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Review

Triple helix formation and the antigene strategy for sequence-specific control of gene expression

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Abstract

Specific gene expression involves the binding of natural ligands to the DNA base pairs. Among the compounds rationally designed for artificial regulation of gene expression, oligonucleotides can bind with a high specificity of recognition to the major groove of double helical DNA by forming Hoogsteen type bonds with purine bases of the Watson–Crick base pairs, resulting in triple helix formation. Although the potential target sequences were originally restricted to polypurine·polypyrimidine sequences, considerable efforts were devoted to the extension of the repertoire by rational conception of appropriate derivatives. Efficient tools based on triple helices were developed for various biochemical applications such as the development of highly specific artificial nucleases. The antigene strategy remains one of the most fascinating fields of triplex application to selectively control gene expression. Targeting of genomic sequences is now proved to be a valuable concept on a still limited number of studies; local mutagenesis is in this respect an interesting application of triplex-forming oligonucleotides on cell cultures. Oligonucleotide penetration and compartmentalization in cells, stability to intracellular nucleases, accessibility of the target sequences in the chromatin context, the residence time on the specific target are all limiting steps that require further optimization. The existence and the role of three-stranded DNA in vivo, its interaction with intracellular proteins is worth investigating, especially relative to the regulation of gene transcription, recombination and repair processes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Triple helix; Antigene strategy; Gene therapy; Targeted mutagenesis; Oligonucleotide; H-DNA

Abbreviations: CNBP, cellular nucleic acid binding protein; IL-2Rα, Interleukin-2 receptor, alpha subunit; IGF-IR, insulin growth factor I receptor; nt, nucleotide; Pu, purine; Py, pyrimidine; (T,mC), oligonucleotide motif composed of thymines and 5-methylcytosines; G-rich, designs the (G,T) or (G,A)-motif; (G,T), oligonucleotide motif composed of guanines and thymines with an antiparallel orientation with respect to the purine strand of the duplex (reverse-Hoogsteen hydrogen bonds); (T,G), oligonucleotide motif composed of guanines and thymines with a parallel orientation with respect to the purine strand of the duplex (Hoogsteen hydrogen bonds); rPu–dPy, duplex formed between an oligoribonucleotide strand composed of purines, and an oligodeoxyribonucleotide composed of pyrimidines; (TCG), guanine and thymine majoritarily; (po), phosphodiester; (pn), N3′→P5′ phosphoramidate; (ps), phosphorothioate; (mp), methylphosphonate; NER, nucleotide excision repair; PNA, peptide nucleic acid; OPC, oxazolopyridocarbazole; TFO, triple helix-forming oligonucleotide; ODN, oligodeoxyribonucleotide; Hprt, hypoxanthine phosphorybosyl transferase

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1. Introduction

During recent years oligonucleotides (ODN) have received considerable attention since they provide a rational way to design sequence-specific ligands of nucleic acids for various purposes. They can be employed as gene inhibitors to investigate the function and regulation of specific genes. They have been successfully used as highly sequence-specific artificial nucleases by attachment of cleaving reagents or by targeting cellular nucleases to chosen sites. They can be developed as therapeutic agents the activity of which relies on the selective modulation of gene expression.

Different strategies can be used to target a sequence in a nucleic acid: the *antisense* and *ribozyme* strategies both rely on the formation of Watson–Crick hydrogen bonds between a specific RNA target and the oligonucleotide or the ribozyme. An oligonucleotide can be used as a decoy to trap DNA binding proteins, thus preventing them from associating to their normal target. Oligonucleotides can also recognize double-helical DNA at specific sequences by forming Hoogsteen or reverse-Hoogsteen hydrogen bonds with purine bases on one of the duplex strands, thereby forming a local triple helix. A single-stranded DNA or RNA sequence can engage in very stable three-stranded structures by establishing both Watson–Crick and Hoogsteen-type hydrogen bonds with *clamp* or *circular* oligonucleotides.

Targeting oligonucleotides to the gene itself (the *antigene* or *triple helix* strategy) presents several advantages as compared to antisense oligonucleotides or ribozymes which are directed to messenger RNAs. There are only two copies (two alleles) of the targeted gene whereas there may be thousands of copies of a messenger RNA. Blocking mRNA translation even by inducing sequence-targeted cleavage of the RNA chain does not prevent the corresponding gene from being transcribed, thereby repopulating the RNA pool. In contrast, preventing gene transcription is expected to bring down the mRNA concentration in a more efficient and long-

lasting way, depending on the residence time of the antigene oligonucleotide on its target sequence and its life-time determined by its nuclease sensitivity. Obviously the simultaneous use of two oligonucleotides one targeted to the gene of interest, the other one targeted to its mRNA, would open new possibilities to control gene expression in a sequence-specific way. One of the difficulties in designing an antigene oligonucleotide resides in the accessibility of the target sequence in the chromatin structure of the cell nuclei. Moreover the problem is not more complex than that of finding a target sequence for an antisense oligonucleotide in a folded mRNA molecule which interacts with cellular proteins. This possibility was first proposed in 1987 by two independent groups of investigators [1,2]. The present review will address some of the issues raised by the development of antigene oligonucleotides as gene-specific regulators.

2. Intermolecular triplex formed on double-helical DNA

2.1. *Triple helix formation: basic rules and canonical motifs*

Triplex formation obeys precise rules imposed by several structural constraints (see [3–6] for reviews). Optimal target sequences must harbor consecutive purines on the same strand since only purine bases are able to establish two Hoogsteen (or reverse-Hoogsteen) type hydrogen bonds in the major groove of DNA (Fig. 1): this is the main restriction to the repertoire of potential target sites. The notion of isomorphism provides additional constraints to the formation of different base triplets in the same structure. When the c-′1 atoms in each strand of the duplex is fixed, the position of the c-′1 atom in the third strand depends on the triplets (Fig. 1). Energy minimization is obtained when combining those triplets whose spatial positions of c-′1 atoms of the third strand are proximal. Several motifs optimal for triplex stability are presented on Fig. 1. The (C,T)-motif involves the formation of isomorphous $C.GxC^+$ and T.AxT base triplets, upon binding of a (C,T)-containing oligonucleotide with a parallel orientation with respect to the purine strand (Hoogsteen hydrogen bonds). Binding is pH dependent because cytosines must be protonated to form two hydrogen bonds with G. The (G,A)-motif involves the formation of C.GxG and T.AxA triplets, upon fixation of a (G,A)-containing oligonucleotide in an antiparallel orientation with respect to the purine strand (reverse-Hoogsteen hydrogen bonds); this triple helix does not depend on pH. A (G,T)-motif involves binding of a (G,T)-containing oligonucleotide, whose orientation depends both on the number of GpT or TpG steps in the third strand and on the length of G and T tracts [7].

2.2. *Recent progress made to increase intermolecular triplex stability and to extend the repertoire of target sequences*

Considerable research efforts are presently devoted to extending the repertoire of potential target sites and increasing the stability of triple helices under physiological conditions. The search for optimal nucleotide analogues involving base, sugar or backbone modifications, or attachment of an intercalating agent, was driven by several goals [3,6]: (a) to increase triplex stability while preserving specificity; (b) to overcome the pH dependence in the (C,T) motif; (c) to extend the repertoire of potential target sequences (see [8] for a review), for example to achieve the recognition of a pyrimidine interrupting an oligopurine sequence [9], or the recognition of two oligopurine motifs located on each of the DNA strands (switch oligonucleotides) [7]; (d) to find substitutes in the third strand for thymines, which gives rise to the less stable T.AxT triplet; (e) to minimize self-associated-structures which compete with triplex formation, especially in the case of G-rich or C-rich oligonucleotides.

A preferential site of intercalation is formed at the triplex–duplex junction [10]. Intercalating agents were conjugated to the 5′- or 3′-end or to internal positions to stabilize triple helices [11,12], especially those containing one or two base-pair interruptions in the purine motif [13]. Several intercalating agents have been developed, some of them provide additional interesting properties for photochemical or chemical activation [3,6] (see Table 1).

A panel of drugs with various binding modes were reported to stabilize triple helices [4] or to promote the formation of otherwise unstable triplex structures

