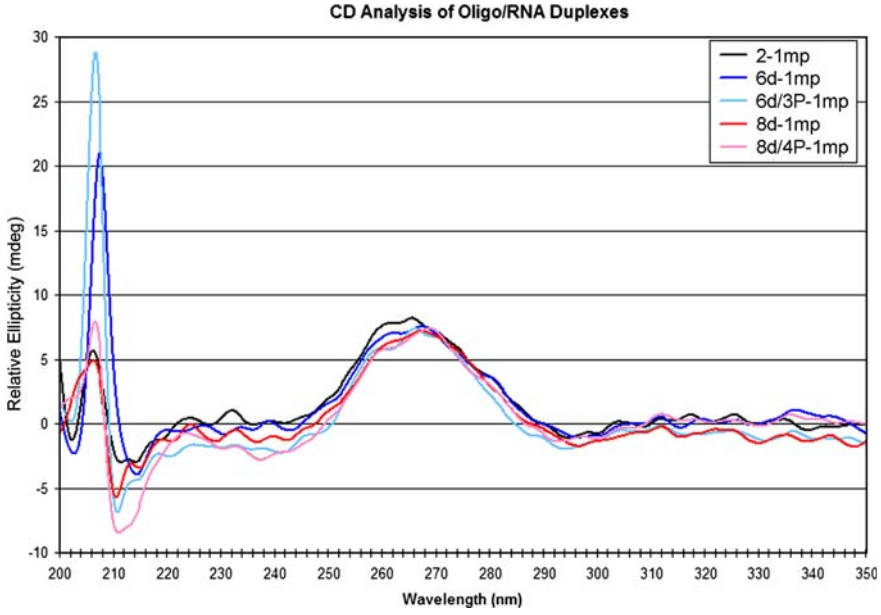


Figure 3. Circular dichroism spectra of **oligonucleotide/c2RNA** duplexes. Spectra were recorded in buffer containing 60 mM Tris–hydrochloride, pH 7.8, 60 mM potassium chloride, and 2.5 mM magnesium chloride at 37 °C.

elipticity at 260 nm and negative elipticity at 210 nm is characteristic of duplexes in the A-type conformation.[25] These results show that although introduction of the propynyl nucleotides stabilizes the hybrid, it does not perturb the overall structure of the duplex to any significant extent.

2.3. Interactions of chimeric oligonucleotides with stem-loop II RNA

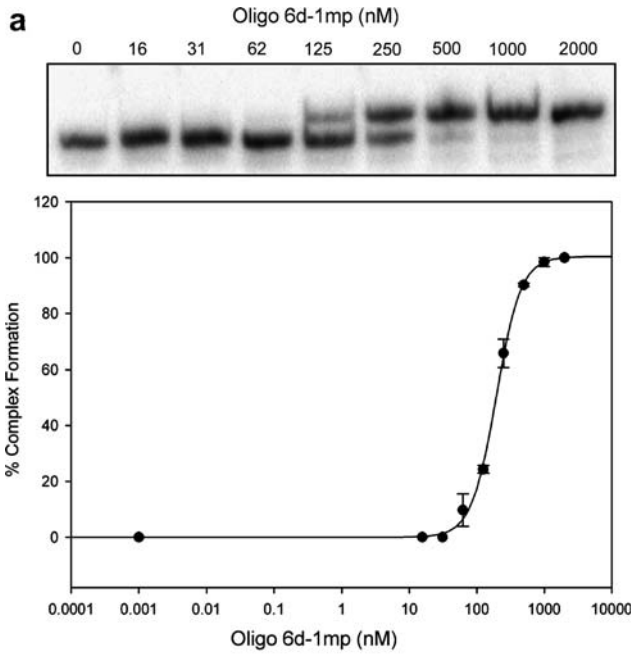
Binding between the chimeric oligonucleotides and RRE stem-loop II RNA was analyzed by electrophoretic mobility shift assays. RRE stem-loop II RNA was prepared by in vitro run-off transcription of a linearized plasmid DNA that contained stem-loop II sequence c-DNA. The resulting RNA was gel-purified, 5′-end labeled with ^{32}P, and incubated with increasing concentrations of oligonucleotides at 37 °C overnight in buffer containing 0.1 M sodium chloride. The oligonucleotide/RNA complexes were separated from free RNA by electrophoresis on non-denaturing gels run at 4 °C. A typical gel and its derived binding isotherm are shown in Figure 4a. Apparent dissociation constants (K_D), which are listed in Figure 4b, were calculated from the binding curve inflection point, which represents the concentration of oligonucleotide required to achieve 50% complex formation.

The chimeric oligonucleotides form complexes with the RRE stem-loop II RNA whose K_D values vary between 185 and 243 nM. These values are 4–5 times higher than the apparent K_D of the **2-1mp/RRE stem-loop II RNA** complex measured under the same conditions.[6] As was the case for the duplexes with **c2RNA**, the binding affinities of the chimeric oligonucleotides that contain 2′-deoxyribo-5-propynylpyrimidines are somewhat higher than those lacking these modified nucleotides.

