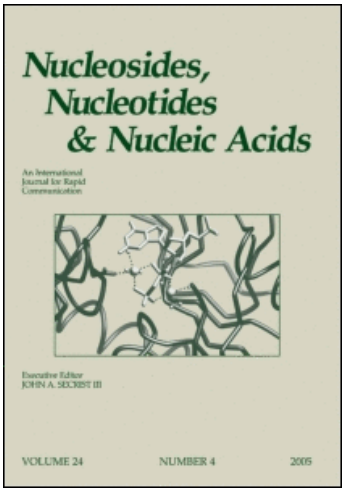

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Jian-Sheng Sun[ab]; Claude Hélène[b]
[a] Laboratoire de Biophysique, USM0503 "Régulation et Dynamique des Génomes", Muséum National d'Histoire Naturelle, Paris, France [b] Laboratoire de Biophysique, USM0503 "Régulation et Dynamique des Génomes", Muséum National d'Histoire Naturelle, Paris cedex 05, France

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Oligonucleotides and Derivatives as Gene-Specific Control Agents[†]

Jian-Sheng Sun[*] and Claude Hélène

Laboratoire de Biophysique, USM0503 "Régulation et Dynamique des Génomes", Muséum National d'Histoire Naturelle, UMR8646 CNRS-MNHN, U565 INSERM, Paris, France

ABSTRACT

The current achievement of genome sequence projects of a dozen eukaryote organisms (including human genome) and the development of functional genomics are providing the basic knowledge required to utilize gene-specific reagents for both basic understanding of cell physiology and therapeutical development. The field of chemical genomics has the ambitious goal of designing molecules that could act selectively on every single gene or gene product in a cell and in vivo. The progress in oligonucleotide-based approaches will be the topic of this review, however, other nucleic acid- and SELEX-based approaches as well as high sequence-specific low molecular weight DNA-specific ligands will also be discussed.

Key Words: Chemical genomics; Gene-chemistry.

[†]Jian-Sheng Sun dedicates this review to the memory of Professor Claude Héléne who died on 11 February 2003, at the age of 65, for his important contributions on nucleic acids at the chemistry-biology interface.

*Correspondence: Jian-Sheng Sun, Laboratoire de Biophysique, USM0503 "Régulation et Dynamique des Génomes", Muséum National d'Histoire Naturelle, UMR8646 CNRS-MNHN, U565 INSERM, 43 rue Cuvier 75231 Paris cedex 05, France; Fax: 33-1-40-79-37-05; E-mail: sun@mnhn.fr.

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INTRODUCTION

The fast and still accelerating pace of the deciphering of the genomic information of an increasing number of organisms (including human genome) together with the development of functional genomics are providing the basic knowledge required to utilize gene-specific reagents for both basic understanding of cell physiology and therapeutical development. The field of chemical genomics has the ambitious goal of designing molecules which could act selectively on every single gene or gene product in a cell and in vivo. Low molecular-weight DNA-binding molecules with limited sequence specificity have been described over the last 50 years and have demonstrated their utility as therapeutic drugs especially in the field of cancer chemotherapy. The progress in oligonucleotide-based approaches will be the topic of this review, however, other nucleic acid- and SELEX-based approaches as well as high sequence-specific low molecular weight DNA-specific ligands will also be discussed.

