Pergamon

Advan. Enzyme Regul., Vol. 38, pp. 181–203, 1998

Printed in Great Britain
0065-2571/98/$19.00 + 0.00

PII: S0065-2571(97)00004-6

TOWARDS THE STRUCTURE-BASED DESIGN OF OLIGONUCLEOTIDE THERAPEUTICS

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INTRODUCTION

Chemically modified oligonucleotides are currently being investigated as antisense and antigene reagents with potential therapeutic applications (1–8). Interference with biological information transfer can occur at a variety of stages. Thus, targeting either mRNA synthesis (transcription—antigene approach) (9) or protein synthesis (translation—antisense approach) (10, 11) may allow a modulation of gene expression (Fig. 1). The great potential of the antisense strategy consists in the high specificity of hybridization between antisense strand and RNA via formation of Watson-Crick base pairs, offering the opportunity of rationally designing nucleic acid drugs. However, several obstacles have to be overcome on the way to a therapeutic exploitation of such antisense compounds (Fig. 2). In the recent years, many problems which hampered the antisense strategy in its initial phase of development have been solved.

Natural DNA and RNA oligonucleotides are rapidly degraded by a variety of cellular nucleases. Hence the search for chemically modified nucleic acids with improved *nuclease resistance*. A great number of compounds with modifications in the backbone, sugar or base moieties has been introduced during the last five years (12–14). An important criterion for the usefulness of a particular modification in an antisense oligonucleotide is whether it combines improved resistance with *enhanced RNA affinity* (15–17). Both features will be helpful for reducing dosage, improving oral absorption and minimizing metabolite toxicity. Examples of modifications with higher nuclease resistance and increased RNA affinity relative to native oligonucleotides are 2′-*O*-methoxyethyl RNA (16, 18–20), 2′-*O*-aminopropyl RNA (21) and N3′ → P5′ phosphoramidate DNA (22, 23) and others (14). At the same time, most modifications with the exception of the first-generation phosphorothioates (24) and phosphorodithioates (25) fail to induce RNase H-mediated RNA degradation. Phosphorothioates are readily available at relatively low cost, are well

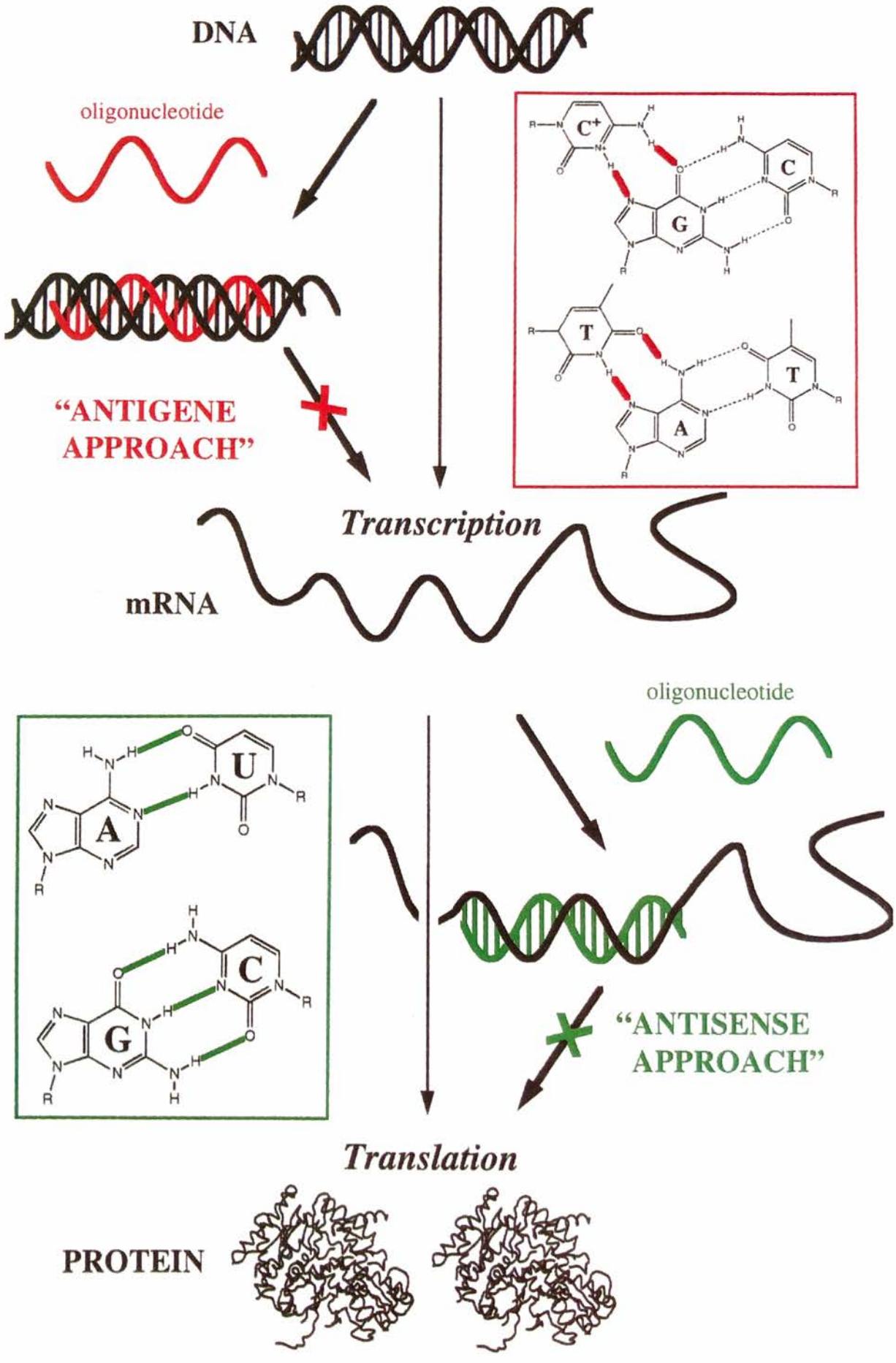
DNA
oligonucleotide
"ANTIGENE APPROACH"
Transcription
mRNA
oligonucleotide
"ANTISENSE APPROACH"
Translation
PROTEIN

Fig. 1.