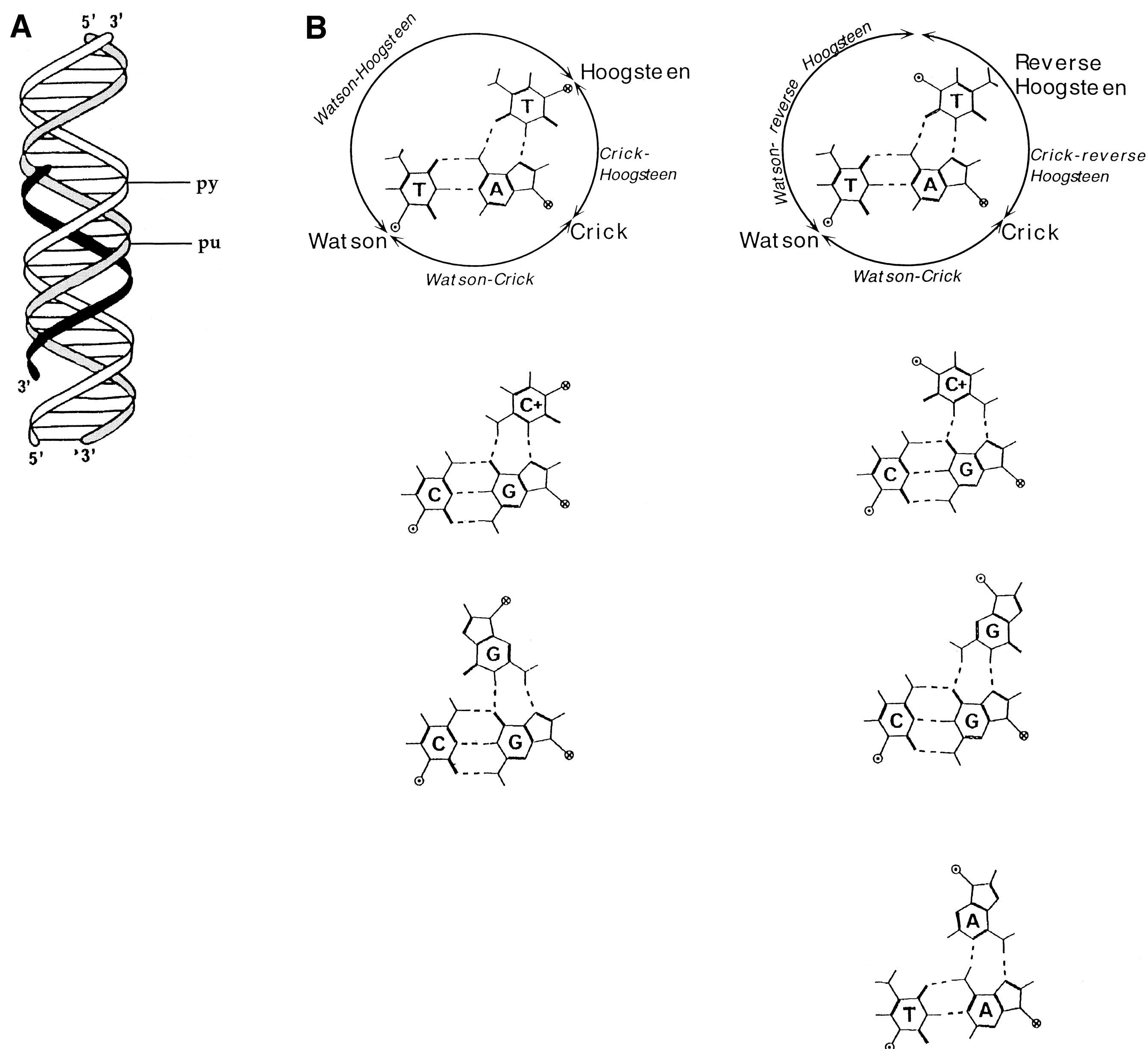

Fig. 1. (A) Intermolecular triple helix schematically represented. The third strand (black thick line) establishes hydrogen bonds with the purine strand of the duplex. In this drawing the third strand establishes hydrogen bonds with the oligopurine (gray) target sequence and runs parallel to it. (B) Base triplets are formed by Watson–Crick T•A and C•G base pairs with T, protonated C, G and A. Hoogsteen bonds are formed in the (C,T)-motif (left), whereas reverse Hoogsteen bonds are formed in the (G,A)-motif (right). The position of C′1 carbon atom of deoxyribose is indicated by circles containing a cross (parallel orientation with respect to the purine strand), or a dot (antiparallel orientation). The grooves of the triple helix are indicated on the top with T.AxT Hoogsteen and reverse-Hoogsteen base triplets.

[14,15] (Table 2). However only a few of them interact specifically with triplex structures. Anthraquinones [16,17], benzopyridoindole derivatives (B[*e*]PI) [18] and benzopyridoquinoxaline (B[*f*]PQ) are tetracyclic aromatic crescent-shaped molecules that interact with the Crick–Hoogsteen base pair of the T.AxT triplet. These compounds were shown to promote triplex formation with various motifs of the third strand (Table 2). Covalent attachment of these molecules to either the 5′ or 3′ end, or to internal

Table 1
Intercalating or activatable reagents covalently linked to TFOs

Dye	Comment	In vitro	In cell culture	References
Intercalating agents				
2-methoxy,6-chloro-acridine		+		[10,186,187]
	IL-2Rα promoter		+	[123]
	SV40 origin of replication		+	[127]
OPC	Sites for HIV-1 viral integrase or non-integrated HIV dsDNA	+		[188,189]
B[e]PI, B[f]PQ		+		[19,20]
Daunorubicin				[190,191]
Photoactivatable agents				
Psoralen		+		[192]
	-β-*galactosidase* (coding sequence)		+	[68]
	-*IL-2Rα promoter*		+	[67]
	-*aromatase* (coding sequence)		+	[69]
	Detection of covalent triplex:			[56]
	-*integrated proviral HIV1DNA*		+	
	-*IL-2Rα promoter*		+	[76]
	Covalent triplex not repaired *luciferase gene* (transcribed untranslated)		+	[132]
	Directed mutagenesis (*SupF* coding sequence)		+	[70,72]
Azido-phenacyl or -proflavin		+		[193]
Aryl-azide		+		[194]
Ellipticine		+		[195]
Fullerene		+		[196,197]
Chlorine		+		[198]
Alkylating agents				
N-2-Chloroethyl-*N*-methylamino benzylamide	*c-fos* promoter		+	[199]
Modified dC	Transcription truncated	+		[91]
Bromoacetyl		+		[200]
Metal complexes				
Fe–EDTA		+		[1,201]
Metallo-porphyrins		+		[202,203]
Desferal conjugates		+		[204]
Ni-tripeptide	PNA	+		[205]
CuII-phenanthroline		+		[206]
Pt II		+		[207,208]
Radioactive constituents				
^{125}I or ^{127}I		+		[209,210]

Intercalating or activatable agents which have been covalently linked to oligonucleotides. Some of them were studied in biological systems in vitro or in cells as indicated in the 'comment' column.

positions of TFOs increases triplex stability to a higher extent than duplex stability [19,20]. Recently pentacyclic BIQ and BQQ were rationally designed as optimal structures from the last two molecules to increase the overlap with all three bases of the base triplets, and were shown to specifically stabilize triplex structures over duplex ones [21,22].

Protection of the oligonucleotide against cellular nucleases can be achieved by changing the anomeric configuration of the glycosidic bond from [β] to [α]

Table 2
Ligands binding to triple helices

Binding mode	Ligand	Motif	References
Minor groove	Polyamine[a]	(T)	[211]
	Berenil	T•AxT and rU•rAxU	[15,212,213]
		dT•rAxrA or dT•rAxdT	[15]
	Distamycin	(T)	[214]
	Netropsin	(T)	[215]
	DAPI	(T)	[15]
	Peptide (lys-X)$_3$	(T,C), (G,A)	[216]
Intercalation	2-Methoxy-6-chloro-acridine[a]	Duplex/triplex junction	[10]
	BET	(T)	[217,218]
	Benzophenazine (NC-182)	(T)	[214]
	Coralyne and derivatives	(T,C) and (T)	[219]
	Quinacrine and derivatives	(T)	[220,221]
	Quinoline	(T,C) and (T)	[222,223]
	Anthraquinone	(T,C)	[186,223–225]
	B[*e*]PI	(G,T) reverse-Hoogsteen	[18,226,227]
		(T,C)	[228]
		(TCG) Hoogsteen	[93]
	B[*e*]PI[a]	(T,C)	[20]
	B[*f*]PQ[a]	(T,C)	[19]

Specific triplex ligands. From left to right are indicated: the interaction mode, the motif of the third strand, and the bibliographic references.
[a]Have been covalently linked to TFOs (see Table 1).

(Fig. 2) [6] without much incidence on triplex stability [23], the orientation of the third strand depending on the sequence [6].

Another modification was obtained by substitution of deoxyribose for ribose in the third strand of the triplex and this resulted in stabilization of triple helices for the (C,U) or (G,U)-motif with a parallel orientation of the third strand with respect to the purine target sequence [3,6,24]. NMR structures were determined for short intramolecular hybrid (D.DxR) triplexes with an RNA third strand in the (U,C) motif [25,26]. For parallel triplexes, only the pyrimidine third strand can be RNA, whereas for antiparallel structures RNA is not well tolerated unless all three strands are RNA [27–29]. Significantly, the (C,U) canonical RNA motif was the only one selected from a pool of random RNA sequences at pH values between 6.5 and 7.4 [30,31]. The stability of triple helices involving 2′-*O*-alkyl analogues is higher than that of oligoribonucleotides [24,32]. The relative stability of all nine combinations of DNA (D) and RNA (R) clearly depends on the sequence and general rules have not yet emerged [24,33–35] except the finding that replacing D to R in the purine strand of the duplex actually gives the most unfavorable combinations. Backbone modification was also exploited to optimize triplex stability while keeping sequence specificity, to minimize self-associations, and furthermore to design oligonucleotides which are resistant towards degradation by cellular nucleases. For this purpose, several families of derivatives were investigated, among which phosphoramidates (pn) and peptide nucleic acids (PNAs) are, at least in vitro, the most promising derivatives (Fig. 2).

