Loop formation in a DNA duplex by hybridization with a phosphate-methylated DNA oligomer. Implications for the use of the hybridization-arrest technique in Alzheimer's disease

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Loop formation in a DNA duplex by hybridization with a phosphate-methylated DNA oligomer. Implications for the use of the hybridization-arrest technique in Alzheimer's disease

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SUMMARY

Hybridization of a phosphate-methylated DNA oligomer to two nonadjacent regions of single- or double-stranded DNA is possible via loop formation of the intervening sequence. For duplex DNA, this loop formation was deduced from hybridization-arrest experiments on the c-*myc* gene in human leukemia cells.

Based on literature data, it is predicted that duplex-loop formation only occurs when more than 100–200 bases are present between the targets. For single-stranded DNA, it is shown that a 10-base loop can be induced by hybridization with an appropriate phosphate-methylated oligomer. The consequences of these results are discussed for Alzheimer's disease, which is related to the absence of an intervening sequence on the mRNA level. Due to the small size of the insert (168 base pairs), no effect on duplex DNA via a looped-out structure is expected.

By free energy calculations on the folded mRNA structures with and without hybridization to target sites around the insert region, it is shown that the mRNA without insert can be selectively blocked by hybridization with a phosphate-methylated oligomer, since looping out in the mRNA with insert is unfavourable.

INTRODUCTION

Phosphate-methylated DNA oligomers have been shown to hybridize strongly with natural single-stranded DNA, due to the absence of interstrand phosphate-phosphate charge repulsions (Moody et al., 1989a). When the target area is chosen in duplex DNA, as occurred in biological experiments with *Escherichia coli* and *Salmonella typhimurium*, an efficient hybridization is also found, leading to a reduced transcription (Moody et al., 1989b, 1990). Ap-

parently, the enhanced strength of hybridization for phosphate-methylated DNA causes a displacement of one of the natural strands. In the present paper, we report evidence for the formation of a tertiairy structure for a DNA duplex via hybridization with a phosphate-methylated oligomer. In this buckled DNA, a part of the duplex is looped out of the hybridization area. The implications of this new hybridization mode for the possible application of phosphate-methylated DNA in Alzheimer's disease (AD) are investigated.

DUPLEX DNA WITH A LOOPED-OUT REGION

The hybridization of phosphate-methylated DNA oligomers with human duplex DNA was studied for acute myeloid leukemia (AML) patient cells, which have a strongly enhanced DNA synthesis due to an overexpression of the regulatory c-*myc* gene (Cole, 1986; Varmus, 1987). It was found that 3 μM of sense and antisense phosphate-methylated 15-mers, targeted at the start of exon 2 in the c-*myc* gene (see fig. 1A), results in a 50% reduction of DNA synthesis as monitored via ^{3}H-thymidine uptake by AML cells (van der Feltz et al., 1990).

Both sense and antisense systems were found to hybridize with the duplex DNA, and block the DNA replication. When a phosphate-methylated 20-mer was employed, which overlaps the last 10 bases of exon 1 and the first 10 bases

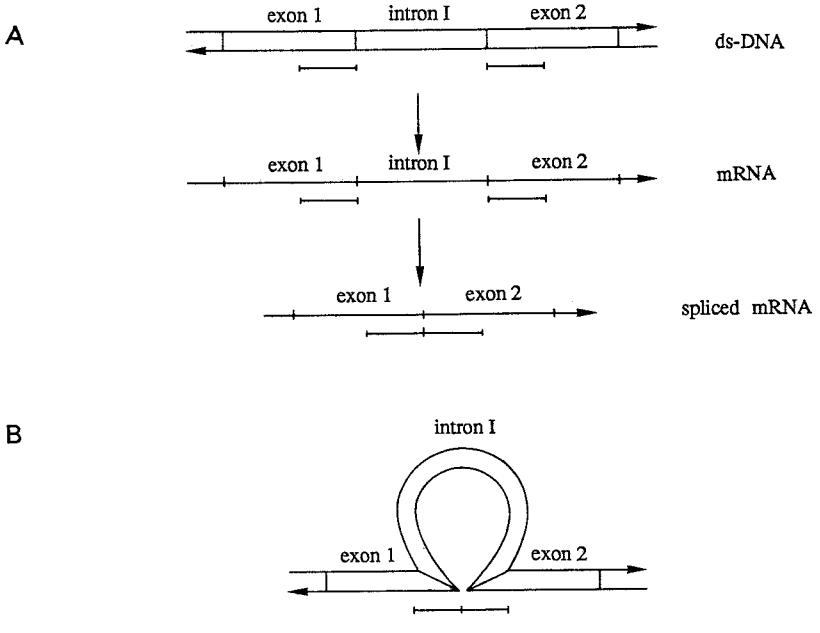

Fig. 1. A: Schematic representation of the c-*myc* gene, where exons 1 and 2 are separated by intron I in the DNA duplex and in unspliced mRNA. After splicing, the exons are adjacent, and the indicated target areas at the end of exon 1 and the start of exon 2 are joined. B: Looped-out structure that arises after hybridization of a phosphate-methylated oligomer to both the target regions in duplex DNA.

