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ABSTRACT

For the purposes of the antisense strategy oligodeoxyribonucleotides can be protected against serum and cell nuclease digestion by tagging at their 3′-end with a sequence naturally forming a very stable hairpin, d(GCGAAGC). This nuclease-resistant hairpin is also known for its high thermostability. We demonstrate in this study that attachment of d(GCGAAGC) at the 3′-end of an oligodeoxyribonucleotide does not hinder hybridization of the 5′-part of this oligonucleotide to a complementary DNA strand. Moreover, the hairpin is in equilibrium between a folded and an open structure, with an energy minimum in favor of pairing if it is possible, even with mismatches.

INTRODUCTION

Some DNA and RNA sequences form unusually stable hairpin structures (1). The DNA fragments d(GCGAAGC) and d(GCGAAGC) form extraordinarily stable DNA mini-hairpins with regard to thermal denaturation, nuclease degradation and denaturing electrophoresis conditions (2–4). They are stabilized through only two G-C base pairs and a non-Watson–Crick G-A pair (5,6). Their high resistance against nucleases makes them useful in the antisense strategy, because unmodified oligodeoxyribonucleotides are rapidly degraded in both serum and cells, primarily by 3′-exonucleases (7). In competition with chemical modification of the phosphate backbone or of the bases, stabilization of antisense oligonucleotides has recently been achieved by tagging at the 3′-end with a sequence naturally forming a hairpin (3,8–10). We have studied, by a fluorescence resonance energy transfer (FRET) technique, the nuclease resistance of an oligonucleotide which is protected on its 3′-end by the hairpin 5′-GCGAAGC (11).

The shortest sequence likely to be unique is 13 bases within the mRNA pool (12). At the moment the usual length of antisense oligonucleotides in clinical trials is ~20 bases (13). In this study we tried to establish whether the 7 bases of the hairpin 5′-GCGAAGC can be taken into account for stabilization of an antisense oligonucleotide. In other words, does pairing of the 5′-part of an oligodeoxyribonucleotide to its complementary mRNA lead to destabilization and therefore opening of its 3′-hairpin part?

Attachment of the GCGAAGC hairpin structure on the 3′-end of a 16mer did not hinder hybridization to its complementary mRNA (3). Nevertheless, the measured $T_m$ of the double strand was the same with and without the hairpin part, indicating lack of further stabilization caused by opening and pairing of the hairpin part. On the other hand, inclusion of a less stable hairpin was shown to increase the $T_m$ (3). However, the method used in this study was not a classical absorbance versus temperature measurement.

Two 19mer oligodeoxyribonucleotides were used in this study. H has on its 3′-end the naturally hairpin-forming sequence GCGAAGC. R is a reference, whose sequence is the same as H except that inversion of two bases in its 3′-end makes it unable to form the hairpin. C(H) is a 29mer which has in the middle a 19 base long sequence complementary to H. However, if the hairpin does not open, pairing is possible only over a 12 bp length. C(R) has in the middle a 19 base long sequence complementary to R: formation of a 19 bp double strand is therefore expected. C_m(H) is the same as H except that in hybridization a mismatch G-A is expected instead of the regular G-C base pair. This mismatch involves the adenine of H, which is known to interact with a guanine through a non-Watson–Crick bond when the hairpin is formed. The G-A mismatch is the most thoroughly studied in solution and was shown to be accommodated into a regular B-helix with minimal perturbation (14). C(11) has a stretch of only 11 bases complementary to each of both 19mers H and R. A short (S) oligomer, the same as H but without the 3′-end GCGAAGC was also used.