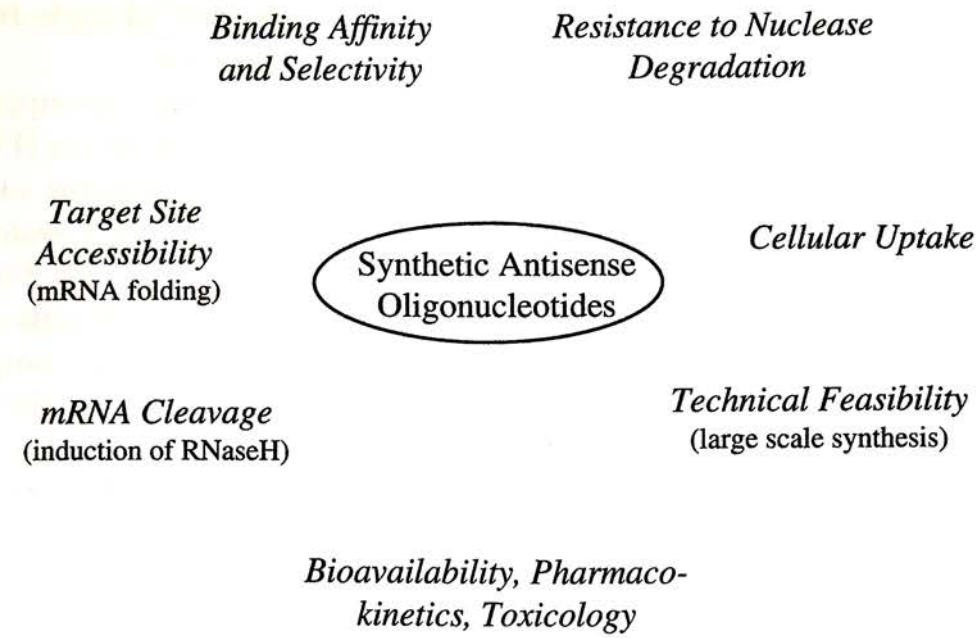

FIG. 2. Key issues for the development of antisense therapeutics [adapted from (19)].

characterized, provide high nuclease resistance and were therefore a logical choice for initial clinical applications of the antisense strategy.

More than 15 phosphorothioate (P = S) oligonucleotides with antiviral, anticancer and antiinflammatory indications have entered human clinical trials (26–29). The average length of these compounds is around 20 bases. While phosphorothioates are performing very well in clinical trials, there are certain pharmacokinetic, pharmacodynamic and toxicological limitations of P = S oligonucleotides. For example, every P = S linkage results in a destabilization of –0.7°C in the UV melting temperature T_M of a modified oligonucleotide (14, 30–34). This translates into a destabilization of roughly 14°C for a fully modified P = S 20mer or a reduction of 3 to 4 orders of magnitude in the dissociation constant. In addition, phosphorothioates have the tendency to bind to serum proteins with high affinity (35). This could be a reason for their toxicity at high concentrations. Finally, P = S oligonucleotides, initially thought to be very stable to nucleolytic degradation, are now known to have only suboptimal stability parameters with half lives of between 1 hr to 24 hr in various animal models and in humans (36, 37). Therefore, it will be crucial to find

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FIG. 1. Simplified scheme of biological information transfer (thin arrows) and two basic ways to interfere with it (thick arrows). The antigene approach (red) attempts to block transcription and the formation of messenger RNA. The antisense approach (green) attempts to block translation and the synthesis of proteins. The high selectivity of pairing via either Watson-Crick or Hoogsteen type hydrogen bonding (see boxes) constitutes the basis for the potential rational design of oligonucleotide drugs.

oligonucleotides with greater resistance to nucleases than phosphorothioates, in particular in view of future oral intake of oligomers.

The basic components of nucleic acid structure, phosphate group, sugar moiety and bases, offer a variety of sites for chemical modification (Fig. 3). Among them the 2′-position has proven to be the most valuable one for several reasons: (1) 2′-modification can confer an RNA-like C3′-*endo* conformation to the antisense oligonucleotide (Fig. 4) (21, 38, 39); (2) 2′-modification can provide nuclease resistance (40); (3) some 2′-modifications result in substantially increased melting temperatures of the complexes between modified strands and their RNA complements. Thus, the 2′-*O*-methoxyethyl (2′-MOE, Fig. 5) RNA modification leads to an increased UV melting temperature T_M of $+1.4°C$/modification with a P = O backbone (20). Interestingly, this modification also exhibits nuclease resistance at approximately the same level as a P = S linkage. For 2′-*O*-alkyls, with increase in size of the alkyl chain, the T_M drops while the nuclease resistance increases (resistance for a P = O backbone, *O*-methyl < *O*-propyl < *O*-pentyl which has similar stability as P = S) (14, 41). The 2′-F modification locks the sugar in a high C3′-*endo* conformation and offers the greatest increase in binding affinity (ΔT_M/modification = + 2.3°C for P = S backbone and +2.7 for P = O backbone) (42). Unfortunately, this modification offers only insufficient resistance to nucleases However, com-

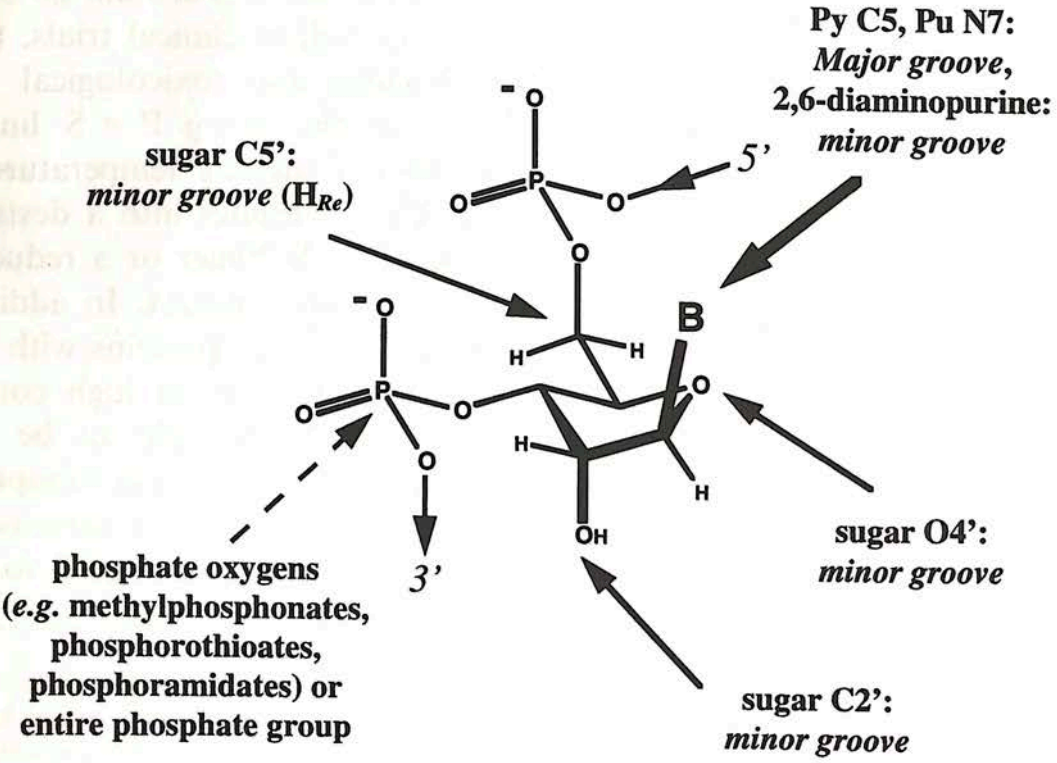

FIG. 3. Possible sites for chemical modification within the nucleic acid framework and their location in terms of helix grooves.

