Correlation of Selective Modifications to a 2′,5′-Oligoadenylate−3′,5′-Deoxyribonucleotide Antisense Chimera with Affinity for the Target Nucleic Acid and with Ability To Activate RNase L†

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The use of an antisense oligonucleotide to address a specific targeted RNA sequence and subsequent localized activation of the 2-5A-dependent RNase (RNase L) to effect selective RNA degradation is a new approach to the control of gene expression called 2-5A-antisense. The previously reported biological activity of the 2-5A:AS chimeric oligonucleotide [p5′(A2′p)$_3$A-antiPKR1], directed against nucleotides 55−73 of the coding sequence of the PKR mRNA, has been used as a point of reference to examine the effect of introducing mismatches into the chimeric oligonucleotide, altering the chain length of the antisense domain of the chimeras, removal of the 5′-monophosphate moiety, shortening the 2′,5′-oligoadenylate domain, and substitution of 3′,5′-linked 2′-deoxyadenosine nucleotides for the 2-5A domain. The general formula for the novel chimeric oligonucleotides is p5′(A2′p)$_3$A2′p(CH$_2$)$_4$p(CH$_2$)$_4$p(5′N3′p)$_m$N, where N is any nucleoside and *m* is any integer. When the biological activity of these new chimeric oligonucleotides was compared to that of the parent chimera, 2-5A-aPKR, for their ability to effect target PKR RNA cleavage in a cell-free and in an intact cell assay, it was determined that there was a close correlation between the activity of 2-5A-antisense chimeras and their affinity (T_m) for a targeted nucleic acid. In addition, there was also a close correlation between activity of the 2-5A-antisense chimeras and their ability to activate the 2-5A-dependent RNase L.

Introduction

We have described a new approach, called 2-5A-antisense (2-5A:AS),[1−6] to regulate gene expression by accelerating the degradation of a targeted mRNA. An antisense oligonucleotide is used to address a specific targeted RNA sequence, and subsequent localized activation of the 2-5A-dependent RNase (RNase L) is relied upon to effect RNA degradation. This selective RNA destruction requires the covalent conjugation of 5′-monophosphoryladenylyl(2′→5′)adenylyl(2′→5′)-adenylyl(2′→5′)adenosine [p5′(A2′p)$_3$A] through two 1,4-butanediol phosphate linker molecules to a 5′-phosphorylated 3′,5′-deoxyribonucleotide. The general formula for this novel chimeric oligonucleotide is p5′(A2′p)$_3$A2′p-(CH$_2$)$_4$p(CH$_2$)$_4$p(5′N3′p)$_m$N, where N is any nucleoside and *m* is any integer. Using such composite nucleic acids, sequence-specific cleavage of a modified human immunodeficiency virus RNA[1] and of mRNA encoding the dsRNA-dependent protein kinase (PKR)[3,4] has been achieved in cell-free systems, as well as ablation in intact HeLa cells of mRNA, protein, and the biologic function of PKR.[3]

Examination of the relationships among structural features and physical, biochemical, and biological properties of such composite nucleic acids is necessary for a full realization of the potential applications of this technology for the following reasons: (1) the 2-5A:AS approach involves the conjunction of two oligonucleotides each of which brings its own modes of potential biological action; (2) the 2-5A component of 2-5A:AS introduces a novel biochemical mode of action to antisense oligonucleotides; (3) it is possible that oligonucleotide−protein and oligonucleotide−nucleic acid interactions may be fundamentally altered by this unique liason of structural partners.

In order to gain insight into some of the structural parameters which govern the cell-free and intact cell activity of such composite nucleic acids, also called 2-5A-antisense chimeras, we have used the biological activity of the 2-5A:AS chimeric oligonucleotide [p5′(A2′p)$_3$A-antiPKR],[3] directed against nucleotides 55−73 of the coding sequence of the PKR mRNA, as a point of reference. Herein we describe the outcomes of altering the chain length of the antisense domain of the chimeras, introduction of mismatches into the chimeric oligonucleotide, shortening the 2′,5′-oligoadenylate domain, and substitution of 3′,5′-linked 2′-deoxyadenosine nucleotides for the 2-5A domain.