Different combinations of 29mer + 19mer or 29mer + 12mer were studied through a set of techniques (non-denaturing gel electrophoresis, $T_m$ measurement, S1 nuclease digestion assay and hydroxylamine modification) in order to assess stability of the hairpin depending on pairing possibilities.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Two 19mer, one 12mer and four 29mer oligodeoxyribonucleotides were synthesized by Genosys: H, d(5′-TCACCTCATCCCGCGG-\text{AAGC}); R, d(5′-TCACCTCATCCGCGCAAGC); S, d(5′-TCACCTCATCC); C(H), d(5′-AATGACCTTCGGCGGATGAGGGTGTTG); C(R), d(5′-AATGAGCTTGGCCGGA TGAGGTGA-\text{GTTGA}); C_m(H), d(5′-AATGACGCTGCGGGGATGAGGGTGTTG); C(11), d(5′-AATGAGCTATGGCAGAGGTGA-\text{GTTGA}).

H forms a hairpin at its 3′-end; this is not the case with the reference R. C(H) [C(R)] is complementary to the sequence of

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Figure 1. Sequences of the four 29mers C(R), C(H), C_m(H) and C(11) and of the two 19mers H and R. The sequences are displayed in such a way as to show the possible pairings. The hairpin part of H is in bold. The dots indicate mismatches.

H [R] over a length of 19 bases. C_m(H) is the same as C(H) except that in hybridization a G-A mismatch is expected instead of the regular G-C pairing. C(11) has a stretch of only 11 bases complementary to each of the 19mers.

All oligonucleotides were solubilized in 10 mM NaCl at a concentration of 1 mg/ml. H and R were heated for 20 min at 80 °C then slowly cooled down. This method has been described as favoring formation of the hairpin (5). Figure 1 presents all possible combinations between 29mers and 19mers. The dots indicate mismatches. The part of H able to fold in the hairpin form is in bold.

Non-denaturing gel electrophoresis

Oligonucleotides C(R), C(H), C_m(H) and C(11) were labeled with T4 polynucleotide kinase (Pharmacia) and [γ-32P]ATP (ICN). One of the radioabeled 29mer oligonucleotides (≤0.1 pmol) plus 30 pmol of the same unlabeled oligonucleotide were mixed with 60 pmol 19mer (H or R) or 12mer (S) in a total volume of 10 µl containing 10 mM Tris buffer, pH 7.3, and 50 mM NaCl. In most of the experiments a common annealing process was carried out in order to speed up hybridization: heating to 80 °C followed by a slow cooling to room temperature. The extent of hybridization after an overnight incubation at room temperature was also checked. Samples (4 µl plus 2 µl 40% sucrose containing bromophenol blue and xylene cyanol FF) were loaded on a non-denaturing 20% polyacrylamide gel. Electrophoresis was carried out without or with temperature monitoring at 4 °C and the gels were autoradiographed.

T_m measurements

The melting profiles of five oligonucleotide combinations have been studied: C(H) + H, C(R) + R, C(R) + H, C(11) + R, C(11) + S. Aliquots of 600 pmol each oligonucleotide in 800 µl 5 mM Tris buffer, pH 7.3, and 20 mM NaCl were allowed to anneal by heating at 80 °C and slow cooling to room temperature. Measured absorbance at 260 nm (A_260) was ~0.4. Melting curves of A_260 versus temperature were collected on a Uvikon 940 spectrophotometer matched in temperature by a Huber-ministat PD415. They were converted to relative absorbance versus temperature by dividing all absorbance measurements by the value of the first absorbance measurement. T_m was determined from differential curves. The heating rate was 3 °C/h and the absorbance was measured from 15 to 80 °C.

S1 nuclease digestion assay

The 19mers R and H were labeled as previously described. Radiolabeled R (or H) plus 110 pmol of the same unlabeled oligonucleotide were mixed with 220 pmol of one of the four 29mers in a total volume of 30 µl 50 mM NaCl. Control experiments without 29mer were also carried out. After annealing, 10 times concentrated S1 buffer was added. S1 buffer comprises 30 mM CH₃COONa (pH 4.6 at 37 °C), 50 mM NaCl and 0.1 mM ZnSO₄.