Phosphorothioate (ps) modification was extensively used in the antisense strategy since it confers resistance to nuclease degradation and the hybrid formed with RNA is still recognized and cleaved by RNase H (Fig. 2). However, triple helices with (ps) oligonucleotides appear at most as stable as their phosphodiester (po) analogues, depending on the motif of the third strand and on the presence of divalent cations. Triplex stability is greatly reduced in the pyrimidine motif by this modification [36–38]. A report established that the regular diastereoisomer Rp configuration was able to form a triple helix in

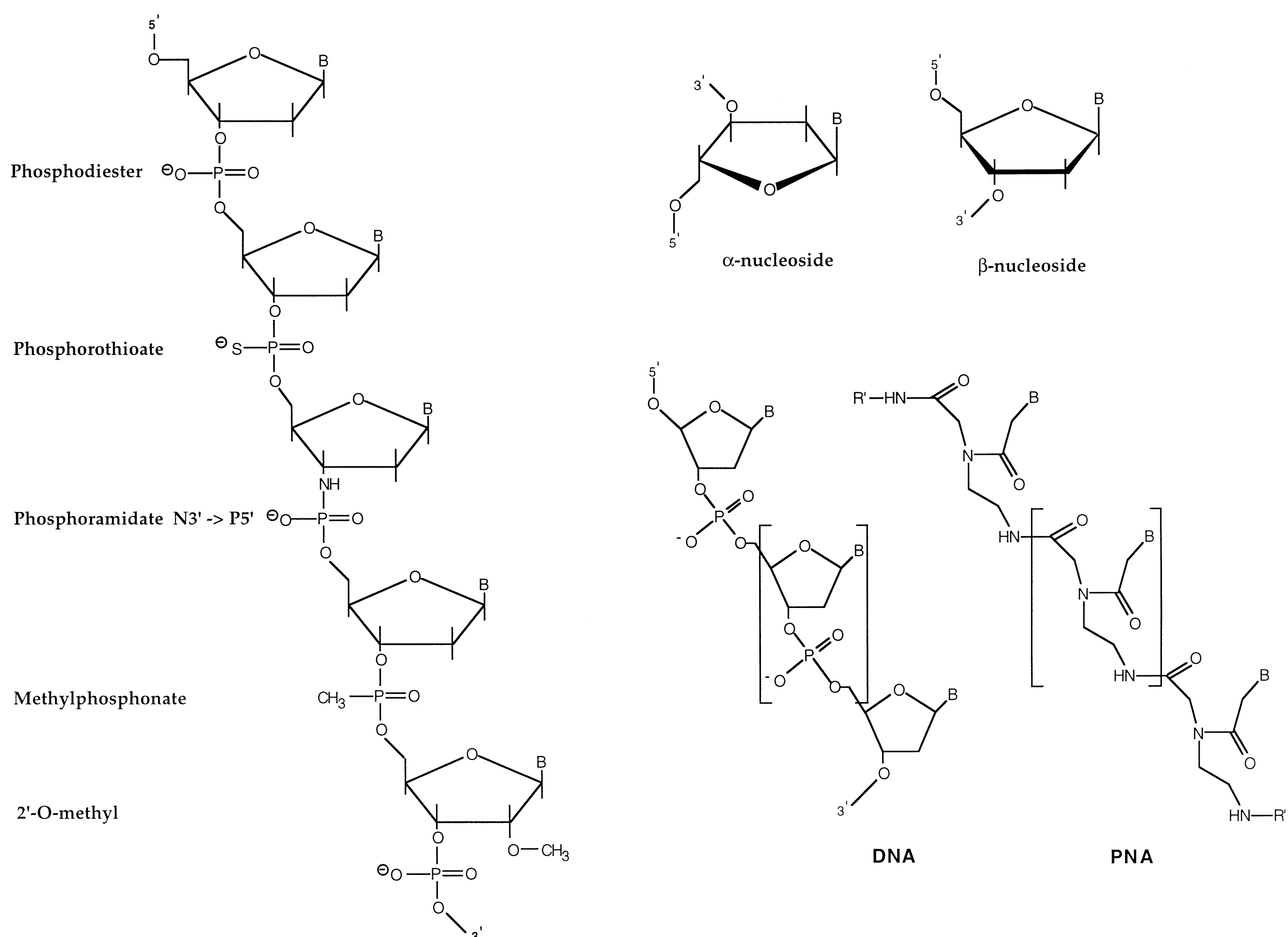

Fig. 2. Different backbone modifications conferring stability towards intracellular nucleases and used in the antigene strategy.

the purine motif, but not in the pyrimidine motif [37]. Derivatives with the (G,A) [37–40] or the (G,T)-motif [41] have been used in cells, exhibiting in some cases more pronounced biological effects compared to their (po) counterpart. However, non-specific effects are suspected due to their propensity to bind to other non-nucleic targets [42].

Among derivatives with neutral backbones, methyl phosphonate (mp) in the purine motif bind to a duplex or a single-stranded nucleic target forming triple helices with higher stability than their phosphodiester counterparts [43] whereas (mp) oligonucleotides containing exclusively thymines did not form stable triple helices [44].

Peptide nucleic acids (PNAs) (Fig. 2) are nuclease-resistant oligomers in which the deoxyribose phosphate backbone is replaced by a homomorphous, achiral and uncharged backbone based on *N*-(2-amino-ethyl)glycine (see [45] for review). Oligopyrimidine PNAs were shown to bind to single- or double-stranded DNA, forming a 2:1 complex with the purine target sequence. In the case of a duplex target, the complementary pyrimidine sequence is displaced, thus resulting in the formation of a P-loop structure (Fig. 3C). Although the binding to a duplex sequence is severely inhibited under physiological ionic conditions, it can be promoted by sequences adopting a cruciform or a D-loop structure even when transitorily formed (see Section 2.4) [46,47]. Strand displacement was also reported with an oligopurine PNA forming a very stable 1:1 complex with the oligopyrimidine sequence [48]. Clamp-PNA composed of pyrimidines were designed so that half of the bases form Watson–Crick base pairs, while the other half

forms Hoogsteen hydrogen bonds with the Watson–Crick duplex (Fig. 3E,F). Targets are composed of single-stranded DNA [49,50], RNA [51,52], or double-stranded DNA [47,49,53]. For a purine target present in long duplex DNA sequences, higher stability was obtained by an increase of the 'on-rate', compared to monomeric PNA [49,50]. An optimal structure was obtained when one pyrimidine motif (Watson–Crick) was parallel (the N-terminal end of the PNA is close to the 5′ side of the oligonucleotide) and the second (Hoogsteen) antiparallel to the purine target strand [50,51].

Recently analogues containing N3′→P5′ phosphoramidate linkages (Fig. 2) were synthesized [54]. This modification highly stabilizes triplex structures formed with (T,C) and (T,G)-motifs bound in a parallel orientation with respect to the oligopurine sequence, but (G,A) or (G,T)-containing (pn) oligonucleotides did not form triple helices in the reverse-Hoogsteen motif, a result similar to that obtained with oligoribonucleotides. Oligophosphoramidates forming Hoogsteen hydrogen bonds seem to have very promising properties, in vitro and in cells [55–57].

2.2.1. Minimization of self-associated structures

The efficacy of triplex formation does not only depend on intrinsic triple helix stability: it can be severely impaired when the oligonucleotide is made unavailable by sequestering in self-associated stable structures. These problems can be circumvented in several ways: (a) choosing the most appropriate third strand motif according to the primary sequence of the target; (b) using base analogues; (c) modifying the backbone; (d) conferring a secondary structure to the oligonucleotide.

Guanine-rich oligonucleotides can form intra- or inter-molecular four-stranded structures involving G-quartets; these structures are favored when the sequences contain repeats of consecutive guanines. (G,A)-oligonucleotides can form other intermolecular structures, such as parallel homoduplexes involving A•A, G•G and G•A base pairs [58]. Monovalent cations, such as K^+ present under physiological conditions, enhance the formation of quadruplexes observed when using G-rich oligonucleotides [59,60], but decrease the formation of a parallel homoduplex in the presence of divalent cations [58]. Partial replacement of guanines by analogues such as 6-thioguanine or 7-deazaguanine was shown to minimize G-quartet formation [60,61].

Cytosine-rich oligonucleotides can form, under acidic pH conditions, four-stranded structures called i-DNA involving hydrogen-bonded pairs between cytosine and protonated cytosine (C•C^+) [62]. Oligoribonucleotides form unstable i-structures and therefore should be preferred to DNA when the oligonucleotide contains repeats of two or more adjacent cytosines [63].

TFOs can be conjugated to a secondary structure that will impair the formation of self-associated structures but not triplex formation:

- clamp or circular oligonucleotides are less prone to self-association [64];
- an additional oligonucleotide 'helper' forming a short duplex can be added at either the 3′ or the 5′ end of the oligonucleotide, thereby reducing its propensity to self-associate [40];
- a short hairpin structure can be introduced at one end of the oligonucleotide in order to prevent self-association [65].

2.2.2. Activatable triple helix

The residence time of a TFO on its DNA target is a determinant parameter to observe a biological response. Dissociation constants (K_d) of some of the triple helices tend towards values comparable to K_d values measured for transcription factors, affording competition with natural regulators of gene expression. The presence of an intercalating agent, or a ligand specific for triple helix, as well as some modification of the backbone can lower the dissociation constant and increase the half-time of the complex [3,4,6,66]. However, the best way to obtain a durable biological effect consists in irreversible modification of the DNA target. In the antisense strategy, RNase H cleaves the RNA target hybridized to the oligonucleotide. In the antigene strategy, no enzymatic activity associated to triple helices has yet been described. However, irreversible modifications can be achieved by attaching a chemical agent that is able under specific conditions, to react either by covalent modification of the bases or by cleavage of the backbone. TFOs have been tethered to a number of activatable groups that can be subdivided in several families (Table 1):

