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THEORETICAL AND EXPERIMENTAL APPROACHES TO DESIGN EFFECTIVE ANTISENSE OLIGONUCLEOTIDES

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1. ABSTRACT

Among the large number of possible antisense oligonucleotides (asODN) against a given target nucleic acid, only a small number of species seems to give rise to satisfactorily strong inhibition of target gene expression in living cells. Therefore much attention is paid to strategies that help to successfully design effective asODN. Here, selected experimental approaches and theoretical concepts will be briefly described that have been developed to increase the probability of success in the use of asODN. Advantages and disadvantages of these strategies will be compared and the relatively new and controversially discussed concept of a theoretical and computer-supported design of effective asODN will be addressed.

2. INTRODUCTION

Antisense oligodeoxyribonucleotides (asODN) are increasingly applied in basic sciences, developmental biology, and molecular medicine as tools to study and to suppress aberrant gene expression and viral functions. The successful use of asODN to suppress gene expression is somewhat limited since only a small portion of all possible antisense species against a given target sequence shows efficacy in the respective test system (1,2). The large number of applications of asODN and detailed analyzed of their mode of action in living cells are not reflected by knowledge about the rules that govern the relationship between their specific sequence and their target structure on the one hand and annealing *in vitro* and efficacy *in vivo* on the other hand. While it is generally assumed that local RNA structures of the target strand determine binding of an asODN and, thereby, its effectiveness not much is known about this structure-function relationship in detail. For example, as little as one or two additional or lacking terminal nucleotides can convert an effective antisense nucleic acid into a completely inactive one (e.g. references: 3,4). These studies can be interpreted such that terminal sequence portions of an antisense strand are more important for annealing than central ones though no specific explanation has been proposed so far.

In principle, insights into the relationship between target structure and efficacy of asODN can be derived from those systems for which knowledge on local target structures and inhibition data with large sets of asODN are available. This idea has been recognized in the field and one attempted to relate the local accessibility for antisense molecules for those target RNA for which solid structural models exist (5-7). In case of unknown target RNA structures, information on their local accessibility for antisense inhibitors has been provided by using libraries of random sequences or antisense oligodeoxyribonucleotide (asODN) species (e.g.: 5,7-10). Alternatively, the accessibility of target RNA can be studied by measuring

annealing kinetics with sets of successively elongated antisense ribonucleotide (asRNA) sequences: if one assumes that one given terminus, either the 5´- or 3´ end of an antisense nucleic acid does not contribute significantly to annealing, then sets of homologous antisense species which are successively elongated at the opposite end may be used to probe in a sort of kinetic probing the local target structures (3,11,12). A major uncertainty of this approach is related to the fact that due to the length of probing species they may adopt stable intramolecular structures which influence annealing by themselves in a significant manner. Conversely, for most kinds of oligomeric DNA species used to probe target RNA, one does not assume very stable secondary structures and, thus, probing studies in their use may be thought to more directly monitor the accessibility of target RNA.

This work aims to introduce into the currently available experimental approaches to identify accessible local target structures and to derive rules for the design of asODN that are statistically more effective than asODN chosen by a random approach. Further, conceivable and new computer-based concepts will be described that might be helpful to manipulate large data sets in the long-term and, thereby, might help to generate and to analyze large amounts of data on the structure-function relationship of asODN.

3. MATERIALS AND METHODS

3.1.Computer algorithms

The program that calculates the local folding potential for a given nucleotide sequence is termed 'foldsplit' and has been described in detail (13,14). It is based on the GCG package (15) and is available within the computer service 'HUSAR' (Heidelberg Unix Sequence Analysis Resources, ref.: 16). External users of 'HUSAR' as well as those who wish to get access to 'HUSAR' and 'foldsplit' respectively, should contact the following E-mail address: *genome@dkfz-heidelberg.de* and ask for the service 'GENIUSnet'. Those who wish to receive 'foldsplit' on request need to own a license agreement for the GCG package. To run the program 'foldsplit', one first defines two variable parameters, the step width and the window size. The window defines the length of the sequence stretch for which the structure with the lowest possible free energy (delta-G) is calculated. Note, that it is the delta-G value that is used and not a particular RNA structure. The distance along which this window is shifted before the next delta-G value is calculated is defined by the step width. In case of a step width that is smaller than the window, the given sequence is scanned by overlapping windows. The folding potential for a given sequence is represented by the plot of the delta-G values versus the sequence position.

