Eur J Cancer, Vol. 27, No. 11, pp. 1466–1471, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Feature Articles

Rational Design of Sequence-specific Oncogene Inhibitors Based on Antisense and Antigene Oligonucleotides

Claude Hélène

Synthetic oligonucleotides can be used to control the expression of specific genes. When targeted to messenger RNAs, oligonucleotides inhibit translation (the antisense strategy). Oligonucleotides can also be targeted to specific sequences of the DNA double helix where they inhibit transcription (the antigene strategy). Both strategies can be applied to control the expression of oncogenes in tumour cells. The mRNAs of several oncogenes have been chosen as targets for antisense oligonucleotides (*myc, myb, bcl2, abl, ras...*). Discrimination between the proto-oncogene and the oncogene can be achieved in the case of *ras* oncogenes where activation results from point mutations in the coding sequence. Regulatory sequences involved in controlling the transcription of oncogenes can also be used as targets for antigene oligonucleotides (*myc, ras*).
Eur J Cancer, Vol. 27, No. 11, pp. 1466–1471, 1991.

INTRODUCTION

CELL TRANSFORMATION leading to tumour development is a multistep process involving the activation of growth-stimulatory pathways (under the control of oncogenes) and the inactivation of growth-inhibitory pathways (under the control of tumour-suppressor genes). Inhibition of oncogenes and/or stimulation of tumour-suppressor genes represent new strategies that should lead to a better understanding of the different steps involved in tumorigenesis and to the development of new therapeutical approaches.

During the past years, oligonucleotides have been developed to selectively modulate gene expression. Two main strategies ought to be considered [1]: in the antisense strategy, the oligonucleotide is targeted to a specific messenger RNA, thereby inhibiting its translation into the corresponding protein; in the antigene strategy, a double-stranded DNA sequence is the target of the oligonucleotide which is then expected to block transcription of the specific gene where the target is located. Figure 1 gives a schematic representation of the different steps of gene expression where oligonucleotides can exert their biological effects. This presentation will not cover antisense RNAs which are obtained by transcription of DNA fragments placed in the reverse orientation as compared to the gene of interest, nor ribozymes, short RNAs that induce catalytic cleavage of their target RNAs [1, 2]. Antisense RNAs can be considered as potential tools in gene therapy approaches rather than in chemotherapy.

Here we will briefly review the basic principles underlying each strategy and then show how they have been applied to the inhibition of specific oncogenes in tumour cells.

Correspondence to C. Hélène, Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, INSERM U.201 – CNRS UA.481, 43 rue Cuvier, 75005 Paris, France.
Received 5 Aug. 1991; accepted 8 Aug. 1991.

A Review Lecture presented at the 11th Meeting of the European Association for Cancer Research (ECCO-6), Genoa, Italy, 3–6 November, 1991.

THE ANTISENSE STRATEGY

Antisense mechanisms

To recognise a specific sequence in a messenger RNA an oligonucleotide with a complementary sequence can be synthesised. The recognition rests on Watson–Crick hydrogen bonding interactions between complementary bases. An mRNA sequence containing A,U,C,G ribonucleotides is selectively bound by an oligodeoxyribonucleotide containing T,A,G,C. The first synthetic molecules used as antisense agents were oligodeoxyribonucleotides [oligo(dN)] because their chemical synthesis was (and still is) much easier than that of their ribo analogues.

The original idea behind the antisense strategy was that upon binding to a complementary sequence on a mRNA, the oligonucleotide would inhibit translation of the mRNA by ribosomes. However, it became clear in the mid 1980s that an oligonucleotide bound to the coding sequence of a mRNA would not be able to stop the translation machinery once it was launched on the mRNA. However, inhibition of translation was observed in different systems: in cell-free translation assays; after microinjection in, e.g. *Xenopus* oocytes; and in cells incubated with oligodeoxynucleotides. There are at least two mechanisms by which oligodeoxynucleotides inhibit mRNA translation: the oligo(dN)-mRNA hybrid is a substrate for an endogenous ribonuclease, called RNase H, which recognises DNA–RNA hybrids and exclusively cleaves the RNA part; or an oligo(dN) bound to the 5'-untranslated region of a mRNA can inhibit binding or sliding of the 40S ribosomal subunit and/or the association of protein factors involved in translation initiation.

The first mechanism is probably an obligatory pathway for the antisense effect when the oligo(dN) is targeted to the coding sequence of the mRNA. The second mechanism is superimposed on the first one when the oligo(dN) is targeted to the 5'-untranslated region of the mRNA.