OLIGONUCLEOTIDE-BASED APPROACHES

Antisense Strategy

The "simple" recognition code that provides the fundamental characteristics of the DNA double helix has led to the design of antisense oligonucleotides.[1,2] Upon binding to a complementary sequence on a targeted messenger RNA (mRNA) they can induce gene-specific inhibition of protein synthesis. The most active antisense oligonucleotides (natural backbone and phosphorothioate analogs) induce sequence-specific cleavage of the targeted mRNA by endogenous RNase H. Several non-RNase H-inducing analogs exhibit properties linked to the processing of mRNA information, e.g., inhibition of the correct assembly of the translation machinery or inhibition of splicing. Until now only PNAs (analogs with a polyamide backbone) have been shown to be able to arrest the translation machinery when targeted to the coding region of the mRNA.[3] Antisense molecules can not only be used to down-regulate protein production, they can also modulate splicing and lead to the production of alternatively spliced mRNAs.[4,5] In all cases the lack of accessibility of the target sequence may severely limit the efficacy of antisense molecules due to folding of the RNA chain and binding of numerous proteins. The development of new oligonucleotide analogs with different chemistries should help overcome the problems associated with cell uptake, compartimentalization inside cells, bio-availability, pharmacokinetics for in vivo applications and therapeutical development. Despite limited but encouraging success in the clinics,[6] the simplicity and the generality of the basic principles underlying the antisense technology makes it very attractive for future development based on both new chemistries and new formulations.

RNA Interference-Based Approach

An alternative to antisense technology has recently emerged; it is originated from a phenomenon named "Post Transcriptional Gene Silencing" first observed

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in transgenic petunia[7] and later in *C. elegans*.[8] It was shown that a double-stranded RNA with sequence identity to a mRNA can induce fast degradation of the cognate mRNA. This phenomenon is named RNA-induced interference (RNAi).[9] RNAi-induced gene silencing depends upon the introduction of double-stranded RNA, which is processed by Dicer enzymes (an RNase III-like ribonuclease which has been discovered in almost all eukaryote kingdoms) into short pieces of double-stranded RNA. These short interfering RNAs (siRNAs), are incorporated into an RNAi-induced silencing complex (RISC), which uses them as a guide to target and destroy complementary mRNAs, and thereby prevent synthesis of the encoded protein.[10–12] All details of the process have not been elucidated yet but recent results indicate that short synthetic double-stranded siRNA (21-23 nt) and even short synthetic antisense RNA oligonucleotide can be incorporated into RISC and achieve the same sequence-specific inhibitory effects as long dsRNAs,[13–15] especially in mammalian cells where long dsRNAs induce other phenomena than RNA interference. The efficacy of the RNAi effect is, in most cases, superior to that of antisense oligonucleotides.

It should be noted that some modified nucleotides incorporated into dsRNA and siRNA are well tolerated by the RNAi machinery.[16,17] This opens the way for tailor-made modifications of synthetic siRNAs to improve their efficiency for in vivo applications, as a promising tool in chemical genomics. Alternatively, the incorporation of nucleotides carrying chemical or photochemical reagents (i.e., 4-thiouracil, 5-bromouracil) in the synthetic siRNAs can trigger site-specific cross-linkage with specific components in RISC and help to unravel the mechanism of action of the RNA interference phenomenon. It has been noted that the degree of RNAi-mediated gene silencing varied markedly from gene to gene and also depends on the particular chosen RNA sequence. Such unpredictable variation is obviously a major impediment for using siRNA in genome-wide application. It should be possible to optimize the selection of the siRNA sequences, provided the molecular details of the interactions between the target gene mRNA, the siRNA and the participating proteins were better known. They are mandatory to apply this promising technique for routine analysis of gene function in higher eukaryotic cells. It can be foreseen that the number of publications in this area will explode in the coming months/years and that a full understanding of the basic mechanisms underlying the RNA interference pathway(s) will lead to new approaches aimed at exploiting this interesting natural phenomenon.