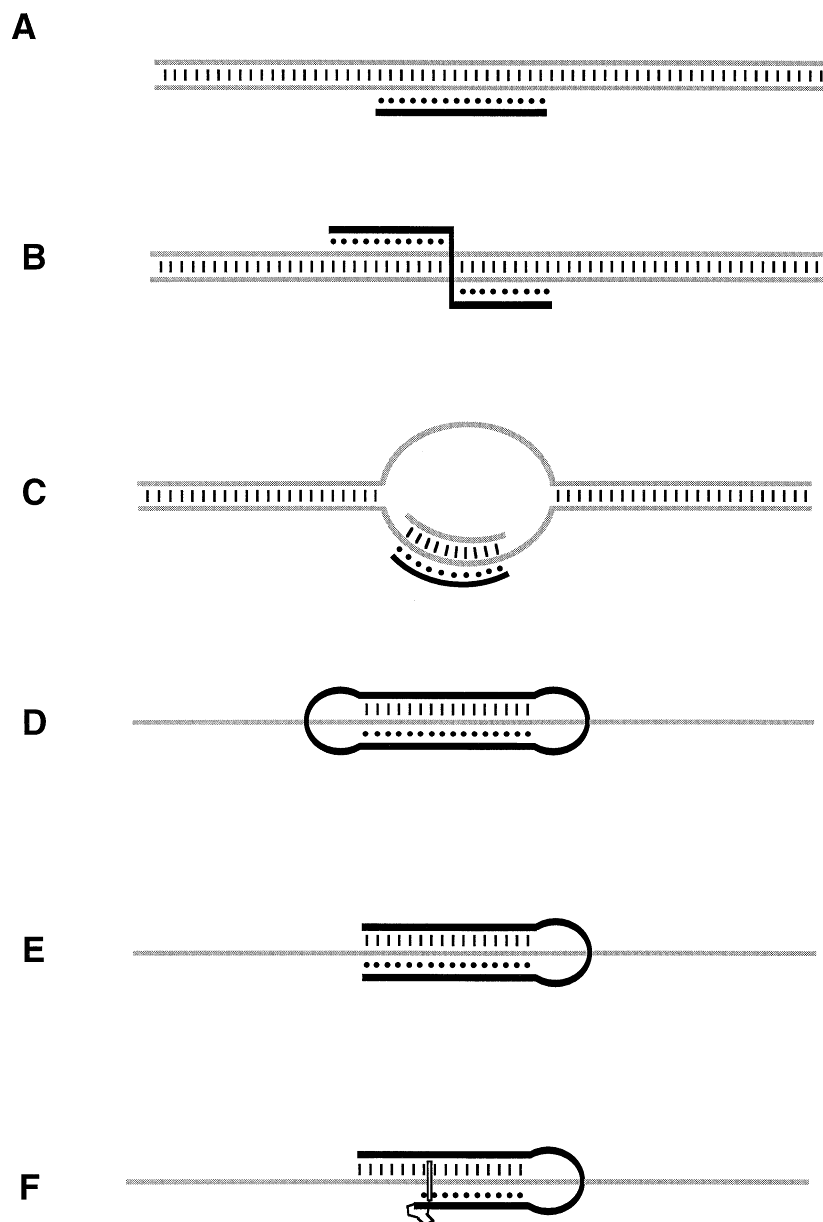

Fig. 3. Schematic representation of several types of intermolecular triplexes. (A) The target oligopurine sequence is located on the lower strand of duplex DNA. Two target oligopurine stretches are present on each strand, the third strand switching from one strand to the other (B). Two oligonucleotides bind to the purine target sequence (C), they can be connected by two loops forming a circular oligonucleotides (D) or with one linker forming a clamp (E,F). Asymmetric clamps preserve an RNA region accessible to RNase H; stabilization can be provided by an intercalating agent (F). Although the target is repesented as a single strand in D–F, a double-stranded target is also conceivable, with strand displacement of the pyrimidine (represented in C). (|) Watson–Crick hydrogen bonds; (●) Hoogsteen (or reverse-Hoogsteen) hydrogen bonds.

Among these compounds psoralen and orthophenanthroline derivatives are remarkable by their in vitro efficacy at inducing triplex-mediated cross-linking and cleavage reactions, respectively, on a duplex target [6]. The design of efficient and selective artificial nucleases functioning in vivo still remains an important goal. A few of these derivatives have a mechanism of action consistent with in vivo action: psoralen [56,67–72] and alkylating agents [73–75] were experimented in living cells; yields of target modification in situ did not exceed 40% [56,75,76].

2.2.3. Enzymatic activation

In the antisense strategy cellular nucleases RNase H [77] or RNase L [78] can be recruited as a third partner to degrade a specific RNA target. DNA-specific nucleases fulfilling a similar role have not been identified yet. An intracellular nuclease activity associated to non-covalent triplex formation was suggested to account for the decreased copy number of preformed triplex plasmid molecules in fibroblast CHO cells [79], leading to transcription inhibition of the reporter gene. However, no direct proof of such a triplex-associated nuclease activity has been documented. Recruitment of a nuclease can be provided by attaching appropriate molecules to the oligonucleotide. Camptothecin, a well known inhibitor of topoisomerase I, was recently conjugated at the 3′-end of short (U*,C)-containing TFOs (where U* designates 5-propynyl-2′-deoxyuridine): a specific cleavage of duplex DNA was induced in vitro in the presence of topoisomerase I [80]. This opens new perspectives for site-directed intracellular nucleolytic activity.

2.3. DNA is accessible to oligonucleotides in cells?

Antisense oligonucleotides may exert their action either in the cytoplasm or in the nucleus. They have to compete with secondary RNA structures and proteins bound to RNAs. TFOs have to reach the nucleus and compete with all proteins binding to DNA. A few reports have unambiguously demonstrated an antisense mechanism involving a nuclear localization of the target. Splicing inhibition of β-globin or E-selectin pre-messenger RNA with oligonucleotides delivered with a cationic lipid [81,82] obviously takes place in the cell nucleus. Accessibility of target sites in the context of chromatin is crucial for the development of the antigene strategy. It is likely that an additional restriction to potential target sequences for triplex formation is linked to chromatin structure, a parameter which is largely unknown for most of potential target sequences. It could be expected that transcriptionally active genes might be accessible to triplex-mediated regulation, especially when the target sequences for oligonucleotides are located in the same DNA domain as transcription factor binding sites. However, it is less obvious that target sites located downstream of the transcription start site would be accessible to TFOs. Recent experiments have provided evidence for such accessibility on both plasmids and endogenous genes.

2.3.1. Plasmidic DNA

Plasmids were first used as models to explore the intracellular stability of preformed triplexes as well as the possibility of their formation inside cells. In vivo footprinting and covalent triplex formation using psoralen conjugates, indicated that preformed (non-covalent) triplexes actually persist in cells. When plasmid and TFOs were delivered separately, triple helix formation inside the cells was detected by different methods: covalent triplex formation was demonstrated either by direct analysis of adducts on the intracellular plasmid [76], or by site-directed mutagenesis [71] when repair processes were involved.

2.3.2. Accessibility of genomic DNA sequences in cell nuclei

The first report describing the accessibility of nuclear DNA sequences to an antigene oligonucleotide dealt with a viral HIV-1 sequence in chronically infected cells [56]. Triplex formation was detected on one of the two viral polypurine PPT sequences (16 bp) present in the integrated HIV-1 proviral genome, within permeabilized cells incubated with a 15-mer (TCG)-psoralen-oligonucleotide [phosphodiester (po) or phosphoramidate (pn) conjugate]. The specificity was demonstrated with a control target containing four mismatches. These results were not markedly affected when transcription of the HIV viral genes was activated by PMA treatment of the cells. The accessibility of a translated sequence in a human endogenous gene coding for the chemokine receptor CCR5, in cells permeabilized by streptolysin O treatment, was revealed by showing that up to 24% of covalently modified target could be detected after treatment with a short (G,A) oligonucleotide linked to an alkylating agent [75]. Site-specific mutations have been recently introduced in the *Hprt* gene of CHO cells by oligonucleotide-psoralen conjugates after UV-irradiation. This result obtained after electroporation of the cells show that the oligonucleotide-psoralen conjugate did reach its target site in the cell nucleus [83].

2.4. Triplexes inhibit biological processes in vitro

Triplexes can be designed to interfere with replication and transcription at different levels. Triplex interference with initiation processes is largely documented: chromatin structure [84,85], transcription factors binding (see [5,6,12,66] for reviews) and RNA polymerase binding [86,87], can be locally controlled by triplex formation.

Triple helices can also interfere with the elongation step of the growing macromolecule. DNA polymerase and RNA polymerase elongation can be hampered by triple-helical complexes. Intermolecular TFOs (17 to 27nt) composed of (T, methylC) were able to transiently block *Escherichia coli* Klenow polymerase-mediated elongation, at nanomolar concentrations [88]. In these experiments the triplex was preformed on the duplex target and the primer extension assay involved strand displacement.

Inhibition of T7 RNA polymerase elongation by a physical blockage via intermolecular triplex formation, at a site located near the transcription start site (+40) was reported in the presence of long (30 or 45 nt) oligonucleotides in the (T,C) or (G,T) motifs [89,90]. Shorter oligonucleotides failed to block RNA polymerase unless modified or conjugated with an alkylating agent [91,92]. Recently, (T,G)- and (T,C)-containing short oligonucleotides directed to the PPT sequence of HIV-1 proviral DNA stabilized either by a tethered acridine at the 5′ end, or in the presence of B[*e*]PI (see Section 2.2) were able to physically stop transcription elongation mediated by phage SP6 and eukaryotic RNA polymerases at a site located far downstream of the transcription start site (400 to 650 nt) [93]. The stabilizing effect of the intercalating agent was dispensable when (pn) oligonucleotides were used instead of (po) derivatives [55,57]. The main advantage of (pn) derivatives over their (po) counterparts is ascribed to a higher residence time of (pn) oligonucleotides on their targets and to their higher stability with respect to enzymatic digestion (Sun and Giovannangeli, unpublished results).