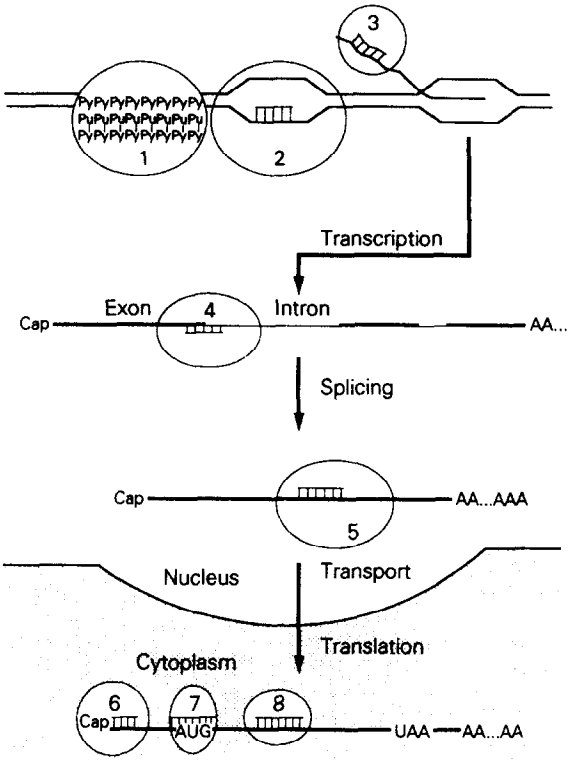

Fig. 1. Summary of the possible sites of sequence-specific action for oligonucleotides along the information flow from DNA to protein in a eukaryotic cell. Oligonucleotides could interfere: (i) with transcription by triple helix formation (1) on sequences containing contiguous purines (Pu) on one strand and pyrimidines (Py) on the other strand, by hybridisation to the locally opened loop created by RNA polymerase (2) or by hybridisation to nascent RNA (3); (ii) with splicing through hybridisation at intron–exon junctions (4); (iii) with the turnover of spliced mRNA via e.g. RNAse H-induced cleavage (5); or (iv) with translation, through inhibition of the binding of initiation factors (6), inhibition of the assembly of ribosomal subunits at the start codon (7) or inhibition of polypeptide chain elongation (8) via RNase H-induced cleavage of the mRNA. Other processes such as capping and polyadenylation, interactions of snRNPs with pre-mRNAs in the splicing machinery, recruitment of stored mRNA into polysomes and nucleic acid–protein complexes are not illustrated. (Reproduced from [1] with permission.)

Specificity of the antisense effect

An antisense oligonucleotide should be designed to recognise a single mRNA species within the mRNA population of a human cell. It has been calculated [3] that its minimum length should be between 11 and 15 depending on its base composition: 11 if it contains only Gs and Cs, 15 if it contains As and Ts [this difference accounts for the higher AT (60%) than GC (40%) content in human DNA]. These numbers were calculated with the assumption that DNA base pairs are randomly distributed in the 4×10^9 bp-long human genome and that about 0.5% of this genome is transcribed as mRNA in a given cell type at a given time. Since 5'CpG3' sequences (but not GpC sequences) are under-represented in the human genome (as in all eukaryotes) an oligonucleotide containing several CpG dinucleotides has a low probability of finding a target complementary sequence in more than one mRNA species (since CpG is self-complementary, it will be found in both the oligonucleotide and its target sequence).

Antisense oligonucleotides should be chosen as short as possible to achieve a higher specificity under physiological conditions. This arises because the effect of mismatches on the binding energy decreases when the oligonucleotide length increases. Therefore under physiological conditions (37°C, ionic conditions prevailing inside cell compartments) a shorter oligonucleotide should yield a higher discrimination between its complementary sequence and a sequence with a single base change. Of course, the oligonucleotide should have a minimum length to avoid its binding to fully complementary sequences that would otherwise be found in several mRNA species, and to achieve a strong enough binding under physiological conditions in order to have an inhibitory effect on the biological function (translation). It should be remembered that AU (TA) base pairs contribute a smaller binding energy than GC (CG) base pairs. Antisense oligonucleotides which have been shown to induce specific biological responses have been usually chosen in the range of 12–20 nucleotides. Shorter oligonucleotides can be used provided their hybrids with mRNAs are stabilised, e.g. by covalent attachment of an intercalating agent (see modified oligonucleotides, below).

Oligonucleotide modifications

Oligonucleotides have been chemically modified to increase their nuclease resistance and their uptake by cells in culture. Oligophosphorothioates and dithioates still induce RNase H cleavage of an RNA target, as do natural phosphodiester backbones. Oligophosphorothioates have been used to inhibit gene expression in several *in vitro* systems and in cell cultures [4–7]. They have been shown to inhibit HIV development. However there is a non-sequence-specific effect of these oligophosphorothioates on acute infection which might be due to their binding to the cell surface receptor CD4 and viral proteins such as reverse transcriptase and gp120 [6]. In contrast, a sequence-specific inhibition is observed in chronically infected cells [7] or if the oligophosphorothioates are added some time after cell infection.

Oligomethylphosphonates which are neutral derivatives (no negative charge on the phosphates) do not sustain RNase H activity on their target mRNA. They are expected to be active only when targeted to non-coding sequences. An oligomethylphosphonate complementary to an exon–intron junction was shown to inhibit splicing of immediate–early genes of herpes simplex virus (HSV1) [8].

In oligo-[α]-deoxynucleotides, the natural [β]-anomers of nucleoside units have been changed to their synthetic [α]-anomers where the base is located on the same side as the 3'-OH with respect to the main sugar plane (as opposed to the 5'-OH in the [β]-anomers). Oligo-[α]-deoxynucleotides do not induce RNase H-mediated cleavage of their RNA substrate. Consequently they are devoid of any antisense activity when targeted to coding sequences but may inhibit translation according to an RNase H-independent mechanism when targeted to the 5'-untranslated region [4, 10, 11].

