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Self-complementary sequence context in mature miRNAs

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ABSTRACT

MicroRNAs (miRNAs) are a class of 19–25 nt long non-coding RNAs that regulate gene expression post-transcriptionally by binding with partially complementary sequences in the 3′-untranslated region (3′-UTR) and inhibiting mRNA translation or by affecting mRNA stability. We have characterized the structures and the equilibrium between hairpin and homo-duplex form of the mature strand of hsa-mir-520h by various concentration and temperature dependent 1D, 2D NMR experiments and those structures correspond well with Mfold-folded and UNAFold-aligned secondary structures. A detailed folding and alignment analysis in physiological conditions of all mature miRNA strands from the complete database of known miRNAs (miRBase) was performed. The statistical analysis of the resulting folding and alignment data showed for the first time the potential of a large number of mature miRNAs to form significant hairpin and/or homo-duplex structures in solution. The self-complementarity of mature miRNAs can provide a mechanistic tuning and a regulatory sophistication to the process of miRNA mediated gene regulation.

Introduction

MicroRNAs (miRNAs) are endogenous, short (19–25 nt) single stranded, non-coding RNAs found in numerous eukaryotic lineages, including plants, insects, vertebrates, and mammals [1–3]. They are evolutionary conserved [4] and they function as regulator of post-transcriptional gene expression [5]. In this way, they control diverse biological processes in cells such as developmental timing [6], differentiation [7], cell proliferation [8], haematopoiesis [9] and apoptosis. There is a growing number of reports that link miRNAs to the regulation of pathways associated with diseases such as cancer [10,11], neurological diseases [12] and most recently also with viral [13] and metabolic [14] disease.

Based on previous work, it is known that primary miRNAs (pri-miRNAs) are derived from the transcription of large, highly structured precursors encoded by cellular genes. Then Drosha cleaves pri-miRNAs into 70–100 nt miRNA precursors (pre-miRNAs) [15,16], which are transported from the nucleus into the cytoplasm by Exportin 5 [17,18]. Subsequently, Dicer processes these pre-miRNAs into siRNA-like miRNA–miRNA* duplexes [19,20]. These duplexes [21] unwind in the cytoplasm, after which the mature guide strands [22] are specifically loaded into microRNP complexes and the passenger strands are degraded [3]. Mature miRNAs reside in the microRNP, likely similar to the RNA-induced silencing complex (RISC) [23–25], which regulates mRNA by sequence specific base pairing with miRNA-complementary sequences in mRNA target [26,27]. It is observed that depending on the level of complementarity between miRNA and the target sequences, the mRNA can either be translationally repressed (if there is only partial alignment) or cleaved in case of full complementarity. Generally in animals translational repression [23,28] seems to be the key method while in plants mRNA degradation [29] is the most likely pathway, however, recently some exceptions have been reported [30].

MicroRNAs are believed to control the expression of thousands of target mRNAs [31,32] and mRNAs are believed to be targeted by multiple miRNA [27]. In order to fully understand the biological functions of miRNAs, it requires knowledge of their mRNA targets. Many bioinformatics approaches have been used to predict mRNA targets [33], and to govern the principle of miRNA target binding [31]. Recently three in silico studies predicted the interaction of miRNA with other miRNA [34], and with genomic repetitive elements like Alu RNA [35–38] and suggested a regulatory role of those interactions on miRNA functions.

When we tried to experimentally characterize such an interaction of hsa-miR-520h mature strand with Alu Jo-RNA by different solution phase biophysical and biochemical measurements, we were unsuccessful to diagnose such interactions. Contrarily, we observed that at higher oligo concentrations, the miRNA which was predicted to interact with Alu-RNA sequences, self-aggregated. This process, which occurs in solution, possibly prevented the mutual interaction between miRNA and Alu target. To study this phenomenon in detail, we studied the conformational feature of the mature strand of hsa-miR-520h by various NMR biophysical experiments, we observed that at low temperature and low concentra-

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tion a less stable hairpin structure is formed which, at higher oligo concentration, forms a stable homo-duplex by breaking the hairpin form. The folded hairpin (Mfold) and aligned homo-duplex (UNAFold) structures for this sequence correspond well with the NMR observations. A detailed folding and alignment analysis for all the mature miRNA strands from miRBase (database of miRNAs) was performed in physiological conditions. The statistical data analysis of the resulting folding and alignments showed the tendency of a large number of mature miRNAs to form significant self-complementary hairpin and/or homo-duplex structures in solution.