† Abbreviations: 2-5A-aPKR refers to the general structure of the 2-5A-antisense chimera of the following formulation: p5′A2′-(p5′A2′)$_2$p5′ApO(CH$_2$)$_4$OpO(CH$_2$)$_4$Op5′d(GTA CTA CTC CCT GCT TCT G)3′. To indicate mismatches in the antisense chain, the prefix m*x* is used where *x* indicates the number of mismatches. To indicate changes in the length of the antisense chain, the prefix n*ty* is employed where *y* designates the antisense chain length in nucleotide units. All of these mismatched and length-altered analogues are also defined in Table 1. A[2′p5′A]$_3$-aPKR refers to the core analogue, as follows: 5′A2′-(p5′A2′)$_2$p5′ApO(CH$_2$)$_4$OpO(CH$_2$)$_4$Op5′d(GTA CTA CTC CCT GCT TCT G)3′. p5′A2′p5′A-aPKR refers to 2-5A-aPKR in which the 2-5A moiety has been shortened to just two nucleotides. spA[2′p5′A]$_3$-aPKR refers to the parent chimera wherein the 5′-monophosphate has been altered to a 5′-thiophosphate. aPKR and p5′aPKR refer to simply the antisense domain, either without or with a 5′-monophosphate; specifically, 5′d-(GTA CTA CTC CCT GCT TCT G)3′ and p5′d(GTA CTA CTC CCT GCT TCT G)3′. Other abbreviations include PKR, dsRNA-dependent protein kinase; 2-5A:AS, 2-5A-antisense.

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Table 1. 2-5A-Anti-PKR Chimeras Effect of Base Mismatching and Chain Length on Activity: Oligonucleotide Design

oligonucleotide	sequence
nt6-2-5A-aPKR	CT CAT C5′−2-5A
nt9-2-5A-aPKR	TC CCT CAT C5′−2-5A
nt12-2-5A-aPKR	TC GTC CCT CAT C5′−2-5A
nt15-2-5A-aPKR	TC TTC GTC CCT CAT C5′−2-5A
sense (mRNA)	5′CAG AAG CAG GGA GTA GTA C3′
antisense	3′GTC TTC GTC CCT CAT CAT G5′−2-5A
m10-2-5A-aPKR	3′GTC AAC CAC GCA CTT GAA C5′−2-5A
m4-2-5A-aPKR	3′GTC TTC CAC CCA CTT CAT G5′−2-5A
m1-2-5A-aPKR	3′GTC TTC GTC CCA CAT CAT G5′−2-5A

Results

2-5A-Antisense Oligonucleotides: Effects of Base Mismatches and Antisense Chain Length on Activity. To evaluate the effect of base mismatching on the physical properties and biological activity of 2-5A-antisense composite nucleic acids, three chimeric oligonucleotides were synthesized, namely, m10-2-5A-aPKR, m4-2-5-A-aPKR, and m1-2-5A-aPKR. These contained 10 and 4 mismatches and 1 mismatch, respectively. The oligonucleotides m4-2-5A-aPKR and m1-2-5A-aPKR contained the mismatches in and near the middle of the antisense domain of the chimera, whereas the mismatched bases in m10-2-5A-aPKR were distributed throughout the antisense region (Table 1). Placement of the mismatches within the inner nucleotides of the antisense moiety would be expected to maximize their destabilizing effect on the hybrid formed with target RNA, as opposed to their placement at the termini of the antisense sequence.[7,8] Terminal placement of the mismatches would be accommodated essentially as an $n - 1$ oligonucleotide and would have minimal effect on helical disruption in the antisense−target RNA duplex.[7,8]

These mismatched and length-altered 2-5A-antisense chimeras were evaluated for their ability to cause the degradation of PKR RNA in a cell-free assay with the purified recombinant human RNase L and in an intact HeLa cell assay. The results of these experiments are shown in Figure 1. It was clear that the ability of 2-5A-antisense chimeras to effect the degradation of the targeted PKR RNA was sensitive to both the extent of mismatching and the chain length of the antisense oligonucleotide. Thus in the in vitro assay with purified RNase (Figure 1, upper panel), degradation of the PKR RNA occurred only with the singly mismatched chimera, m1-2-5A-aPKR, and not with the chimeras with either 4 or 10 mismatches. This behavior may be most directly linked to the decrease in T_m which occurs with the increasing mismatches. The intact HeLa cell results, however, revealed a decreased tolerance for mismatches in the intact cell system since even the singly mismatched chimera did not effect RNA degradation under these conditions (Figure 1B, upper panel, lane 3).