The mechanism of blockage of RNA polymerase II transcription complex was also investigated using modified non-covalent or covalent psoralen conjugated oligonucleotides: whereas transcription termination was observed with 5′ acridine or psoralen-monoadduct derivatives, psoralen biadducts (cross-linked triplexes) allowed the formation of very stable elongation complexes stalled at the cross-linked site [94].

Linear or clamp PNAs composed of pyrimidines were also shown to stop RNA polymerases in vitro, only when hybridized to the transcribed strand, producing a truncated transcript [52,95–97]. Furthermore, successive cycles of transcription were shown to promote binding of PNA clamps (pyrimidine T-rich motif) to the oligopurine target of DNA duplex, with formation of a stable P-loop structure, leading to the concept of suicidal transcription [47].

2.4.1. Other examples of triplex interference with DNA- or RNA-binding proteins

Helicases and topoisomerases are involved in transcription, replication, recombination and repair processes and it is essential to know how triplex structures interfere with these activities. Triplex is able to inhibit topoisomerase II-mediated cleavage of DNA, as long as its site overlaps a sequence located between positions −3 to +7 relative to the cleavage site [98]. Intermolecular 26-mer (G,T) triplexes were reported to be unwound by T4 bacteriophage helicase [99]. Triplexes appear to be less sensitive to eukaryotic helicases, since a (T,C) triple helix was shown to be resistant to unwinding by SV40-associated helicase under physiological conditions, except if a non-paired 3′ tail was present [100,101].

Recruiting proteins via triplex formation can have interesting applications as anti-repressors, since triplexes targeted to a repressor binding site can favor transcription factor binding and activate gene transcription (see Section 2.5.2). Triple helices formed with oligopyrimidine PNAs can promote RNA polymerase binding by forming P-loop structures similar to unwound DNA duplex in the initiation complex: functional transcription can take place from these artificial promoters [102]. Bifunctional oligonucleotides were proposed to locally recruit transcription factors via triplex formation, by providing a recognition site for a transcription factor included in an adjacent hairpin [65]. Moreover the possibility to attract intracellular nucleases was already mentioned (cf. Section 2.2.3).

In a recent report, triplex formation on two sites separated by 10 nucleotides, using a tandem 15 (G,T)

Table 3
Inhibition of transcription in cells using triple-helix-forming oligonucleotides

Target	Triplex formation	Oligonucleotide modification	Control	Biological effect, % inhibition of transcription	References
Regulatory sequences					
Plasmid					
•Progesterone-response elements	TH n.p.	38-mer GT po 3′-cholesterol	38-mer GT scramble (15% inhib. 20 μM)	30% (10 μM) 50% (20 μM)	[112]
•*c-pim-1*	TH p.	13-mer GA po/ps 3′-amine	13-mer GCA (2 mismatches)	20% (15 μM)	[40]
•Rat-a1(I)collagen	TH p. ex vivo footprint	30-mer GA po	20-mer ATCG scramble	50%	[229]
		18-mer GA po/ps 3′-amine	18-mer ATCG scramble	50%	[38]
•IL-2Ra NF-kB, SRF binding sites	TH p. or n.p. ex vivo detection of covalent TH	15-mer TmC po 5′-psoralen or acridine	Target mutated in TH site	Psoralen: 100% TH p., 70% (20 μM TH np.)	[67,76,124]
		15 (C,T) PNA 2:1 complex	no NFkB sites	70% (3.3 μM)	[230]
•Ig heavy chain repressor site	Ex vivo footprint	41-mer GT po 3′-amine	Target mutated in TH site	Activation (×2.6)	[125]
Endogenous gene					
•*c-myc* (hnRNPK, CNBP, SP-1)		27-mer GA po	27-mer CT scramble	50% (25 μM)	[118]
		37-mer GT po	37-mer GT scramble	65% (10 μM)	[120]
•IL-2Ra		28-mer GT po 3′-propylamine	31-mer GT scramble	32% (20 μM)	[231]
•aldehyde dehydrogenase (SP-1)		21-mer GT ps/po	21-mer GT scramble (≠base composition)	80% (0.6 μM)	[41]
•*GMCSF*		15-mer GT po	15-mer GT scramble (≠base composition)	70% (2.4 μM)	[110]
•*HER-2* (*NEU*)		28-mer GA po	28-GA inverted sequence 28-CT, 28-GA scramble	42% (2 μM) 59% translation inhib. (0.22 μM)	[106]
Transcribed, untranslated sequences					
Plasmid					
•Luciferase (5′ untranslated)	Covalent TH preformed in vitro	19-mer GT po 5′-Psoralen	Same constituents but without TH formation	84% (30 μM)	[132]
•T-antigen RNA 5′ untranslated	2:1 complexes	13-mer CT PNA microinjected	Mutant target (60% inhib)	100% (2 μM)	[113]
•First intron				80% (2 μM)	
Endogenous gene					
•IGF-1R and IGF-1(3′ untranslated)		Oligopurine RNA transcribed in situ	Control vector: CT insert, without insert	100%	[160]
•TNF 3rd intron		27(G,T) 3′ cholesterol	Scramble, inverse	75% (2 μM)	[111]

Table 3 (*continued*)

Target	Triplex formation	Oligonucleotide modification	Control	Biological effect, % inhibition of transcription	References
Coding region					
Plasmid					
•β-Galacto-sidase	Covalent TH preformed in vitro	15-mer TmC po 5′-psoralen	15-mer TmC (2 mismatches)	70% (12 μM)	[68]
•T-Antigen RNA exon 2	2:1 complexes	13-mer CT PNA microinjected		60% (2 μM)	[113]
Endogenous gene					
•SV40 T antigen		15, 20-mer CT PNA microinjected	10-mer CT PNA, β-gal expression	Inhibition of T Ag expression	[95]

n.p., not preformed; p., preformed; po, phosphodiester; ps, phosphorothioate; np, N3′→P5′ phosphoramidate.
Examples of antigene strategy applied to regulatory sequences, transcribed untranslated 3′ or 5′ sequences or coding sequences. In each case the type of triplex-forming derivative is indicated and the controls with regard to the target and oligonucleotides are mentioned. Indications refer to the conditions of triplex formation (preformed or not, covalent triplex) and triple helix detection inside cells.

oligonucleotide connected by a linker of variable length was shown to induce DNA-bending toward the minor groove with a controlled angle [103,104]. This could have interesting applications to study the influence of structural constraints on various biological processes.

2.5. Triple helices in living cells

2.5.1. Vectorization: how to drive oligonucleotides toward the nucleus

Most of reported biological effects with triplexes involve procedures known to increase DNA transfection efficiency. Different strategies are developed:

1. Association of the oligonucleotide to cationic lipids [41,79,105,106] or condensing agents of DNA such as polyethyleneimine (PEI) [107]
2. Modification of cell permeability (electroporation or treatment with streptolysin, digitonin or isolecithin) which is damageable to cell viability [56,69,108–110]
3. Modification of the oligonucleotide by coupling to a peptide [74] or another group promoting penetration, such as cholesterol [111,112].

Special methods have to be adapted to increase the delivery of non-ionic derivatives such as PNA or (mp) oligonucleotides. Microinjection [95,113], cell permeabilization [108] and coupling to a cell-penetrating peptide [114] are the only methods that have been used until now.

2.5.2. Biological effects described in cell cultures treated by TFO

An increasing number of reports attempt to relate triplex formation evaluated by in vitro experiments to a modified level of expression of the targeted gene upon cell treatment with TFOs. However, the mechanism of action of the oligonucleotide is very difficult to ascertain inside cells. Sequence-specific effects can be obtained by mechanisms other than the expected one. This is easily explained by the fact that some oligonucleotides adopt highly structured conformations that can be recognized by specific proteins; G-rich oligonucleotides are particularly prone to such effects [115,116]. In most of the reports, control oligonucleotides of different length or composition, or with disrupted tracts of $(G)_n$ are not satisfactory and definitive demonstration of a triplex-mediated mechanism is generally lacking. The best control experiment actually consists in using the same TFO but a mutated target sequence affecting triplex stability but not gene expression [67]. This is easy to achieve when the target gene is carried by a plasmid but more difficult with endogenous genes.

As promoter sequences are accessible to regulatory factors, TFOs (most of which were purine-rich, espe-

cially (G,T)-oligonucleotides), were reported to repress the transcription of various genes (Table 3). However, none of the publications provides unambiguous evidence that transcription inhibition is due to triplex formation except for (i) plasmid-borne genes and (ii) covalent triplexes.

Historically, the c-*myc* oncogene was the first example of modulation of the expression of a cellular gene using the triplex strategy in vitro [117], then in cell cultures [118]. Transcription initiation from one of the promoter (P1) is regulated by an upstream G-rich sequence which can form an intermolecular triple helix with a third strand oligonucleotide (nuclease sensitive element (NSE) or III1 region). Several positive regulators have been shown to bind either the single-stranded oligopyrimidine and oligopurine sequences or the double-stranded promoter sequence [119]. Addition of (G,A) or (G,T) TFOs to the cell medium culture resulted in both disappearance of DNase I sensitive sites and inhibition of transcription [118,120]. However, a mechanism other than triplex formation was subsequently proposed: titration of one of the transcription activator (CNBP) by the purine-rich TFO could fully account for the observed decrease of c-*myc* RNA transcription [121,122]. This illustrates the difficulty to provide evidence for a triplex-mediated mechanism inside cells.

One example of using a mutant sequence as a control is provided by a study of transcription inhibition of the gene coding for the alpha subunit of interleukin-2 receptor (IL-2Rα) obtained with 15 mer-(T,C) acridine or psoralen conjugates; intracellular covalent triplex was measured in parallel [67,76,123, 124]. In these experiments a control plasmid mutated in the triplex site was unable to bind TFO and was neither affected with regard to transcription activity nor to binding of regulatory proteins.