Since RNase H has a strict requirement for β-deoxyribo derivatives with phosphodiester or phosphorothioate backbones, it was of interest to determine whether mixed oligonucleotides would still induce RNase H cleavage. RNase H requires only 4–5 base pairs to achieve cleavage of the RNA part of an RNA–DNA hybrid. Oligonucleotides containing nuclease resistant backbones (phosphoramidites) at both the 5'- and 3'-ends and 4–5 phosphodiesters in the centre are still active at inducing RNase H-mediated cleavage of their RNA substrate while being highly resistant to exonucleases [12].

All the modifications mentioned above confer resistance to nuclease attack and therefore improve oligonucleotide bioavailability in both biological fluids and intracellular compartments. However, they do not change appreciably their ability to cross cell membranes.

Other modifications have been introduced into oligonucleotides to improve their antisense efficacy. We early introduced intercalating agents as terminal substituents to increase the stability of oligonucleotide–mRNA complexes. At the same time 3′-substitution of the oligonucleotide provided a protection against 3′ → 5′ exonucleases which are the most active nucleases in plasma and inside cells. In addition, the intercalating agent enhanced cell uptake. All these properties make oligonucleotide–intercalator conjugates interesting substances to inhibit mRNA translation. They have been shown to prevent the cytopathic effect of influenza virus [13] and simian virus 40 [14] on cells in culture. Trypanosomes, the parasites responsible for sleeping sickness, were killed by an oligonucleotide–intercalator conjugate targeted to the common sequence which is present at the 5′-end of all trypanosomal mRNAs [15].

Oligonucleotides can be substituted by hydrophobic groups such as cholesterol [16, 17]. These derivatives bind to lipoproteins: this binding increases their lifetime in plasma and opens the possibility that their cellular uptake occurs via LDL receptors.

Oligonucleotides can also be attached to reagents that can induce irreversible reactions in their target sequence [1, 18, 19]. Upon binding to a complementary sequence, the reagent can be activated either chemically or photochemically to induce reactions such as cleavage of the target sequence or crosslinking of the two nucleic acids. A discussion of these different types of reactions is beyond the scope of this review [1, 18, 19].

ONCOGENES AS TARGETS FOR ANTISENSE OLIGONUCLEOTIDES

Oncogenes can be selected as targets for antisense oligonucleotides. If translation of an oncogene mRNA is blocked, then one should expect to alter cell behaviour depending on whether the oncogene is involved in cell immortalisation or in cell transformation.

Discrimination between proto-oncogene and activated oncogene

There are several situations where the activated oncogene can be selectively inhibited without any effect (or with weaker effects) on the corresponding proto-oncogene.

Firstly, when activation results from a point mutation. This has been documented in the case of the Ha-*ras* oncogene from T24 cells (a bladder carcinoma cell line) where Ha-*ras* is activated by a point mutation on codon 12 [20]. An antisense oligonucleotide targeted to the mutated sequence induced RNAse H cleavage of the mutated mRNA, whereas a much weaker effect was seen on the normal mRNAs. An inhibition of T24 cell proliferation was demonstrated. The discrimination between mutated and normal sequence is due to the destabilisation of the oligonucleotide–mRNA hybrid resulting from a mismatch at the point mutation site. Alternatively a ribozyme can be used to discriminate a mutant from the normal HA-*ras* mRNA [21]. The mutant RNA which had a GUU codon at position 12 was cleaved by the ribozyme whereas the normal RNA which contained a GGU codon was not changed.

Secondly, when activation results from translocation, creating a new fusion gene. This occurs, e.g. in chronic myelogenous leukaemia (CML) where a *bcr-abl* hybrid gene arising from a translocation between chromosomes 9 and 22 is responsible for cell transformation [22]; in acute promyelocytic leukaemia (APL) where a t(15;17) translocation fuses the retinoic acid receptor α (*RARα*) gene to the *myl* gene, resulting in the synthesis of a *myl/RARα* fusion mRNA [23]; and in B-cell lymphomas where a t(14;18) translocation generates chimeric mRNAs involving a *bcl2*-immunoglobulin fusion [24]. Antisense oligonucleotides have been targeted to the *abl* [25] and *bcl 2* [26] mRNAs. A recent report describes the selective antisense effect of an oligonucleotide targeted to the region involved in fusion [27]. This is an interesting potential target since the antisense oligonucleotide would be expected to bind the chimeric mRNA after translocation but not the individual mRNAs of the two genes in cells where the translocation process has not occurred.

Thirdly, when transcription is initiated from an aberrant promotor without translocation. In many Burkitt lymphomas, c-*myc* transcription starts in the first intron of the normal gene. Consequently the mRNA contains sequences which are not found in the normal c-*myc* mRNA. It was recently shown that a 21-mer antisense oligonucleotide directed against the intron sequence was able to inhibit proliferation of tumour cells that expressed the abnormal transcript but not cells containing the normal transcript [28].

Fourthly, alternatine splicing creates a new mRNA species containing sequences which are not found in the original mRNA. There is no evidence for this kind of mechanism in proto-oncogene activation yet available. If such a mechanism occurred, it would provide potential target sites for antisense oligonucleotides that are selective for the tumour cells.

Inhibition of oncogene expression

An antisense oligonucleotide targeted to an oncogene mRNA can be expected to have selective effects on tumour cell proliferation even when the target is also present in normal cells. This occurs in many cases of cell transformation where abnormal expression of the proto-oncogene is sufficient to deregulate cell growth as a result of e.g. gene amplification or enhanced transcription (or translation).

