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Antisense-Induced Guanine Quadruplexes Inhibit Reverse Transcription by HIV-1 Reverse Transcriptase

Masaki Hagihara, Lisa Yamauchi, Akiko Seo, Keisuke Yoneda, Mayo Senda, and Kazuhiko Nakatani*

Department of Regulatory Bioorganic Chemistry, The Institute of Scientific and Industrial Research, Osaka University, Ibaraki 567-0047, Osaka, Japan

Received April 15, 2010; E-mail: nakatani@sanken.osaka-u.ac.jp

Abstract: Guanine quadruplex structures in DNA and RNA affect normal cellular processes such as replication, recombination, and translation. Thus, controlling guanine quadruplex structures could make it possible to manipulate the biological function of nucleic acids. Here, we report a novel antisense strategy using guanine-tethered antisense oligonucleotides (g-ASs) that introduces an RNA−DNA heteroquadruplex structure on RNA templates in a predictable and sequence-specific manner, which in practice effectively inhibited reverse transcription on a variety of RNA sequences, including the HIV-1 RNA genome. Reverse transcriptase-mediated enzymatic analysis, together with other biophysical analyses, elucidated a cooperative binding of duplex and quadruplex in g-AS−RNA complexes. The remarkable ability of g-ASs to inhibit reverse transcription could make possible the development of novel anti-retroviral gene therapies based on blocking the replication of RNA genomes to complementary DNA, which is a critical step for integration into the host's genome.

Introduction

Regulation of gene expression by short oligonucleotides (antisenses) is a promising approach for gene therapies that perturb RNA structures and inhibit subsequent association with the translation machinery.[1,2] At present, various mRNAs, such as Bcl-2,[3,4] H-ras,[5] and PKC-α,[6] have been targeted by antisenses for potential clinical use, because overexpression of these proteins has been implicated as playing a crucial role in the maintenance of several types of cancers. In fact, antisenses that specifically bound to mRNA of these proteins suppressed protein translation. Decreasing intratumoral protein levels thereby resulted in the inhibition of tumor cell growth, migration, and tumor angiogenesis.

Over the past decades, numerous modified antisenses have been developed to improve gene regulation efficiency by enhancing the resistance of nucleases and hybridization efficiency.[7,8] Chemically reactive antisenses can induce an irreversible covalent cross-link with target mRNAs and thereby effectively inhibit protein expression. In addition, photoreactive antisenses can irreversibly react with and/or induce site-directed cleavage of mRNAs upon irradiation.[9−11] Another class of bifunctional antisenses has been recently reported that anchored exonic splicing enhancers in the antisenses and successfully recruited positively acting factors to mimic splicing reactions *in vitro* and *in vivo*.[12]

Intrinsic secondary structures in DNA/RNA produced by runs of guanines, e.g., guanine quadruplex structures, showed a significant effect on biological processes, including translation[13,14] and reverse transcription.[15,16] Besides being observed in native DNA/RNA, guanine quadruplex structures are found in artificial nucleic acids receptors (aptamers). It has recently been reported that folding guanine-rich DNA aptamers into intrastrand quadruplex structures suppressed HIV-1 reverse transcriptase (*RTase*) activity *in vitro* with high affinity.[17,18] Thus, controlled

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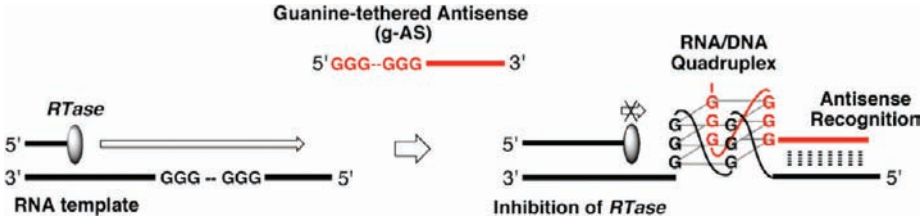

Figure 1. Inhibition of reverse transcription by an antisense-induced RNA−DNA quadruplex. A guanine-tethered antisense (g-AS, shown in red) hybridized to a target RNA, and then contiguous guanines in both the RNA and the g-AS associated to form a quadruplex that inhibited *RTase* reaction.

introduction of quadruplex structures in DNAs/RNAs would provide opportunities for potential regulation of biological processes.

Here, we report a novel antisense strategy using guanine-tethered antisense oligonucleotides (g-ASs) that introduce an RNA−DNA heteroquadruplex structure[19] at a designated sequence on RNA targets. We designed g-ASs that consisted of two functionally independent domains: one was an antisense domain that bound to the target RNA at its binding site like conventional antisense DNAs, and the other was a contiguous guanine run at the 5′-end of the antisense domain that assembled into heteroquadruplex structures together with a guanine-rich region in target RNAs. Combination of antisense recognition and self-assembly of contiguous guanines afforded predictable and sequence-specific DNA−RNA heteroquadruplex structures, which in practice effectively inhibited reverse transcription on a variety of RNA sequences, including the HIV-1 RNA genome (Figure 1).