Although many reports deal with triple helix interference with transcription activators, a unique report illustrates the potential role of TFOs as *antirepressors* of gene transcription, by competing with a negative regulatory factor [125]. Increased gene activity was obtained with TFO treatment, but not when a control construct mutated in the adjacent activator site was used (see Table 3).

In the coding region two potential targets are constituted by both genomic DNA and the corresponding messenger RNA: if the targeted oligopurine sequence belongs to the coding strand and exhibits some mirror symmetry, the TFO can also exert its effect by Watson–Crick pairing to the RNA target. The respective contribution of antigene, antisense or other effects is not easy to evaluate [69,95,113,126].

Only very few studies deal with the antigene strategy applied to endogenous genes (Table 3). Irrelevant targets and scrambled or mutant oligonucleotides were generally used as controls, so that the mechanism was not definitely ascertained. In most cases gene regulatory sequences were chosen as target.

Viral sequences were also targeted by antigene oligonucleotides. Oligopyrimidine PNAs directed to the coding region inhibited *T antigen* synthesis whether the target sequences were included in a plasmid construct [113] or incorporated in the cellular genome [95]. On the basis of in vitro experiments, the biological effect was ascribed by the authors to physical blockage of both transcription and translation processes [95]. An oligothymidylate–acridine conjugate directed to the SV40 replication origin proved to be efficient at inhibiting viral replication in CV1 cells [127] but this biological response may be due to other mechanisms than triple helix formation on the targeted sequence. HIV-1 transcription was inhibited in lymphocytes cells by (T,G)-rich 38-mer oligonucleotides directed against the transcription initiation or SP1 binding sites [128]. However, in the light of recent results reported on (G,T) oligonucleotides it appears that this effect should rather be ascribed to a different mechanism involving a direct interaction between the HIV-1 integrase and TFOs.

2.5.3. Triple helix processing ex vivo

The antigene strategy applied in cell cultures raises the question of the possible recognition of the triple helix structure by intracellular proteins. Covalent triplexes could exert an inhibitory effect on repair due to the oligonucleotide part of the damage which may cover and inhibit protein binding sites; it might recruit other proteins recognizing the triplex structure, that would either cooperate with or inhibit repair proteins.

2.5.3.1. Recognition of triplex structures by intracellular proteins. Kiyama and Camerini-Otero [129] first reported the partial purification of a human protein on an affinity column carrying a poly(dT)•

poly(dA)xpoly(dT) triplex. A protein specific for triplex structures was also detected in HeLa nuclear extracts using an intramolecular triplex as a probe, with either a (C,T) or a (G,A)-third strand [130] or a covalent triplex [131]. A similar triplex-binding activity was also found with a (G,T) Hoogsteen third strand in human lymphocytes and epithelial cell extracts, as well as in yeast cell extracts (Guieysse, unpublished results). Purification of this protein and investigation of its role in stabilization of triple helices and in other biological processes are currently under way.

2.5.3.2. Intracellular processing of covalent triples. Contradictory results have emerged from different studies on the repair and processing of covalent triple helices inside cells. This probably reflects the complex interplay of different parameters which were shown to influence repair in the case of free psoralen adducts, such as the origin of the cell line, the location of the target (replicated, transcribed or non-transcribed sequence, coding or non-coding strand) and the structure of the adduct. In the case of a covalent triplex, the cross-linked oligonucleotide can inhibit the repair process in a length dependent way.

Several reports have shown that covalent triplexes are not repaired in cells: psoralen adducts preformed at a specific site via triplex formation on a plasmid carrying the regulatory region of IL-2Rα were not repaired but instead, were found to persist with the oligonucleotide part protected from degradation for at least 72 h in lymphocytic cell lines (Guieysse, unpublished results). Similar results were obtained in vitro with HeLa cell extracts. Transcription inhibition in HeLa cells was also maintained for 72 h as reported by Musso et al., using a psoralen-(T,G) parallel TFO cross-linked to the 5′ untranslated sequence of the luciferase gene. A direct analysis confirmed the presence of covalent triplexes [132]. Experiments performed on a replicating plasmid showed that a double cross-link in the *supF* gene sequence with a 30-mer (G,A) TFO substituted at both ends with a psoralen, was weakly repaired (10% survival) in a B-cell line, in contrast with adducts obtained with a monosubstituted oligonucleotide [133].

In contrast, others have found that covalent triplexes are repaired in cells: Degols et al. showed that covalent triplexes formed in the coding sequence of the *β-galactosidase* gene on a plasmid vector were repaired in human HeLa cells or rodent COS cells, but not in a human fibroblast cell line deficient in repair (complementation group F) [68]. Similarly, triplex-induced psoralen adducts (80% biadducts) targeted to the coding sequence of the *SupF* gene with a 11-mer (G,A,T)-containing oligonucleotide conjugate, was efficiently repaired in eukaryotic cell; the yield of the replicated progeny vector was about 50% [70,134].

2.5.3.3. Triple helices as a tool for directed mutagenesis. Covalent triple helix formation appears as an ideal tool to induce a localized damage in genomic DNA. Model systems were developed and directed mutagenesis was clearly demonstrated with a plasmid shuttle vector replicating in T-lymphocytes or COS cells. An oligonucleotide was covalently linked in vitro to the coding region of the *SupF* gene (position 170). Psoralen-linked (G,A,T) 21-mer oligonucleotides were first reported to induce targeted mutations (2–5%) in the coding sequence of the *SupF* gene via triple helix formation [71]. While plasmid survival was not affected in the case of psoralen-monoadducts, it was significantly lowered (50%) for cross-linked plasmids [134]. A 30- to 160-fold increase of mutation rate compared to free psoralen was reported both in prokaryotic and in eukaryotic cells [70,72]. A majority of point mutations were located at the site of adduct formation and could be satisfactorily explained by a repair of the damage by the nucleotide excision repair (NER) mechanism for bi-adducts, or by translesion synthesis for mono-adducts. Mutation at other sites or deletions which occurred at a low level remain unexplained. It was surmised that access of repair proteins could be inhibited when the length of the third strand increases. Covalent triplexes longer than 15 base triplets could not complete the whole cycle of excision repair. The mutation pattern of the *SupF* gene was best explained in the context of NER assuming (i) a preferential incision of the purine strand where the furan moiety of psoralen was cross-linked, and (ii) a triplex mediated inhibition of excision process on majoritarily the purine strand. The role of other processes than NER to explain the restoration of *SupF* activity is still controversial [68,71,135].

The possibility to induce mutations in endogenous genes is presently under investigation and models have been now established with oligonucleotide-psoralen directed against human *Adenosyl Phosphoribosyl Transferase* (*Aprt*) gene [136,137] or in the *SupF* gene integrated in the genome of transgenic mice [138]. A recent paper by Majumdar et al. [83] describes mutation induced in the *Hprt* gene using a long (23 nt) oligopyrimidine containing an internal intercalating agent to stabilize the triplex in addition to the psoralen attached at the end of oligonucleotide to induce the cross-link.

Most importantly, non-covalent triplexes were also shown to induce both point mutations and deletions more than 10 fold above the spontaneous mutation rate, by a process which probably involves excision and transcription-dependent repair. These results were obtained on *SupF* gene with (G,A) TFOs [139] and more recently with short clamp-PNA [140], carried by a plasmid shuttle vector or a mouse chromosome, respectively. This raises the question of the possible recognition of a non-covalent triplex structure as a structural defect by repair systems.

2.5.4. Intramolecular recombination promoted by a covalent triplex

Recently, a covalent triplex was reported to enhance recombination processes between two tandem *supF* genes present on a plasmid construct, in mammalian cells. Psoralen linked to an (A,G) 30-mer oligonucleotide was directed to the 3′ part of the proximal *supF1* gene. The oligonucleotide part was clearly essential considering the importance of its length evidenced by the absence of recombination with a 10-mer or a psoralen-derivatized (A,G) 13-mer with a cleavable disulfide linker [109]. Reciprocal and non-reciprocal recombination events were both detected, the second process probably involving the prior occurrence of double-strand breaks.

3. Triple helix formation on single-stranded nucleic acid: 2:1 complexes, clamp and circular oligonucleotides

3.1. Concept

Single-stranded DNA and RNA also constitute potential targets for triple helix formation (Fig. 3) [141,142]. Clamp oligonucleotides [142] (Fig. 3E,F) can bind to a single-stranded RNA target with higher affinity compared to the two corresponding oligonucleotides [64,143]. Circular oligonucleotides [141,144,145] (Fig. 3D) provide additional entropic gain (see [146] for review). Moreover, the absence of free ends prone to exonuclease attack can confer a greater stability ex vivo.

An oligopurine *RNA* sequence can be recognized by a clamp or circular oligopyrimidine RNA [71,147] or by a DNA clamp containing a (T,G) parallel third strand [142,143]. In the last case an acridine derivative was attached to the 3′ end (Fig. 3F). This is the only example until now where a D.R*D triplex combination exhibits a higher stability compared to D.D*D. Asymmetric clamps can be designed to extend the recognition to a mixed sequence adjacent to a small oligopurine sequence: the longer Watson–Crick strand can provide additional specificity and stability [52], extend the range of potential target sequences (Fig. 3F), and offers a window of RNase H sensitivity in the case of (po) or (ps) derivatives. Asymmetric PNA clamps also bind to sequences containing at one end a very short purine tract (as few as 5–6 consecutive purines).