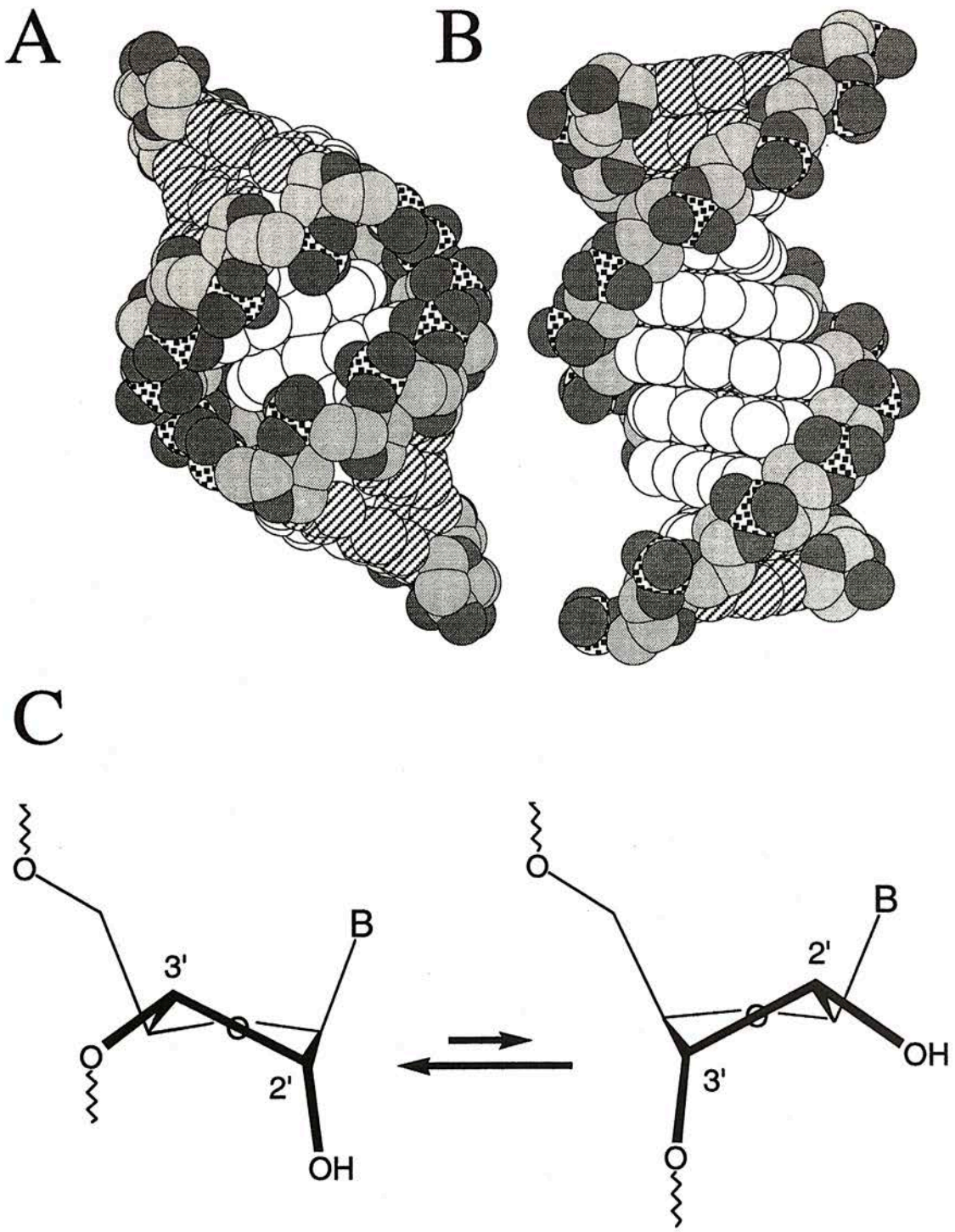

FIG. 4. The two fundamental right-handed conformations adopted by double-stranded oligonucleotides. (A) A-form, RNA and DNA. (B) B-form, DNA. Sugar atoms are shown in light grey, phosphate groups are shown in dark grey (oxygen atoms) and stippled in black (phosphorus atoms). Major groove base atoms are drawn as open spheres and minor groove base atoms are drawn as dashed spheres. (C) Two basic sugar puckers, C3′-*endo* (left, RNA-like) and C2′-*endo* (right, DNA-like).

bining the 2′-F modification with an N3′ → P5′ phosphoramidate DNA backbone produces exceptionally high RNA affinity (ΔT_M/modification = + 4.0°C) and enhanced nuclease resistance (43). Finally,