Results and Discussion

g-ASs Confer Particular Structures That Interfere with *RTases*. Quadruplex structures generally require four runs of as few as three contiguous guanines by self-assembly through Hoogsteen hydrogen bonds between each of the four guanine bases in the presence of potassium cation.[20−22] First, we used an RNA template containing two runs of three contiguous guanines in 5′-**GGG**UUA**GGG**-3′ to see whether quadruplexes can be organized by simply mixing with a g-AS and to determine the effect of quadruplexes on reverse transcription by a *RTase*-mediated stop assay (Figure 1). In the *RTase* stop assay, an *RTase* proceeds along RNA templates until the enzyme encounters stable RNA structures. Inhibition of the *RTase* reaction on the RNA templates results in the production of truncated complementary DNA products, which can be detected by polyacrylamide gel electrophoresis analyses.[15]

Addition of a g-AS containing five guanines (**GGGGG**-AS) at 10 μM did not produce paused bands (Figure 2a, lane 7), whereas those containing six and seven contiguous guanines (**GGGGGG**-AS and **GGGGGGG**-AS, respectively) did at the 3′-side of the GGG region in the RNA template, with faint bands around a hybridization region (lanes 8 and 9). Substitution of two guanines in the seven contiguous guanines by two thymines, leaving only one GGG (**GT**GGG**TG**-AS), resulted in the complete disappearance of the paused products (lane 10), whereas substitution of one guanine by one thymine (**GGG**T**GGG**-AS), leaving two GGGs, effectively blocked the *RTase* reaction (lane 11). The length of a thymine spacer between two GGGs sensitively affected the amount of paused bands (lanes 12−14). The antisense and the contiguous guanines needed to be covalently connected to exert sequence-specific inhibitory effects on reverse transcription (Figure 2b). In fact, no apparent paused band derived from inhibition of reverse transcription was observed in the presence of either 10 μM contiguous guanine region (5′-**GGG**T**GGG**-3′) lacking the antisense sequence (lane 8) or a 1:1 mixture of the guanine region and the AS domain (lane 9).

We next evaluated whether the stability of RNA−DNA heteroduplexes affected the inhibitory effect on reverse transcription by sequential deletion of an antisense domain from the 3′-end, with the guanine-rich region (5′-**GGG**T**GGG**-3′) kept unchanged (Figure 2c). With fewer than a nine nucleotides in the antisense domain (lanes 12−20), no paused band was observed at either the guanine-rich region or the antisense region. A paused band at the guanine-rich region appeared starting with a 9-nucleotide antisense domain (lane 10) and became most intense with g-AS-T1 (lane 6), having a 14-nucleotide sequence.

Titration experiments with the g-AS-T1 showed a concentration-dependent inhibition of reverse transcription (Figure 2d). With the RNA template (0.3 μM), addition of g-AS-T1 at 0.3 μM produced only a faint paused band (lane 7). Increasing the concentration of the g-AS-T1 clearly resulted in the formation of paused band (lanes 8 and 9), and addition of 3 μM g-AS-T1 completely interfered with the DNA synthesis by *RTases*, leading to disappearance of the full-length products (lane 10). Taken together, these data suggest that inhibition of reverse transcription could be attributed to formation of stable RNAs-g-AS and association of four GGG units in close proximity and that a covalent connection of the antisense domain and the guanine-rich region was essential for the effective inhibition of reverse transcription.

Association of Four GGG Units Induces a Putative Guanine Quadruplex Formation. A possible induced structure on RNA templates by association of g-ASs was investigated by spectroscopic analyses of the model assembly of RNA−DNA (Figure 3). Circular dichroism (CD) spectra of an RNA−DNA hybrid lacking guanine regions (rG0 and dG0) exhibited a typical A-form structure, showing a broad positive peak around 270 nm and a weak negative peak around 250 nm (Figure 3a, red line).[23] On the other hand, the spectra of the RNA−DNA hybrid involving two runs of GGG sequence in each strand (rG2 and dG2) showed a strong positive peak at 270 nm and a weak negative peak at 240 nm, characteristic of a parallel quadruplex formation.[24]