The *myc* oncogenes have received a great deal of attention. The human promyelocytic cell line HL 60 has been used as a model because it overexpresses c-*myc* as a result of gene amplification and can be induced to differentiate into either monocytes or granulocytes by various agents. This differentiation is associated with a decrease in c-*myc* protein synthesis. A 15-mer antisense oligonucleotide targeted to the first five codons of the c-*myc* mRNA was shown to induce a sequence-specific reduction in c-*myc* expression which was accompanied by inhibition of cell proliferation [29, 30] and by granulocytic differentiation [31]. By "walking" along the human c-*myc* mRNA with 15-mer oligonucleotides, the highest antisense efficacy was obtained when the target was the 5′-cap untranslated region [32]. The N-*myc* oncogene was also inhibited by a 15-mer antisense oligonucleotide targeted to the translation initiation site in a neuroepithelioma cell line [33]. The results provided a clear example of the sequence selectivity of the antisense effect because the closely related c-*myc* gene was not affected by the anti-N-*myc* oligonucleotide. The activity of anti-*myc* oligonucleotides is enhanced when they are linked to poly-L-lysine and administered to cells in culture internary complexes with heparin [34].

The role of the c-*myb* gene in a number of cells has been addressed by using antisense oligonucleotides to reduce c-*myb* protein expression (e.g. in T-cell leukaemias, normal T-cells and bone marrow progenitors, myleoid leukaemia). A 18-mer oligonucleotide targeted to codons 2–7 was shown to inhibit proliferation of myeloid leukaemia cell lines [35] as well as DNA synthesis in T-leukaemia cells. The anti-*myb* oligonucleotide was further utilised to investigate early erythroid differentiation

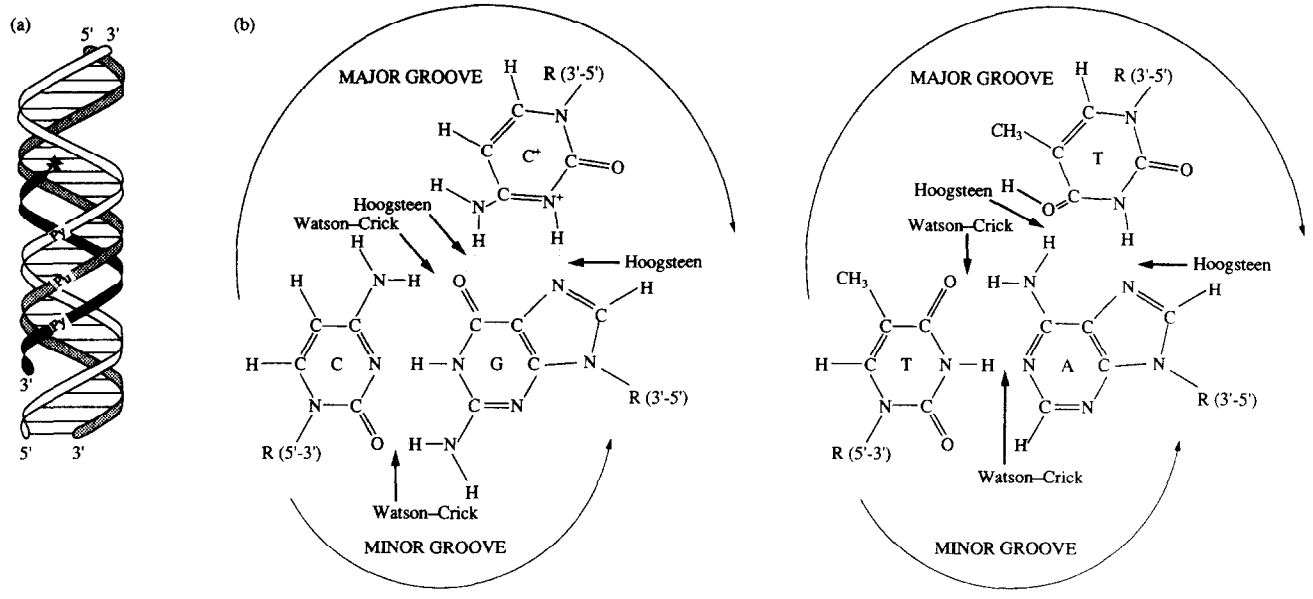

Fig. 2. Oligonucleotide targetting to double-stranded DNA. (a) A homopyrimidine oligonucleotide bound to the major groove of double helical DNA at a homopurine.homopyrimidine sequence. The oligonucleotide carries a reactive group ( ) that can be used to induce irreversible reactions on both strands of the double helix. (b) Triple helix formation involves Hoogsteen hydrogen bonding of thymine and protonated cytosine to Watson–Crick A.T and G.C base pairs, respectively. (Reprinted from [1] with permission.)

[36]. It was shown that c-*myb* activation is associated with the progression of progenitors into the S phase of the cell cycle and with an enhanced activity of DNA polymerase α. Normal and leukaemic haematopoietic cells manifest differential sensitivity to inhibitory effects of c-*myb* antisense oligodeoxynucleotides [37], suggesting that such oligonucleotides could be used as *ex vivo* bone marrow purging agents.