Antigene Strategy

The design of molecules that recognize specific sequence on the DNA double helix would provide interesting tools to interfere with DNA information processing at an early stage of gene expression. The ability to specifically manipulate gene expression has wide range of applications in experimental biology and in gene-based biotechnology and therapeutics. It is an important challenge in biological and biomedical sciences. The so-called antigene strategy was first contemplated by two seminal studies almost 15 years ago with the description of triplex-forming oligonucleotides (TFOs) that can bind to the major groove of oligopyrimidine•oligopurine

sequences in duplex DNA by hydrogen bonding with purine bases.[18,19] During the past decade, a large effort has been devoted to modify TFO in order to improve mostly binding properties to their cognate target sequences in a cellular environment. TFOs that have been shown to be active in cell cultures are:

- G-rich TFOs with either 3′-modified phosphodiester or phosphorothioate backbone interacting by reverse Hoogsteen hydrogen bonding (so-called (G,A)/(G,T)- or purine-motif triple helix).
- T-rich TFOs interacting by Hoogsteen hydrogen bonding (so-called (T,C)/(T,C,G)- or pyrimidine-motif triple helix) with full length modified backbone containing either N3′ → P5′ phosphoramidate linkage (phosphoramidate TFOs) or pseudopeptide linkage (triplex-forming PNA), or with alternatively modified backbone containing 2′-O, 4′-methylene-bridged locked nucleic acids (LNA-containing TFOs).

Some (G,A)-containing TFOs form very stable triplexes. The stability of these triplexes is highly-dependent on the target sequence and high G content seems to be mandatory.[20] A recent spectroscopic study was made by an original design of triplex systems to compare directly the thermodynamic parameters of triplex formation for purine-rich TFOs (in reverse Hoogsteen motif) with pyrimidine-rich TFOs (in Hoogsteen motif) for the same target sequence: a low ΔH was found for purine-motif triplexes whose formation is entropy driven, whereas pyrimidine-motif triplex formation is enthalpy driven.[21] This observation explains why purine-motif triplexes may still be observed at high temperatures. At present, the strongest limitation of G-rich TFOs is their ability to form competing self-associated structures (intermolecular homoduplex or G-quadruplex). A limited incorporation of 6-thioguanine can prevent G-quadruplex formation but decreases slightly TFO binding.[22] Further chemical modifications should increase the intracellular potency of these G-rich TFOs.

Phosphoramidate oligonucleotides have been successfully used as antisense agents both in cell cultures and in vivo since they bind strongly to their complementary RNA target.[23] It appears that this class of modified oligonucleotides has a favorable intracellular and tissue distribution, and makes them a good candidate for in vivo applications. It has also been shown that N3′ → 5′P *thio*-phosphoramidates exhibit an efficient anti-telomerase activity and produce gradual telomere shortening followed by cellular senescence and/or apoptosis in the majority of cancer derived cell lines tested so far.[24] N3′ → P5′ phosphoramidate TFOs have been demonstrated to bind very strongly to double-stranded DNA by triplex formation in Hoogsteen configuration, whereas reverse Hoogsteen interaction was not detected for the investigated sequences.[25] Kinetic study showed that the triplex formed with phosphoramidate TFO has a long lifetime (unpublished data). This can explain why phosphoramidate TFOs can efficiently inhibit transcription at the elongation step in vitro and in cell cultures.

PNA binding by strand invasion requires DNA strand opening and has been shown to be inhibited or strongly decreased under physiological salt conditions.[26] The development of dimeric PNAs (bis-PNA) was found to enhance the strand

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invasion capacities of these PNAs. Triplex-forming bis-PNA have been described to induce intracellular action, which might indicate that PNA strand invasion may be more efficient inside cells.[27,28] Cellular uptake of PNAs still raises major problems. Standard procedures using cationic carriers are inefficient for uncharged PNAs; permeabilization or electroporation seem to be more appropriate techniques but more original and less invasive approaches are needed to fully exploit the properties of PNAs, for example, the utilization of PNA-peptide conjugates.[29]