of exon 2 (see fig. 1A), hybridization seems possible only on the level of spliced mRNA, i.e., for an antisense system. Nevertheless, both the sense and antisense 20-mers were equally active (50% inhibition of DNA synthesis at 3 μM), while a random phosphate-methylated 20-mer had no effect. It must therefore be concluded that the DNA duplex assumes a tertiairy structure, where the two exons are adjacent and hybridization with the sense phosphate-methylated 20-mer can occur (see fig. 1B), while intron I is looped out. This situation is similar to structures reported earlier in the R-loop method (Thomas et al., 1976), where duplex DNA is partially opened with 70% formamide, and the spliced mRNA is allowed to hybridize with the template DNA strand. This can occur only for the exons containing the structural information, and the introns are looped out in the form of duplex DNA. This type of DNA structure has been found for introns of 500–600 base pairs (Tilghman et al., 1978), but a lower limit exists for the intron length due to the maximal curvature that can be accommodated by duplex DNA.

Based on molecular mechanics calculations, DNA with introns less than 100–200 base pairs is not expected to assume a looped-out structure (Sussman and Trifonov, 1978; Lewitt, 1978). In contrast to the artificial R-loop technique, phosphate-methylated oligomers hybridize strong enough to duplex DNA to allow spontaneous formation of buckled structures, provided that the intron is long enough. For the c-*myc* intron I, which has a length of 1652 base pairs, a looped-out duplex can be assumed (see fig. 1B). The ability of phosphate-methylated DNA oligomers to hybridize with two target regions simultaneously, while looping out the intervening sequence, was investigated on the single-strand DNA level with UV hyperchromicity experiments. For the phosphate-methylated 16-mer d(CAT.GAA.TCC.TAG.CAG.T) and its natural complement d(ACT.GCT.AGG.ATT.CAT.G), it was established that the duplex$\rightleftarrows$coil transition temperature (T_m) is 49°C at a DNA concentration of 2 μM in a buffered aqueous solution (pH 7). From the slope of the melting curve, it was calculated that the enthalpy of dissociation (ΔH^0) equals 6.2 kcal/mol per base pair, using the formula as described in Materials and Methods. The phosphate-methylated 16-mer was then hybridized with the natural 26-mer d(ACT.GCT.AGG.TAA.GTA.CAG.GAT.TCA.TG), in which a 10-base intervening sequence is present between two 8-base target areas. Now, a T_m of 31°C was found at a DNA concentration of 2 μM under similar conditions. Calculation of the total ΔH^0 for all base pairs gave a value of 94.4 kcal/mol, which is consistent only with the presence of 16 base pairs (5.9 kcal/mol per base pair) in a looped-out structure (see fig. 2A).

Hybridization to only one of the target areas in the 26-mer would involve 9 or 10 base pairs (see fig. 2B), and would give a much lower total ΔH^0 value (maximum 62 kcal/mol for 10 base pairs, vide supra). The decrease in T_m value from 49°C to 31°C is consistent with the presence of a strained 10-base loop in the duplex. These experiments show clearly, that phosphate-methylated DNA hybridizes sufficiently strong to natural DNA to induce loop formation. This new possibility of hybridization is important when phosphate-methylated DNA

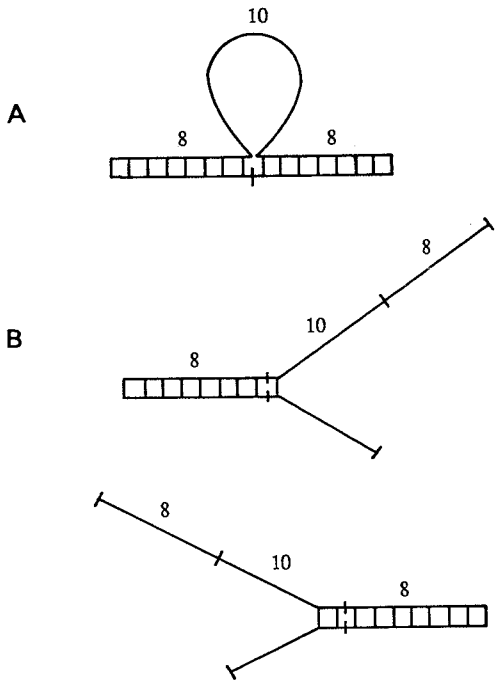

Fig. 2. Possible structures for the duplexes between the phosphate-methylated 16-mer and the natural 26-mer (see text). A: Looped-out structure with 16 base pairs. B: Base pairing occurs only with one of the target areas, involving either 9 or 10 base pairs.