After addition of 1.4 U S1 nuclease (Sigma)/µg DNA (15), i.e. 3.6 U in hybridization experiments and 0.84 U in control experiments, the reaction was incubated for 15 min at 37 °C. After precipitation with ethanol the samples were resuspended in a 98% formamide, 10 mM EDTA buffer, pH 8, containing dyes. The sequencing gels contained 50% urea and 20% polyacrylamide in TBE buffer. An (A+G) Maxam–Gilbert reaction was also performed (16).

Hydroxylamine modification

The 29mers C(R), C(H) and C_m(H) were labeled as previously described. Radiolabeled 29mer plus 60 pmol of the same unlabeled oligonucleotide were mixed with 120 pmol 19mer (H or R) in a total volume of 20 µl 10 mM Tris buffer, pH 7.3 and 50 mM NaCl. After annealing, 4 µg carrier DNA were added to
each sample and hydroxylamine (HA) (Fluka) modification carried out as previously described (17). An aliquot of 75 µl 4 M HA [pH adjusted to 6 by addition of diethylylmelamine (Fluka)] was added to each sample. After 20 min incubation at 20°C (similar results were obtained at 37°C) the reaction was terminated by ethanol precipitation. After piperidine (Aldrich) treatment (15 min at 90°C) (15) the samples were resuspended in 98% formamide, 10 mM EDTA buffer, pH 8, containing dyes and analyzed on a 20% polyacrylamide denaturing (50% urea) gel in TBE buffer. A dimethylsulfate (Aldrich) (G) Maxam–Gilbert reaction was performed in order to identify the autoradiographic bands (16).

RESULTS AND DISCUSSION

Non-denaturing gel electrophoresis

Figure 2 shows non-denaturing gel electrophoresis of hybridization experiments carried out at room temperature (Fig. 2a) or after heating at 80°C (Fig. 2b) (see Materials and Methods). Both processes gave the same results.

Let us first consider the simplest cases. C(R) + R, where C(R) has in the middle a 19mer sequence fully complementary to R, is expected to form a double helix of 19 bp with single strand extensions at both the 5′- and 3′-ends (Fig. 1). C(11), C(H), C m(H) and C(R) are single strands. As expected for a double strand, C(R) + R is retarded compared with single strands (Fig. 2a and b). The slight retardation of C(11) in comparison with the other single strands [C(H), C m(H) and C(R) in Fig. 2b] is very likely explainable by its different 5′-end base composition (some adenines instead of guanines and a range of alternating purines and pyrimidines). Many DNA sequences indeed lead to compression or retardation effects (18,19).

Apart from these straightforward cases, there are three kinds of bands, migrating as double strands, single strands or retarded single strands. C(H) + H as well as C m(H) + H migrate exactly like the double-strand reference C(R) + R (Fig. 2a and b). Because of the unusual stability of the hairpin, H was a priori expected to hybridize to C(H) or C m(H) over a length of 11 or 12 bases. The base complementarity is with 12 bases but the hairpin could lower the stability of its neighboring base pair (position G13; see Fig. 1). Therefore, for C(H) + H and C m(H) + H we expected an electrophoretic behavior comparable with that of C(11) + S, C(11) + H and C(11) + R, which are hybridizable over a length of 11 bp. However, these three combinations migrate like the single strand C(11) (Fig. 2a and b). Probably the T m of hybridization over such a short length of base pairs does not allow stabilization during electrophoresis carried out without temperature monitoring. This will be confirmed in the following sections. Anyway, C(H) + H and C m(H) + H are obviously stabilized over >11 bp.

C(H) + R (Fig. 2a and b), C m(H) + R and C(R) + H (Fig. 2b) migrate as retarded single strands. C(H) + R is slightly more retarded and, more particularly in Figure 2a, appears as a blurred spot. In the cases of C(H) + R and C m(H) + R hybridization can occur over the whole length of R except for two mismatches, GG and CC (Fig. 1). There is a third mismatch close to the 3′-end of R in the case of C m(H) + R. The greater number of bases able to hybridize likely increases the T m of the resulting double strands (see the following section), but not sufficiently to allow stabilization throughout electrophoresis. The two DNA strands dissociate during migration, positioning the labeled C(H) or C m(H) in an intermediate location.