Changes in the length of the antisense domain of the 2-5A-antisense chimeras also had a dramatic effect on the ability of the chimeras to catalyze RNA degradation. As seen in Figure 1, in both the cell-free system with purified RNase L and the intact HeLa cell experiments, the chimera with the shortened 15-mer antisense domain, nt15-2-5A-aPKR, was equipotent with the parent 19-mer, 2-5A-aPKR. However, all the shorter 2-5A-antisense chimeras, nt12-, nt9-, and nt6-2-5A-aPKR (results not illustrated) were without any discernible

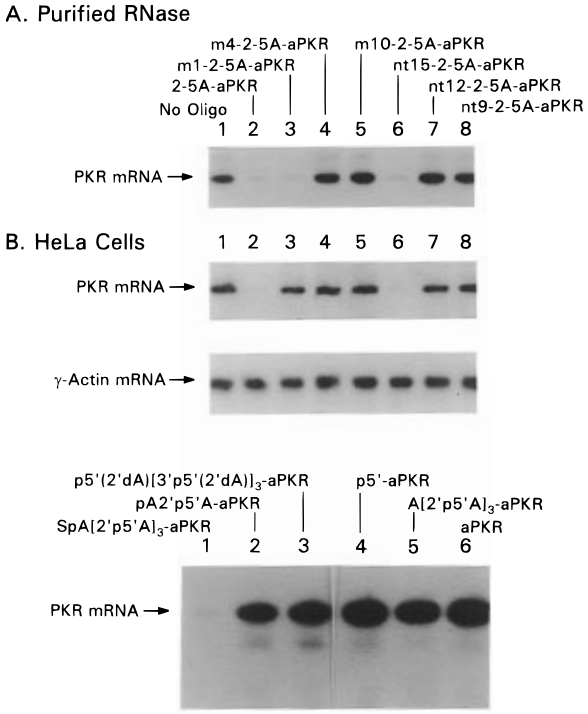

Figure 1. (A) In vitro cleavage of PKR RNA by various 2-5A-antisense oligonucleotides. For these experiments, 25 nM 5′-terminus ^{32}P-labeled PKR mRNA was incubated with 24 nM purified human RNase L at 37 °C for 30 min in the presence of 25 nM respective 2-5A-antisense chimera as indicated. Autoradiograms of dried gels are shown: lane 1, no 2-5A-antisense chimera; lane 2, 2-5A-aPKR; lanes 3−5, m1-2-5A-aPKR, m4-2-5A-aPKR, m10-2-5A-aPKR, respectively; lanes 6−8, nt15-2-5A-aPKR, nt12-2-5A-aPKR, nt9-2-5A-aPKR, respectively. (B) Selective ablation of PKR mRNA in HeLa cells as determined by RNase protection assay. HeLa cells were incubated for 4 h in the presence or absence of 2 μM indicated oligonucleotide, mRNA was extracted from the cells, and after annealing with the appropriate RNA to PKR or to γ-actin and RNase digestion, the reaction mixtures were electrophoresed on 6% polyacrylamide/urea gels and the resulting bands quantitated by Phosphorimager scanning. The lane contents correspond to the labeling in panel 1A.

activity in both assays. None of the above chimeras caused degradation of γ-actin mRNA in the intact HeLa cell assays (Figure 1B, lower panel), thereby further demonstrating the specificity of the 2-5A-antisense approach.

We next examined the affinity of these modified 2-5A-antisense chimeras for nucleic acids with complementary base sequences. The effect of mismatching on helical stability was gauged by the measurement of the melting temperatures (T_m) as determined by UV absorbance−temperature (see Table 2). For these experiments, we judged the effect of mismatching on stability by determining the melting temperature of the hybrid formed with the corresponding DNA sense oligonucleotide as a model. For most experiments, the sense PKR DNA 19-mer oligonucleotide (5′CAGAAGCAGGGAGTAGTAC3′) was used rather than the complementary RNA because the DNA was more readily available; however, in some cases, the trends in stability were also checked with complementary PKR RNA. For comparison, the T_m of the duplex of the parent chimera, 2-5A-aPKR, with its DNA 19-mer sense sequence was 66.0 °C as compared to a T_m of 66.2 °C for the duplex formed between the unmodified DNA antisense sequence alone