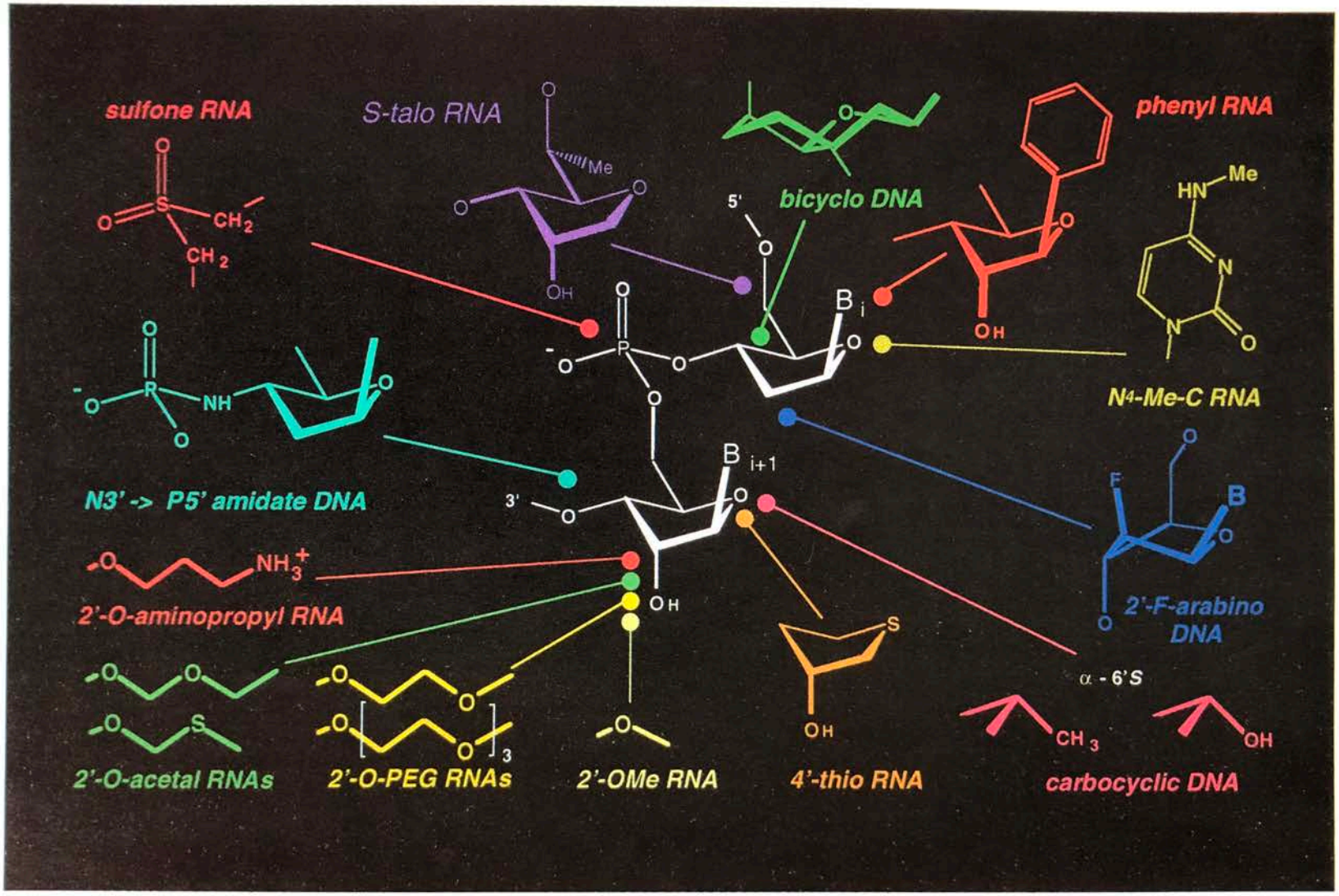

FIG. 5. Structures of chemically modified nucleosides employed in X-ray crystallographic studies by our laboratory in collaboration with several research groups in industry and academia.

the zwitterionic 2′-*O*-aminopropyl (2′-AP, Fig. 5) RNA modification exhibits the highest nuclease resistance among oligomers with a phosphodiester backbone (21).

The failure to induce *RNaseH-mediated RNA degradation* by all of the above second-generation antisense modifications may initially constitute a disadvantage in terms of activity (44–46). However, as shown by recent studies, stable hybridization can overcome the lack of RNaseH induction via a steric block mechanism. Thus, using a series of uniformly modified 2′-MOE/P = O 20mer oligonucleotides, an *RNase H-independent* antisense oligonucleotide was found to show greater activity than its parent 2′-deoxy P = S oligomer in targeting the 5′-cap region of human ICAM-1 transcript in HUVEC cells (17). Similar results have been achieved with DNA phosphoramidate oligonucleotides (47). A further approach to overcome this limitation of many modifications is the so-called GAPmer technology (48–50). In such gapmer oligonucleotides, 2′-modifications were placed only at the terminal ends ("wings"), leaving a phosphorothioate window in the middle for RNaseH activation. Such a gapmer was demonstrated to have four-fold greater activity in reducing C-raf mRNA in T_{24} cells compared with a P = S oligomer (16). In this system as well as in others, the observed *in vitro* biological activity more closely correlates with nuclease resistance than binding affinity. In another study, a 20mer analog with 2′-AP/P = O at the 3′-end was electroporated into A549 cells. The 2′-AP-modified oligonucleotide showed a four-fold increased potency in mRNA assays relative to the parent P = S oligomer and a two-fold increase in potency in c-raf protein assays (21). The sulfur content of the oligonucleotide was lowered by 55%, potentially reducing side effects observed with many P = S oligonucleotides.

In most cases it is not feasible to determine via RNA secondary structure prediction the regions within an mRNA that are accessible to pairing with an antisense oligomer. However, by *targeting* a particular mRNA at numerous sites, including the 5′-untranslated, coding and 3′-untranslated regions, it is usually possible to select oligonucleotides with particular activity in terms of downregulation (16, 51). *Transport, pharmacokinetics* and *toxicology* issues have been investigated in some detail with first and second generation modifications and a number of studies with animals as well as clinical trials with humans have indicated that the doses required for activity may be tolerated without serious complications (16, 36, 37, 52). Although all clinical trials are being conducted with first generation phosphorothioate oligonucleotides at the moment, high expectations exist for future trials with second generation antisense oligonucleotides currently under development. However, an appreciation of the dramatic improvements in many aspects of the antisense approach should not lead one to conclude that there is a magic class of nucleic acid analogs which could

be applied against virtually any target. Thus, despite the fact that a large number of promising modifications has been put forward in the past few years, there can be no doubt that more research will be necessary to establish the existing modifications in terms of therapeutic applications and to identify novel modifications with optimal RNA-affinity and nuclease resistance features.

In collaboration with several groups in industry and academia, we have initiated efforts to better understand the RNA affinity displayed by individual nucleic acid modifications (39, 53). We believe that a dissection of the detailed chemical and structural causes for the favorable thermodynamic self-pairing and RNA hybridization exhibited by a number of promising oligonucleotide analogs is best achieved by a combination of high-resolution structures with precise thermodynamic parameters. This will permit a rigorous *structure-stability* and *structure-activity analysis*. X-ray crystallography is the ideal tool to study the three-dimensional structure of nucleic acid fragments and their hydration and ionic environment. Towards this end, a growing number of oligonucleotides containing one, two or only chemically modified residues has been crystallized in the past two years in our laboratory and their structures have been determined at resolutions of between 1.5 Å to 2.8 Å or structure determinations and refinements are under way. It is hoped that the creation of a structural data base for oligonucleotide analogs will be of great help both in the immediate future for improving the efficacy of the next generation of antisense compounds to undergo clinical trials as well as in the long run for providing the tools for a rational design of high-affinity antisense drugs. This chapter provides a summary of our overall strategy and describes selected first results.

MATERIALS AND METHODS

Choice of modifications. In order for a detailed analysis of how chemical modification affects nucleic acid structure and stability to be meaningful, it should include analogs with changes comprising the three basic constituents of nucleic acid structure, phosphate group, sugar and bases. Since the determination of a high-resolution crystal structure of a nucleic acid fragment can be a challenging task, it is important to have a detailed knowledge of the self-pairing and RNA-binding properties of a particular analog prior to embarking on the structural work. A qualitative structure-stability correlation is greatly facilitated if a modification has either strongly stabilizing or destabilizing features. A further criterion for a particular analog to be selected for a structural analysis is its accessibility. The chances for obtaining crystals of an analog or a mixed RNA-analog or DNA-analog oligomer can certainly be improved if one is able to pro-

duce a variety of either all-modified or chimeric oligomers with incorporation sites for modified building blocks at a number of locations. This requires that the synthetic route for producing a chemically modified oligomer be relatively easy and furnish high yields. Moreover, it should be compatible with established solid phase synthesis methods for producing DNA and RNA oligonucleotides. An overview of the chemically modified nucleosides used in our crystallographic studies thus far is depicted in Fig. 5. These include molecules with altered backbones [bicyclo-DNA (54, 55), sulfone RNA (56), N3′ → P5′ phosphoramidate DNA (57), 5′-talo-RNA (58)], sugar moieties [6′-α-substituted carbocyclic DNAs (53), 2′-*O*-methyl RNA (59) and a flurry of other 2′-*O*-modified RNAs, 2′-F-arabino DNA, 4′-thio RNA] and bases [phenyl RNA (60), N4-methyl-cytosine].