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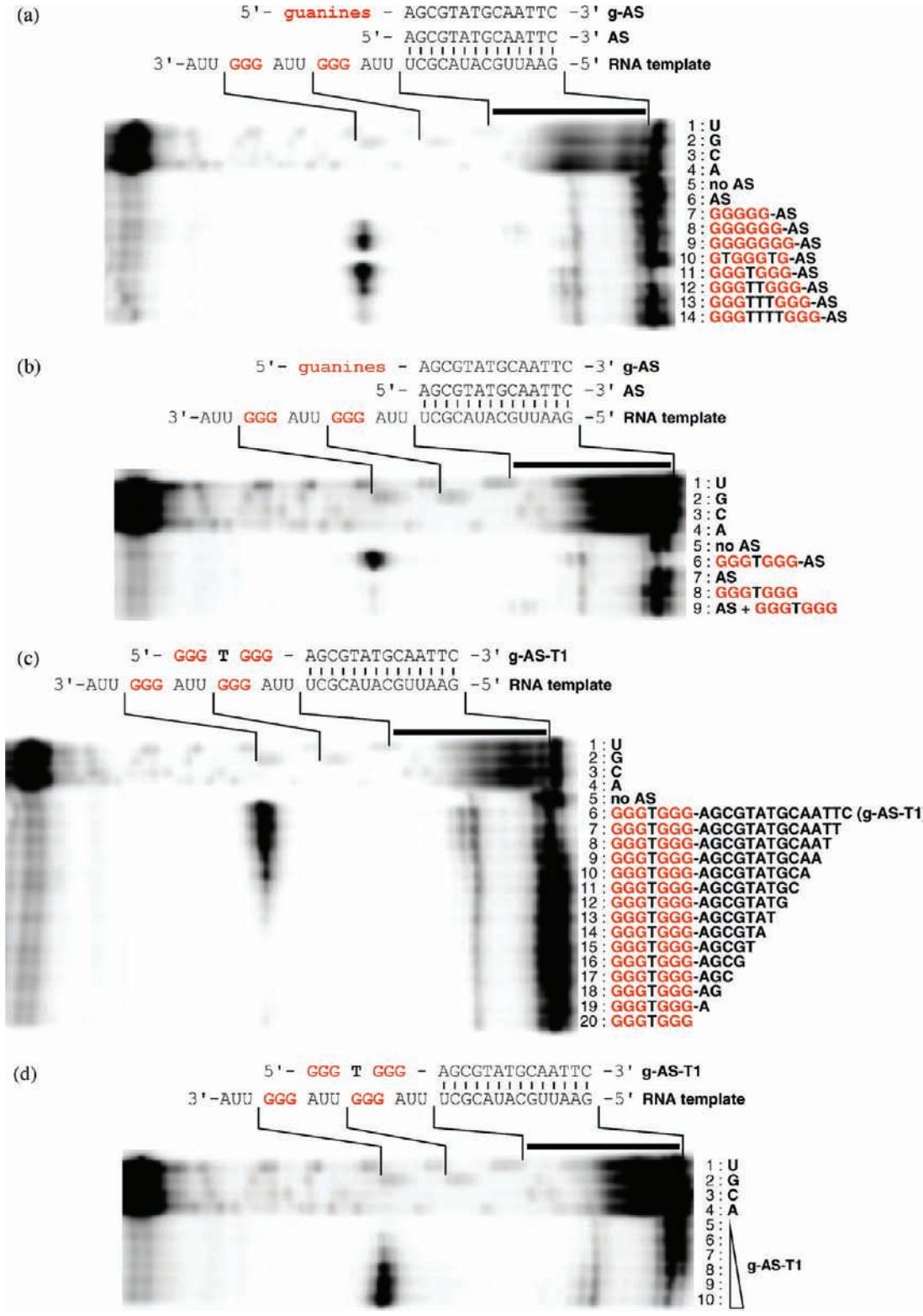

Figure 2. (a) Guanine-tethered antisense-inhibited reverse transcription of an RNA template bearing two runs of the GGG sequence. (b) The necessary covalent linkage of the AS and the guanine-rich region. (c) Effects of the length of antisenses on the inhibition of reverse transcription. (d) Concentration-dependent inhibition of reverse transcription by addition of g-AS-T1 (0, 0.1, 0.3, 1, 3, and 10 μM). An RNA−AS heteroduplex sequence is shown in the figure. A bold line indicates the AS hybridization site. Lane markers U, G, C, and A indicate the bases on the template RNA.

Analysis of the melting of RNA−DNA hybrids by spectral changes at 260 and 295 nm demonstrated the formation of duplexes and quadruplexes, respectively.[25] The rG0-dG0 hybrid showed a duplex melting at 45.9 ± 0.3 °C (Figure 3b, red line). The melting temperature (T_m) of the rG2-dG2 hybrid measured at 260 nm increased to 56.8 ± 0.3 °C (black line), which was almost identical to that of the quadruplex (60.9 ± 0.3 °C) measured at 295 nm (Figure 3c, black line), suggesting a