Table 2. 2-5A-Anti-PKR Chimeras Effect of Base Mismatching and Chain Length on T_m

oligonucleotide	target sequence	T_m (°C)
nt6-2-5A-aPKR	PKR DNA[a]	≪25[e]
nt9-2-5A-aPKR	PKR DNA	36.0
nt12-2-5A-aPKR	PKR DNA	53.4
nt15-2-5A-aPKR	PKR DNA	59.2
2-5A-aPKR[a]	PKR DNA[b]	66.0
2-5A-aPKR[a]	PKR RNA[c]	71.8
aPKR[d]	PKR DNA[b]	66.2
aPKR[d]	PKR RNA[c]	70.6
m10-2-5A-aPKR	PKR DNA	≪25
m4-2-5A-aPKR	PKR DNA	35.0
m1-2-5A-aPKR	PKR DNA	58.8

[a] Standard 2-5A-aPKR chimera 19-mer. [b] PKR DNA: 5′CAG AAG CAG GGA GTA GTA C3′. [c] PKR RNA: 5′r(CAG AAG CAG GGA GUA GUA C)3′. [d] Antisense oligomer without 2-5A or linker: 5′d(GTA CTA CTC CCT GCT TCT G)3′. [e] Estimated precision: ±0.2 °C.

with its corresponding sense DNA oligonucleotide. On the other hand, when the 2-5A-aPKR was annealed to the corresponding sense RNA oligonucleotide, the resulting duplex had a T_m of 71.8 °C, an increase of approximately 6 °C due to the presence of the RNA strand. This compared to a T_m of 70.6 °C for the complex of the unmodified aPKR DNA with the complementary RNA.

The introduction of one mismatch (m1-2-5A-aPKR), corresponding to the loss of one AT base pair, resulted in a T_m of 58.8 °C when the oligonucleotide was complexed to the corresponding sense DNA oligonucleotide (Table 2). This represented a decrease of 7.2 °C as compared to the parent perfectly matched DNA duplex of 2-5A-aPKR with its target. The 2-5A-antisense chimera with four mismatches, m4-2-5A-aPKR, corresponding to the loss of three AT base pairs and one GC pair, formed a duplex with the sense PKR DNA oligomer with a resultant T_m of 35.0 °C. This corresponded to a 31°C decrease in helix stability compared to the completely matched duplex. Finally, when the 2-5A-antisense chimera m10-2-5A-aPKR was mixed in stoichiometric equivalence with the sense PKR DNA oligonucleotide, no complex formation could be detected as low as 25 °C in the buffer of 0.15 M NaCl, 0.01 M sodium cacodylate, and 0.001 M $MgCl_2$.

Four additional chimeric 2-5A-antisense oligonucleotides were synthesized for evaluation of the stability of their duplexes, if any, with the sense PKR DNA and to evaluate the effect of antisense domain chain length on activity. The antisense domain of the parent 2-5A-antisense chimera was truncated by removal of sequences from both the 5′- and 3′-termini (Table 1). Thus, to obtain nt15-2-5A-aPKR, three nucleotides were excised from the 5′-terminus of the antisense sequence and one nucleotide was deleted from the 3′-terminus, resulting in the deletion of two AT base pairs and two GC pairs. The oligonucleotide nt12-2-5A-aPKR was generated by further shortening nt15-2-5A-aPKR by three nucleotides from the 3′-terminus for a total loss of four AT pairs and three GC pairs. Likewise nt9-2-5A-aPKR and nt6-2-5A-aPKR were obtained by further deletions of nucleotides from the 3′-terminus of the antisense domain, corresponding to a five AT/five GC pair deletion and a six AT/seven GC base pair deletion, respectively. Subsequent T_m experiments were carried out under the same conditions as employed for the mismatched series described above.