Although incorporation of deoxyribonucleotides decreases binding affinity, it appears to increase the specificity of binding. Binding experiments between the chimeric oligonucleotides and RRE stem-loop II RNA were repeated at 37 °C in the presence of a 1000-fold molar excess of non-specific competitor yeast tRNA. A specificity ratio was determined as described by Luedtke and Tor,[26] by dividing the apparent K_D obtained in the presence of non-specific competitor tRNA by the apparent K_D obtained in its absence. Oligonucleotides that exhibit highly specific binding would be expected to have specificity ratios near 1.0, whereas those with less specificity would have ratios greater than 1. As shown in Figure 4b, with the exception of **6d/3P-1mp**, the specificity ratios of the chimeric oligonucleotides are very close to 1, and are less than half the value of the specificity ratio of **2-1mp**.

2.4. Oligonucleotide-induced RNase H hydrolysis of RRE stem-loop II RNA

The experiments described above show that the chimeric oligonucleotides are capable of binding to the highly structured RRE stem-loop II RNA target with a high degree of specificity and relatively high affinity under



b

Oligo	K_D(nM)	SEM (nM)	Specificity Ratio
2-1mp	45.9	3.4	3.2
6d-1mp	191.7	8.1	1.3
6d/3P-1mp	171.3	6.5	1.8
8d-1mp	242.7	12.0	1.2
8d/4P-1mp	185.1	10.3	1.3

Figure 4. Interaction of chimeric oligonucleotides with RRE stem-loop II RNA. (a) EMSA gel and associated binding isotherm for **6d-1mp/RRE stem-loop II RNA** complex at 37 °C. (b) Apparent dissociation constants (K_D) of oligonucleotide/RRE stem-loop II RNA complexes. The standard error of the mean values (SEM, $n \geqslant 3$) and specificity ratios (RRE specificity ratio = $K_D^{(RRE+1000\times tRNA)}$/average $K_D^{(RRE)}$) are also shown.

essentially physiological conditions. Binding presumably initiates by interaction of the 3′-end of the oligonucleotide, which consists of 2′-*O*-methylribonucleotide residues, with the 3-nucleotide loop and proceeds via invasion of the base-paired stem. This opens up the stem and positions the deoxyribonucleotide patch on the 3′-side of the stem creating a DNA/RNA hybrid that can be hydrolyzed by RNase H.

The ability of the chimeric oligonucleotides to activate RNase H-mediated hydrolysis of RRE stem-loop II RNA was analyzed in vitro using *Escherichia coli* RNase H1. This enzyme is highly analogous to human RNase H1 and is readily available in purified form.[27,28] ^{32}P-Labeled RRE stem-loop II RNA was preincubated overnight at 37 °C with a large excess of the chimeric oligonucleotides prior to incubation with RNase H. Aliquots were removed at various times and the products analyzed on denaturing polyacrylamide sequencing gels. A typical gel is shown in Figure 5a. The positions of the RNase H mediated hydrolysis products were