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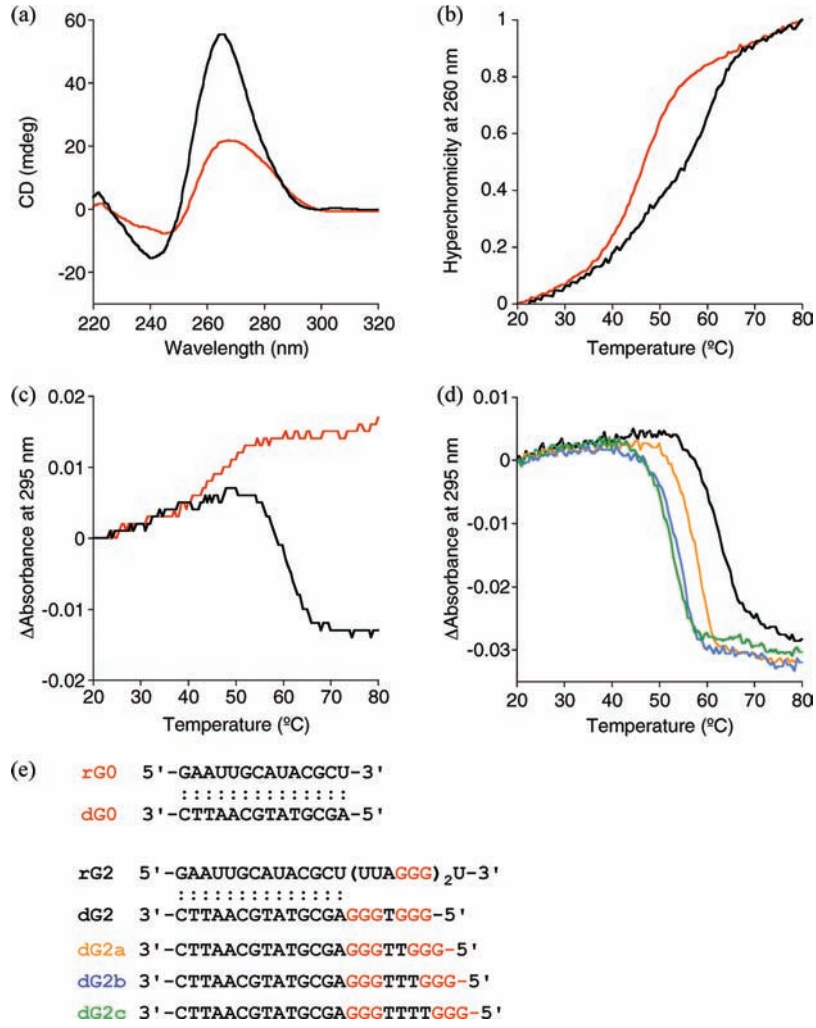

Figure 3. Biophysical properties of RNA−DNA hybrids modified with runs of contiguous guanines. (a) CD spectra of rG0 and dG0 (red) and rG2 and dG2 (black). UV−melting profile of RNA−DNA hybrids measured at (b) 260 and (c) 295 nm. (d) UV−melting profile of the RNA−DNA hybrids with various thymine spacers measured at 295 nm. Key: rG2-dG2, black; rG2-dG2a, orange; rG2-dG2b, blue; rG2-dG2c, green. (e) Oligonucleotide sequences used in these analyses.

cooperative melting of the duplex and the quadruplex in the rG2-dG2 hybrids.

To evaluate the relationship between the formation of DNA−RNA heteroquadruplexes and sequences in the guanine-rich region, we carried out the T_m measurement at 295 nm with g-ASs having various thymine spacers between the guanine runs (Figure 3d). The analyses proved that the melting temperatures of quadruplexes sensitively depend on the length of the thymine spacers, with the stability of the quadruplex decreasing in the order **GGG**T**GGG**-AS (62.9 ± 0.3 °C), **GGG**TT**GGG**-AS (58.0 ± 0.2 °C), **GGG**TTT**GGG**-AS (54.4 ± 0.5 °C), and **GGG**TTTT**GGG**-AS (52.8 ± 0.3 °C). This order was consistent with the results obtained by the *RTase* stop assay (Figure 2a, lanes 11−14).

g-ASs Interfere with Reverse Transcription on Various RNA Templates. Our concept of g-ASs is based on the cooperative formation of DNA−RNA heteroquadruplexes with the assistance of hybridization of antisenses. The formation of heteroquadruplexes between g-ASs and RNA templates may not be limited to two GGGs in each strand. If different combinations were effective, the g-AS concept would be applicable to a variety of RNA sequences. To investigate the g-AS-induced inhibition of reverse transcription on a variety of RNA sequences, we next tested an RNA template containing three GGG sequences (5′-**GGG**UUA**GGG**UUA**GGG**-3′) (Figure 4). Addition of a g-AS containing one and two contiguous guanines (**G**-AS and **GG**-AS, respectively) at 10 μM did not produce paused bands (Figure 4b, lanes 7 and 8), whereas that containing three contiguous guanines (**GGG**-AS) markedly produced paused bands at the 3′-side of the GGG region in the RNA template, with faint bands around a hybridization region (lane 9). Substitution of one guanine in the middle of GGG by a thymine (**GT G**-AS) resulted in the complete disappearance of the paused products (lane 10). The length of thymine spacer between the GGG sequence and the antisense did not significantly affect the amount of paused bands (lanes 11−15). A covalent linkage between the guanine-rich region and the antisense domain also was found to be essential for the efficient inhibition of reverse transcription in heteroquadruplexes involving a combination of one GGG run in the antisense and three GGG runs in the RNA strand (Figure 4c).