is targeted at duplex DNA with a deleted region, in such a way that hybridization can only take place after the deletion has brought two parts of the target area together (cf. the splicing process in fig. 1A). It must be taken into account in these cases that hybridization to duplex DNA without the deletion can also occur via a structure with a looped-out region.

IMPLICATIONS FOR ALZHEIMER'S DISEASE

In patients with AD, a deposition of A4 protein into placques on nerve cells is characteristic for the molecular pathology (Müller-Hill and Beyreuther, 1989). It has been reported that AD is related to the increased presence of one specific mRNA (APP_{695}) which codes for an amyloid protein precursor (APP) of 695 amino acids (Palmert et al., 1988). Normally, a longer mRNA (APP_{751}) dominates, containing the so-called KPI insert which codes for a protease inhibitor protein that prevents the proteolytic breakdown of APP into A4 protein (amino acids 597–638 in the 695 amino-acid precursor, see fig. 3) (Ponte et al., 1988).

The absence of the KPI region in APP_{695} therefore leads to a too large production of A4 protein, which is then deposited on nerve cells. Hybridization-arrest with phosphate-methylated DNA could be used to block translation of

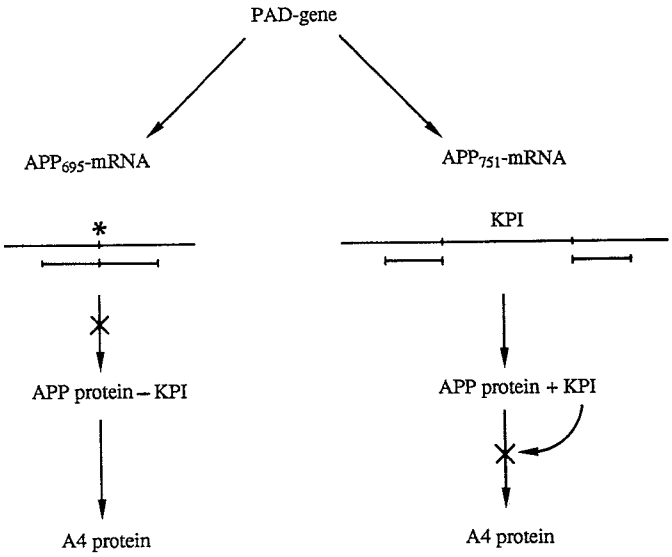

Fig. 3. Expression of the Precursor for Alzheimer's Disease A4 amyloid precursor gene (PAD). In AD patients, the left route predominates and leads to an overproduction of the A4 protein that forms amyloid placques. A target region for a phosphate-methylated oligomer is indicated, which will block the translation of the APP_{695} mRNA exclusively, since it overlaps the junction point (*). This also follows from the free energy calculations on the mRNA structures (vide infra).

the overabundant APP_{695} mRNA exclusively, when the target region is chosen to overlap the junction point of the flanks around the KPI insert (see fig. 3). However, the APP_{751} mRNA can easily assume a folded structure that loops out the KPI region, leading to hybridization of a junction-targeted phosphate-methylated oligomer not exclusively to APP_{695} mRNA. A similar structure may be assumed by the parent DNA duplex (which always contains the KPI insert). On the DNA duplex level, we feel that hybridization via a looped-out structure will not occur, because the KPI region is only 168 base pairs long, which is a borderline case (vide supra). In order to assess the possibility of looped-out structures on the RNA level, we performed stability calculations for both mRNAs with and without a simulated antisense oligomer present. Regions of 234 bases were used, either from APP_{751} (bases 939–1172; numbering according to Ponte et al., 1988) or from APP_{695} (bases 771–989 and 1158–1172) up to 15 bases downstream of the junction point for the normal folding pattern. To these mRNA fragments, a six-adenosine loop and a 30-base antisense RNA region are added at the downstream end (aimed at the 30 bases around the junction point), while 36 bases are removed at the upstream end to preserve the length (see fig. 4). This construction enables the 30-base antisense region to fold back and hybridize with the 30-base target area around the junction point.