Let us go back to the cases of C(H) + H and C m(H) + H, which migrate exactly like the double-stranded C(R) + R. It is clear from the previous results that C(H) + H and C m(H) + H are not only hybridized over 11 or 12 bp or even over 19 bp with two or three mismatches. The obvious conclusion is that the hairpin opens and fully hybridizes to the 29mer, even when this implies a non-Watson–Crick G-A bond [C m(H)].

Lastly, the combination C(R) + H (Fig. 2b) is a retarded single strand. This means that stabilization occurs over >11 bp. Therefore, the hairpin also opens in this case and hybridization involves all possible Watson–Crick bonds on both sides of the two GG and CC mismatches.

Therefore, the presence of a strictly complementary DNA strand is not essential to energetically favor opening of the hairpin and, even with two mismatches, the 3′-end of H has a higher affinity for hybridization with complementary DNA than for preservation of the hairpin structure. This set of results relativizes the ‘extraordinary’ stability of the hairpin with regard to temperature increase, denaturing electrophoretic conditions and exonuclease activity (2–4). However, it is necessary to confirm
Melting curves

Figure 3 presents the melting profiles of C(H) + H and C(R) + R with their corresponding differential curves (inserts a and b). Similar $T_m$ values were determined in both experiments: 52 ± 2°C for C(H) + H and 55 ± 2°C for C(R) + R. Figure 3 also presents the melting profiles of C(R) + H, C(11) + R and C(11) + S. The $T_m$ values are 32 ± 2°C for C(R) + H (c), 25 ± 2°C for C(11) + R (d) and 27 ± 2°C for C(11) + S (e). C(R) + R is our double strand reference with a stretch of 19 bp. Its $T_m$ is comparable with that of C(H) + H, confirming opening of the hairpin: once more, we have hybridization over a stretch of 19 bp.

The $T_m$ of C(R) + H, which migrated as a retarded single strand (see above), is 32°C. We suggest that the $T_m$ of a double strand of 19 bp except for two mismatches does not allow stabilization throughout electrophoresis, which is of the order of 2 h. It has been shown that after a run of several hours at 150 V the temperature reached by a 6% polyacrylamide gel is close to 43°C (20). Therefore, it is likely that when electrophoresis is carried out without temperature monitoring the temperature achieved by the gel is below the $T_m$ of C(R) + H at the beginning of the run and above it at the end of the run.

The lowest $T_m$ values (−26°C, considering the 2°C standard error) have been calculated for C(11) + R and C(11) + S, for which hybridization can only occur over a length of 11 bp. These combinations have been shown to migrate as single strands, which is logical, since 26°C is likely quickly reached in the polyacrylamide gel during electrophoresis.

S1 nuclease digestion assay

S1 nuclease cleaves single-stranded DNA and does not show any base-specific recognition pattern. It has a pH optimum in the acid range, below pH 5 (15). Figure 4 presents the nuclease S1 cleavage patterns of, on the one hand, H alone as a control or after hybridization with C(11), C(R), C(H) and C_m(H) and, on the other hand, R alone as a control or after hybridization with C(11), C(R), C(H) and C_m(H).