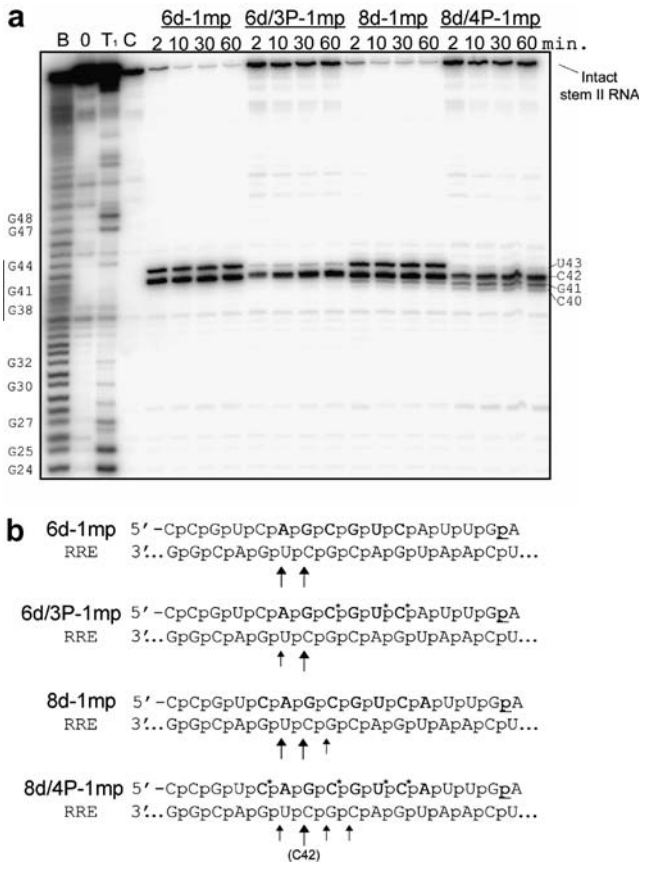


Figure 5. RNase H mediated hydrolysis of RRE stem-loop II RNA. (a) Sequencing gel showing RNase H hydrolysis of RRE stem-loop II RNA in the presence of each of the chimeric oligonucleotides or **2-1mp** (lane C). The RNA was also treated with sodium bicarbonate (lane B); RNase T_1 buffer (lane 0), or RNase T_1 (lane T_1). The positions of Gs identified in T_1 digest are indicated at the left side of the gel, where the vertical line indicates the binding region of the deoxyribonucleotide patch of **6d-1mp**. (b) Hybrids formed between chimeric oligonucleotides and RRE stem-loop II RNA. The 2′-deoxyribonucleotides are in bold and the 5-propynyl modified nucleotides are indicated by an asterisk. The large and small arrows indicate major and minor sites, respectively, of RNase H mediated hydrolysis.

compared to oligonucleotides generated by chemical (sodium bicarbonate) or enzymatic (RNase T_1) digestion of the RRE stem loop II RNA. The positions of the G residues, as provided by the T1 digest, are shown on the left hand side of the gel, and the respective alignment of the complementary deoxyribonucleotide patch is indicated by the vertical line.

Each of the chimeric oligonucleotides formed hybrids that were hydrolyzed by RNase H. As expected, **2-1mp**, which lacks a deoxyribonucleotide patch, failed to form an RNase H cleavable hybrid. The products generated in the presence of the chimeric oligonucleotide correspond to RNase H-induced hydrolysis at positions complementary to the deoxyribonucleotide portion of each oligonucleotide. The main site of hydrolysis in all of these reactions occurs at C42 of the RRE stem-loop II RNA. Oligonucleotides **6d-1mp** and **8d-1mp** also produced a product corresponding to hydrolysis at U43.

Figure 5b shows an alignment of the chimeric oligonucleotides with their RNA target and the corresponding positions of RNase H hydrolysis. The observed positions of hydrolysis are consistent with reported studies where *E. coli* RNase H-mediated cleavage of chimeric-oligo/RNA hybrids occurred at positions opposite the 5′-deoxy junction in the oligonucleotide.[14,29–31] Oligonucleotide **6d-1mp** promotes hydrolysis at both U43 and C42, and the target is almost completely cleaved within 2 min. Substitution of three 5-propynyl nucleotides into this sequence, **6d/3P-1mp**, results in significantly reduced hydrolysis at both sites, and the reaction is only 35% complete after 1 h. Increasing the size of the deoxyribonucleotide patch, as in **8d-1mp**, results in a small amount of hydrolysis at G41, in addition to the major products at U43 and C42. Oligonucleotide **8d-1mp**, like **6d-1mp**, promotes rapid cleavage of the target, and hydrolysis is almost complete within 2 min. Incorporation of four 5-propynyl residues into **8d-1mp** to give **8d/3P-1mp** causes reduced hydrolysis at U43 and C42, but increased hydrolysis at G41, and the appearance of a new product corresponding to hydrolysis at position C40. However overall hydrolysis is still relatively slow compared to that promoted by **8d-1mp**, and the reaction is only 55% complete after 1 h.

There is a very significant reduction in the rates of hydrolysis promoted by the 5-propynyl modified oligonucleotides compared to their unmodified counterparts. Under the conditions of the experiments, all of the accessible RNA target should be bound by the oligonucleotides, and consequently oligonucleotide turnover would not be required in order to achieve complete hydrolysis of the target. In order for hydrolysis to occur, the oligonucleotide must remain bound to the target long enough to allow the enzyme to bind and cleave the hybrid.[32] Gel electrophoresis experiments run at 37 °C suggest that the off-rate of the propynylated oligonucleotides is considerably slower than that of the unmodified chimeric oligonucleotides (data not shown). Therefore, it appears that differences in the rates of dissociation of the hybrid cannot account for the observed differences in the rates of hydrolysis. As evidenced by the CD results, the propynyl groups appear to cause no significant structural changes to the duplex that would result in perturbation of RNase H binding. Furthermore, the 5-propynyl moieties which project into the major groove of the duplex would not be expected to sterically interfere with RNase H binding, which occurs in the minor groove.

Therefore, the reduced RNA hydrolysis by RNase H with the propynyl-containing chimeric oligos most likely reflects a decreased ability of the RNase H enzyme to carry out hydrolysis on the propynyl-containing duplex target. The flexibility of the sugar–phosphate backbone in the DNA/RNA hybrid is thought to be important for accommodating the RNase H cleavage reaction and the concomitant structural changes of enzyme and substrate. Modifications that reduce this flexibility by raising the activation-energy barrier for these structural changes would be expected to decrease the rate of target hydrolysis.[7,33] It seems possible that changes in sugar

conformation, as well as the increased base-stacking interactions provided by the 5-propynyl pyrimidines, could result in a more rigid hybrid with the target RNA that is not readily amenable to the restructuring required for efficient hydrolysis of the RNA strand. Paradoxically then, the properties of the propynyl-modified chimeras which enhance duplex stability with the target adversely affect RNase H catalyzed hydrolysis of the resulting hybrid.