An opposite combination of four GGG—one GGG on the RNA template and three on the g-AS—was further investigated for the inhibition of the *RTase* reaction (Figures 5). Addition of a g-AS containing nine contiguous guanines (**GGGGGGGGG**-AS) at 10 μM did not produce paused bands (Figure 5b, lane

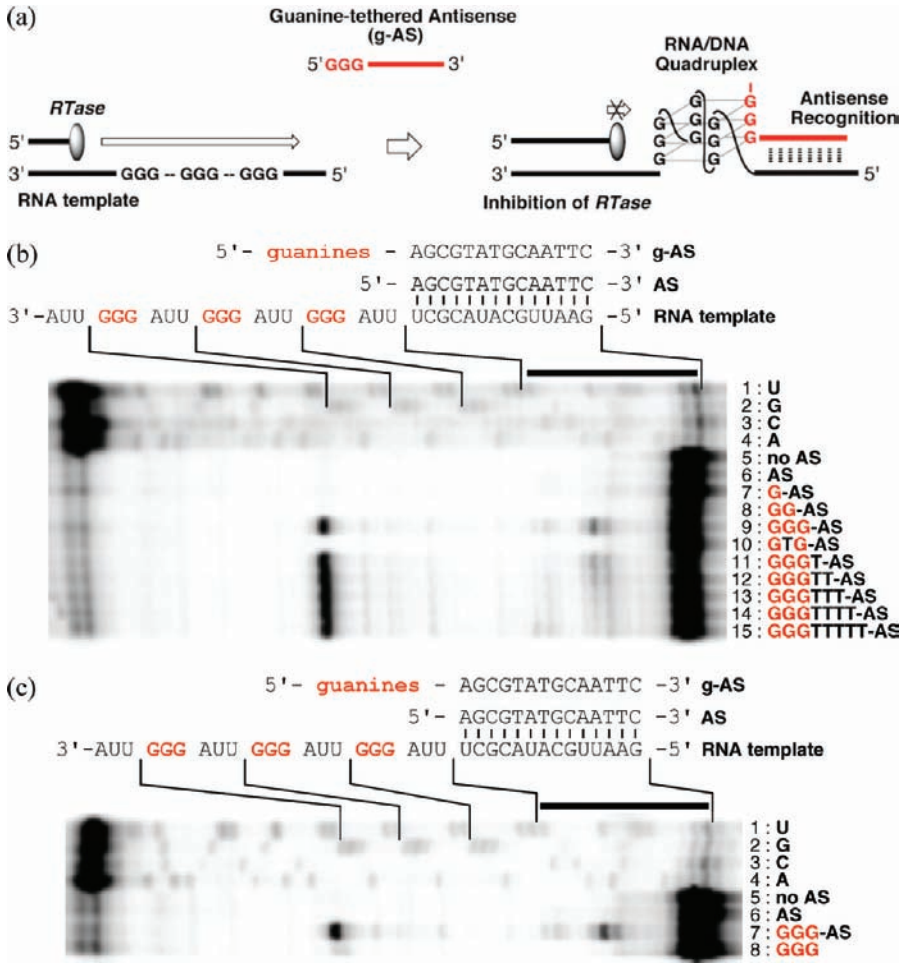

Figure 4. (a) Antisense-induced RNA−DNA quadruplex involving three runs of GGG sequence in the RNA strand and one GGG run in the antisense strand. (b) Guanine-tethered antisense-inhibited reverse transcription on an RNA template bearing three runs of GGG sequence. (c) The necessary covalent linkage of the AS and the guanine-rich region. An RNA−AS heteroduplex sequence is also shown. A bold line indicates the AS hybridization site. Lane markers U, G, C, and A indicate the bases on the template RNA.

7), whereas insertion of two single thymine spacers between three GGG units in nine contiguous guanines (**GGG**T**GGG**T**GGG**-AS) markedly produced paused bands at the 3′-side of the GGG region in the RNA template, with faint bands around a hybridization region (lane 8). The length of thymine spacers between three GGG units sensitively affected the amount of paused bands (lanes 9−11). Remarkably, both TTT and TTTT spacers resulted in the complete disappearance of the paused bands (lanes 10 and 11). Again, a covalent connection between three runs of GGG and the antisense was essential for the quadruplex formation that efficiently inhibited *RTase* reaction in the RNA template containing one GGG run (Figure 5c).

The g-ASs-induced quadruplex structures on RNA templates were also confirmed by spectroscopic analyses of the model assemblies of RNA−DNA (Figure 6). CD analyses of the RNA−DNA hybrids involving three GGG runs in the RNA strand and one GGG run in the DNA strand (rG3-dG1), and one GGG run in the RNA strand and three GGG runs in the DNA strand (rG1-dG3), gave spectra similar to those observed for the RNA−DNA hybrid of rG2 and dG2 (Figure 6a), with a strong positive peak at 270 nm and a weak negative peak at 240 nm, suggesting a parallel quadruplex formation.

The melting temperatures of the duplex of the rG3-dG1 hybrid (56.7 ± 0.6 °C) and the rG1-dG3 hybrid (62.3 ± 1.3 °C) measured at 260 nm were almost identical to those of the quadruplexes of the rG3-dG1 hybrid (58.0 ± 0.1 °C) and the rG1-dG3 hybrid (65.7 ± 0.3 °C) measured at 295 nm, respectively (Figure 6b,c and Table 1), suggesting a cooperative melting of the duplex and the quadruplex in these RNA−DNA hybrids. Taken together, the formation of heteroquadruplexes between g-ASs and RNA templates could be applicable to a variety of RNA sequences.