Calculation of the thermodynamically most stable structures with the FOLD program (Zuker and Stiegler, 1981) showed clearly, that for APP_{751} free energy rises when the normal folded structure is disrupted to allow hybridization with

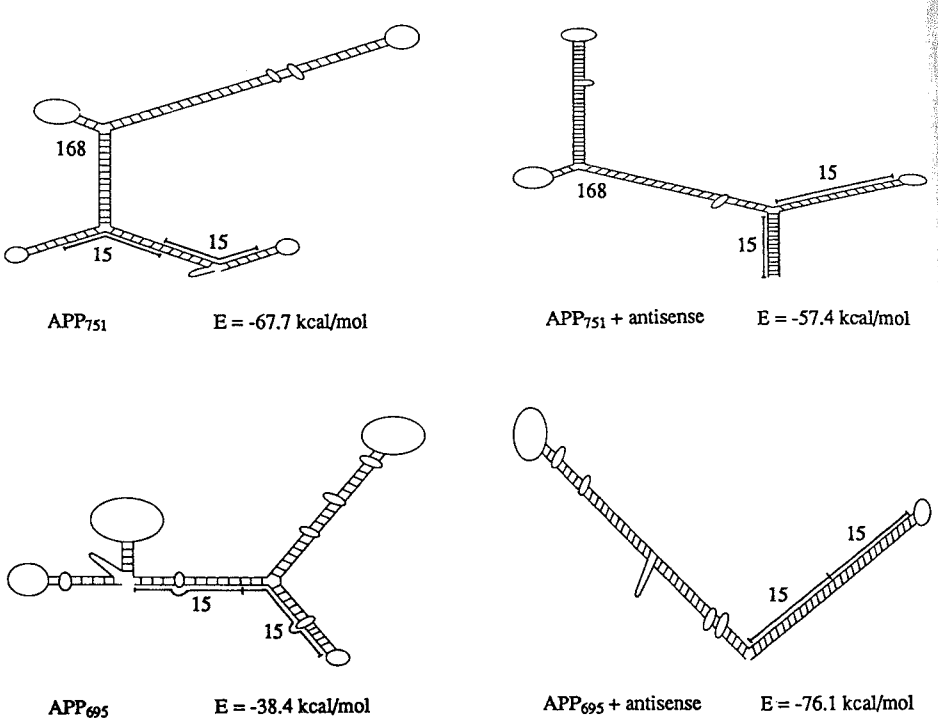

Fig. 4. Optimal folded structures for the mRNAs APP_{751} and APP_{695}, without an antisense region present (left), and with an antisense region attached (right). The two 15-base target areas are indicated, and the 168-base insert in APP_{751}. Free energies are given for all structures.

the joined flank regions of the looped-out KPI insert. In contrast, APP_{695} assumes a more stable structure when the antisense fragment hybridizes with the target region (see fig. 4). The present calculations use a 30-base antisense RNA fragment as a model for a shorter phosphate-methylated DNA oligomer, since the hybridization to mRNA will be stronger for the neutral DNA. Based on the calculational results, it seems reasonable to assume, that a phosphate-methylated DNA aimed at the junction point in APP_{695} will exclusively hybridize with this mRNA, and will not interfere with APP_{751}. Therefore, it appears to us that phosphate-methylated DNA may be inhibitory for the nerve-cell damage in AD patients.

MATERIALS AND METHODS

Phosphate-methylated DNA oligomers were synthesized as described by Moody et al. (1989a). Natural DNA oligomers were prepared on an Applied Biosystems 381A DNA synthesizer, and purified by alcohol precipitation. UV melting curves were measured on a Perkin-Elmer 124 spectrophotometer at a wavelength of 260 nm. Samples were prepared by mixing the DNA solutions at room temperature.

All experiments were performed in a 2 mM TrisHCl/0.1 mM EDTA buffer solution with 0.1 M NaCl. The T_m value of duplexes between phosphate-methylated and natural DNA is known to be independent of the NaCl concentration (Moody et al., 1989a). The present concentration was chosen, because it gives well-defined UV hyperchromicity effects. To obtain accurate melting curves, the temperature was raised at 1°C/min and the extinction was recorded every 2°C. These data were analyzed with a computer program that calculates the best-fitting sigmoidal curve with the equation for the fraction single-stranded DNA f (Marky and Breslauer, 1987):

$$f = 2/\left[1 + \left\{ 1 + 8e^{(\Delta H^0/R)(1/T - 1/T_m)} \right\}^{1/2}\right]$$

to give T_m and ΔH^0.

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