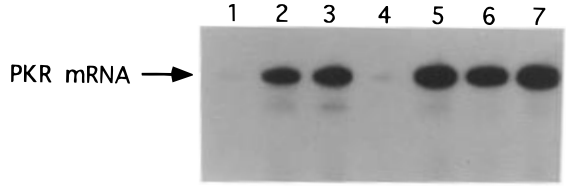

Figure 2. Cleavage patterns of PKR mRNA using various 2-5A-antisense analogues with modifications in the 2-5A domain. The experimental conditions were the same as that provided in Figure 1A. Autoradiograms of dried gels are displayed: lane 1, sp5′A[2′p5′A]$_3$-aPKR; lane 2, pA2′p5′A-aPKR; lane 3, p5′(2′dA)[3′p5′(2′dA)]$_3$-aPKR; lane 4, p5′aPKR; lane 5, A[2′p5′A]$_3$-aPKR; lane 6, aPKR.

As presented in Table 2, successive shortening of the antisense sequence of the chimeras was reflected in a decreasing stability of the chimera's duplex with complementary PKR DNA 19-mer (Table 2). Thus, proceeding through the series nt15-2-5A-aPKR, nt12-2-5A-aPKR, nt9-2-5A-aPKR, and nt6-2-5A-aPKR, the T_m dropped by 6.8, 12.6, 30, and more than 41 °C, respectively, as compared to the parent 19-mer 2-5A-aPKR. The nonlinearity of decrease was expected from the varying GC content of the length-changed chimeras (vide infra).

When the T_m values for the above four length-altered congeners and that for parent 2-5A-aPKR were projected from the calculation method of Breslauer et al.,[9] good agreement with experimental values was noted. According to this calculation, parent **2-5A**-aPKR would possess a T_m of 67.7 °C, whereas the calculated T_m's for nt15-2-5A-aPKR, nt12-2-5A-aPKR, and nt9-2-5A-aPKR were 60.2, 55.2, and 33.9 °C, respectively. The differentials between calculated and experimental T_m's were therefore +1.7, +1.0, +1.8, and −2.1 °C for 2-5A-aPKR, nt15-2-5A-aPKR, nt12-2-5A-aPKR, and nt9-2-5A-aPKR, respectively. Thus, under these conditions, the 2-5A moiety did not exhibit a significant destabilization of derived duplexes with complementary nucleic acid.

2-5A-Antisense Chimeras: Effects of 2-5A Domain Structural Alterations on Activity. Several modifications to the structure of the 2-5A component have been carried out in order to define the role of RNase L in the biological activity of the 2-5A-antisense chimeras. Three specific congeners were synthesized for this purpose: namely, the 2-5A core analogue, A[2′p5′A]$_3$-aPKR; the 2-5A dimer derivative, pA2′p5′A-aPKR; and a 3′,5′-phosphodiester-linked 2′-deoxyadenosine congener, p5′(2′dA)[3′p5′(2′dA)]$_3$-aPKR. Aside from the indicated modifications, all of these had the same structure and sequence as the parent 19-mer, 2-5A-aPKR.

Figure 2 presents the results of the in vitro cell-free assay with purified human recombinant RNase L using pA2′p5′A-aPKR (Figure 2, lane 2), p5′(2′dA)[3′p5′(2′dA)]$_3$-aPKR (Figure 2, lane 3), and A[2′p5′A]$_3$-aPKR (Figure 2, lane 5) as compared to a 5′-thiophosphorylated version[3,10] of the parent chimera 2-5A-aPKR, namely, sp5′A[2′p5′A]$_3$-aPKR (Figure 2, lane 1). In addition, two unmodified antisense DNAs were included, specifically, aPKR (Figure 2, lane 6) and p5′aPKR (Figure 2, lane 4). The latter differs from aPKR only in bearing a 5′-terminal monophosphate group.

As previously reported,[3] sp5′A[2′p5′A]$_3$-aPKR (Figure 2, lane 1) was fully active in causing the degradation of PKR mRNA. However, shortening the 2-5A domain to

a dinucleotide to give pA2′p5′A-aPKR (Figure 2, lane 2), substitution of 2′-deoxyadenosine combined with changes to the 2′,5′-linkages to give p5′(2′dA)[3′p5′-(2′dA)]$_3$-aPKR (Figure 2, lane 3), deletion of the 5′-thiophosphate group to give A[2′p5′A]$_3$-aPKR (Figure 2, lane 5), or complete deletion of the 2-5A and linker moieties to give aPKR (Figure 2, lane 6) and p5′aPKR (Figure 2, lane 4) resulted in a complete loss of PKR target RNA cleavage ability. Previously, the core chimera, A[2′p5′A]$_3$-aPKR, had been shown to be devoid of activity in intact HeLa cells.[3]