Synthesis of modified oligonucleotides. All nucleic acid analogs for structural studies are being produced by collaborators from several companies as well as academic and government institutions. Past and present collaborations include the central research laboratories of Novartis Pharma Inc., Basel, Switzerland (2′-*O*-methyl RNA, 2′-*O*-modified RNAs, 6′-α-substituted carbocyclic DNAs and others); Ribozyme Pharmaceuticals Inc., Boulder, CO (5′-talo-RNA, 4′-thio RNA, phenyl RNA, N4-methyl-cytosine and others); ISIS Pharmaceuticals Inc., Carlsbad, CA (2′-*O*-aminopropyl RNA and others); Dr. Sergei Gryaznov at Lynx Therapeutics Inc., Hayward, CA (N3′ → P5′ phosphoramidate DNA); Dr. Victor E. Marquez at the Laboratory of Medicinal Chemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD (2′-F-arabino DNA); Prof. Steven A. Benner at the Dept. of Chemistry, University of Florida, Gainesville, FL (sulfone RNA); and Prof. Christian Leumann at the Organic Chemistry Institute, University of Berne, Switzerland (bicyclo-DNA). All oligonucleotides are purified with reverse phase HPLC and ion exchange chromatography.

Choice of sequence. For an oligonucleotide sequence to be suitable for structural studies of nucleoside analogs, it has to fulfil certain criteria. Firstly, its native structure has to be known in sufficient detail so that the structures of modified and native fragments can be compared later on. Secondly, it should be relatively tolerant with regards to incorporation of modified building blocks at a variety of sites. Thus, a natural DNA or RNA sequence may furnish densely packed crystals which diffract to high resolution, but crystals cannot be grown anymore of the modified strand because of lattice distortions due to the presence of the modified residues. Thirdly, crystals of a suitable template sequence and its modified versions should diffract to high resolution, usually 2 Å or better. This will allow

detailed analysis of the ionic and water environment of the oligomer in the crystal. There is ample evidence from our own studies and those by others that hydration and ions can crucially contribute to the overall thermodynamic stability of natural DNAs and RNAs as well as their analogs (39, 61).

Selection of incorporation sites for chemically modified nucleosides within a template sequence. The selection of suitable incorporation sites for nucleoside analogs within a sequence is demonstrated in Fig. 6. The decamer sequence d(GCGTATACGC) is useful for assessing the structural features of 2′-*O*-modified analogs, since the presence of RNA or RNA-analog residues drives the decamer duplex into an A-conformation, the helix form adopted by RNA and RNA-DNA hybrids (62–64). The RNA octamer r(CCCCGGGG) was used for assessing structural perturbations caused by modified RNA residues whose effects on the cleavage activity of hammerhead ribozymes were investigated by our collaborators at Ribozyme Pharmaceuticals Inc. Examples of such modified building blocks are talo-RNA, 4′-thio RNA, phenyl-RNA, 2′-*O*-methylthiomethylene RNA and N4-methyl-cytidine RNA (Fig. 5). The structure of the native RNA octamer had previously been solved in two crystal forms (65, 66) and its hydration was analyzed at 1.4 Å resolution (61). Similarly, we have established nucleoside analog incorporation sites within the Dickerson-Drew DNA dodecamer (CGCGAATTCGCG) that will not affect the favorable crystallization properties displayed by this sequence (39, 53).

Crystallization. In the recent years, tremendous progress has been made in terms of the determination of suitable crystallization conditions for oligonucleotides and proteins. For the crystallization of nucleic acid analogs, we rely on sparse matrix crystallization screens which are either commercially available (67–69) or for which details were published (70–72). In addition, we use established conditions for the crystallization of nucleic acid fragments, such as sodium cacodylate buffer with a range of pH values, a selection of divalent metal cation salts, spermine tetrahydrochloride and 2-methyl-2,4-pentanediol (MPD) as the precipitant.

X-ray diffraction data collection, structure determination and refinement. Data collections are usually performed at −170°C by transferring the crystals directly into a cold nitrogen stream on either in-house rotating anode/image plate equipment or at the DND-CAT (Dupont-Northwestern-Dow Consortium Access Team) beamline of the Advanced Photon Source Synchrotron at Argonne National Laboratory outside Chicago. Data are merged using standard software and in most cases the structures can be solved by the Molecular Replacement method. The structure refinements