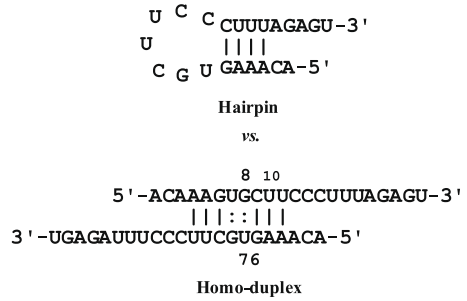

Fig. 1. Hairpin and homo-duplex form of hsa-miR-520h mature strand.

Materials and methods

NMR samples. RP-HPLC purified 22 nt miRNA (mature strand of hsa-miR-520h) oligo was purchased from Eurogentec S.A., Belgium and used as such to prepare NMR sample solution. Samples were annealed prior to NMR experiments by briefly heating at 70 °C and snap cooling on ice to promote hairpin formation. Samples were lyophilized and re-dissolved in 100% D_2O or in a 90%/10% mixture of H_2O/D_2O. The pH was adjusted to 6.5 by adding few μl of NaOH solution (0.1 N) in D_2O.

NMR experiments. Proton NMR spectra were recorded in H_2O:D_2O (9:1) using different concentrations of miRNA and various experimental temperatures in Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TXI HCN Z gradient cryoprobe (BioMacs). The water signal was suppressed by excitation sculpting with gradient pulse (Puls Program: zgesgp). A 2D NOESY spectrum was recorded in H_2O:D_2O (9:1) at 10 °C using WATERGATE water-suppression with a sweep width of 13,200 Hz in both dimensions, 128 scans, 4 K data points in t2 and 512 FIDs in t1 dimension. The data were apodized with a shifted sine-bell square function in both dimensions. Proton spectra in D_2O were also measured (with water pre-saturation) at different temperatures (5–70 °C) in a Bruker Avance II 500 MHz spectrometer equipped with 5 mm TXI HCP Z gradient probe to study non-exchangeable protons.

Folding and alignment analysis. Sequences of all miRNA mature strands were obtained from the mirBase [39] (Release 11.0) – comprehensive miRNA database (http://microrna.sanger.ac.uk/sequences/). The sequences were classified into groups as Virus, Mouse, Human, Animal and Plant. Only unique mature miRNA sequences were considered in each category and the repetition of same sequences from different species within a group was deleted. Mfold [40] program version 3.2 (http://mfold.bioinfo.rpi.edu/) was used to compute the folding free energies at 37 °C temperature and 1 M sodium ion concentration for all the sequences towards formation of hairpin structures. The free energies and estimated melting temperatures for all mature miRNA sequences to form homo-duplex structures were computed by UNAFold [41] program (Version 3.5) at 37 °C temperature, 1 M sodium ion concentration, 5 μM RNA strand concentration. The folding free energies for hairpins and the melting temperatures for homo-duplexes were used to arrange the sequences in each category to find out most probable and least probable sequences towards their ability to form self-complimentary structures. Histograms were created with the analyzed data for each group in order to get an idea about the distribution of different miRNA sequences towards the formation of self-complementary hairpin and homo-duplex structure.