Let us first notice the faster migration of the 19mer H in comparison with that of the 19mer R. This is due to the more compact structure of the hairpin (4). We have already observed this under denaturing as well as native gel electrophoresis conditions (11). After 15 min incubation with S1 nuclease at 37°C most samples have been almost fully digested. We see only a small remaining amount of 19mer in the control lanes H and R and in the lanes C(11) + H, C(R) + H, C(11) + R, C(H) + R and C_m(H) + R (see for comparison the intensities of the 19mer bands in the other three lanes), C(11) + H and C(11) + R, which can only hybridize over a length of 11 bp, have $T_m$ values around 26°C. Since S1 nuclease cleavage is carried out at 37°C, they dissociate and digestion of single-stranded H and R is observed. C(R) + H, C(H) + R and C_m(H) + R migrated as retarded single strands (see above): hybridization has been considered to occur over 19 bp except for two or three mismatches. The $T_m$ of C(R) + H is ∼32°C. A $T_m$ slightly below the incubation temperature linked with low pH conditions leads to destabilization of the duplexes and, once more, S1 nuclease cleaves the single strands H and R.

Hydroxylamine modification

Hydroxylamine (HA) reacts with exposed cytosine residues at neutral and acid pH in a way that allows further cleavage of the backbone by piperidine. It is highly reactive with single-stranded DNA and practically unreactive with double-stranded DNA having a uniform structure (17). The assessment of cytosine protection in the different 29mer–19mer combinations is another way to check hairpin opening.

Figure 5 presents the HA reactivity of C(R), C(H) and C_m(H), each of them alone as a control or after hybridization with H or R. Cytosines stand out from the degradation background. C(R) has three cytosines in positions 7, 12 and 13 (Fig. 1). As foreseen, all these positions are modified by HA in the single-strand control. The same electrophoretic pattern is observed in the case of C(R) + H, for which a non-Watson–Crick G-A bond takes the place of the regular G-C bond) perform in exactly the same way as the reference double-stranded C(R) + R. This strengthens our assumption that in the presence of DNA the hairpin opens and, if possible, hybridizes to the complementary strand. We can explain the faint cleavage observed by the fact that the bases at the ends of a double-stranded oligonucleotide are statistically little paired and accessible to S1 nuclease digestion.

In contrast, C(H) + H, C_m(H) + H and C(R) + R are largely uncleaved by S1 nuclease and only faint bands corresponding to possible loss of the 3′-terminal bases are observable. All these oligonucleotide combinations have been shown to hybridize over a length of 19 bp, with a $T_m$ >50°C. Once more, C(H) + H and C_m(H) + H (for which a non-Watson–Crick G-A bond takes the place of the regular G-C bond) perform in exactly the same way as the reference double-stranded C(R) + R. This strengthens our assumption that in the presence of DNA the hairpin opens and, if possible, hybridizes to the complementary strand. We can explain the faint cleavage observed by the fact that the bases at the ends of a double-stranded oligonucleotide are statistically little paired and accessible to S1 nuclease digestion.
Both the experiments involving C(H) and C_m(H) gave the same results. C(H) and C_m(H) have three cytosines at positions 7, 10 and 12. All these sites are modified by HA in the single-stranded C(H) and C_m(H). C10 and C12 are protected when the 29mers are hybridized with H. Protection of C10 from HA is an additional proof of hairpin opening. If the hairpin remained in its folded form, steric hindrance could perhaps protect the C12 but not the C10 site. C7 reactivity has already been discussed above.

Intermediate protection is observed when the 29mers are hybridized with R: C10 is now reactive but C12 is still partially protected. Hybridization is expected to occur over 19 bp except for two mismatches in the case of C(H) and three in the case of C_m(H), the third in the already unpaired end of the double-stranded oligonucleotide.

Although of the same expected stability, C(R) + H appears in this experiment less stabilized than C(H) + R and C_m(H) + R. This will be confirmed in the next section.

Native electrophoresis at low temperature

The electrophoresis experiments presented in the first section allowed us to characterize three kinds of fragments migrating as single strands, double strands and retarded single strands. Some of the single strands and all retarded single strands result from hybrids having a T_m below the temperature of the electrophoretic run, which therefore dissociate during the course of the experiment. We carried out electrophoresis under non-denaturing conditions at 4°C in order to stabilize these hybrids and possibly discriminate between apparently similar stabilities.