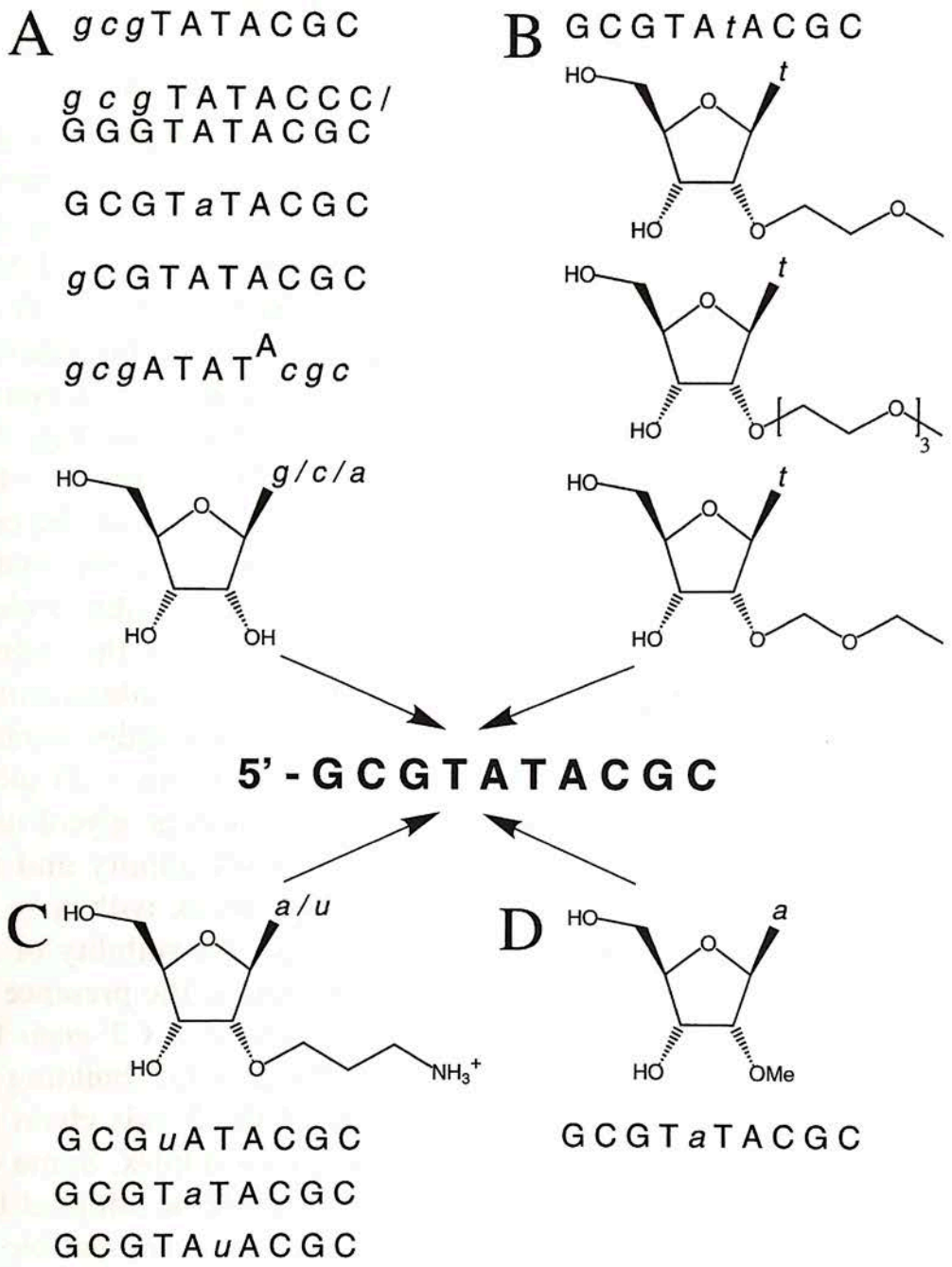

FIG. 6. Incorporation sites for ribonucleosides or 2′-*O*-modified nucleosides within a DNA decamer sequence (highlighted, deoxynucleosides in capital font) that produced well diffracting crystals. Decamer fragments featuring this alternating purine-pyrimidine sequence and containing at least one RNA or modified RNA residue (lower case, in italics) all appear to adopt an A-form duplex in the crystal (39, 59, 62).

are performed with standard programs, using constraint least-squares methods and simulated annealing. With chemically modified nucleic acid fragments, the parameter and topology files have to be adapted (connectivity, bond lengths and angles, torsion angles, charges) to fit the individual modifications. These parameters can often be determined on the basis of crystal structures of modified nucleosides [e.g. (60)].

RESULTS AND DISCUSSION

X-ray Crystallography of Chemically Modified Oligonucleotides

We used three template sequences for incorporating chemically altered building blocks which are being evaluated as second generation antisense modifications or are being studied in the context of hammerhead-type ribozymes (Fig. 5). We relied on the DNA decamer d(GCGTATACGC) to assess the structural consequences of the presence of single 2′-*O*-methoxyethyl (2′-MOE), 2′-*O*-[methyltri(oxyethyl)], 2′-*O*-ethoxymethylene (Fig. 6B) and 2′-*O*-aminopropyl ribonucleotides (Fig. 6C). Crystals of these decamers d(GCGTAt/uACGC) (t/u = modified residue, Fig. 7A–C) diffract to high resolutions of around 1.6 Å when flash-frozen at −170°C. Such high resolution structures will allow accurate analyses of the conformations of the 2′-*O*-substituents and will reveal details of the hydrogen bonding interactions between substituents and first shell water molecules. A qualitative and perhaps quantitative rationalization of the individual stabilizing or destabilizing influences of the RNA 2′-*O*-substituents will likely require such details. Relative to RNA, oligonucleotides containing the 2′-MOE RNA modification with 2′-*O*-$(CH_2)_n$-O-CH_3 (n = 2) substituents (Fig. 6B, top) or a related one featuring three ethylene glycol units in its 2′-*O*-substituent (Fig. 6B, middle) show high RNA affinity and nuclease resistance (14, 18–20). Conversely, the building block with n = 1 (see above formula and Fig. 6B, bottom) does not affect the stability of modified oligonucleotides in a significant way. In both cases, the presence of an electronegative 2′-oxygen should favor the adoption of a C3′-*endo* RNA-like sugar pucker. Moreover, *gauche* effects in the 2′-MOE building block (absent when n = 1) may lead to arrangements of the 2′-side chain in the minor groove that are consistent with formation of a duplex. Some of the multiple side chain conformations that can in principle be adopted by the 2′-*O*-ethoxymethylene substituent (n = 1) may be incompatible with duplex formation. Similarly, it was observed that long 2′-*O*-alkyl side chains lead to a strong destabilization of the duplex, possibly as a result of steric crowding and distortion of first shell hydration (14, 41). Now, with structures of relative to RNA stabilizing and somewhat destabilizing 2′-*O*-modifications at hand, we should be able to more precisely determine the role of the individual effects in terms of RNA affinity.

The zwitterionic 2′-*O*-aminopropyl (2′-AP) RNA modification (Fig. 5, middle left) is highly nuclease resistant, but thermodynamically slightly less stabilizing than the 2′-MOE RNA modification. As in the case of building blocks with ethylene glycol units, we have grown crystals of d(GCGuauACGC decamers featuring single 2′-AP RNA modifications (individual modification sites in lower case font, see Fig. 6C; crystal depicted in Fig. 7C). The consequences of the presence of covalently

