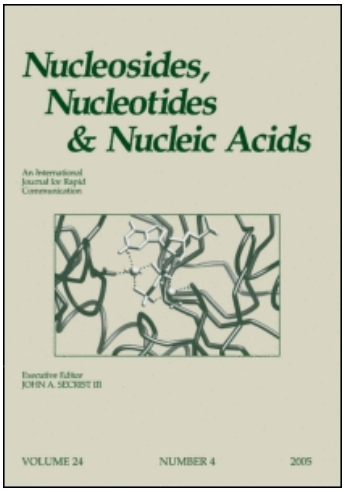

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Paul F. Torrence[a]; Wei Xiao[a]; Guiying Li[a]; Shahrzad Khamnei[a]; Krystyna Lesiak[a]; Avudaiappan Maran[b]; Ratan Maitra[b]; Aseem Kumar[b]; Beihua Dong[b]; Bryan R. G. Williams[b]; Robert H. Silverman[b]
[a] Section on Biomedical Chemistry, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD [b] Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, Ohio

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TARGETING RNA FOR DEGRADATION WITH A (2',5')-OLIGOADENYLATE ANTISENSE CHIMERA

Paul F. Torrence*, Wei Xiao, Guiying Li, Shahrzad Khamnei, Krystyna Lesiak, Avudaiappan Maran§, Ratan Maitra§, Aseem Kumar§, Beihua Dong§, Bryan R. G. Williams§, and Robert H. Silverman§
Section on Biomedical Chemistry, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892
§Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195.

Abstract: A novel method is described to selectively cleave RNA by harnessing the 2-5A-dependent ribonuclease.

Antisense oligonucleotides hold considerable promise both as research tools for inhibiting gene expression and as exogenous agents for the treatment of a myriad of human diseases. The 2-5A system, on the other hand, is a natural defense mechanism which mediates some of the antiviral effects of interferon. We have developed[1-3] a novel and effective strategy for cleaving unique RNA sequences with 2-5A-dependent RNase, an endoribonuclease which mediates inhibitory effects of interferon on some virus infections, and is activated by 5'-phosphorylated, 2',5'-linked oligoadenylates known as 2-5A, [p_n5'A2'(p5'A2')$_m$p5'A], resulting in the cleavage of single-stranded RNA, predominantly after UpUp and UpAp sequences.

To direct 2-5A-dependent RNase to cleave unique RNA sequences, p5'A2'p5'A2'p5'A2'p5'A was covalently linked to antisense oligonucleotide to yield a chimeric molecule (2-5A:AS). As a prototypical 2-5A-antisense chimera, we synthesized **pA_4:dT_{18}.**

p5'A2'(p5'A2')$_2$p5'A2'p1[$CH_2(CH_2)_2CH_2$]4p1[$CH_2(CH_2)_2CH_2$]4p5'dT3'(p5'dT3')$_{16}$p5'dT

(pA_4:T_{18})

Chimeric 2-5A-antisense constructs were undiminished in their ability to bind to the 2-5A-dependent RNase when compared with unmodified 2-5A. Constructs such as **pA_4:T_{18}** formed stable complexes with the complementary poly(A) and had similar stability (as determined by melting temperature) as oligo(dT)$_{20}$.poly(A) itself. Moreover, a complex of

poly(A) with **$pA_4:T_{18}$** was bound to the 2-5A-dependent endonuclease as well as unmodified 2-5A.

To determine the ability of the 2-5A:AS chimera, **$pA_4:T_{18}$**, to induce site-directed RNA cleavage, a target RNA was constructed containing an internal 3',5'-oligo(rA) tract. Hybridization between the $(dT)_{18}$ in the 2-5A:AS with the oligo(rA) in the RNA would, according to our hypothesis, attract 2-5A-dependent RNase to the target RNA, resulting in a highly specific cleavage event. The experimental target, TAR:A_{25}:vif RNA, was produced *in vitro* from a pSP64-derived plasmid containing a partial cDNA for the HIV vif protein interrupted with 25 dA residues. A control TAR:vif RNA was constructed with the dA_{25} tract deleted. These RNA species, which lack 3'-poly(rA) tails, were radiolabeled at their 3'-termini with [5'-^{32}P]-pCp by T4 RNA ligase and gel purified prior to assay.

The 2-5A:AS strategy for site-directed RNA cleavage was tested in a cell-free system consisting of a postribosomal supernatant fraction of human lymphoblastoid Daudi cells which exhibited basal levels of 2-5A-dependent RNase as detected by a radiobinding assay. The cleavage reaction was performed after adding $pA_4:T_{18}$ and purified radiolabeled TAR:A_{25}:vif RNA to the Daudi cell extract (without preannealing) followed by incubation at 30^0. In the absence of added oligonucleotide, the TAR:A_{25}:vif RNA was degraded with a half-life of about 90 min as measured by image analysis of the autoradiogram . Addition of the 2-5A:AS, $pA_4:T_{18}$, to the cell-free system resulted in the nearly quantitative conversion of the intact RNA to a specific cleavage product. The cleavage reaction was observed with as little as 25 nM and was optimal at 250 nM of $pA_4:T_{18}$.