2.5. Stability of chimeric oligonucleotides in serum

Previous studies from our laboratory have shown that oligo-2′-*O*-methylribonucleotides that terminate with a 3′-methylphosphonate linkage, such as **2-1mp**, are remarkably stable to hydrolysis by nuclease activities found in mammalian serum and can be recovered intact from mammalian cells in culture after cationic lipid mediated delivery.[34] This resistance to degradation was attributed to the methylphosphonate linkage, which can prevent hydrolysis by 3′-exonucleases, and the 2′-*O*-methylribose backbone, which appears to be inherently more resistant to nuclease degradation than the 2′-deoxyribose backbone. In order to examine the stability of the chimeric oligonucleotides in serum, the 5′-[^{32}P]-labeled oligonucleotides were converted to their 2-aminoethylphosphoramidate derivatives. This modification prevents removal of the label from the oligonucleotide by phosphatase activities present in the serum. The modified oligonucleotides were incubated with cell culture medium containing 10% fetal calf serum and the products of the reaction were analyzed by denaturing polyacrylamide gel electrophoresis as a function of time. The results are presented in Figure 6.

All of the chimeric oligonucleotides possessed high serum nuclease resistance with half-lives greater than 24 h. This stability, which is similar to that of **2-1mp**, may be compared to that of the unmodified oligodeoxyribonucleotide, **d-T**$_{15}$, whose half-life is less than 30 min under the same conditions. The central patch of six deoxyribonucleotides in **6d-1mp** does not compromise nuclease resistance, nor does incorporation of the propynyl-modified deoxyribonucleotides in **6d/3P-1mp**. However, increasing the size of the patch to 8 deoxyribonucleotides in **8d-1mp** does appear to slightly increase susceptibility to degradation, although the half-life for this oligonucleotide is still greater than 24 h. Interestingly this susceptibility can be diminished by incorporation of the propynyl modified deoxyribopyrimidines as in **8d/3P-1mp**. Overall, these results suggest that the methylphosphonate modified chimeric oligonucleotides are sufficiently stable for use in cell culture experiments.

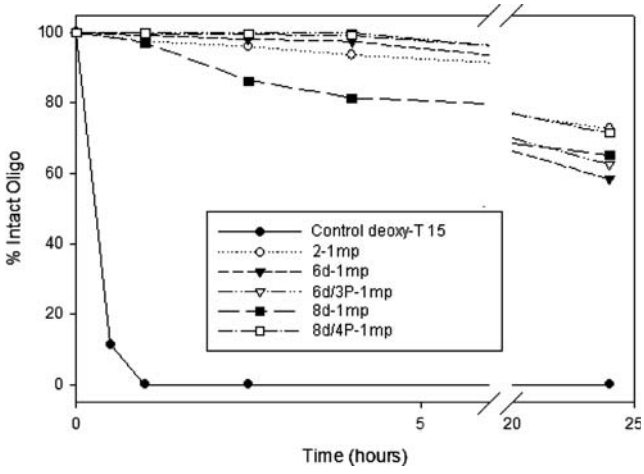


Figure 6. Stabilities of chimeric oligonucleotides in 10% fetal calf serum. The ^{32}-P labeled 5′-(2-aminoethyl)phosphoramidate derivatives of the oligonucleotides were incubated in medium containing 10% serum for the indicated lengths of time. The reaction mixtures were analyzed by polyacrylamide gel electrophoresis and the percent intact oligonucleotide, **dT**$_{15}$ (●), **2-1mp** (○), **6d-1mp** (▼), **6d/3P-1mp** (▽), **8d-1mp** (■), or **8d/3P-1mp** (□), plotted as function of time.

2.6. Inhibition of Rev-dependent reporter gene expression by chimeric oligonucleotides in cell culture

The results described above demonstrate that the anti-RRE chimeric oligonucleotides are capable of promoting RNase H-mediated hydrolysis of RRE stem-loop II RNA and that they are resistant to degradation in serum-containing medium. Of the chimeric oligonucleotides shown in Figure 1b, **6d-1mp** appears to possess the best combination of binding affinity, ability to promote RNase H-directed hydrolysis, and serum stability. Therefore, we next tested the ability of this oligonucleotide to interfere with Rev-dependent CAT gene expression in HEK 293T cells in culture. To carry out these experiments we used a set of mammalian expression vectors originally described by Coburn and Cullen.[35] The expression vector, p-RRE/CAT, contains an intron that encodes sequences for the chloramphenicol acetyl transferase gene (CAT) and the HIV-1 RRE. When expressed in cells in the absence of Rev protein, the resulting transcript remains in the nucleus where the intron is spliced out and degraded. When the cells are cotransfected with p-RRE/CAT and p-REV, a vector that encodes Rev protein, the RRE/CAT transcript is transported to the cytoplasm where it is translated.