Discussion and Conclusions

On the basis of previous experiments with the prototype 2-5A-antisense chimera p5′A[2′p5′A]$_3$-[O(CH$_2$)$_4$-Op]$_2$-(dT)$_{18}$,[2] we concluded that the addition of the pendant 2-5A tetramer moiety and the two butanediol residues had no significant effect on the stability of the duplex of parent (dT)$_{18}$ oligonucleotide with target poly(A).[2] The present experiments, using an antisense sequence of a more typical nature containing all four DNA bases, confirm that initial conclusion. The T_m of the 2-5A-aPKR duplex with a DNA target (PKR DNA, Table 2) was only 0.2 °C less than that of the corresponding unmodified duplex aPKR−PKR DNA (Table 2). This difference was not significant according to the precision of T_m determination. The corresponding duplexes with the RNA target sequence also revealed little stability change; however, the T_m for 2-5A-aPKR−PKR RNA measured 1.2 °C greater than the T_m for the unmodified aPKR−PKR RNA. In addition, the T_m's for the progressively shortened 2-5A-aPKR chimeras were in reasonable agreement with calculated values.[9] Thus, we did not observe a significant effect of the covalent addition of 2-5A-butanediol phosphate linker to the parent antisense sequences, and we certainly could not document any effect that would be expected to affect markedly their biological activity.

For 2-5A-antisense chimeras with chain length changes in the antisense domain, there was a good correlation between affinity to the target sequence and in vitro and in vivo biological activity. For mismatched 2-5A-antisense oligonucleotides, there was also a correlation of activity with target affinity; however, this correlation diverged in the case of the 2-5A-antisense chimera bearing a single mismatch. The m1-2-5A-aPKR chimera effected PKR RNA cleavage in the cell-free assay with purified recombinant RNase L but failed to bring about PKR RNA cleavage in the intact HeLa cell system. The implication of this divergence is not presently clear since the cell-free assay was not structured to detect quantitative differences in cleavage efficiency among the different chimeras. It is possible that a difference in chimera cleavage efficiency might be expressed in the intact cell assay wherein the RNase L concentration would be much more limiting.[11] The intact cell single mismatch or point mutation specificity might be an advantage if an RNA target were the result of a single-base mutation that would have to be discriminated from the normal RNA. This could conceivably be the situation for application of 2-5A-antisense to oncogene-induced cancers or autosomal dominant genetic diseases.[12] On the other hand, the restricted activity of the mismatched chimera might be disadvantageous when the target would be a potentially rapidly mutating RNA, as in HIV or influenza virus.

The cell and cell-free RNA cleavage activity of the remaining chimeras examined provided corroboration of the role of RNase L in activity of 2-5A-antisense. For instance, the core analogue, A[2′p5′A]$_3$-aPKR, would be expected to be inactive if RNase L is the primary mechanism by which RNA degradation occurs, since such 5′-dephosphorylated derivatives are orders of magnitude less effective activators of RNase L than 2-5A (e.g., p5′A2′p5′A2′p5′A) itself, if in fact they are activators at all.[6,13,14] Likewise, the dimeric congener pA2′p5′A-aPKR would be expected to be devoid of activity since the dinucleotide p5′A2′p5′A does not activate RNase L because it is bound several orders of magnitude less effectively than the active 2-5A trinucleotide.[6,13,14] Finally, the chimera p5′(2′dA)[3′p5′(2′dA)]$_3$-aPKR would be inactive since it includes two modifications known to cause loss of RNase L binding and activation abilities: (1) the presence of 3′,5′-phosphodiester bonds[6,15] and (2) the absence of a free 3′-hydoxyl moiety on the adenosine nucleotide second from the 5′-terminus.[6,16] These latter chimeric oligonucleotides, with 2-5A domain structural alterations, additionally provide useful control 2-5A-antisense congeners that can be used in intact cell experiments. In other words, just as antisense mismatch and/or scramble sequences can be employed to determine the specificity of antisense oligonucleotide action,[17] chimeras with the foregoing modifications can be used to ascertain the participation of RNase L in intact cell experiments targeting selected mRNAs.