An oligopyrimidine *RNA* sequence can be efficiently targeted by two (mp) oligopurine [148] or by oligonucleotides forming a hairpin-loop or a dumbbell structure (the double-helical region binds the oligopyrimidine RNA as the third strand of the triple-helical complex) [64].

Stabilization of DNA or RNA structures by triplex formation is achievable in vitro and could be used to trap, purify and study specific nucleic structures and their associated functions [149,150].

3.2. Inhibition of replication and translation processes

3.2.1. polymerases

Clamp oligonucleotides with a r(U,G) or d(T,G) third strand directed to the PPT (polypurine tract) sequence of the HIV-1 viral RNA, were designed to interfere with initiation of plus-strand DNA synthesis by HIV reverse transcriptase [142,151]. Triplex formation by (G,T)- or (C,T)-oligonucleotides on the site of hybridization of a purine-rich primer was shown to prevent initiation of T7-DNA poly-

merase elongation [152] as long as the 3′-end of the primer did not protrude by more than two bases from the triplex structure [153].

Clamps made of *2′Omethyl* oligoribonucleotides [154], or acridine and psoralen conjugates [143] can block DNA-polymerase elongation on a single-stranded purine target. Two oligopurine *methylphosphonates* forming a triplex on a pyrimidine target [148] and T-rich monomeric or clamp *PNAs* which bind to a short purine sequence [51,95] were described to efficiently block reverse transcription elongation. PNAs composed of pyrimidines also act as roadblocks to several types of DNA-polymerases [97,155,156]. Interestingly a pentadecamer of mixed sequence forming a 1:1 PNA–RNA complex proved to be as efficient as the clamp PNA to block reverse transcriptase [51] but not Taq polymerase [97] elongation.

Accessibility of the PPT target for HIV1 RNA complexed with nucleoproteins in isolated viral particles was also demonstrated [151], as clamps promoted similar arrests of reverse transcriptase elongation in particles, as those observed in vitro.

3.2.2. RNA processing

PNAs able to form a triplex on RNA (2:1 complexes) were targeted to a sequence cloned in the T-antigen 5′ untranslated region [113]. Upon microinjection of PNAs and plasmid in CV1 cells, the complexes formed were shown to completely inhibit T antigen activity after microinjection in cells. A mutant construct with a single mutation was shown to be inhibited to a lesser extent (40% inhibition). 2:1 complexes formed in intronic sequences also exhibited efficient inhibitory effects. The inhibition mechanism probably involves interference with RNA processing, although interaction with DNA was not excluded.

3.2.3. Translation machinery

Several reports have shown that triplex structures can physically block translation elongation. Translation inhibition was reported using two (mp) oligopurines which bind in opposite orientation to the oligopyrimidine RNA target (next to the AUG start codon of the CAT gene) [148]. A physical blockage of translation was also obtained in vitro, with decamer T_{10} PNAs forming a triplex with the oligopurine target present in RNA [95]. With pyrimidine-rich clamp PNAs, triplex formation on as few as five nucleotides was enough to provide inhibition of the ribosomal elongation complex, resulting in the appearance of a truncated protein. The inhibition was more substantial if the ribosomal complex first encountered the loop part of the PNA clamp [52]. In contrast, asymmetric clamp PNAs directed against the promyelocytic leukemia/retinoic acid receptor fusion region had no effect on translation. Mixed sequence PNAs which could only form 1:1 complexes had insufficient stabilities to allow inhibition at sites located more than a few nucleotides downstream from the translation start site [52,97].

4. Pharmacology versus gene therapy

Giving the cells the means to produce their own regulatory oligoribonucleotide may prove an interesting approach in the antigene strategy, as it has in the antisense field [157]. Appropriate DNA vectors can be constructed to generate RNA transcripts in situ in the nucleus in such a way that the RNA sequence allows for triple helix formation on a targeted DNA sequence [158,159]. This strategy was first tested to regulate insulin-like growth factor-1 (IGF-1) and its receptor gene at the transcriptional level [160,161]. The 27 nucleotide-long purine target sequence was located in the 3′ untranslated region of IGF-1R whereas the 23 bp-long sequence in the IGF-1 gene was located in the 5′UTR sequence. Constructs generating RNA transcripts containing the oligopurine or the oligopyrimidine sequence were transfected in rat glioblastoma cells and stably transfected clones were selected. Only the RNAs containing the oligopurine sequence were shown to down regulate IGF-1R and IGF-1. The postulated triplex-mediated mechanism, although not sustained by in vitro data (triple helices with a third (G,A) RNA strand were actually not detected on a DNA target duplex), cannot be excluded if secondary structures of the RNA transcript and endogenous proteins stabilize this type of triplex inside cells. Clonal cells down-regulated for IGF-1 or IGF1-R were then expended and reinjected in tumor-bearing animals, inducing tumor regression.

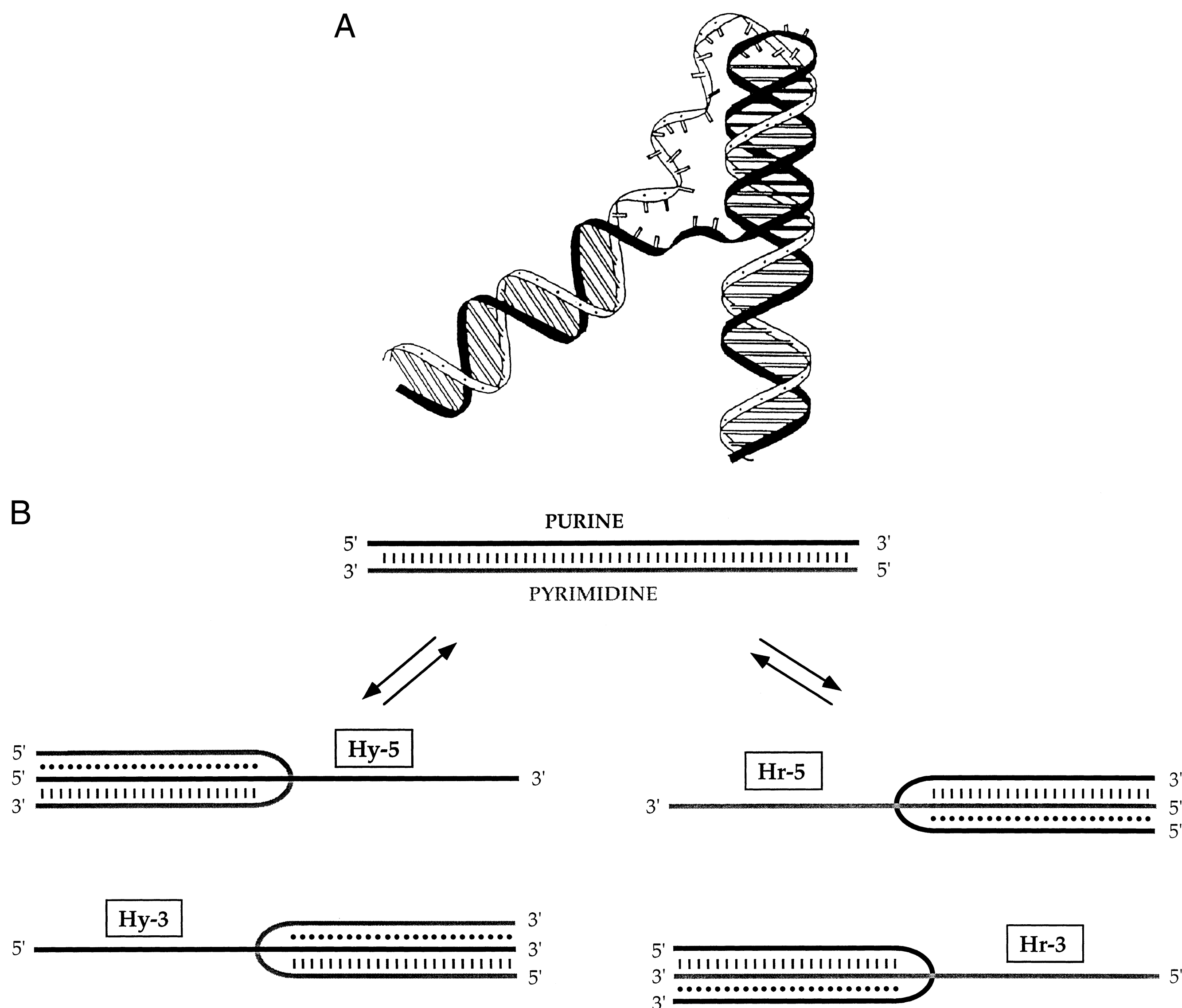

Fig. 4. (A) Schematic representation of an intramolecular triplex. (B) The four different isomers obtained in the case of an oligopyrimidine•oligopurine motif presenting a mirror symmetry. Hy-3 and Hy-5 have a pyrimidine, whereas Hr-5 and Hr-3 (also called H*) have a purine motif in the third strand.

5. Intramolecular triplexes

Oligopurine sequences presenting a mirror symmetry can potentially adopt an intramolecular structure called H-DNA, involving both a triplex and a single-stranded structure [162,163] (Fig. 4A). These structures were detected in prokaryotic [164] and eukaryotic cells [165], on extrachromosomal or genomic DNA [166]. If their roles are not yet elucidated, growing evidence is in favor of their implication in chromatin structure and condensation phenomena. Several examples show that potential H-forming DNA sequences are also critical for gene expression, even though direct proof is still lacking to establish a correlation between H-DNA structures and gene expression within intact cells. A recent report gives evidence that antibodies specific for H-DNA can inhibit global transcription and replication processes on isolated nuclei or in permeabilized cells [167]. Furthermore, oligopurine sequences are over-represented in eukaryotic genomes [168], and especially in regulatory sequences [169–171], where non-B

structures are suspected on the basis of their sensitivity to S1 nuclease (NSE elements) or hypersensitivity to DNase I.