Cells were transfected with a Lipofectamine 2000 complex of p-RRE/CAT and p-REV in the presence of increasing concentrations of **6d-1mp** complexed with Oligofectamine. As a control, we also tested **6dr-1mp**, a chimeric oligonucleotide preparation whose patch of 6 deoxyribonucleotides consists of random sequences. Transfection was allowed to proceed for 4 h. The medium was then removed and fresh medium lacking both the vectors and the oligonucleotide was added. Incubation was continued for 16 hrs, at which time the cells were lysed and the lysate was assayed for CAT activity. The CAT activity at each oligonucleotide concentration was normalized to the number of cells used and was compared to the CAT activity in cells not exposed to the oligonucleotide. The results are shown in Figure 7.

As shown in Figure 7, **6d-1mp** inhibits CAT expression in the HEK 293 T cells in a dose dependent manner over the concentration range of 10–300 nM oligonucleotide, whereas inhibition is not seen in the presence of the randomized oligomer, **6dr-1mp**, over the same

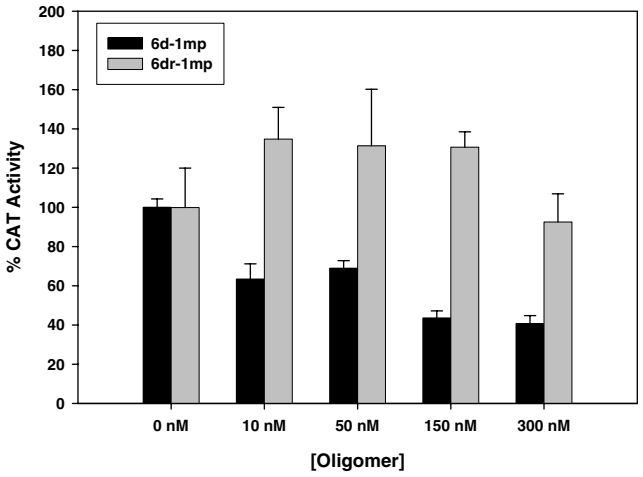


Figure 7. Effects of chimeric oligonucleotides on RRE-mediated CAT expression in HEK 293T cells in culture. HEK 293T cells were transfected with increasing concentrations of an Oligofectamine complex of **6d-1mp** or **6dr-1mp** and a Lipofectamine complex of expression plasmids p-RRE/CAT and p-REV for 4 h at 37 °C, followed by a 16-h incubation with fresh medium containing 10% fetal bovine serum. Cell lysates were prepared and assayed for CAT expression and for protein concentration. CAT expression was normalized to the protein concentration. The results are the average obtained from two complete sets of experiments, in which each oligomer concentration was carried out in triplicate.

concentration range. These results suggest that the observed inhibition is dependent upon the oligonucleotide sequence and is not simply due to interference with the transfection process by the chimeric oligonucleotide/Oligofectamine complex.

The level of inhibition observed for **6d-1mp** is very similar to that seen previously for **2-1mp**, a methylphosphonate modified oligo-2′-*O*-methylribonucleotide that can sterically block Rev/RRE interaction, but which cannot support RNase H-mediated target degradation.[6] In theory, **6-1mp** could inhibit CAT expression by both sterically blocking Rev protein/RRE interactions and by degrading its RRE target. However, given the 4-fold lower binding affinity of **6d-1mp** versus **2-1mp** for RRE stem-loop II RNA, it seems unlikely that the chimeric oligonucleotide could inhibit as effectively as **2-1mp** by a strictly steric blocking mechanism. The observation that **6d-1mp** inhibits as well as **2-1mp** at comparable concentrations therefore suggests that the chimeric oligonucleotide functions at least in part by the alternative RNase H-mediated degradation pathway.

3. Conclusion

The Rev response element of HIV-1 is a particularly attractive therapeutic target because mutations in this region of the HIV genome result in loss of virus viability.[4,5] Consequently the virus would not be expected to be able to circumvent the anti-viral activities of drugs that target this region by forming 'escape mutants', a problem that is encountered with drugs that target viral activities such as reverse transcriptase or protease. Unlike more conventional therapeutics, it is relatively straightforward to design antisense oligonucleotides that can interact directly with RRE RNA. The studies presented here show that anti-RRE chimeric oligonucleotides can be designed that take advantage of the relatively high binding affinities of the 2′-*O*-methylribonucleotide backbone and the nuclease resistance imparted by a 3′-terminal methylphosphonate linkage. Oligonucleotides of this type can function essentially in a catalytic manner and thus have the potential of being effective inhibitors, even at low concentrations. Delivery of antisense oligonucleotides to cells in culture and in vivo remains an important problem. Although cationic lipids proved to be effective in the experiments reported here, they are cumbersome to work with, can be somewhat toxic to cells, and are not applicable at the organism level. Alternative strategies in which the antisense oligonucleotides are conjugated to cell penetrating peptides[36–40] are currently being investigated.[34] With the advent of more effective delivery systems, chimeric oligonucleotides of the type described here could have potential therapeutic applications.