In conclusion, we have provided evidence herein (1) that there is a close correlation between the activity of 2-5A-antisense chimeras and their affinity for a targeted nucleic acid and (2) that there is also a close correlation between activity of the 2-5A-antisense chimeras and their ability to activate the 2-5A-dependent RNase L. Thus, in both cell-free and intact cell circumstances, there was an absolute requirement for RNase L involvement in the activity of 2-5A-antisense chimeras. These determinations may be used as principles in future experiments with 2-5A-antisense oligonucleotides.

Experimental Section

Oligonucleotide Synthesis. Synthesis of oligonucleotides was accomplished using the phosphoramidite approach to solid-phase DNA/RNA synthesis modified to permit incorporation of 1,4-butanediol phosphate linkers and 2′,5′-internucleotide linkages.[2,18] 5′-Phosphorylation was carried out with 2-[[2-[(4,4′-dimethoxytrityl)oxy]ethyl]sulfonyl]ethyl 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite (Glen Research, Sterling, VA) as described.[2,18]

Oligonucleotide Purification.[2,18] The HPLC system consisted of a Beckman System Gold software controlling an IBM PS/2 computer, two 110B solvent delivery modules, and a 167 UV/vis variable wavelength detector (set to operate at 260 and 280 nm) (or the equivalent of this HPLC system). Anion exchange HPLC was used for purification of synthetic oligonucleotides, under our set of conditions, a Nucleogen DEAE 60-7 column (4 × 125 mm). The elution program was a linear gradient of 10−100% buffer B in buffer A in 30 min and then isocratic at 100% buffer B (flow rate = 1 mL/min). Buffer A was 20 mM KH_2PO_4 (pH 7.0) in H_2O/CH_3CN (8:2, v/v), and buffer B was 20 mM KH_2PO_4 in 1 M KCl, pH 7.0. Oligonucleotide preparations were desalted by C-18 Sep-Pak cartridges.

Oligonucleotide Characterization. Snake Venom Phosphodiesterase Digestion. Standard 2-5A-antisense oligonucleotides were analyzed by digestion with snake venom phosphodiesterase under conditions described previously.[2,18]

An analytical Ultrasphere ODS [reversed-phase C_{18}, 4.6 × 250 mm, flow rate = 0.5 mL/min, 2% B isocratically for 20 min followed by a linear gradient of 2−50% B over 5 min and then isocratic elution at 50% B for 10 min with solvent A as 100 mM $(NH_4)_2PO_4$, pH 5.5, and solvent B as MeOH/H_2O (1:1)] was employed to determine enzymic digests of the products.

Capillary Gel Electrophoresis. Conditions for CGE have been described previously.[2,18]

UV Absorbance−Temperature Profiles (Melting Curves). These were obtained using a Hewlett-Packard 8452 diode-array spectrophotometer and were measured at 260 nm in a buffer of 0.15 M NaCl, 0.01 M sodium cacodylate (pH 7.5), and 1 mM $MgCl_2$. The concentration of oligonucleotide was 2 μM in each strand. The temperature was increased at a rate of 1 °C/min, and the sample was held at each temperatutre for 1 min before the absorbance was recorded. Background and volume corrections were made according to the HP proprietary software of the instrument, and the T_m curve was constructed using a spline algorithm also specified by the system software. The T_m value reported was determined by the first-derivative method using the Savitsky−Golay algorithm of the HP software. Repeated determinations of the same samples led to a precision estimate of ±0.2 for the T_m determination. Extinction coefficients were determined using the method of Puglisi and Tinoco.[19]