The *ras* family of oncogenes has been investigated as a potential target for antisense oligonucleotides. In the experiments reported in the preceeding paragraph it was shown that an antisense oligonucleotide or a ribozyme directed towards a region comprising the mutation could discriminate between the proto-oncogene and the oncogene [20]. All three *ras* genes (Ha-*ras*, Ki-*ras*, N-*ras*) are activated by point mutations and therefore are amenable to the same strategy. *ras* mRNA sequences that are outside the point mutation have also been used as targets for antisense oligonucleotides. The 5'-cap untranslated region of the Ha-*ras* gene appears to be the most efficient target for maximum inhibition of p21 synthesis [38]. A 11-mer oligo methylphosphonate was also shown to inhibit p21 expression in 3T3 cells transformed by LTR-activated Ha-*ras* [39]. The targets are identical in both the normal and the mutated genes when the oligonucleotide does not overlap the mutation; therefore no discrimination is expected between the proto-oncogene and the oncogene.

Cellular receptors, cytokines, growth factors, etc. can be chosen as targets for antisense oligonucleotides aimed at inhibiting cell proliferation. This has been particularly well demonstrated when autocrine growth loops are involved. Both colony stimulating factor CSF-1 [40] and its receptor encoded by the c-*fms* gene [41] can be inhibited by antisense oligonucleotides resulting in inhibition of proliferation of autocrine cells [40] or differentiation of HL 60 cells induced by phorbol esters [41]. Certain human myeloma cell lines are characterised by an IL-6 autocrine growth loop which can be blocked by an anti-IL-6 oligonucleotide [42]. The growth of IL-2 and IL-4-dependent cell lines can also be selectively inhibited by antisense oligonucleotides [43]. Antisense oligonucleotides targeted to basic fibroblast growth factor (bFGF) inhibit malignant melanoma cell proliferation and colony formation in soft agar [44].

THE ANTIGENE STRATEGY

In the antisense approach, the oligonucleotide is targeted to a messenger RNA and is thus expected to inhibit translation. Viral RNAs can also be used as targets for complementary oligonucleotides that will inhibit either replication or viral gene expression [45]. More recently it has become clear that DNA itself could be the target for oligonucleotides that would inhibit the first step of gene expression, namely transcription [46, 47]. We have called this new approach the "antigene" strategy [1]. The molecular basis for the recognition of a DNA double helical sequence by an oligonucleotide is summarised in Fig. 2. Thymine and protonated cytosine can form two hydrogen bonds with A.T and G.C Watson–Crick base pairs, respectively, generating base-triplets T.A × T and C.G × C^+ where the dot indicates Watson–Crick hydrogen bonding in the base pairs T.A and C.G and the cross refers to the so-called Hoogsteen hydrogen bonds represented on Fig. 2. Therefore, an oligonucleotide containing T and C can recognise a homopurine.homopyrimidine sequence on duplex DNA and form a local triple helix. It binds to the major groove of DNA in a parallel orientation with respect to the homopurine sequence. Oligonucleotides containing G and T or G and A nucleotides can also bind to homopurine.homopyrimidine sequences of DNA [48, 49]. Although the base triplets C.G × G and T.A × T or T.A × A are not isomorphous (whereas T.A × T and C.G × C^+ are isomorphous) G/T and G/A oligonucleotides may form more stable triple helices under physiological conditions because they do not require base protonation. In contrast, cytosine protonation (favoured by low pH) is required to form the C.G × C^+ base triplet. Replacement of cytosine by 5-methylcytosine allows more stable complexes to be formed at neutral pH.

In order to recognise a single sequence on double helical DNA in human cells, a triple helix-forming oligonucleotide must have a minimum length of about 17 bases [3]. Triple helix formation by oligonucleotides is strongly stabilised when oligonucleotides

are covalently linked to intercalating agents [50]. Triple-strand forming oligonucleotides can also be equipped with substituents that can induce irreversible reactions in their target sequence, such as crosslinking of the oligonucleotide or cleavage of the DNA strands [46, 47, 51, 52]. Recently we have shown that the two strands of DNA can be crosslinked by a psoralen-oligonucleotide conjugate under UV irradiation at the specific sequence where the oligonucleotide forms a triple helix [53].

Oligonucleotide-directed triple helix formation inhibits association of sequence-specific DNA binding proteins such as restriction enzymes or transcription factors [48, 54–56]. It is thus expected that triple helices will interfere with biological processes such as transcription and replication. A triple helix-forming oligonucleotide does block transcription of the c-*myc* gene because it inhibits binding of a transcription factor to one of the regulatory sequences upstream of the transcription start site [48]. More recently, oligonucleotide-directed triple helix formation was shown to inhibit the synthesis of the mRNA of IL-2 receptor subunit α in cultured cells [57]. Replication of simian virus 40 DNA was reported to be inhibited in CV 1 cells treated with an oligonucleotide–intercalator conjugate [14]. In all cases it remains to be demonstrated that the effects observed on cells in culture are due to triple helix formation and not to binding of the oligonucleotide to other targets. A direct proof of triple helix formation *in vivo* might use oligonucleotides carrying reactive groups that can be activated to induce an irreversible reaction (e.g. crosslinking) at the specific site on DNA where the oligonucleotide is bound.