4. Experimental

Protected 2′-*O*-methylribonucleoside-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropylamino) phosphoramidites, protected ribonucleoside β-cyanoethylphosphoramidites, 5′ nucleoside-derivatized controlled pore glass supports, 4,5-dicyanoimidazole, 1-*H*-tetrazole, Cap Mix A, Cap Mix B, and oxidizing solution were purchased from Glen Research Inc., Sterling, VA. Protected 2′-*O*-methylribonucleoside-3′-*O*-(*N*,*N*-diisopropylamino) methylphosphonamidites were purchased from ChemGenes Corp., Ashland, MA. The exocyclic amino groups of the C and G amidite reagents were protected with acetyl and isobutyl protecting groups, respectively. 5-Ethylthio-tetrazole was purchased from VWR. In vitro transcription of RNA was performed using components from Promega's Riboprobe-System SP6. Plasmid sequencing was done by the Johns Hopkins University School of Medicine Department of Biological Chemistry Synthesis and Sequencing Facility. MALDI analysis was performed in the AB Mass Spectrometry/Proteomics Facility at Johns Hopkins School of Medicine (www.hopkinsmedicine.org/msf/) with support from a National Center for Research Resources shared instrumentation Grant 1S10-RR14702. OPTI-MEM and DMEM cell culture media were obtained from GIBCO, Inc.; Lipofectamine 2000 and Oligofectamine were purchased from Invitrogen, Inc.; reporter lysis buffer, β-GAL assay buffer, and *n*-butyl CoA were purchased from Promega, Inc.; the BCA protein assay kit was purchased from Pierce Chemical Co.; and [^{14}C]-chloramphenicol was purchased from Amersham/GE Health Care Products, Inc. The mammalian expression vectors used to examine the effect of the anti-RRE oligonucleotide on CAT expression were a generous gift from Dr. Brian Cullen, Duke University: p-RRE/CAT, original name pDM128/RRE; p-REV, original name pcREV; and p-IL-2, original name pBC12/CMV.[35]

4.1. Oligonucleotide synthesis

The oligonucleotides, whose sequences are shown in Figure 1b, were synthesized on an ABI Model 392 DNA/RNA synthesizer using phosphoramidite procedures similar to those used previously to prepare psoralen-conjugated chimeric oligonucleoside methylphosphonates.[41] The concentrations of the protected nucleoside methylphosphonamidite and nucleoside β-cyanoethylphosphoramidite solutions were 0.15 M, the coupling time was 120 s, and the activating agent was 0.25 M 4,5-dicyanoimidazole. The methylphosphonate containing oligonucleotides were removed from the support by incubation in 0.4 mL of concentrated ammonium hydroxide for 2 h. at room temperature and the residue obtained after evaporation of the supernatant was treated with a solution containing 10 μL of water, 22.5 μL of acetonitrile, 22.5 μL of 95% ethanol, and 50 μL of ethylenediamine for 6 h at room temperature. The solution was neutralized with 600 μL of ice-cold 2 N hydrochloric acid and desalted using C-18 SEP PAK. Oligonucleotides **6d-1mp**, **6d/3P-1mp**, **8d-1mp**, and **8d/4P-1mp** were each purified by HPLC on a Dionex DNAPac PA-100 strong anion exchange column using a linear gradient of 0.0–0.5 M sodium chloride in a solution containing 10% acetonitrile in 0.1 M Tris, pH 7.8. The purified oligonucleotides were desalted on C-18 SEP PAK cartridges and analyzed by MALDI-TOF mass spectrometry: **6d-1mp** (*m*/*z*: calcd 5096.9, found 5095.5); **6d/3P-1mp** (*m*/*z*: calcd 5210.9, found 5210.8); **8d-1mp** (*m*/*z*: calcd 5036.9, found 5036.7); **8d/4P-1mp** (*m*/*z*: calcd 5188.9, found 5188.5). Oligonucleotide extinction coefficients were calculated using program based on nearest neighbor interactions.[42]

The RNA target used for thermal melting analysis, **c2RNA**, was synthesized using standard automated β-cyanoethylphosphoramidite chemistry. The concentration of the protected ribonucleoside β-cyanoethylphosphoramidites was 0.1 M, the coupling time was 450 s, and the activator was 5-ethylthiotetrazole. The oligoribonucleotide was partially deprotected by incubating the support with 0.4 mL of a solution containing concentrated ammonium hydroxide/95% ethanol (3:1) ratio for 24 h. at room temperature and 3 h at 55 °C. The supernatant was evaporated and the residue was treated with 150 μL of triethylamine-trihydrofluoride at room temperature for 24 h to remove the *t*-butyldimethylsilyl groups. The deprotected oligomer was precipitated by addition of 400 μL methanol, collected by centrifugation, and the pellet was washed twice with 400 μL of methanol. The oligoribonucleotide was purified by SAX HPLC using a 30-min linear gradient of 0.0–0.6 M ammonium chloride in buffer containing 25 mM HEPES, pH 6.8, and 0.5% acetonitrile, at a flow rate of 1.5 mL/min. The purified oligonucleotide was desalted on a C-18 SEP PAK.