In Vitro Synthesis and Purification of RNA and Labeling for Cleavage Assays. Cloned human cDNAs for PKR[20] and 2-5A synthetase (a complete coding region cDNA for the human 2-5A synthetase produced from a 1.8 kb mRNA obtained form D. Gewert of Wellcome Research Lab, Kent, U.K.) in linearized plasmids were transcribed with T7 RNA polymerase (Bethesda Research Laboratories). The respective RNA products were treated first with phosphatase to remove 5′-terminal phosphate groups before 5′-end labeling. This was accomplished by incubating for 30 min at 37 °C 15−16 μg of RNA with 1.5 units of calf intestinal phosphatase (Boehringer) in a total volome of 25 μL of CIP buffer. The CIP was then digested with proteinase K. After phenol:$CHCl_3$:isoamyl alcohol deproteinization, the RNA was precipitated with 70% ethanol, washed with ethanol, and dissolved in RNase-free H_2O. 5′-End-labeling of RNA was accomplished with T4 polynucleotide kinase (2 U; U.S. Biochemicals) and [γ-^{32}P]ATP (50 μCi, 3000 Ci/mol). After deproteinization with phenol:$CHCl_3$:isoamyl alcohol, the RNA was passed through a Sephadex G-50 column to remove nucleotides and then precipitated from aqueous solution with ethanol. The RNA then was gel-purified. First**,** the RNA solution was diluted with an equal volume of gel−sample buffer (deionized 80% formamide containing 10 mM EDTA, pH 8.0, 1.0 mg/mL xylene cyanol, and 1.0 mg/mL bromophenol blue). Then the sample was heated to 90−95 °C for 3 min and immediately put on ice. Electrophoresis was on 6% polyacrylamide/8 M urea gels in TBE (90 mM Tris-borate, pH 7.5, 2 mM EDTA, pH 8.0) (gel dimensions: 8 × 7.5 × 0.075 cm) at 300 V for 1 h.

After electrophoresis, the gel was exposed to Kodak X-OMAT film at room temperature for 5−15 min. The film was developed and aligned to the gel by use of radioactive ink markers, and the portion of the gel corresponding to the full length transcriptase was excised using a sterile razor blade. The gel fragment was minced with the blade, and the gel pieces were placed in an 1.5 mL tube and incubated overnight with 300−400 mL of buffer (0.5 M ammonium acetate, pH 5.2, 1.0 mM magnesium acetate, and 1.0% sodium dodecyl sulfate) at room temperature. The RNA-containing solution was extracted twice with equal volumes of phenol−chloroform (1:1) and chloroform:isoamyl alcohol (24:1) and then ethanol precipitated. The RNA was used as soon as possible (a maximum of 3 days after preparation) to avoid autoradiolysis. Its specific activity was in the range of 10 000−20 000 cpm/μg.

RNA Cleavage with Recombinant RNase L. Reactions were constituted as follows: 14 μL of buffer (25 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 8 mM β-mercaptoethanol, 100 mM KCl, 0.5 mg/ml leupeptin, and 50 μM ATP), 2 μL of oligonucleotide solution (final concentration 25 nM), 2 μL of ^{32}P-end-labeled RNA (3000−5000 cpm a final concentration of 25 nM), and 2 μL of RNase L[21] (10 μg). Incubation was for 30 min at 37 °C. The reaction was terminated by the addition of an equal volume of gel−sample buffer. After the sample was boiled for 5 min, 7−8 μL of each sample was loaded on large 8% polyacrylamide/8 M urea gels (30 cm × 40 cm × 0.04 cm) and run for about 2 h. The gel was transferred to Whatman 3 MM paper, dried, and exposed overnight to film at −80 °C.

Cell Culture and Oligonucleotide Treatment of Cells. HeLa cells were maintained at 37 °C in Dulbecco's MEM containing 10% fetal calf serum and penicillin/streptomycin in 10 cm dishes. Cells at 80% confluency were treated with 2 μM 2-5A-antisense chimeric oligonucleotides in 5 mL of fresh medium for 4 h.

Preparation of RNA from Intact Cells and RNase Protection Assay. After oligonucleotide treatment of cells, the cells were washed with ice-cold PBS and harvested by scraping into 1.0 mL of PBS. The cell pellet was obtained after centrifugation at 700 rpm, and the RNA was isolated using RNazol reagent exactly as described in the instructions provided by the manufacturer (Tel-Test, Inc., Friendswood, TX).

Preparation of PKR antisense RNA probe and γ-actin antisense RNA was carried out as described.[3,22] RNase protection assays were carried out as shown earlier.[3] The RNA probes (3 × 10^5 cpm) labeled with [α-^{32}P]CTP were hybridized with 50 μg of total RNA from cells at 45 °C for 16 h. After hybridization, the RNA was digested with RNases A and T1 for 30 min at 30 °C. Subsequently, digestion with proteinase K was carried out for 15 min at 37 °C. The RNA products were then purified by phenol extraction and separated in a 6% polyacrylamide/urea gel. The lengths of the protected fragments were 409 and 145 nucleotides for PKR and γ-actin, respectively.

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