LNA is an RNA derivative in which the ribose ring is constrained by a methylene linkage between the 2′-oxygen and the 4′-carbon. This bridge results in a locked 3′-*endo* sugar puckering and thus reduces the conformational flexibility of the ribose and increases the local organization of the phosphate backbone. This conformational restriction enhances binding affinity for complementary sequences with high sequence selectivity. It confers nuclease resistance and cannot activate RNase H.[30] LNA-DNA chimera were used as antisense oligonucleotides to target a G-protein coupled receptor (DOR) and produced a physiologic response when injected directly into mice.[31] Injection of LNA were well tolerated by the mice suggesting that their in vivo toxicity should be mild. It is also shown that partially LNA substituted TFOs can significantly stabilize triplex formation whereas the full length LNA TFO fails to form a triplex.[32] Modeling study suggests that this is due to the difference in helical parameters between the rigid and pre-organized LNA TFO and the DNA double helix (unpublished data). One LNA substitution every 2-3 nt in TFOs seems to be optimal. In addition, for mixed LNA-DNA TFOs, a LNA5′-3′DNA step is energetically more favorable than a DNA5′-3′LNA step (unpublished data). In vitro and ex vivo studies show that appropriately designed LNA-containing TFOs can inhibit transcriptional elongation at least as efficiently as phosphoramidate TFOs. Further developments of these conformationally constrained nucleotide derivatives should provide an exciting chemical approach for the control of gene expression and for a broad range of biotechnological and therapeutical applications.

A recent kinetic study by surface plasmon resonance showed that the pyrimidine-motif triplex formation is consistent with a $5' \rightarrow 3'$ directional nucleation-zipping mechanism.[33] The directionality is likely dictated by the right-handedness of the DNA double helix which provides a stronger base stacking interaction at the 5′ duplex-triplex junction than that at the opposite junction. Other evidence suggests that this is likely a common feature for all triplex motifs. The understanding of the mechanism of triplex formation paves the way for a better and rational design of TFOs at the time when genome-wide sequence information of an increasing number of genomes becomes accessible for gene-specific targeting approaches.

As described above, TFOs have been extensively modified especially in their backbone, therefore it becomes more appropriate to name them triplex-forming molecules (TFMs). The high sequence-specificity and high affinity of TFMs have been exploited to down-regulate or up-regulate transcription by binding to their target oligopyrimidine•oligopurine sequences, or to induce directed mutagenesis and to promote homologous recombination as well as to direct modification on genomic DNA at selected gene *loci*. TFMs are therefore powerful tools for gene-specific chemistry. The main achievements are (see Ref.[34] for review and the references therein):

Triplex-Mediated Modulation of Transcription

TFOs-mediated inhibition of transcription has been reported in vitro and in cell cultures either through competion with the binding of transcription factors at promoter sites during the initiation phase or by arresting physically the transcription machinery during the elongation phase.[25,35,36] The demonstration of the postulated triplex-mediated mechanism was provided by the use of mutant oligopyrimidine•oligopurine target sequence. The accessibility of TFOs to the transcriptionally active target genes under chromatin environment was demonstrated by the use of psoralen-TFO or nitrogen mustard-TFO conjugates.[37,38] The observation of triplex-mediated down-regulation of expression of integrated genes into cellular chromosomes or endogenous genes on native chromosomes were additional proofs of TFO's accessibility to their target sequences.[39,40] Even though triplex formation is presently restricted to oligopyrimidine•oligopurine sequences, the fact that these sites can be located downstream of the transcription start site, including exons and introns, considerably increases the number of potential target genes for triplex-based strategies. In addition, activation of transcription has been achieved by using a TFM covalently tethered to activation domains.[41] This suggests that a TFM-effector conjugate may be capable of selectively up-regulating expression of a target gene, provided that the conjugates were appropriately designed and optimized. The approaches of triplex-mediated down- or up-regulated gene expression of selectively targeted genes could be useful to dissect biological mechanisms or to alter phenotypes of cells and organisms.