These results demonstrated that inhibition of reverse transcription exclusively depended on quadruplex formation by association of four units of unpaired GGG in the RNA−g-AS complex. Furthermore, these results showed that g-AS-induced quadruplex structures were sensitive to the length of the antisense domain (Figure 2c) and the length of the thymine spacer between GGGs (Figure 2a, lanes 11−14 and Figure 5b, lanes 8−11), but not the length of the spacer between GGG and the antisense (Figure 4b, lanes 11−15). These results agreed well with the observation that both loop length and sequence play important roles in quadruplex folding and stability.[26−28]

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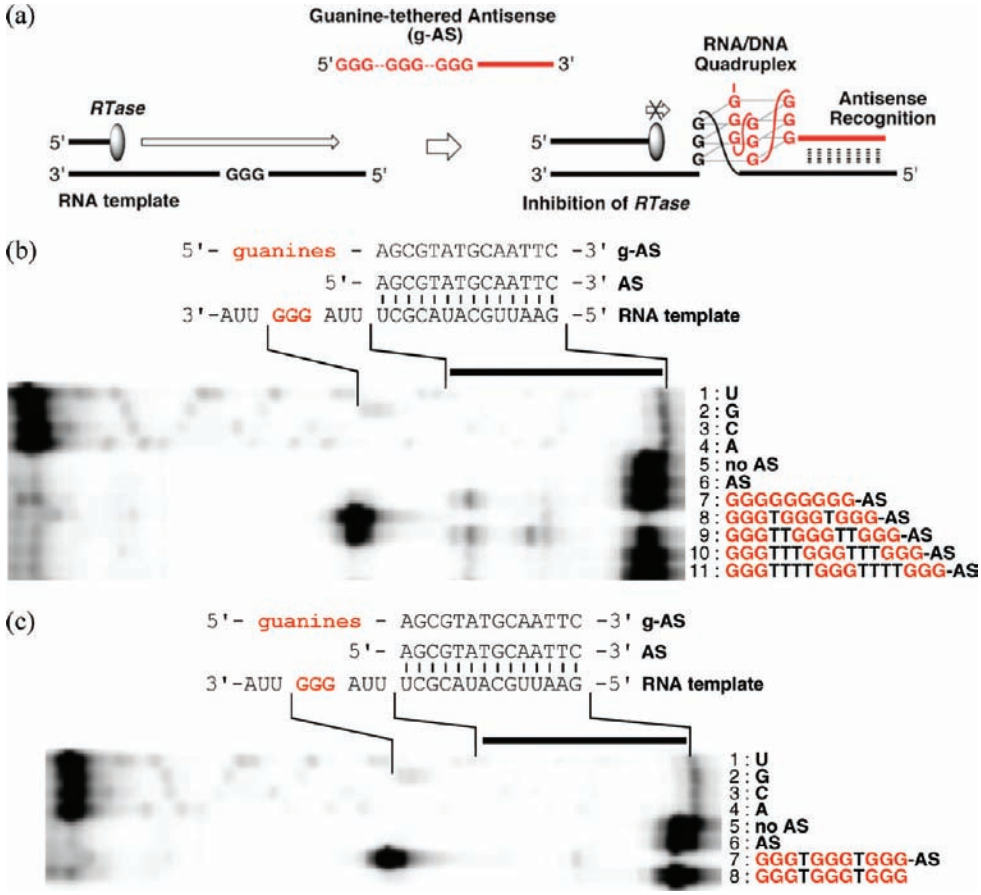

Figure 5. (a) Antisense-induced RNA−DNA quadruplex involving one GGG run in the RNA strand and three runs of GGG in the antisense strand. (b) Guanine-tethered antisense-inhibited reverse transcription on an RNA template bearing one run of GGG sequence. (c) The necessary covalent linkage of the AS and the guanine-rich region. An RNA−AS heteroduplex sequence is also shown. A bold line indicates the AS hybridization site. Lane markers U, G, C, and A indicate the bases on the template RNA.