4.2. Oligonucleotide 5′-(2-aminoethyl)phosphoramidates

Oligonucleotides **dT_{15}**, **2-1mp**, **6d-1mp**, **6d/3P-1mp**, **8d-1mp**, and **8d/4P-1mp** were each converted to their 5′-aminoethylphosphoramidate derivatives as follows. Each oligonucleotide (200 nmol) was incubated with 5 units of T_4 polynucleotide kinase in 10 μL of buffer that contained 60 μM γ-^{32}P-ATP (specific activity, 100 Ci/mmol), 50 mM Tris, pH 7.6, 10 mM magnesium chloride, and 10 mM mercaptoethanol for 60 min at 37 °C. A 14 μL aliquot of each reaction solution was diluted with 140 μL of 0.1 M imidazole buffer, pH 6.0, and 16 μL of 1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and the solution was incubated at room temperature for 4 h, after which 6.4 μL of ethylenediamine and 13 μL of 12 N hydrochloric acid were added. The pH of the solution was adjusted to 7.4 by further addition of hydrochloric acid and the solution was incubated overnight at room temperature. The 5′-aminoethylphosphoramidite derivatized oligonucleotides were desalted on G-25 Microspin columns purified by electrophoresis on 20 × 20 × 0.75 cm gels containing 20% acrylamide, 7 M urea, 90 mM Tris, 90 mM boric acid, and 0.2 mM ethylenediamine-tetraacetate buffered at pH 8 (TBE). The oligonucleotides were extracted from the gel by incubation with 1 mL of 0.1 M ammonium acetate in 20% aqueous acetonitrile overnight at 37 °C and then desalted by G-25 Sephadex microspin columns.

4.3. Synthesis and characterization of RRE stem-loop II RNA

RRE stem-loop II RNA (see Fig. 1a) was prepared by in vitro transcription run-off of an EcoR1 cut pGEM3Z plasmid (Promega, Inc.) containing RRE template DNA[6] Polymerase slip at the 3′-end produced two transcripts 86 and 87 nucleotides in length both of which contained the 69-nt RRE RNA sequence (underlined): 5′-GAAUACUCAAGCUAAGCACUAUGGGCGCAGCGUCAAUGACGCUGACGGUACAGGCCAGACAAUUAU-UGUCUGGUUAGUGCAGAAUUX-3′ (where X denotes the nucleotide added by polymerase slip). Because there were no differences in oligonucleotide binding affinity between the two transcription products (data not shown), the two transcripts were purified as one on a 12% denaturing polyacrylamide gel run at 800 V until the loading dye had run off the bottom. However, for clarity in the RNase H assay, the two transcripts were separated on an 18 × 32 cm 12% polyacrylamide gel run under denaturing conditions at 400 V for 24 h. Only the shorter transcript was used for the RNase H experiments. Each transcript was characterized by digestion with sodium bicarbonate, pH 9.0, and by T_1 enzyme digestion, and were found to be identical with the exception of the additional 3′ nucleotide on the longer transcript.[6] The pattern of oligonucleotides seen in the T_1 digests also agreed with the secondary structure predicted by M-fold.[43]

4.4. Thermal denaturation experiments

A solution containing 1 μM each of **c2RNA** and the oligonucleotide in 1 mL of buffer containing 50 mM MOPS (3-(N-morpholino)propane sulfonic acid), pH 7.0, and 100 mM sodium chloride was heated at 65 °C for 15 min and slow cooled to room temperature. Absorbance versus temperature measurements were carried out using a thermostat-controlled Cary 3 E UV–visible spectrophotometer (Varian). The samples were

heated at a rate of 0.4 °C/min. and data were collected at 0.25 °C intervals. Melting temperatures (T_m) were determined from the first derivative of the data sets.

4.5. Circular dichroism (CD) spectra of oligonucleotide/c2RNA duplexes

Solutions containing 1 μM anti-RRE oligonucleotides and 1 μM **c2RNA** in 110 μL of buffer containing 60 mM Tris–hydrochloride, pH 7.8, 60 mM potassium chloride, and 2.5 mM magnesium chloride were heated at 90 °C for 2 min and slow cooled to 37 °C. CD spectra were recorded at 37 °C between 200 and 350 nm on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature controller.

4.6. Oligonucleotide binding studies

Oligonucleotide binding studies were carried out in 10 μL solutions containing 1 nM ^{32}P-labeled RRE stem-loop II RNA, 0-2 μM oligonucleotide, 100 mM sodium chloride, 10 mM Tris, pH 7.5, and 0.5 mM EDTA. The solutions were incubated overnight at 37 °C and after addition of 1 μL of 80% glycerol, electrophoresed on an 8% nondenaturing polyacrylamide gel run at 4 °C and 200 V for 5.5 h. Phosphorimage analysis (Image Quant, Molecular Dynamics) of the dried gels was used to quantitate percent complex formation for each oligonucleotide concentration. Binding data obtained from at least three experiments were plotted as percent complex formation versus the log of oligonucleotide concentration, and fit to a 3-parameter Hill equation (Sigma Plot) to determine the apparent dissociation constant (K_D) for each oligonucleotide as well as standard error of the mean (SEM, $n \geqslant 3$). Binding specificity was determined by repeating the binding experiments in the presence of 1000-fold molar excess of yeast tRNA. Specificity ratios were calculated by dividing the K_D in the presence of the yeast tRNA by the K_D in its absence (RRE specificity ratio $= K_D^{(RRE+1000\times tRNA)}/K_D^{(RRE)}$).