Triplex-Directed Gene Manipulations

Directed mutagenesis in mammalian cells has been the focus of intense research because of their promising applications for gene correction and engineering. Recent developments have shown that RNA-DNA chimeric oligonucleotides, small fragments of single- and double-stranded DNA as well as DNA vector can effectively perform targeted alteration of genomic sequences. The use of both circular and linear RNA-DNA chimeric oligonucleotide (RDO) has been shown to induce gene conversion in cell cultures and in vivo, but their mechanism of action is still largely unknown and their efficiency seems variable and unpredictable.[42–44] Homologous recombination may be an ideal way for gene conversion since it is usually an error-free process. Several methods (SFHR/SDF, vector DNA) have been developed to facilitate gene replacement and gene knockout by site-specific homologous recombination, but the efficiency is usually low.[45,46] Therefore the development of tools for targeted modification of genomic DNA remains a topic of great interest, and will have important implications for gene therapy and development of transgenic animals.

Triplex-based strategies are among the few methods that allow for a rational design of molecules for targeted homologous recombination and gene correction. TFMs have been used to target mutations or to promote homologous recombination at selected sites in cells and in vivo via the induction of a DNA repair-dependent process (see Ref.[47,48] for review and the references therein). It has been shown that TFMs either alone (both TOFs and bis-PNA) or tethered to a DNA-damaging agent (TFO-psoralen conjugates) can induce a variety of spectrum of point mutations and

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deletion/insertion around triplex sites located either in an extrachromosomal vector or in chromosomal DNA, with low but measurable frequency. They can also stimulate homologous recombination and gene conversion within and between extrachromosomal vectors at selected sites in mammalian cells and in vivo.[49] Triple helix-induced mutagenesis seems to be associated with nucleotide excision repair or transcription-coupled repair pathways, however the understanding of the precise mechanism requires further investigations.

TFOs have been used to guide homologous donor DNA (DD) to its intended target site on an extrachromosomal gene and to position it for efficient information transfer via homologous recombination and/or gene conversion.[50,51] In this approach, TFO was covalently tethered to DD through a linker. The effectiveness of the TFO-DD conjugate could be explained by: (i) an increase in the local concentration of DD; and (ii) a stimulation of DNA repair by triple helix formation that could provoke recruitment of proteins involved in homologous pairing, strand exchange and/or recombination.[52] Recently, a new method named GOREC (for Guided hOmologous RECombination) has been described.[53] It shares similar gene targeting strategy but differs by the non covalent attachment (Watson-Crick base pairing) of the donor DNA to an adapter oligonucleotide which is covalently linked to the TFO. This modular concept allows to guide not only an oligonucleotide (ODN, RDO) but also a DNA fragment (either single- or double-stranded) to the target site for homologous replacement. Therefore, the target site is not restricted to the vicinity of the triplex site as is the case for the TFO-DD conjugate and can be at a remote site. It has even shown that the TFO accelerates D-loop formation between DD and target DNA in the presence of RecA protein, and both triplex and D-loop are formed in the joint molecule (an obligatory intermediate and a limiting step in the homologous recombination process) in vitro.[53] Recent data have shown that PCR-amplified 759-nt single-stranded or 759-bp double-stranded donor DNA fragments guided by a TFO were able to restore the expression of mutant eGFP gene by correcting a stop codon on a transfected plasmid in CHO cells.[54] It remains to determine quantitatively the efficiency of the GOREC method to perform gene correction at a number of endogenous genes (e.g., HPRT, HBB, tyrosinase, CFTR, etc.) under chromatin context.

Triplex-Based Gene Chemistry

Triplexes are very attractive for biotechnological applications because they open new ways to manipulate double-stranded DNA in a sequence-specific manner. They have been successfully applied to purify plasmids used in gene therapy.[54] The method is based on a triple helix affinity chromatography technique.[55] It avoids toxic chemicals and allows purification of plasmids whatever their size, provided they contain a triplex site and have potential for scale-up. An approach using bis-PNA to non-invasively label plasmid has been described.[56] It is a useful tool to investigate, in real time and in living cells, the factors affecting plasmid intracellular traffic, biodistribution and those influencing the potency of gene delivery systems for gene transfer into cell cultures and in vivo. The circularization of an oligonucleotide around double-stranded DNA, i.e., a DNA plasmid, is possible thanks to triple helix