Results

NMR characterization of homo-duplex

Formation of the stable self-aggregated homo-duplex structure of hsa-miR-520h mature strand (Fig. 1) in higher oligo concentrations was experimentally characterized by two main observations (1) the appearance of two hydrogen bonded imino proton peaks in proton NMR spectra (H_2O:D_2O solvent) at chemical shifts (δ) 10.9 and 11.9 p.p.m which are the characteristics of the central GU wobble base pairs in the duplex form and (2) by a symmetric 2D NOESY imino-imino (hydrogen bonded) correlation (Fig. 2) which is a typical observation of this kind of symmetric duplex.

Concentration dependency of self-aggregation

The concentration dependency (Fig. S1) of the hydrogen bonded imino proton signals of the homo-duplex further gives evidence in favor of self-aggregation as inter-strand process. At 20 °C it was found that the self-aggregation occurred on or above 0.3 mM oligo concentration, as indicated by the appearance and the gradual increase in the imino peak intensity at chemical shifts (δ) 13.9, 13.4, 11.9 and 10.9. When the concentration was 0.88 mM or more, signals from additional hydrogen bonded imino protons appeared at δ 14.2, 13.6, 13.2, 12.4, 11.5, 10.4 and 10.1 p.p.m, which is most probably caused by the formation of higher ordered aggregates of the homo-duplex form by sticking of large single stranded ends. At concentration of 0.1 mM or less, there is no significant structure formation at 20 °C as no imino peaks were observed.

Thermal stability of the homo-duplex

The temperature dependency (Fig. S2) of the hydrogen bonded imino proton signals at 1.7 mM oligo concentration indicated that

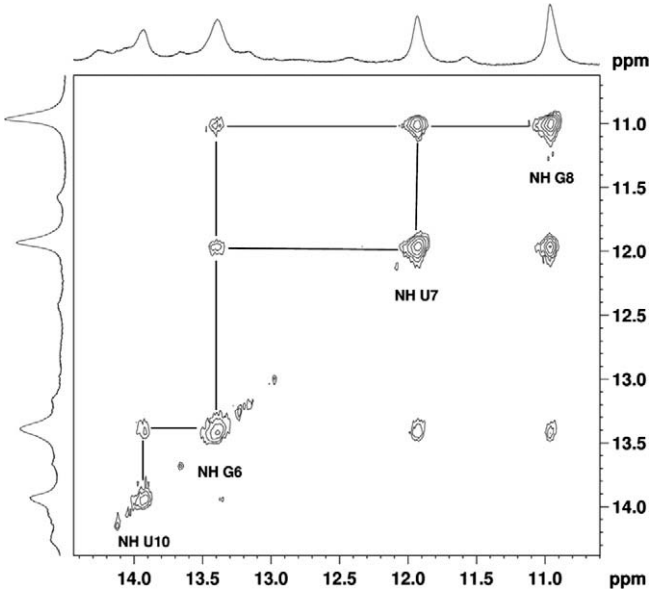

Fig. 2. Imino (Hydrogen bonded) region of the ^1H–^1H 2D NOESY spectra of 1.7 mM miRNA sample in H_2O:D_2O (9:1) solvent at 10 °C.

the breaking of higher ordered aggregates occurs first followed by melting of the duplex structure. Initial increase of temperature up to 25 °C increases the intensity and sharpness of the imino signals because of slower relaxation associated with the formation of lower effective molecular weight duplex species. Then further heating above 30 °C gradually melts the duplex form as indicated by broad imino signals and the subsequent disappearance of the imino peaks.

NMR identification of hairpin form and its thermal stability

The 0.1 mM oligo sample at 20 °C did not show any imino peaks. However, when the sample was cooled to 5 °C and measured with higher number of scan, a distinct hydrogen bonded imino proton profile appeared (Fig. S3) for the hairpin structure which has no GU base paired imino proton signals and which were different from the imino proton signals of the homo-duplex form (1.7 mM, 5 °C). At relatively low (0.1 mM) oligo concentration the temperature dependency (Fig. S4) of imino proton signals is rather simple and only the melting of hairpin form around 15–20 °C is observed.