As expected, combinations migrating as double strands in the previous experiments [C(H) + H, C_m(H) + H and C(R) + R] still migrate in the same way (Fig. 6). Among fragments which first migrated as retarded single strands, C_m(H) + R and C(H) + R now migrate as double strands, whereas C(R) + H is more complex: the sample is double stranded for the most part but there is a faint blurred spot migrating as single strand or retarded single strand. Therefore, C(R) + H is less stable than C(H) + R or C_m(H) + R, which had already been observed in the HA modification experiments (see above). C(H) + R, C_m(H) + R and C(R) + H are hybridizable (if H opens) over a length of 19 bp except for two or three mismatches. Our assumption is as follows: the temperature of the run is now below the T_m of these combinations and allows hybridization to occur throughout the experiment. Nevertheless, with two GG and CC mismatches, evolution of the hairpin towards its energy minimum, which corresponds to pairing with a complementary strand, is not complete: the hairpin fluctuates between its folded and open forms. Therefore, a low amount of C(R) + H is hybridized over a length of only 11 bp (the hairpin remaining in its folded form) and migrates as single strand or retarded single strand because of its lower T_m.

C(11) + S, C(11) + H and C(11) + R migrated as single strands without temperature monitoring. Let us remember that hybridization is then expected to occur over a length of 11 bp. Now, with temperature monitoring of the electrophoresis buffer at 4°C, C(11) + R, C(11) + H and C(R) + S migrate as an equilibrium between double and single strands. Therefore, the T_m of these combinations, which is ~26°C, is likely very close to the temperature of the electrophoretic run. T_m is actually the temperature for which half of the oligonucleotides are double stranded and half single stranded. The double-stranded C(R) + S migrates further than the other double strands because of the shorter length of S compared with H and R (12 bases instead of 19). We again find retarded migration of C(11), which had already been observed in the case of the single strand and is now observable for double-stranded C(11) + R and C(11) + H. This had been explained by the different base composition of the 5'-end of C(11) compared with the other 29mers.

If we look carefully at C(11) + H, the band corresponding to the double-stranded fragments has two components of about the same intensity. Once again [see the discussion of C(R) + H above] we assume that the hairpin fluctuates between open and folded forms. Whereas the hairpin tends towards pairing when possible [C(H) + H and C_m(H) + H], even with mismatches [C(R) + H] it does not reach its energy minimum either in its folded form or in its open form when pairing is impossible [C(11) + H]. Because of its more compact structure, the folded form of H accelerates migration of C(11) + H (see above and ref. 11); this is not the case for the open form of H.

Conclusion

In conclusion, we have shown that when the extraordinarily stable mini-hairpin d(GCGAAGC) is attached at the 3'-end of an oligodeoxynucleotide, pairing of the 5'-part of this oligonucleotide with a complementary DNA strand over a length
of ∼12 bp is unhindered, leading to destabilization of the 3’-hairpin structure. The energy minimum of the hairpin corresponds in that case to pairing to a complementary DNA strand [C(H)], even with a G-A mismatch [Cm(H)]. With two GG and CC mismatches the hairpin fluctuates between the folded and open forms [C(R)]. The energy minimum still favors hybridization and most hairpins open. Lastly, when pairing is impossible [C(11)] the hairpin is in equilibrium between its open and folded forms.

All these results strengthen the relevance in the antisense strategy of oligonucleotides protected by such hairpins at their 3’-end. First of all, the hairpin protects the oligonucleotide against nuclease digestion. It does not hinder hybridization and, finally, it possibly contributes to pairing. It would be of interest to confirm these results with a complementary RNA instead of DNA.

DNA–RNA hybrids are generally considered to be more stable to thermal denaturation than their DNA–DNA counterparts (14). However, the reality is more complex and, in fact, the relative stability of these duplexes varies as a function of the sequence (7). Anyway, we are justified in expecting the stability of the antisense oligonucleotide complexed with mRNA to be in the same region as with DNA (22).

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