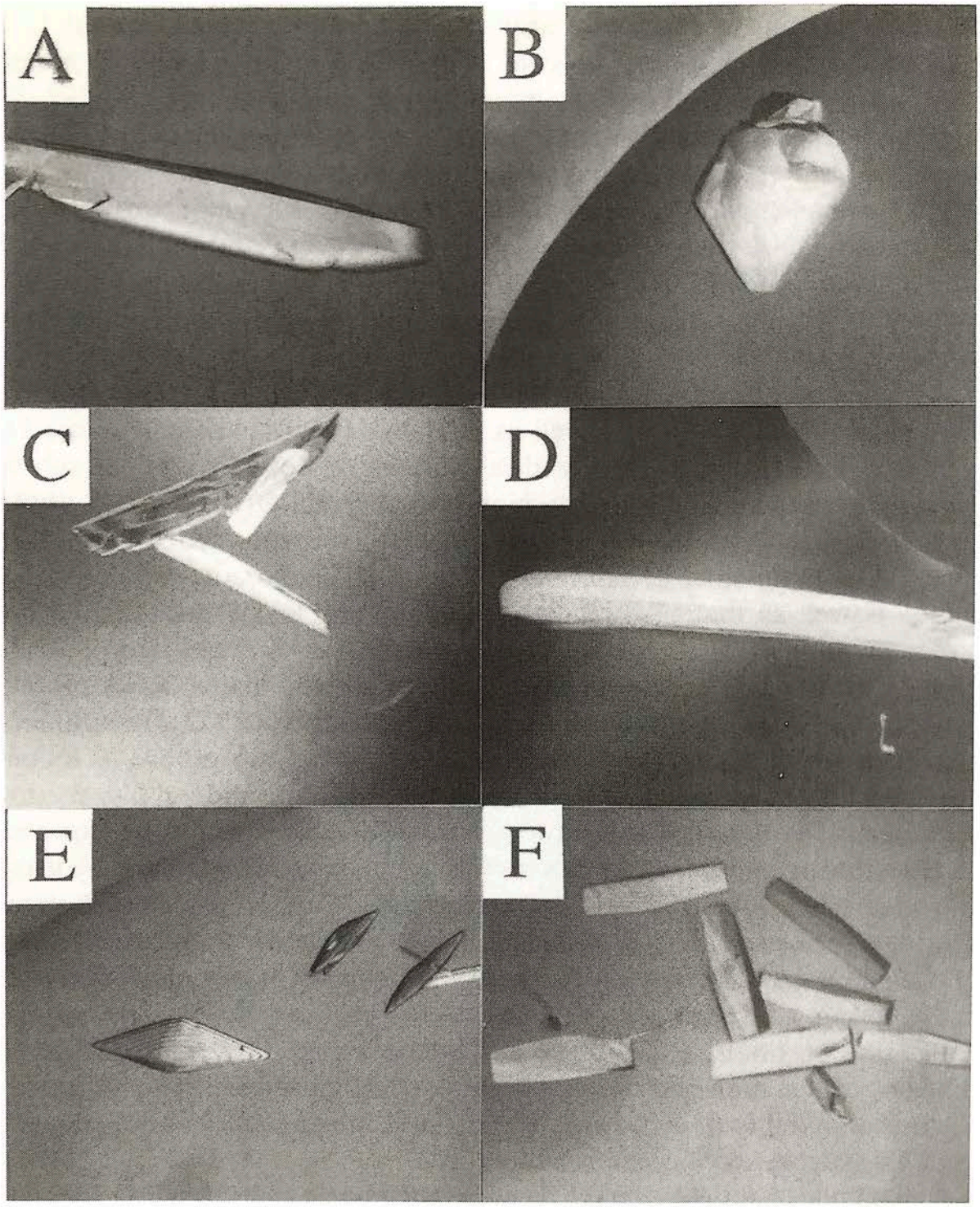

FIG. 7. Crystals of selected chemically modified nucleic acid fragments. Modified A-form DNA decamers with sequence 5′-d(GCGTAtACGC), (A) t = 2′-*O*-[methyltri(oxyethyl)] ribothymidine and (B) t = 2′-*O*-ethoxy-methylene ribothymidine. (C) Modified A-form DNA decamer with sequence 5′-d(GCGTAuACGC), u = 2′-*O*-aminopropyl ribouridine. (D) Modified B-DNA dodecamer with sequence 5′-d(CGCGAAttCGCG), t = 6′-α-hydroxyl carbocyclic thymidine. (E) Modified RNA octamer with sequence 5′-r(CCsCGGGG), s = 2′-*O*-methylthiomethylene ribocytidine. (F) N3′ → P5′ phosphoramidate DNA dodecamer with sequence 5′-d(CnpGnpCnpGnpAnpAnpTnpTnpCnpGnpCnpG).

bound positively charged residues on the overall duplex structure have not been studied in great detail so far. For example, it is unclear whether the positively charged aminopropyl chain protrudes into the groove (21), or if

the amino group is positioned near the floor of the groove, linked to a base function via a water molecule. Alternatively, the protonated amino group [pK_a = 9.4 (21)] could be positioned in the vicinity of a negatively charged phosphate group in the backbone. All these questions can now be addressed in more detail, based on the crystallographic data.

Two other template sequences which produced diffraction quality crystals are the DNA dodecamer 5′-CGCGAATTCGCG and the RNA octamer 5′-CCCCGGGG. In order to determine incorporation sites for carbocyclic residues (Fig. 5, lower right) that would still produce high quality crystals, we carried out a selection similar to the one shown in Fig. 6 for the above DNA decamer (39, 53). Although incorporation of isolated modified residues as well as stretches of modifications (tetramer) produced crystals, only those with either one or both thymidines substituted were suitable for collecting high-resolution data (> 2.5 Å). A photo of such a crystal of the dodecamer with two incorporated 6′-α-hydroxy carbocyclic thymidines is depicted in Fig. 7D. The structure of this dodecamer as well as that of a related one containing two 6′-α-methyl carbocyclic thymidines were solved at 2.1 and 2.5 Å resolution, respectively (53). They provided qualitative explanations for the higher RNA affinity conferred by the 6′-α-hydroxy carbocyclic building block (73). In addition, they revealed distinct patterns of hydration in the minor groove as a consequence of the presence of either the 6′-α-hydroxy and or 6′-α-methyl substituents. Although the hydrophobic methyl group would be expected to alter the arrangement of water molecules more profoundly, the hydroxy substituent led to a more significant distortion of minor groove hydration. Through formation of hydrogen bonds to the base functions at the floor of the minor groove, the hydroxyl groups replaced water molecules normally forming a continuous spine along the groove (74, 75). The ensuing enthalpic contribution as well as an entropic gain as a consequence of releasing water molecules from the groove are plausible explanations for the higher stability of RNA pairing observed for oligonucleotides containing 6′-α-hydroxy carbocyclic residues.

In a similar fashion, we have determined suitable incorporation sites for modified RNA residues in an RNA duplex. An example of a successful crystallization is depicted in Fig. 7E. A single 2′-*O*-methylthiomethylene ribocytidine (Fig. 5, lower left) was incorporated at position 3 of the octamer r(CCCCGGGG). Several other RNA modifications were incorporated either at this site or within the purine stretch and yielded well diffracting crystals.

The N3′ → P5′ phosphoramidate DNA dodecamer 5′-d(CnpGnpCnpGnpAnpAnpTnpTnpCnpGnpCnpG) furnished the first high-resolution crystal structure of an all-modified nucleic acid fragment (57). Crystals of this oligonucleotide are depicted in Fig. 7F, and the

structure of the phosphoramidate DNA modification is shown in Fig. 5 (left). This crystal structure has provided answers to a number of initially puzzling observations concerning the chemical and pairing properties of N3′ → P5′ phosphoramidate DNAs (O3′ replaced by an amino group). Despite being a DNA analog, phosphoramidate DNA appears to adopt a conformation similar to that of duplex RNA (23, 76). It displays extremely stable self-pairing and hybrid duplexes between phosphoramidate DNA and RNA are thermodynamically more stable than RNA duplexes (22, 23). However, while N3′ → P5′ phosphoramidate DNA forms stable duplexes, replacement of the 5′-oxygen by an amino group prevents duplex formation. Moreover, combining the 2′-*O*-methyl modification with the amidate backbone leads to a reduction of RNA affinity, although 2′-*O*-methyl P = O RNA is thermodynamically considerably more stable than RNA [e.g. (14)]. However, combining the N3′ → P5′ phosphoramidate backbone with the 2′-fluoro substitution produces oligonucleotides with extraordinary RNA affinity and relatively high nuclease resistance (43). The unusual arrangement of anions around the amidate backbone in the crystal has provided insight into the electronic structure of the N3′ → P5′ phosphoramidate DNA backbone (57). Thus, an anomeric effect between the N3′ lone electron pair and the polarized P-O5′ bond is probably the reason for the conformational rigidity of the amidate duplex. In addition, this effect explains why the phosphoramidate moiety is chemically less reactive than the phosphodiester moiety, rendering N3′ → P5′ phosphoramidate DNA more resistant to nucleases. Overall, this crystal structure along with the observed extensive hydration of the phosphoramidate backbone has exposed several feasible sources of both the enhanced RNA affinity and the higher nuclease resistance of N3′ → P5′ phosphoramidate DNA.