NMR biophysical experiments thus confirm the structures (Fig. 1) of the mature hsa-mir-520h strand, which were predicted by Mfold and UNAFold. However, the signals of aromatic and sugar protons (proton spectra in D_2O solvent) of miRNA were temperature dependent (Fig. S5). It was observed that, as we increased the sample temperature, the lines became more resolved, sharp and around 50 °C only the single stranded form is predominantly present. At low temperatures and high oligo concentrations, due to the formation of higher ordered aggregates which are having relatively lower relaxation time, due to the existence of single stranded and double stranded regions in the structures, the NMR spectra have severely overlapped and broad signals that are weak in intensity. The same sequence of mature strand is on interplay between hairpin and homo-duplex forms. The hairpin form exists at low oligo concentration, where NMR sensitivity is significantly low and no further detailed assignment and structural investigation was possible for either the hairpin or the homo-duplex structure.

Folding and alignment analysis showed many miRNA mature strands are self-complementary

Mfold and UNAFold calculations for self-complementarity of all the mature miRNA sequences and subsequent statistical analysis result in the following main observations. (a) The percentages of mature miRNAs to fold into hairpin structures, which are having a negative folding free energies, are in the case of Virus 78.34%, Mouse 74.02%, Human 73.36%, Animal 70.49%, Plant 68.69%. (b) Similarly, the percentages of mature miRNAs to form self-aggregated homo-duplex structure, which are having melting temperature above 37 °C, are for Virus 66.24%, Mouse 59.18%, Human 57.29%, Animal 55.25%, Plant 52.32%. (c) In each category, typical distribution plots (Figs. 3 and S6–S12) were obtained when the number of mature miRNAs were plotted with folding free energies and melting temperature of the homo-duplexes. (d) The most probable change in free energy (*DG*) value and melting temperature (T_m) of the folding and alignment distributions of all the categories of mature miRNAs are respectively within −0.8 to −1.6 kcal/mol and around 40 °C temperature. (e) It is also observed that 50% of mature miRNAs are moderately low to very low self-complementary.

The structures generated by the programs showed that many sequences are highly self-complementary and potentially adopt hairpin and/or homo-duplex structures in solution at near physiological conditions. In many of the cases, the mature miRNA sequences which fold in hairpin forms at low oligo concentrations, form homo-duplexes at higher oligo concentrations. All the homo-duplexes are symmetric and can have some GU wobble base pairs. The homo-duplexes, in many cases, have a central internal loop or two internal loops at the two opposite ends of the duplex, and in some cases, are almost full or partly complementary without having internal loops. In some of the hairpin structures, bulged nucleobases or mismatched bases appear in the stem, while in majority of the hairpins, stems are highly complementary without bulges or mismatches. The best self-complementary homo-duplex and hairpin of the miRNA categories are depicted in Figs. 4 and S13, respectively. Some additional representative self-complementary mature human miRNA homo-duplexes and their corresponding hairpin forms are presented in the supporting information (Fig. S14).

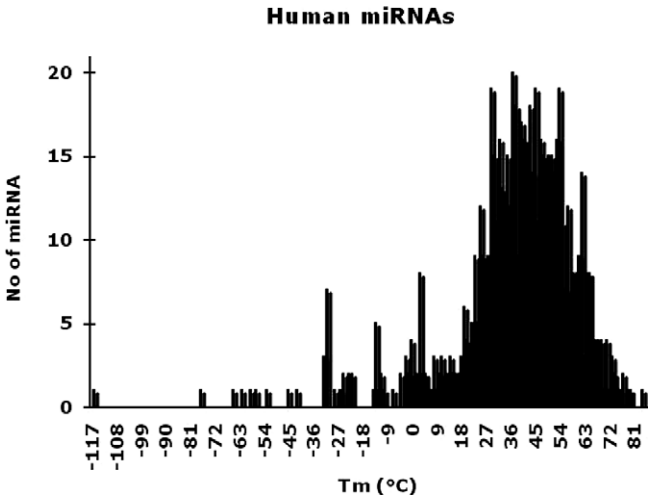

Fig. 3. Self-alignment distribution (Histogram) of mature strands of all human miRNAs.