As discussed above in the case of the antisense strategy, triple helix-forming oligonucleotides can be modified to make them resistant to nucleases. We have shown that oligo-[α]-deoxynucleotides can bind to the DNA double helix at homopurine.homopyrimidine sequences [46, 58, 59]. Substitution of the [α]-anomers of nucleotide units to the natural [β]-anomers make these oligonucleotides highly resistant to nucleases.

The development of triple helix-forming oligonucleotides as potential tools to control gene expression at the transcriptional level is still in its infancy. The only published application dealing with oncogene inhibition used the c-*myc* oncogene as a target *in vitro* [48]. Recent experiments suggest that inhibition also occurs in cells treated with a triple helix-forming oligonucleotide [60]. The forthcoming years will certainly see a burst in publications dealing with the antigene strategy applied to specific cellular genes, including oncogenes, as was observed in the mid 1980s with the antisense strategy.

The problems faced by antigene oligonucleotides as far as potential therapeutical applications are concerned are similar to those raised by antisense oligonucleotides. Both cell uptake and intracellular distribution ought to be improved to increase the efficacy of oligonucleotides. In the antisense strategy the target is either the mRNA in the cytosol or the pre-mRNA in the nucleus; in the antigene strategy the DNA target is located in the nucleus. Oligonucleotides appear to be taken up by endocytosis and therefore a large part of the oligonucleotide might be trapped in endocytic vesicles within the cytoplasm [61]. Recent experiments have shown that upon microinjection in cells, oligonucleotides are rapidly concentrated in the nucleus [62]. Therefore, the mode of oligonucleotide delivery is expected to play an important role in determining the efficacy of their biological effects. There are many steps dealing with pharmacokinetics, tissue distribution, bioavailability, delivery, systems etc. which are being addressed by several laboratories [63]. The results presently available provide a rational basis for the development of highly selective therapeutical approaches. Tumour-specific targets can be identified but it remains to be demonstrated whether inhibition of expression of these target genes can be achieved *in vivo* and whether this approach can qualify as useful cancer chemotherapy.

1. Hélène C, Toulme JJ. Specific regulation of gene expression by antisense,sense and antigene nucleic acids. *Biochim Biophys Acta* 1990, **1049**, 99–125.
2. Rossi JJ, Sarver N. RNA enzymes (ribozymes) as antiviral therapeutic agents. *Trends Biotechnol* 1990, **8**, 179–183.
3. Hélène C, Toulme JJ. Control of gene expression by oligodeoxynucleotides covalently linked to intercalating agents and nucleic acid-cleaving reagents. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. London, Macmillan, 1989, 137–172.
4. Cazenave C, Stein CA, Loreau N, *et al.* Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides *Nucleic Acids Res* 1989, **17**, 4255–4273.
5. Stein CA, Cohen JS. Phosphorothioate oligodeoxynucleotide analogues. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. London, Macmillan, 1989, 97–117.
6. Agrawal S, Sarin PS. Antisense oligonucleotides: gene regulation and chemotherapy of AIDS. *Adv Drug Delivery Rev* 1991, **6**, 251–270.
7. Matsukura M, Zon G, Shinozuka K, *et al.* Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against *rev* (*art/trs*) in chronically infected cells. *Proc Natl Acad Sci USA* 1989, **86**, 4244–4248.
8. Kulka M, Smith CC, Aurelian L, *et al.* Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. *Proc Natl Acad Sci USA* 1989, **86**, 6868–6872.
9. Cazenave C, Loreau N, Thuong NT, Toulme JJ, Hélène C. Rate of degradation of [α]- and [β]-oligodeoxynucleotides in *Xenopus* oocytes. Implications for anti-messenger strategies. *Nucleic Acids Res* 1987, **15**, 10507–10521.
10. Bertrand JR, Imbach JL, Paoletti C, Malvy C. Comparative activity of α- and β-anomeric oligonucleotides on rabbit β-globin synthesis: inhibitory effect of cap targeted α-oligonucleotides. *Biochem Biophys Commun* 1989, **164**, 311–318.
11. Boiziau C, Kurfurst R, Cazenave C, Roig V, Thuong NT, Toulme JJ. Inhibition of translation initiation by antisense oligonucleotides via an RNase-H independent mechanism. *Nucleic Acids Res* 1991, **19**, 1113–1119.
12. Dagle JM, Andracki ME, Devine RJ, Walder JA. Physical properties of oligonucleotides containing phosphoramidate-modified internucleoside linkages. *Nucleic Acids Res* 1991, **19**, 1805–1810.
13. Zerial A, Thuong NT, Hélène C. Selective inhibition of the cytopathic effect of type A influenza viruses by oligodeoxynucleotides covalently linked to an intercalating agent.*Nucleic Acids Res* 1987, **15**, 9909–9919.
14. Birg F, Praseuth D, Zerial A, *et al.* Inhibition of simian virus 40 DNA replication in CV-1 cells by an oligodeoxynucleotide covalently linked to an intercalating agent. *Nucleic Acids Res* 1990, **18**, 2901–2908.
15. Verspieren P, Cornelissen AWCA, Thuong NT, Hélène C, Toulme JJ. An acridine-linked oligodeoxynucleotide targeted to the common 5′ end of trypanosome mRNas kills cultured parasites. *Gene* 1987, **61**, 307–315.
16. Boutorin AS, Gus'kova LV, Ivanova EM, *et al.* Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3′-terminus and their interaction with DNA within mammalian cells. *FEBS Lett* 1989, **254**, 129–132.
17. Letsinger RL, Zhang G, Sun DK, Ikeuchi T, Sarin PS. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc Natl Acad Sci USA* 1989, **86**, 6553–6556.
18. Knorre DG, Valssov VV, Zarytova VF. Oligonucleotides linked to reactive groups. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. London, Macmillan, 1989, 173–210.
19. Hélène C, Le Doan T, Thuong NT. Sequence targeted photochemical reactions in single-stranded and double-stranded nucleic acids