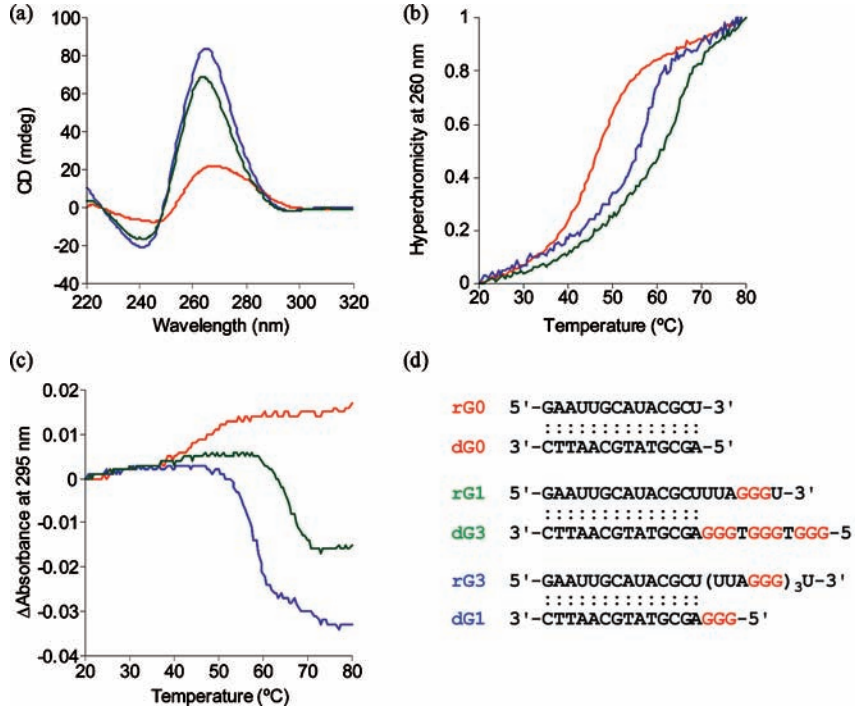

Figure 6. Biophysical properties of RNA−DNA hybrids modified with runs of contiguous guanines. (a) CD spectra and (b,c) T_m curves of the hybrids of rG0 and dG0 (red), rG1 and dG3 (green), and rG3 and dG1 (blue) detected at (b) 260 and (c) 295 nm. (d) Oligonucleotide sequences used in these analyses.

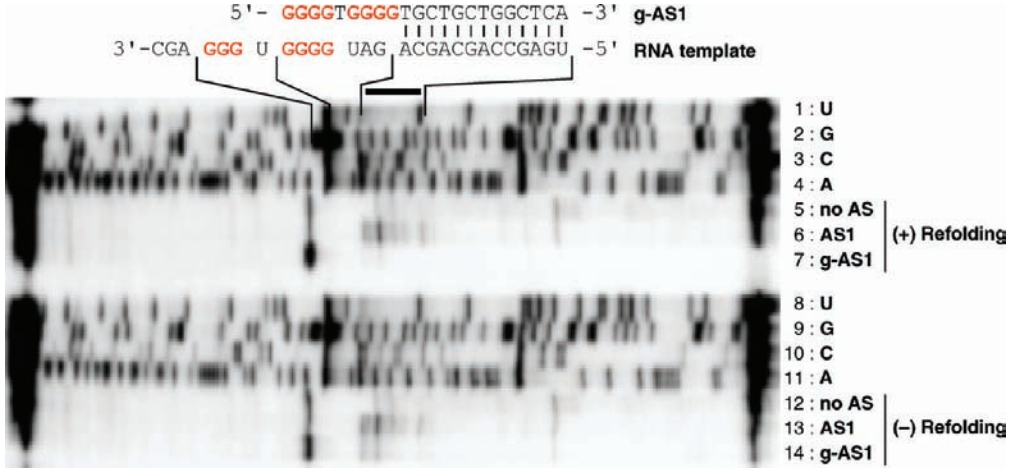

Figure 7. Guanine-tethered antisenses inhibited reverse transcription by HIV-1 *RTase* exclusively at the 3′-side of the guanine-rich region (5′-**GGGG**U**GGG**-3′) of a partial genomic RNA. Reaction mixtures were heated at 80 °C for hybridization of g-ASs (lanes 5−7) or incubated at 37 °C (lanes 12−14) prior to reverse transcription. A bold line indicates the antisense's hybridization site. Lane markers U, G, C, and A indicate the bases on the template RNA.

Table 1. Melting Temperatures of the DNA−RNA Hybrids Modified with Runs of Contiguous Guanines[a]

combination of RNA−DNA hybrids	T_m, °C duplex at 260 nm	quadruplex at 295 nm
rG0-dG0	45.9 ± 0.3	N.D.
rG1-dG3	62.3 ± 1.3	65.7 ± 0.3
rG2-dG2	56.8 ± 0.3	60.9 ± 0.3
rG3-dG1	56.7 ± 0.6	58.0 ± 0.1

[a] All data shown are mean ± standard deviation of three independent experiments. N.D.: not determined.

G-AS Interferes with HIV-1 *RTase* on the HIV-1 Genome Templates. Next, we studied possible inhibition of the *RTase* reaction on an HIV-1 genome template by the guanine-tethered antisenses. A genomic RNA sequence from HIV-1 coding a part of negative factor (NEF) protein (nucleotides 8337−8494), involved in viral replication and pathogenicity *in vivo*,[29] was chosen as a target because the selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) analysis of the entire HIV RNA genome characterized an unstructured region around the NEF-coding region.[30]

We designed a guanine-tethered antisense (g-AS1) that targeted a guanine-rich region (5′-**GGGG**U**GGG**-3′) in the NEF-coding region. The g-AS1 (5′-**GGGG**T**GGGG**-TGCTGCTGGCTCA-3′) consisted of a 13-mer antisense domain, AS1 (5′-TGCTGCTGGCTCA-3′), that hybridized three bases upstream of the guanine-rich region and a guanine-tether (5′-**GGGG**T**GGGG**-3′) at the 5′-terminus. With HIV-1 *RTase*, AS1 lacking the guanine tether produced only a little amount of truncated products around a hybridization region (Figure 7, lane 6). In contrast, the g-AS1 completely blocked reverse transcription, even at 0.3 μM concentration (lane 7), resulting in disappearance of the full-length product with concomitant appearance of truncated products at the 3′-side of the guanine-rich region.