formation: after triplex formation the free ends of the TFOs that are not involved in DNA binding were joined thus creating a circular DNA molecule catenated to the plasmid.[57] Such 'padlock' oligonucleotides may provide a method to attach a molecular tag in an irreversible way without covalent linkage (topological link) to supercoiled plasmid DNA, and likely to large DNA fragments (e.g., BAC). Such a gene chemistry approach may find applications in the development of new techniques for detecting double-stranded DNA[58] also for gene therapy applications by coupling signaling molecules (e.g., nuclear localization signals or other peptides) to the DNA in order to improve its in vivo bio-availability and expression.[59,60]

It is now well established that a triplex can be formed within cells, but the intracellular fate of such a complex is still largely unknown. In particular, it would be interesting to direct efficient DNA damage where triplex is formed. This should enhance the efficacy of the antigene approach and could be considered as an equivalent to the RNase H-mediated RNA cleavage often involved in the antisense approach. Different strategies have been contemplated. TFOs have been conjugated to DNA reagents such as adduct inducers (psoralen), alkylating agents (nitrogen mustard) and cleaving reagents (EDTA•Fe^{2+}, OP•Cu^{2+}) (see[61] for review and the references therein). TFOs can be covalently tethered to a substance (e.g., topoisomerase inhibitors) that is able to recruit a cellular enzyme (e.g., topoisomerase I or II) with a DNA cleavage activity that would induce irreversible damage at the triplex site.[62–64] Covalent linkage between topoisomerase poisons and a short TFO may decrease undesirable toxic effects of the free drugs when used in cancer chemotherapy, by conferring them sequence specificity.

Intramolecular Triple Helices and Elucidation of Their In Vivo Roles

Intramolecular triplexes (named H-DNA) were first found in plasmid DNA at the mirror repeats of oligopyrimidine•oligopurine sequences, provided the appropriate base triplets were formed as in the intermolecular triplexes (see Ref.[65] for review and the references therein). As intramolecular triplex formation involves the disruption of half of the symmetry-related duplex and the subsequent folding back of one of these single-stranded regions (which serves as the third strand in H-DNA), negative supercoiling (the topological constraint in natural DNA) is the driving force of such a DNA strand rearrangement (which can be schematized as $2+2 \rightarrow 3+1$ transition). H-DNA has been well studied in vitro but their in vivo roles, if any, still remain elusive. As DNA undergoes a characteristic conformational change upon H-DNA formation and forms a sharply bent double helix which is often observed when DNA-regulatory proteins bind to DNA, it is tempting to postulate that H-DNA could act as a molecular switch to modulate gene expression in a structure-dependent manner in vivo, and that cellular proteins could specifically recognize triple-helical DNA and stabilize H-DNA. Actually, triplex-binding proteins have been found in *Saccharomyces cervisiae* and in Hela cell extracts.[66–68] A number of proteins with triplex-binding activity has been identified in Hela nuclear extracts by protein 2D-electrophoresis and mass spectrometry.[69] They may reveal novel triplex-associated functions and mechanisms of action in vivo. An alternative and complementary approach has been carried out. It is based on the triplex-specific ligands

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which has been discovered and developed with the primary aim to improve triplex stability. Site-specific DNA damages can thus be triggered by triplex formation where a low molecular weight triplex-specific ligand tethered to a DNA reagent specifically cleaves DNA at the triplex site. A highly specific triplex ligand (a benzoquinoquinoxaline derivative, BQQ) previously reported to be a strong triplex stabilizer was linked to an EDTA molecule. In the presence of Fe^{2+}, this conjugate cleaved double-stranded DNA where triplex was formed.[70] This type of molecule may find a wide range of applications in site-specific cleavage of DNA in triplex-based strategies. In addition, its selectivity for triple-helical structures should make it useful for probing the presence of H-DNA, and thus helping to elucidate the suspected role of such structures in the control of gene expression.