by oligonucleotide-photosensitizer conjugates. In: Nielsen PE, ed. *Photochemical Probes in Biochemistry*. Amsterdam, Kluwer, 1989, 219–229.
20. Saison-Behmoaras T, Tocque B, Rey I, Chassignol M, Thuong NT, Hélène C. Short modified antisense oligonucleotides directed against Ha-*ras* point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *EMBO J* 1991, **10**, 1111–1118.
21. Koizumi M, Hayase Y, Iwai S, Kamiya H, Inoue H, Ohtsuka E. Design of RNA enzymes distinguishing a single base mutation in RNA. *Nucleic Acids Res* 1989, **17**, 7059–7071.
22. Champlin RE, Golde DW. Chronic myelogenous leukemia: recent advances. *Blood* 1985, **65**, 1039–1047.
23. De The H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature* 1990, **347**, 558–561.
24. Seto M, Jaeger U, Hockett RD, *et al.* Alternative promoters and exons,somatic mutation and deregulationof the *bcl-2*-lg fusion gene in lymphoma. *EMBO J* 1988, **7**, 123–131.
25. Caracciolo D, Valtieri M, Venturelli D, Peschle C, Gewirtz AM, Calabretta B. Lineage-specific requirement of c-*abl* function in normal hematopoiesis. *Science* 1989, **245**, 1107–1110.
26. Reed JC, Stein C, Subasinghe C, *et al.* Antisense-mediated inhibition of *BCL* 2 protooncogene expressionand leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligonucleotides. *Cancer Res* 1990, **50**, 6565–6570.
27. Degols G, Leonetti JP, Mechti N, Lebleu B. Antiproliferative effects of antisense oligonucleotides directed to the RNA of c-*myc* oncogene. *Nucleic Acids Res* 1990, **19**, 945–948.
28. McManaway ME, Neckers LM, Loke SL, *et al.* Tumour-specific inhibition of lymphoma growth by an antisense oligodeoxynucleotide. *Lancet* 1990, **335**, 808–811.
29. Wickstrom EL, Bacon TA, Gonzalez A, Lyman GH, Wickstrom E. Human promyelocytic leukemia HL-60 cell proliferation and c-*myc* protein expression are inhibited by an antisense pentadecadeoxynucleotide. *Proc Natl Acad Sci USA* 1988, **85**, 1028–1032.
30. Holt JT, Redner RL, Nienhuis AW. An oligomer complementary to c-*myc* mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol Cell Biol* 1988, **8**, 963–973.
31. Bacon TA, Wickstrom E. Daily addition of an anti-c-*myc* DNA oligomer induces granulocytic differentiation of human promyelocytic leukemia HL-60 cells in both serum-containing and serum-free media. *Oncogene Res* 1991, **6**, 21–32.
32. Bacon TA, Wickstrom E. Walking along human c-*myc* mRNA with antisense oligonucleotides: maximum efficacy at the 5′ cap region. *Oncogene Res* 1991, **6**, 13–19.
33. Rosolen A, Whitesell L, Ikegaki N, Kennett RH, Neckers LM. Antisense inhibition of single copy N-*myc* expression results in decreased cell growth without reduction of c-*myc* protein in a neuroepithelioma cell line. *Cancer Res* 1990, **50**, 6316–6322.
34. Szczylik C, Skorsky T, Nicolaides NC, *et al.* Selective inhibition of lukemia cell proliferation by *BCR-ABL* antisense oligodeoxynucleotides. *Science* 1991, **253**, 562–565.
35. Anfossi G, Gewirtz AM, Calabretta B. An oligomer complementary to c-*myb*-encoded mRNA inhibits proligeration of human myeloid leukemia cell lines. *Proc Natl Acad Sci USA* 1989, **86**, 3379–3383.
36. Valtieri M, Venturelli D, Care A, *et al.* Antisense *myb* inhibition of purified erythroid progenitors in development and differentiation is linked to cycling activity and expression of DNA polymerase α. *Blood* 1991, **77**, 1181–1190.
37. Calabretta B, Sims RB, Valtieri M, *et al.* Normal and leukemic hematopoietic cells manifest differential sensitivity to inhibitory effects of c-*myb* antisense oligonucleotides: an *in vitro* study relevant to bone marrow purging. *Proc Natl Acad Sci USA* 1991, **88**, 2351–2355.
38. Daaka Y, Wickstrom E. Target dependence of antisense oligodeoxynucleotide inhibition of c-Ha-*ras* p21 expression and focus formation in T24-transformed NIH3T3 cell. *Oncogene Res* 1990, **5**, 279–289.
39. Brown D, Yu ZP, Miller P, *et al.* Modulation of *ras* expression by anti-sense, nonionic deoxyoligonucleotide analogs. *Oncogene Res* 1989, **4**, 243–252.
40. Birchenall RM, Ferrer C, Ferris D, *et al.* Inhibition of murine monocyte proliferation by a colony-stimulating factor-1 antisense oligodeoxynucleotide. Evidence for autocrine regulation. *J Immunol* 1990, **145**, 3290–3296.