Other possible strategies consist in using oligonucleotides to prevent the binding of specific factors to the single-stranded part of H-DNA, by trans-competition with the same motif [121] or by using an oligonucleotide complementary to the single-stranded part [165]. The elucidation of the role of H-DNA in gene regulation will require further work with model systems in which H-structure formation can be controlled by oligonucleotides or triplex-specific proteins or ligands such as the intercalating agents described earlier in this review. Much information is expected from identification and cloning of triplex- or H-specific proteins.

6. Three-stranded structures containing two homologous sequences

Three-stranded structures in which two strands of identical sequences have the same orientation (called R-DNA), are possibly involved as intermediates in the recombination process. These structures can be formed independently of the primary nucleotidic sequence in the presence of specific proteins belonging to the recombinase family, such a *E. coli* RecA. Recently, it was shown that an R-structure could form intramolecularly in the absence of protein but in the presence of stabilizing ligands such as propidium iodide, or divalent cations such as Mn^{2+} or spermidine [172–174]. The stability of R-intramolecular structures is increased if the homologous strands are purine-rich. R-structures are distinguished in the RecA complex (extended triplex) from those obtained upon deproteinization (collapsed triplex) [175]. The nature of the interactions between all three strands is not yet elucidated [176,177].

These studies recently gained special interest in the light of results reporting mutation correction by gene targeting in cell cultures using short double-stranded chimeric deoxy- and 2′*O*-methyl oligonucleotides as the source of wild-type gene sequence. Treatment of B lymphoblastic cells with oligonucleotides could transform the mutant *β-globin* gene carrying a single mutation in the sixth codon to wild-type genotype with a significant yield [105]. Correction of the *alkaline phosphatase* gene carried by a plasmid was obtained using the same method in human hepatoma cells [178]. Although the mechanism of mutations is not yet definitely ascertained, repair is likely involved instead of recombination [179]. These results are encouraging and offer an alternative to the use of viral-based vectors in gene therapy, even though this strategy has met with some difficulties to be reproduced in different cell lines.

7. Conclusion and perspectives

The demonstration [1,2] that oligonucleotides can recognize the major groove of DNA in a sequence-specific way has opened interesting applications in different fields of molecular and cellular biology.

1. Affinity columns based on triple helix formation can achieve purification of plasmidic DNAs with an efficacy which is better than any other presently available technology. Use is made of the pH-dependence of triplex stability with oligopyrimidine third strands containing cytosines. The complex is retained at acidic pH and is eluted from the column at basic pH.
2. Triple helix-forming oligonucleotides can be used as probes of DNA sequences without any need for opening the double helix as compared to oligonucleotides complementary to either one of the two DNA strands. Therefore they might find interesting applications in the development of diagnostic techniques.
3. Oligonucleotides forming triple helices are good tools in vitro to cut long DNA fragments, including chromosomes, at specific sites. A methylation-restriction enzyme system can be used to select a cleavage site either by oligonucleotide-directed triple helix formation or by recA-mediated three-stranded complex formation. Alternatively a triplex-forming oligonucleotide can be attached to a DNA cleaving reagent to target the cleavage reaction to a specific site. The Cu^{2+}-phenanthroline chelate proved to give a high yield of double-strand cleavage in vitro.
4. A psoralen oligonucleotide conjugate can be employed to target a cross-linking reaction to a specific site on DNA; if the oligonucleotide carries a

biotin at the other end (as compared to the psoralen) the covalent complex can be fished out by avidin-coated beads and used for further experiments as recently shown on a study of stalled RNA-polymerase complexes [94].

5. Psoralen-oligonucleotide conjugates have been shown to be good probes of DNA accessibility in the nucleus of living cells. The oligonucleotide can be delivered to the cell by permeabilization with digitonin or streptolysin O, by electroporation or using cationic lipids. Following UV-irradiation of the cells the DNA is extracted and cross-linking is revealed either by inhibition of restriction enzyme cleavage of the targeted sequence or by PCR experiments. Alternatively an alkylating agent can be attached instead of psoralen to the oligonucleotide. Psoralen-oligonucleotide conjugates have the advantage of controlling the reaction in time and should prove useful to probe DNA-sequence accessibility at different steps of the cell cycle or when the cells are treated by any reagent that modifies the cell physiology.
6. Site-directed mutagenesis can be induced inside cells, e.g., using psoralen-oligonucleotide conjugates that can be photochemically cross-linked to their target site. Mutations are induced during the repair processes which are triggered by the sequence-specific DNA damages.
7. If the yield of the irreversible reactions induced by an oligonucleotide carrying an activatable group could get close to 100% the triplex technology could provide a new strategy to knock-out specific genes in a controlled way (e.g., UV irradiation with psoralen-oligonucleotide conjugates).

The main goal of developing the antigene strategy, as discussed in this review, is to control in a sequence-specific manner the expression of a selected gene in a living cell. The advantages of the antigene strategy as compared to the antisense strategy are several-fold. (i) At the molecular level there are only two targets per cell (the two alleles of the targeted gene) instead of up to thousands of copies of a messenger RNA species. (ii) The effect of triplex-forming oligonucleotides occurs at the transcription level inhibiting the synthesis of the pre-messenger RNA. All RNA species arising from alternative splicing reactions are no longer synthesized. (iii) Inhibiting mRNA translation through a physical mechanism or an RNase H-dependent process does not arrest transcription and therefore the mRNA pool is continuously repopulated. Therefore, either the antisense oligonucleotide should have a catalytic mode of action or should be present at a sufficient concentration for a long enough period of time (or provided in a repeated fashion) to expect a permanent down-regulation of protein synthesis. Of course there might be cases where a transient inhibition of gene expression may induce an irreversible process so that the biological response does no longer require the action of the antisense oligonucleotide. A permanent inhibition of transcription requires that either the oligonucleotide induces an irreversible reaction or the oligonucleotide–DNA triple helical complex survives for a long enough time. The biological response will depend on whether cell division is arrested or not and on the effect on transcription inhibition on cell fate (apoptosis, quiescence) and on the kinetics of the biological response. We do not know yet what will be the effect of triple-helical complexes on DNA-replication. On a model system (SV40 replication in COS cells) we have not detected any inhibition of replication if the oligonucleotide was not cross-linked to its target (using a psoralen-oligonucleotide-conjugate) (S. Diviaco, C. Giovannangeli, C. Hélène, unpublished results).

The main limitation of the triplex strategy – whatever the contemplated applications – remains the requirement for an oligopurine target sequence in DNA. There are several ways to extend the range of recognition sequences but it still remains a challenge to design nucleotide analogues that would recognize all four base pairs of DNA when reading the major groove (A.T, T.A, C.G, G.C) in a way similar to what has been recently achieved for the sequence-specific recognition of the minor groove by hairpin polyamides [180,181]. However, it should be kept in mind that oligopurine sequences are over-represented in the human genome, and the whole gene including control regions and non-coding sequences (introns) is a potential target for triplex-forming oligonucleotides and not only coding sequences. Therefore it is likely that several target sequences for antigene oligonucleotides will be formed in all genes even with the present limitation of sequences available for triplex formation. Eight-ring hairpin polyamide molecules

developed until now will probably target many sequences in a complex genome since it was already surmised that 17 bp is minimal to afford the recognition of a unique target in human genome. The design of longer polyamide molecules that perfectly match longer target sequences might require sophisticated chemical synthesis [182]. DNA-binding peptides, involving zinc fingers among other motifs, can achieve specific sequence recognition of base pairs both in the minor and major grooves, although no general amino-acid/base-pair code is available yet.

The demonstration that oligonucleotide modification such as phosphoramidates, not only protects the oligonucleotide against nucleases, but also allows for tight binding to DNA and minimal binding to proteins show that the major objectives of the antigene strategy are attainable. The nucleus of living cells appears to be a favorable compartment for oligonucleotides. Antisense oligonucleotides have been shown to inhibit splicing of pre-mRNA, a mechanism which implies a nuclear mode of action [144,145]. Microinjection experiments have shown that oligonucleotides in the cytoplasm directly localize in the cell nucleus [183,184]. Intermolecular triplexes were shown to be formed and to persist in living cells. The accessibility to TFOs of a DNA target sequence within the chromatin is now demonstrated, confirming the feasibility of modulating gene transcription by an antigene approach [56]. Linked to activatable reagents, TFOs can be used to produce localized damages in specific sequences of the genome: long-term effects require that this damage persists in the cells, including its transformation into a local mutation. The development of active reagents conjugated to TFOs, able to function with high efficacy inside living cells, together with the study of damage repair in a triple-helical context is still a challenge.

Gene therapy is conceivable by antigene strategy with approaches similar to those already developed for antisense or ribozymes. Ex vivo treatment by activatable oligonucleotides or in situ RNA production can be followed by subsequent reimplantation of treated cells in vivo. The sequence-specific effect of an oligonucleotide on a unique gene can affect regulation of several other genes if it is involved in autocrine cell proliferation or in cross-regulation networks of gene expression. This is clearly so when the targeted gene is involved in a cascade of signal transduction pathways or if it belongs to a network of regulatory genes. The IGF-1 system provides an interesting example where down regulation of IGF-1 or its receptor by antisense and antigene approaches leads to dramatic changes in the level of expression of several genes, including overexpression of nexin 1 or MHC class I molecules [161]. These phenomena may lead to unexpected responses which may be of immense potential therapeutical interest, e.g., the induction of an immune response against glioblastoma [161] or hepatocarcinoma [185] in syngenic animals.

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