NUCLEIC ACID AND SELEX-BASED APPROACHES

Besides oligonucleotide-based approaches, other nucleic acid-based approaches have also been extensively developed. They mainly originate from the discoveries of naturally occurring nucleic acid complex and PCR amplification method. It is well known that nucleic acids can perform various tasks in living systems that range from the storage and transfer of genetic information to the catalysis of biochemical reactions that are essential to life. The relatively simple and repetitive composition of RNA and DNA molecules makes them easy to synthesize and manipulate outside the confines of cells. This provides the possibility to evaluate and to explore the ability of nucleic acids to perform molecular recognition and catalysis tasks for use in basic research and in biotechnological as well as therapeutical applications. The most significant discoveries and recent advances are summarized below, but the list is not exhaustive:

- Some RNA molecules, called ribozymes, have intrinsic enzyme-like activity first discovered in *Tetrahymena* 20 years ago. They can catalize chemical reactions in the complete absence of protein cofactors (see Ref.[71] for review and the references therein). In addition to their well-known RNA cleavage function, RNA catalysts probably effect a number of key cellular reactions. This versatility has lent credence to the idea that RNA molecules may have played a central role in the early stages of life (RNA world). The so-called hammerhead ribozyme made of about 40 nucleotides is the smallest of the naturally occurring ribozymes. Recent experiments show that the hammerhead motif is the most efficient than that can be selected from randomized pools of RNA suggesting that the natural ribozymes might evolve in multiple rounds since life occurs on Earth.[72] It is interesting to note that the further shortened hammerhead ribozymes (as short as 22-nt) by use of oligonucleotide linkers instead of a stem-loop II region can form homo- or heterodimers that are still very active, called minizymes.[73]
- Aptamers are oligonucleotides derived from an in vitro evolution process called SELEX.[74] Aptamers have been evolved to bind small ligands or proteins which are associated with a number of disease (see Ref.[75] for review and the references therein). Using this method, many powerful antagonists,

or agonists of proteins (enzymes, receptors, etc.) have been found. Modified nucleotides are necessary for long-lived aptamers in blood in order to produce antagonistic effects in vivo. Further conjugation of the aptamers with high molecular weight vehicles can keep them in the circulation from hours to days and inhibit in vivo physiological functions known to be associated with their target proteins. A new approach (aptamer arrays) aimed at diagnosing diseases at an early stage is envisioned by use of photo-cross-linkable aptamers that will allow the covalent attachment of aptamers to their cognate proteins, with very low backgrounds from other proteins in body fluids.

- Allosteric ribozymes are engineered RNAs that operate as molecular switches whose rates of catalytic activity are modulated by the binding of specific effector molecules. It is comprised of distinct effector-binding (aptamer) and catalytic (ribozyme) domains through an optimized linker by ''allosteric selection,'' a molecular engineering process that combines modular rational design and in vitro evolution strategies (see Ref.[76] for review and the references therein). Future development of engineered allosteric ribozymes are promising as biosensor components and as controllable genetic switches.
- DNA in its single-stranded form has the ability to fold into complex three-dimensional structures that can be served as highly specific receptors or catalysts. Untill recently, only protein enzymes and ribozymes are known to be responsible for biological catalysis, but deoxyribozymes (DNAzyme) that can kinetically rival with ribozymes have been a long-standing goal of bioorganic chemists. The advent of SELEX and related combinatorial methodologies has enabled the discovery of new enzymatic activities that could not be easily predicted or synthesized. Nevertheless, the quest for a synthetic RNaseA mimic, as defined as a scaffold appended with cationic amines and catalytic imidazoles has long remained elusive, particularly in terms of turnover. A truly catalytic divalent metal cation-independent DNAzyme synthetically modified with imidazoles and cationic amines has recently been reported to be an RNaseA mimic.[77] This new advance demonstrates how synthetic organic chemistry, when merged with combinatorial selection, can result in a new class of ''hybrid'' nucleic acids with additional functionality, DNAzymes, that meets the ongoing synthetic challenges for developing relatively small biomimetic catalysts.