41. Wu J, Zhu JQ, Han KK, Zhu DX. The role of the c-fms oncogene in the regulationof HL-60 cell differentiation. *Oncogene* 1990, **5**, 873–877.
42. Schwab G, Siegall CB, Aarden LA, Neckers L, Nordan RP. Characterization of an interleukin-6-mediated autocrine growth loop in the human multiple myeloma cell line U266. *Blood* 1991, **77**, 587–593.
43. Harel-Bellan A, Durum S, Muegge K, Abbas A, Farrar W. Specific inhibition of lymphokine biosynthesis and autocrine growth using antisense oligonucleotides in Th1 and Th2 helper T cell clones *J Exp Med* 1988, **168**, 2309–2318.
44. Becker D, Meier CB, Herlyn M. Proliferation of human malignant melanomas is inhibited by antisense oligonucleotides targeted against basic fibroblast growth factor. *EMBO J* 1989, **8**, 3685–3691.
45. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978, **75**, 280–284.
46. Le Doan T, Perrouault L, Praseuth D, *et al.* Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-α-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res* 1987, **15**, 7749–7760.
47. Moser HE, Dervan PB. Sequence-specific cleavage of double helical DNA by triple helix formation. *Science* 1987, **238**, 645–650.
48. Cooney M, Czernuszewicz G, Postel EH, Flint SJ, Hogan ME. Site-specific oligonucleotide binding represses transcription of the human c-*myc* gene *in vitro*. *Science* 1988, **241**, 456–459.
49. Beal PA, Dervan PB. Second structural motif for recognition of DNA by oligonucleotide-directed triple-helix formation. *Science* 1991, **251**, 1360–1363.
50. Sun JS, Francois JC, Montenay-Garestier T, *et al.* Sequence-specific intercalating agents: intercalation at specific sequences on duplex DNA via major groove recognition by oligonucleotide-intercalator conjugates. *Proc Natl Acad Sci USA* 1989, **86**, 9198–9202.
51. Francois JC, Saison-Behmoaras T, Barbier C, Chassignol M, Thuong NT, Hélène C. Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. *Proc Natl Acad Sci USA* 1989, **86**, 9702–9706.
52. Fedorova OS, Knorre DG, Podust LM, Zaritova VF. Complementary addressed modification of double-stranded DNA within alternary complex. *FEBS Lett* 1988, **228**, 273–276.
53. Takasugi M, Guendouz A, Chassignol M, *et al.* Sequence-specific photo-induced cross-linking of the two strands of double-helical DNA by a psoralen covalently linked to atriple helix-forming oligonucleotide. *Proc Natl Acad Sci USA* 1991, **88**, 5602–5606.
54. Maher LJ, Wold B, Dervan PB. Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation. *Science* 1989, **245**, 725–730.
55. Francois JC, Saison-Behmoaras T, Thuong NT, Hélène C. Inhibition of restriction endonuclease cleavage via triple helix formation by homopyrimidine oligonucleotides. *Biochemistry* 1989, **28**, 9617–9619.
56. Hanvey JC, Shimizu M, Wells RD. Site-specific inhibition of EcoRI restriction/modification enzymes by a DNA triple helix. *Nucleic Acids Res* 1990, **18**, 157–161.
57. Orson FM, Thomas DW, McShan WM, Kessler DJ, Hogan ME. Oligonucleotide inhibition of IL2Rα mRNA transcription by promoter region collinear triplex formation in lymphocytes. *Nucleic Acids Res* 1991, **19**, 3435–3441.
58. Praseuth D, Perrouault L, Le Doan T, Chassignol M, Thuong NT, Helene C. Sequence-specific binding and photocrosslinking of α and β oligodeoxynucleotides to the major groove of DNA via triple-helix formation. *Proc Natl Acad Sci USA* 1988, **85**, 1349–1353.
59. Sun JS, Giovannangeli C, Francois JC, *et al.* Triple-helix formation by α oligodeoxynucleotides and α oligodeoxynucleotide-intercalator conjugates. *Proc Natl Acad Sci USA* 1991, **88**, 6023–6027.
60. Orson FM, Thomas DW, McShan WM, KesslerDJ, Hogan ME. Oligonucleotide inhibition of IL2Rα mRNA transcription by promoter region collinear triplex formation in lymphocytes. *Nucleic Acids Res* 1991, **19**, 3435–3441.
61. Jaroszewski JW, Cohen JS. Cellular uptake of antisense oligodeoxynucleotides. *Adv Drug Delivery Rev* 1991, **6**, 235–250.
62. Leonetti JP, Mechti N, Degols G, Gagnor C, Lebleu B. Intracellular distribution of microinjected antisense oligonucleotides. *Proc Natl Acad Sci USA* 1991, **88**, 2702–2706.
63. Zon G. Oligonucleotide analogues as potential chemotherapeutic agents. *Pharmacol Res* 1988, **5**, 539–549.