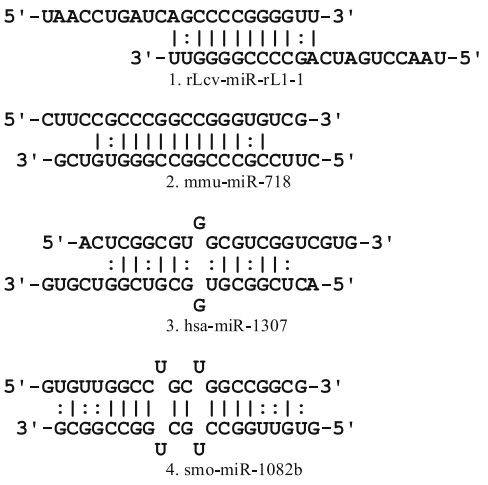

Fig. 4. The best self-complementary homo-duplex of all (1) Virus (T_m = 82.7 °C) (2) Animal (T_m = 100.5 °C) (3) Human (T_m = 83.1 °C) 4) Plant (T_m = 87.0 °C) miRNA mature strand homo-duplexes.

Discussion

The mature miRNAs are generally being considered as single stranded, and to the best of our knowledge, there is no report demonstrating self-complementarity in mature miRNAs. With NMR biophysical studies we have characterized the self-complementary

secondary structures of the hsa-miR-520h mature strand, and with Folding-alignment analysis we have shown that the self-complementary sequence in more than 50% of mature miRNAs drives the formation of hairpin and/or homo-duplex structures in solution.

Most of miRNA–mRNA interactions downregulate gene expression post-transcriptionally, even though there are some recent exceptional reports where miRNAs showed upregulation [42,43] of gene expression. A substantial variation in the degree of gene regulation (from less than 2-fold to greater than 10-fold) has been observed, depending on the specific miRNA–mRNA target combination. The determinants that define the different magnitudes of regulation remain to be defined. Whether the degree of translation of such mRNAs is determined by the accessibility of mRNA target sites to miRNAs or by other factors like involvement of various proteins that specifically bind with UTR regions and thus alter the mRNA structure to modulate the interaction between miRNA and mRNA, is unknown. Recently a study [44] showed the role of messenger RNA structure in microRNA target recognition and suggested that binding of the RNA-induced silencing complex is largely controlled by the thermodynamics of RNA–RNA interactions [45,46].

Our finding of mature miRNA secondary structures and the differential extent of self-complementarity can have a conformational role to modulate miRNA–mRNA interactions and thus can explain the different degree of genetic regulation for the specific miRNA involved in the regulation process. This can contribute to the mechanism by which nature selected sequences for some miRNAs to modulate its binding affinity with their mRNA targets. The self-aggregation in certain numbers of miRNAs can be a way by which miRNA:miRNA* duplexes unwind easily and thus facilitate the loading of the mature miRNA strand in miRNA RISC. It can also provide specificity for the interaction of miRNAs with particular mRNA by preventing the possibility of binding of other miRNAs and complementary genomic RNAs with miRNAs. The high tendency for self-aggregation may also be a defense system for foreign miRNAs, and may suggest that there are no free miRNAs in a cell.

Generally, it is observed that in some mature miRNA self-complementary hairpin and/or homo-duplex structures, the mRNA complimentary 5′-seed (7 nt) sequences are freely available for miRNA–mRNA interaction, while in others complimentary 5′-seeds are inaccessible as they are involved in the hydrogen bonding of hairpin and/or homo-duplex stem. It deserves more detailed further investigation to find out how the self-complementarity of mature miRNA affects the mutual interaction of miRNA with mRNA targets and other RNAs, which can target mature miRNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.072.

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