4.7. RNase H experiments

A protocol adapted from Galarneu et al.[27] was followed. Reaction mixtures (25 μL) containing 1 nM ^{32}P-labeled RRE RNA and 800 nM oligonucleotide in 60 mM Tris–hydrochloride, pH 7.8, 60 mM potassium chloride, and 2.5 mM magnesium chloride were equilibrated overnight at 37 °C after which 1.25 units of *E. coli* RNase H enzyme were added and incubation was continued at 37 °C. Aliquots (5 μL) were removed at the indicated time; inactivated by the addition of 5 μL of denaturing gel loading buffer containing 10 mM EDTA, and then stored at −20 °C. The reaction products were analyzed by electrophoresis on a 20% denaturing sequencing gel run at 1800 V, until the xylene cyanol dye ran 22 cm into the gel. Phosphorimage analysis of the dried gels was used to quantitate the reaction products.

4.8. Oligonucleotide stability in serum

The [^{32}P]-labeled 5′-aminoethylphosphoramidate derivatized oligonucleotides (1.1×10^5 cpm) were each dissolved in 10 μL of RPMI medium supplemented with 10% fetal calf serum and incubated at 37 °C. One microliter aliquots were removed at various times, diluted into 4 μL of 90% formamide gel loading buffer, and analyzed on a 20% denaturing polyacrylamide gel. Phosphorimage analysis (Image Quant, Molecular Dynamics) of the gels was used to quantitate the percentage of intact oligonucleotide for each time point.

4.9. Inhibition of RRE-mediated CAT expression in HEK 293T cells

Two milliliter of a 2×10^5 cells/mL suspension of HEK 293T cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM/FCS) was seeded into 35 mm six-well culture plates. Three wells were seeded for each concentration of oligonucleotide. The plates were incubated for 2 days at 37 °C in a 5% CO_2 atmosphere at which point the cells were approximately 75% confluent. On the day of the experiment, a vector cocktail containing 650 ng of p-RRE/CAT, 1950 ng of p-REV, and 2535 ng of p-IL-2 in 130 μL of TE buffer was prepared. A 97.5 μL aliquot of the vector cocktail was diluted into 4778 μL of OPTI-MEM. A solution containing 38.6 μL of Lipofectamine 2000 and 4836 μL of OPTI-MEM was incubated for 5 min and mixed with the vector cocktail/OPTI-MEM solution, after which incubation was continued for an additional 20 min at room temperature. Solutions were prepared that contained 0, 60, 300, 600 or 1200 nmol of oligonucleotide in 540 μL of OPTI-MEM. A solution containing 144 μL of Oligofectamine and 216 μL of OPTI-MEM was incubated for 10 min at room temperature and 60 μL aliquots of this solution were then added to each oligonucleotide solution, after which the solutions were incubated for 20 min at room temperature. The medium was removed from the cells and 1.3 mL of OPTI-MEM was added followed by 200 μL of the oligonucleotide/Oligofectamine solution and 500 μL of the vector cocktail/Lipofectamine solution. The cells were incubated for 4 h at 37 °C in a 5% CO_2 atmosphere, the medium was removed, the cells were washed once with 2 mL of DMEM/FCS, 2 mL DMEM/FCS was added, and incubation was continued overnight. The medium was removed from the cells, the cells were washed with 2 mL of calcium/magnesium-free phosphate-buffered saline (PBS), and lysed with 400 μL of reporter lysis buffer for 15 min at room temperature. The lysates were transferred to 1.5 mL Eppendorf tubes on ice, vortexed for 15 s, and then centrifuged at 13,000 rpm for 3 min at 4 °C. The supernatants were transferred to new Eppendorf tubes, frozen on dry ice, and stored at −20 °C. Protein assays were carried out on 10 μL of lysate using a BCA protein assay kit. Assays for CAT expression were carried out on 50 μL of cell lysate as follows. The lysates were diluted with 75 μL of a solution containing 25 μg of *n*-butyl CoA and 0.086 μCi of [^{14}C]-chloramphenicol, specific activity 59 Ci/mmol, and incubated for 2 h at 37 °C. The reaction mixture was then extracted with 300 μL of mixed xylenes and the xylene extract was extracted with 100 μL of 0.25 M Tris, pH 8.0. A 150 μL aliquot of the xylene solution was counted in a liquid scintillation counter. The counts

were normalized to the amount of protein present in each lysate.

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