To determine the exact sites of cleavage of the TAR:A_{25}:vif RNA induced by $pA_4:T_{18}$, a primer-extension DNA synthesis reaction was performed on the RNA cleavage products with reverse transcriptase. Consistent with previous results, levels of intact RNA, as measured by the full-length primer extension products, were greatly reduced after incubating the cell-free system in the presence of $pA_4:T_{18}$ in comparison to incubating RNA in the absence of added oligonucleotides, or with $A_4:T_{18}$ or with (2'-5')p_3A_3. The RNA cleavage sites were determined by comparing the migration of the primer extension products and DNA sequencing products performed with the same primer annealed to plasmid containing the TAR:A_{25}:vif sequence. The results showed that $pA_4:T_{18}$ induced multiple cleavages within the oligo(rA) tract of the RNA.

In order to extend the 2-5A-antisense technology to a natural mRNA in intact cells, we targeted for cleavage the mRNA to "PKR" [4], the dsRNA-dependent protein kinase (p68 kinase, DAI, P1 kinase), in human HeLa cells. PKR is an interferon-inducible protein which regulates protein synthesis[5] and which functions as a tumor suppressor factor[6,7].

The PKR mRNA site chosen for the target of the 2-5A-antisense chimera was 60-78 nucleotides from the start codon of the PKR mRNA in a region predicted by computer analysis to be single stranded. Studies with the protein kinase inhibitor, 2-aminopurine, have suggested that PKR is involved in dsRNA-mediated activation of the interferon-β gene and in the viral induction of interferon genes. Double-stranded RNA treatment of cells activates transcription factor NF-κB, probably as a result of the phosphorylation of its inhibitory protein, IκB-α, by PKR. The following 2-5A-antisense chimera directed against the PKR mRNA thus was selected for synthesis for potential ablation of the PKR mRNA, PKR protein, and thus dsRNA signaling through PKR.

p5'A2'p5'A2'p5'A2'p5'A2'-C_4-p-C_4-p5'GTA CTA CTC CCT GCT TCT G3'

pA4-antiPKR

In this structure, the linkage between the 2',5'-oligoadenylate moiety and the 3',5'-deoxyribonucleotide antisense sequence was the same as described previously[1]; namely, two 1,4-butanediol molecules linked by phosphodiester bonds and to the 2-5A moiety through a phosphodiester bond through the 2'-hydroxyl of the 2'-terminal adenosine and to the antisense sequence via the DNA's 5'-phosphate group.

PKR mRNA decay was induced by addition of 2 μM chimeric oligonucleotide to cultures of HeLa cells with no facilitation of uptake. PKR mRNA was detected by reverse transcription-coupled polymerase chain reaction. No PKR mRNA could be detected after incubation of the cells for 4 hours with 2-5A-antisense oligonucleotide. In contrast, antisense oligo itself (with no appended 2-5A), a 2-5A-antisense chimera to a region in HIV RNA, and the chimera pA_4-pBu-p-Bu-$(dA)_{18}$, had no effect on PKR RNA levels. To determine if the 2-5A-antisense oligo was truly selective for PKR mRNA, levels of β-actin mRNA were also determined using the same method. No oligonucleotide tested depleted the amount of β-actin mRNA.

Cellular levels of PKR activity were determined by functional assays after twice daily treatments with various chimeras for 3.5 days. Treatment of cells with pA_4-antiPKR reduced PKR activity to undetectable levels. In contrast, the chimeras pA_4-sensePKR and A_4-antiPKR, had no effect on PKR activity.

To determine if dsRNA signaling was affected by the depletion of PKR activity, electrophoretic mobility shift assays were performed after cells were incubated in the presence or absence of different chimeric oligos (added twice daily for 3.5 days with the addition of poly(I).poly(C) for the final 12 hours. The PRDII element of the human interferon-β promoter was employed as a probe because it binds to NF-κB. A specific complex was formed with this probe when a nuclear extract of ds-RNA-treated cells was

used, but not when an extract from control cells was used. No PRDII complex was seen when cells were preincubated for 3 days in the presence of **pA4-antiPKR** prior to the addition of poly(I).poly(C). In contrast, the control chimeras, pA4-sensePKR and A4-antiPKR, failed to block the dsRNA-mediated activation of NF-κB.

Thus, the 2-5A-antisense strategy is able to cause ablation of a natural mRNA in intact cells without the intervention of any uptake enhancement modality. This mRNA destruction is specific for the targeted mRNA, both in intact cells and with the purified human 2-5A-dependent RNase. Furthermore, we have demonstrated the ablation of the target protein, and we have shown the utility of the 2-5A-antisense strategy by providing direct evidence that PKR is an inducer-receptor for dsRNA.

In essence, conjugation of 2-5A and antisense provides 2-5A-dependent RNase with a dramatically enhanced specificity of action by appending a new and specific substrate RNA binding domain to the endonuclease-2-5A complex. Viewed in this context, the 2-5A:AS strategy for the selective destruction of RNA species would have immediate and widespread application as an RNA equivalent of DNA restriction endonucleases. Although practical barriers to therapeutic usefulness, such as delivery and cell uptake remain a problem with this concept as with other related nucleic acid strategies, 2-5A:AS, because of its specificity, versatility, and potency, is a promising and novel approach to the control of gene expression through targeted RNA destruction.

These chimeric 2-5A-antisense oligonucleotides harness the 2-5A-dependent RNase by activating and directing it to an RNA target. Preliminary experiments have provided evidence that 2-5A-antisense acts catalytically. These attributes continue to suggest that 2-5A-antisense can be a versatile, specific, and powerful agent for the targeted destruction of RNA. By specifically blocking the synthesis of disease-causing proteins, 2-5A-antisense chimeras may prove to be of therapeutic value in a range of human diseases.

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