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(54) Title: MODULATION OF EXON RECOGNITION IN PRE-MRNA BY INTERFERING WITH THE SECONDARY RNA STRUCTURE

(57) Abstract: The invention provides a method for generating an oligonucleotide with which an exon may be skipped in a pre-mRNA and thus excluded from a produced mRNA thereof. Further provided are methods for altering the secondary structure of an mRNA to interfere with splicing processes and uses of the oligonucleotides and methods in the treatment of disease. Further provided are pharmaceutical compositions and methods and means for inducing skipping of several exons in a pre-mRNA.

Title: Modulation of exon recognition in pre-mRNA by interfering with the secondary RNA structure.

The invention relates to the fields of molecular biology and medicine. More in particular the invention relates to the restructuring of mRNA produced from pre-mRNA, and therapeutic uses thereof.

The central dogma of biology is that genetic information resides in the DNA of a cell and is expressed upon transcription of this information, where after production of the encoded protein follows by the translation machinery of the cell. This view of the flow of genetic information has prompted the pre-dominantly DNA based approach for interfering with the protein content of a cell. This view is slowly changing and alternatives for interfering at the DNA level are being pursued.

In higher eukaryotes the genetic information for proteins in the DNA of the cell is encoded in exons which are separated from each other by intronic sequences. These introns are in some cases very long. The transcription machinery generates a pre-mRNA which contains both exons and introns, while the splicing machinery, often already during the production of the pre-mRNA, generates the actual coding region for the protein by splicing together the exons present in the pre-mRNA.

Although much is known about the actual processes involved in the generation of an mRNA from a pre-mRNA, much also remains hidden. In the present invention it has been shown possible to influence the splicing process such that a different mRNA is produced. The process allows for the predictable and reproducible restructuring of mRNA produced by a splicing machinery. An oligonucleotide capable of hybridising to pre-mRNA at a location of an exon that is normally included in the mature mRNA can direct the exclusion of the thus targeted exon or a part thereof.

In the present invention means and methods are provided for the design of appropriate complementary oligonucleotides. To this end the

invention provides a method for generating an oligonucleotide comprising determining, from a (predicted) secondary structure of RNA from an exon, a region that assumes a structure that is hybridised to another part of said RNA (closed structure) and a region that is not hybridised in said structure (open structure), and subsequently generating an oligonucleotide, which at least in part is complementary to said closed structure and which at least in part is complementary to said open structure. RNA molecules exhibit strong secondary structures, mostly due to base pairing of complementary or partly complementary stretches within the same RNA. It has long since been thought that structures in the RNA play a role in the function of the RNA. Without being bound by theory, it is believed that the secondary structure of the RNA of an exon plays a role in structuring the splicing process. Through its structure, an exon is recognized as a part that needs to be included in the pre-mRNA. Herein this signalling function is referred to as an exon inclusion signal. A complementary oligonucleotide of the invention is capable of interfering with the structure of the exon and thereby capable of interfering with the exon inclusion signal of the exon. It has been found that many complementary oligonucleotides indeed comprise this capacity, some more efficient than others. Oligonucleotides of the invention, i.e. those with the said overlap directed toward open and closed structures in the native exon RNA, are a selection from all possible oligonucleotides. The selection encompasses oligonucleotides that can efficiently interfere with an exon inclusion signal. Without being bound by theory it is thought that the overlap with an open structure improves the invasion efficiency of the oligonucleotide (i.e. increases the efficiency with which the oligonucleotide can enter the structure), whereas the overlap with the closed structure subsequently increases the efficiency of interfering with the secondary structure of the RNA of the exon, and thereby interfere with the exon inclusion signal. It is found that the length of the partial complementarity to both the closed and the open structure is not extremely restricted. We have observed high efficiencies with oligonucleotides

with variable lengths of complementarity in either structure. The term complementarity is used herein to refer to a stretch of nucleic acids that can hybridise to another stretch of nucleic acids under physiological conditions. It is thus not absolutely required that all the bases in the region of complementarity are capable of pairing with bases in the opposing strand. For instance, when designing the oligonucleotide one may want to incorporate for instance a residue that does not base pair with the base on the complementary strand. Mismatches may to some extent be allowed, if under the circumstances in the cell, the stretch of nucleotides is capable of hybridising to the complementary part. In a preferred embodiment a complementary part (either to said open or to said closed structure) comprises at least 3, and more preferably at least 4 consecutive nucleotides. The complementary regions are preferably designed such that, when combined, they are specific for the exon in the pre-mRNA. Such specificity may be created with various lengths of complementary regions as this depends on the actual sequences in other (pre-)mRNA in the system. The risk that also one or more other pre-mRNA will be able to hybridise to the oligonucleotide decreases with increasing size of the oligonucleotide. It is clear that oligonucleotides comprising mismatches in the region of complementarity but that retain the capacity to hybridise to the targeted region(s) in the pre-mRNA, can be used in the present invention. However, preferably at least the complementary parts do not comprise such mismatches as these typically have a higher efficiency and a higher specificity, than oligonucleotides having such mismatches in one or more complementary regions. It is thought that higher hybridisation strengths, (i.e. increasing number of interactions with the opposing strand) are favourable in increasing the efficiency of the process of interfering with the splicing machinery of the system.

The secondary structure is best analysed in the context of the pre-mRNA wherein the exon resides. Such structure may be analysed in the actual RNA.

However, it is currently possible to predict the secondary structure of an RNA molecule (at lowest energy costs) quite well using structure-modelling programs. A non-limiting example of a suitable program is RNA mfold version 3.1 server (Mathews et al 1999, J. Mol. Biol. 288: 911-940). A person skilled in the art will be able to predict, with suitable reproducibility, a likely structure of the exon, given the nucleotide sequence. Best predictions are obtained when providing such modelling programs with both the exon and flanking intron sequences. It is typically not necessary to model the structure of the entire pre-mRNA.

The open and closed structure to which the oligonucleotide is directed, are preferably adjacent to one another. It is thought that in this way the annealing of the oligonucleotide to the open structure induces opening of the closed structure, annealing progresses into this closed structure. Through this action the previously closed structure assumes a different conformation. The different conformation may result in the disruption of the exon inclusion signal. However, when potential (cryptic) splice acceptor and/or donor sequences are present within the targeted exon, occasionally a new exon inclusion signal is generated defining a different (neo) exon, i.e. with a different 5' end, a different 3' end, or both. This type of activity is within the scope of the present invention as the targeted exon is excluded from the mRNA. The presence of a new exon, containing part of the targeted exon, in the mRNA does not alter the fact that the targeted exon, as such, is excluded. The inclusion of a neo-exon can be seen as a side effect which occurs only occasionally. There are two possibilities when exon skipping is used to restore (part of) an open reading frame that was disrupted as a result of a mutation. One is that the neo-exon is functional in the restoration of the reading frame, whereas in the other case the reading frame is not restored. When selecting oligonucleotides for restoring reading frames by means of exon-skipping it is of course clear that under these

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