LOW MOLECULAR WEIGHT AND ENGINEERED DNA-SPECIFIC LIGANDS

It is noteworthy that during the past 7 years, specific ligands of double-stranded DNA have been developed. They are based on the understanding of molecular interactions unraveled by X-ray and NMR structures of a number of ligand-DNA and protein-DNA complexes. Two families of ligands which exhibit strong binding and high sequence-specificity of DNA double helix have been the focus of

intensive developments:

- Small synthetic molecules consisting of hairpin polyamides that mainly recognize short DNA sequence via the minor groove of DNA. Pyrrole, hydroxypyrrole, imidazole and other amino acid derivatives are used to generate the polyamide hairpin so that recognition of all four base pairs depends on a code of side-by-side aromatic amino acid pairings in the minor groove (see Ref.[78] for review and the references therein). They have been shown to interfere efficiently with the processing of gene expression in vivo. Continuous efforts have been devoted to further expend the repertoire of DNA sequence that can be targeted by hairpin polyamides in order to achieve gene-specific recognition. The combination of polyamides and polypeptides that are engineered on the basis of naturally occurring DNA minor binding motifs of proteins should help to attain this ultimate goal.
- The structural feature of one of the families of DNA binding proteins, namely, the zinc finger proteins, has been used to design poly-finger peptides displaying naturally occurring zinc finger domains as modular building blocks in a polypeptide chain (see Ref.[79] for review and the references therein). The polyfinger peptide units specifically recognize DNA base triplets $(XNN)_n$ sequence motifs (where $X = G$ or T; $N = A$, T, G, C and $n \leq 6$). For cell biology applications, these protein motifs are produced in situ using a DNA vector that contains all information to synthesize the polypeptide.

CONCLUSION

Oligonucleotides are sequence-specific nucleic acid ligands that have successfully been used to down-regulate or up-regulate and to alter gene expression at various stages of information transfer and processing from genomic DNA to proteins. They can perform gene-specific genomic modification and induce directed mutagenesis and homologous recombination of targeted genes. The explosion of genomic information and genome analysis using bioinformatics tools provides a wide basis for developing such gene-specific tools for chemical genomics in order to achieve the ultimate goal of designing molecules which could act selectively on every single gene or gene product in a cell and in vivo.

A better understanding of oligonucleotide cellular traffic is necessary to design appropriate carriers that could increase oligonucleotide-specific in vivo activity. It can be anticipated that a substantial increase in intracellular efficacy of triplex-induced effects will be achieved by modifications that could improve not only triplex stability but also nuclease resistance, cellular uptake and absence of notable toxicity of TFMs in the same way as it has been described for antisense oligonucleotides. These optimizations should enhance their efficiency so that they become powerful tools in the post-genome era, when new components of DNA-dependent pathways have to be molecularly characterized, or to probe pathways of cellular DNA metabolism, such as chromatin remodeling, transcription or DNA repair.

Further identification and characterization of protein specific binding to peculiar nucleic acid structures in conjunction with the development of new efficient

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structure-specific ligands could lead to comprehension of the roles of DNA structures in vivo and contribute to the discovery of structure-related gene regulation processes.

Chemical approaches to design DNA sequence- and structure-specific ligands have opened new avenues to exploit genomic information and to provide new tools for functional genomics and novel candidates for therapeutic development. Most of the works carried so far has dealt with in vitro and cellular studies. In vivo applications of TFMs are still in their infancy and recent results in mice[49] have provided a first hint at achieving this goal. The field of is still lagging behind the development of antisense oligonucleotides[80] and now of siRNA.[81] But gene-specific ligands have their own potential applications that amply justify further in vivo investigations.

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