The inhibition of *RTase* would depend on the efficiency of hybridization of g-ASs to the target RNAs. We next tested whether an annealing step is crucial for the inhibition of *RTase* reactions by g-ASs. Omitting the initial refolding in the presence of g-AS1 also gave intense paused bands with little decreased efficiency (lane 14). These data show that g-AS1 could hybridize to the target site and induce quadruplex structures on the RNA template, even without the initial refolding process, because of the accessible structure around the NEF-coding region. Together, the addition of guanine tethers to ASs in fact potentiated the efficacy of the inhibiting effect on reverse transcription by HIV-1 *RTase*.

Conclusions

In summary, we have described a novel guanine-tethered antisense strategy to confer *RTase*-resistant quadruplex structures anywhere in sites with at least three contiguous guanines of target RNAs. The g-AS-induced quadruplex structures on RNA templates interfered with the process of elongation by HIV-1 *RTase*, substantially different from the *RTase* binding aptamers that exhibited quadruplex structures and acted directly on the enzyme activity. Effective inhibition of the *RTase* depended exclusively on the stability of the RNA−DNA heteroquadruplex structures. We clearly showed that the stability of the heteroduplex between RNA template and antisense domain and the spacer length between GGGs in both g-ASs and RNA templates exclusively affected the stability of the heteroquadruplex structures. In contrast, the spacer length between the antisense domain and the guanine-rich domain had little effect on the stability of the heteroquadruplexes. The remarkable ability of guanine-tethered antisenses to inhibit reverse transcription could make possible the development of novel anti-retroviral gene therapies based on blocking the replication of RNA genomes to DNA, which is a critical step for integration into the host's genome. Additional studies will give further insight into this challenge.

Experimental Section

Materials. Oligonucleotides were purchased from Invitrogen and Japan Bio Services Co. Ltd.

RNA Preparation. A double-stranded template DNA for RNA transcription was prepared from oligonucleotides by PCR amplification with a T7 promoter primer (5′-TAATACGACTCACTATAGAA-3′) and a 3′-DNA primer (5′-TCCAACTATGTATACCTG-3′). The resulting DNAs were transcribed to single-stranded RNA by the AmpliScribe T7 Transcription Kit (Epicenter). RNAs

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were purified with 8% polyacrylamide gel containing 6 M urea. The RNA concentration was determined by UV spectroscopy.

Reverse Transcriptase (*RTase*) Stop Assay. A reaction mixture of template RNA (0.3 μM), 5′ Texas Red-labeled 3′-DNA primer (0.1 μM), and antisenses (10 μM) was heated to 80 °C for 3 min and cooled to ambient temperature. To test the inhibition of HIV-1 *RTase*, 0.1 μM HIV-1 RNA template and 0.3 μM antisenses were used. ReverTra Ace *RTase* (TOYOBO) or recombinant HIV-1 *RTase* (Ambion), $MgCl_2$, and dNTPs were added to the reaction mixture, and the reaction was carried out for 30 min. The reaction products were purified and analyzed on a Hitachi SQ5500E automated sequencer. The sequencing markers were prepared with the SequiTherm EXCEL II DNA Sequencing Kit (Epicenter) according to the supplier's recommended protocols.

Thermal Denaturation Profiles. UV−melting experiments were carried out with RNA−DNA hybrids (5 μM) in 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM KCl. The absorbance of the sample was monitored at both 260 and 295 nm from 10 to 95 °C with a heating rate of 0.5 °C/min. T_m values were calculated using the median method. RNA−DNA hybrids were heated to 80 °C and slowly cooled to ambient temperature prior to measurements.

Circular Dichroism Spectral Studies. CD experiments were carried out on a J-725 CD spectrometer (JASCO) using a 1.0 cm path length cell. CD spectral changes of RNA−DNA hybrids (5 μM) were measured in 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM KCl. RNA−DNA hybrids were heated 80 °C and slowly cooled to ambient temperature prior to measurements.

Preparation of the Partial Genomic RNA from HIV-1. Double-stranded DNA corresponding to the NEF-coding region (8337−8494), originating from HIV-1 vector pNL4-3,[31] was chemically synthesized and cloned into pMD19 cloning vector (TaKaRa). The partial HIV-1 genomic RNA was prepared as described above. The sequence of RNA used in this study is 5′-GGUGGCAAGUGGUCAAAAAGUAGUGUGAUUGGAUGGCCUGCUGUAAGGGAAAGAAUGAGACGAGCUGAGCCAGCAGCAGAUGGGGUGGGAGCAGUAUCUCGAGACCUAGAAAAACAUGGAGCAAUCACAAGUAGCAAUACAGCAGCUAACAAUGCUGCcagguauacauaguugga-3′. The guanine-rich region is underlined, and the hybridization site for the Texas Red-labeled DNA primer is shown in lowercase letters.

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