Structure–stability Correlations

Such correlations require precise three-dimensional models of modified nucleic acids as well as the corresponding thermodynamic data, either in the form of UV-melting temperatures T_M or explicit enthalpic and entropic data. ΔG, ΔH and ΔS may be obtained from concentration-dependent equilibrium melting curves or from calorimetric measurements. Detailed analysis of T_M data for some 200 modifications has led to several rules of how to modify DNA in order to improve hybridization to RNA targets (14). (1) Shift the sugar conformation into the C3′-*endo* pucker mode adopted by the ribose in an A-form duplex. Examples are the 2′-F and 2′-OR (R being either a short alkyl chain or containing one or multiple ethylene glycole linkers). (2) Preorganize the backbone for an A-type conformation. Examples are N3′ → P5′ phosphoramidate DNA, the amide modification [(77, 78), and reviewed in (13)] and the MMI backbone

(79, 80). (3) Improvement of stacking through addition of a polarizable group to the base heterocycle. Examples are 5-propyne pyrimidine (15) and the amino-ethyl-3-acrylimido pyrimidine (14) modifications. (4) Increase the number of hydrogen bonds. An example is 2,6-diaminopurine which can form three hydrogen bonds with U. (5) Neutralize the negative phosphate charges. Examples are peptide nucleic acid (81–83) and the morpholino modification (84).

In addition to such guidelines for the successful design of alternative high RNA affinity modifications, high resolution crystal structures may reveal the precise origins of gains or losses in terms of RNA pairing stability with a particular modification. Individual contributions may arise from improved hydration, interactions with cations and anions, a variety of stereoelectronic effects and others. A detailed analysis of the hydration of DNA and RNA suggested that a crucial contribution to the higher stability of RNA pairing is due to the extensive hydration of the 2′-hydroxyl groups [(61) and references cited therein]. Therefore, it can be expected that hydration will also play an important role in the altered stabilities observed for 2′-*O*-modified residues.

Thus far we have studied the structures of antisense molecules (e.g. 2′-MOE and 2′-AP RNA) almost exclusively in the context of oligomers containing one or two modifications. It is obvious that structural analysis of oligonucleotides with an isolated modified building block per strand will not provide a full understanding of the role of the modification for stability and activity. In many cases cooperative effects were observed or incorporation of modified purines affected duplex stability differently from incorporation of pyrimidine residues. The crystal structure of the all-modified N3′ → P5′ phosphoramidate DNA dodecamer duplex has provided a detailed picture of the chemistry behind the extraordinary thermodynamic stability of this modification. Clearly, such details would have been inaccessible with just a dimer structure or an isolated O3′ → N3′ substitution in an otherwise all P = O backbone.

Although the exceptional RNA affinity of the 2′-F modification is well established, its origins are not understood. 2′-AP RNA, 2′-MOE RNA and 2′-F RNA all show elevated affinity for RNA relative to both DNA and RNA. However, we would expect the origins of the stability gains to be quite different in the three cases. While all three 2′-substituents promote the adoption of A-form C3′-*endo* sugar puckers as a consequence of their electronegativity, the influence of hydration on the overall stabilities is far less clear. The crystal structure of a DNA decamer duplex with single 2′-MOE substitutions per strand revealed coordination of water molecules to the ether oxygens of the ethylene glycol moiety. It is an open question whether consecutive stretches of 2′-MOE-modified residues can confer more water onto backbone and minor groove through formation

of water bridges between either intra- or inter-strand 2′-MOE substituents or both. Similarly, the effects of positive charges on the hydration of backbone and minor groove in the case of the 2′-AP RNA modification are only poorly understood at the moment. Nothing is known in terms of the importance of fluorine as a hydrogen bonding acceptor in the RNA duplex context. Finally, it is noteworthy that using X-ray crystallography, it will usually be easier to establish the origins for improved RNA affinity than for reduced affinity. This is simply due to the fact that it may often be impossible to crystallize oligonucletides with a modification that strongly destabilizes the duplex.

SUMMARY AND PERSPECTIVES

RNA affinity. Owing to the low resistance of natural DNA and RNA to cellular nucleases, hundreds of nucleoside analogs were synthesized in the last years and the RNA affinities and nuclease resistances of modified oligonucleotides have been explored. Correlation of the structure of a modification with its activity in the oligonucleotide context has provided several rules as how to design a modification with improved RNA affinity (14). We are carrying out X-ray crystal structure determinations of oligonucleotides which contain chemically modified building blocks or consist entirely of nucleotide analogs. Beyond a chemical interpretation of the pairing properties of modified oligonucleotides, high-resolution structures can provide more detailed insight into the electronic and conformational properties of a particular modification and how it affects its environment. In addition, the role of water and ion coordination in the modulation of RNA affinity can be studied. While structure determination of an oligonucleotide featuring a single modified residue is generally straightforward, the crystallization of all-modified nucleic acid fragments is a more challenging goal. Determination of three-dimensional structures of oligonucleotide analogs is expected to become an essential tool in the design of novel nucleic acid modifications with optimal RNA affinity.

Nuclease resistance. With regards to high *in vitro* and *in vivo* biological activity of antisense oligonucleotides, resistance to degradation by nucleases may be more important than RNA binding affinity. Why a certain modification confers improved nuclease resistance is currently not understood. Interestingly, the 2′-F modification provides high RNA affinity but protects only insufficiently against degradation. The 2′-AP RNA modification is less stabilizing, but despite a P = O backbone it displays very high nuclease resistance. By comparison, the 2′-MOE RNA modification can be ranked in between the two, in terms of both affinity and resistance. Thus, in addition to investigations into the causes of the higher RNA affi-

nity of certain oligonucleotide analogs, we need to understand what causes the higher nuclease resistance of some modifications. Both issues are crucial for the successful introduction of improved antisense drugs based on the first-generation analogs and are of importance for other areas of nucleic acid based therapeutics, including ribozyme technology (85), antigene technology (86) and aptamer technology (87, 88). As with RNA binding affinity, a structure-based approach may provide an understanding of the causes underlying superior nuclease resistance. This could be achieved by analyzing the three-dimensional structures of complexes between short all-modified nucleic acid fragments and selected 3′-exonucleases.

ACKNOWLEDGEMENT

I would like to thank former and current coworkers in my laboratory for their relentless efforts and continuous assistance, especially Dr. Stefan Portmann and Dr. Valentina Tereshko. I am grateful to Mr. Scot T. Wallace for help with preparation of the manuscript. Our work is supported by grants from the National Institutes of Health (R01 GM55237) and the The Petroleum Research Fund of the American Chemical Society (PRF♯ 32434-AC4). We are grateful to the Northwestern University Medical School for supporting structural research through the Macromolecular Crystallography and Biochemical Computation Resource.

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