3.2. Generation of a limited antisense sequence space

The transcription of target RNA *in vitro* is performed enzymatically from template DNA which usually is linearized plasmid DNA. Prior to *in vitro* transcription the template plasmid is linearized by a restriction enzyme that preferentially produces 5´-protruding ends or blunt ends. T7 RNA polymerase (Boehringer Mannheim) is used for *in vitro* transcription from plasmid DNA or PCR products which can also be used as templates. Five micro-g of linearized template DNA are transcribed *in vitro* as described elsewhere (3). After adding of 20 micro-l 100 mM $MgSO_4$ the mixtures are treated with 20 U DNaseI (Boehringer Mannheim) and the transcripts are purified by gel filtration (Sephadex G-50-medium, Pharmacia; TE-buffer [10 mM Tris/HCl pH 8, 1 mM EDTA]). For *in vitro* transcription of radiolabeled RNA 0.5 micro-g of template DNA are used under the same conditions as described above. Unlabeled nucleoside triphosphates ATP, CTP, GTP at final concentrations of 1.25 mM, unlabeled UTP at a final concentration of 0.04 micro-M and 20 micro-Ci of [alpha-^{32}P]UTP (3000 Ci/mmol, Amersham Life Science, Braunschweig) are included in 20 micro-l reactions. RNA is purified by gel filtration as described (17).The 5'-ends of *in vitro* transcribed RNA (10 pmole) are ^{32}P-labeled by dephosphorylation with calf intestinal phosphatase and subsequent rephosphorylation with 50 micro-Ci of [gamma-^{32}P]ATP (3000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) as described (18). 3'-labelling of RNA is performed as described by Barrio *et al.* (1978). Briefly, 5 pmole of RNA are incubated for 14 hours at 15°C with 330 micro-M ATP, 50 mM HEPES-buffer pH 8.3, 20 mM $MgCl_2$, 10 units T4 RNA ligase (Boehringer Mannheim) and 50 micro-Ci of [^{32}P]pCp (3000 Ci/mmol; Amersham Life Science, Braunschweig) in a final volume of 20 micro-l. RNA is purified by gel filtration.

A mixture of antisense RNA species that carry a ^{32}P label at one end and which are successively shortened at the opposite end is generated by partial alkaline hydrolysis from full length RNA as described (3). Briefly, 2.5 pmole of end-labelled RNA in TE-buffer are heated with 1.5 volumes of 0.5 M $NaHCO_3$ to 96°C for 12 to 14 minutes, then chilled on ice and desalted by gel filtration. After ethanol precipitation, the RNA is dissolved in TE-buffer. Subsequently, the mixtures of RNA species are heated to 75°C for 10 minutes and cooled slowly to 37°C before using in hybridization assays.

3.3. Selection of fast annealing antisense species

The selection method to identify fast-hybridizing antisense RNA species was described recently (3,19). Briefly, 5'- or 3'-labelled hydrolyzed mixtures of parental antisense RNA are incubated with target RNA (1 to 10 pmole) in a final volume of 20 micro-l of a solution containing 100 mM NaCl, 20 mM Tris/HCl pH 7.4 and 10 mM $MgCl_2$ at 37 °C. At different time points of incubation, 3 micro-l aliquots are transferred into 30 micro-l stop buffer (50 mM Tris/HCl pH 8; 15 mM EDTA, 8 M urea, 0.04% bromophenolblue, 0.04% xylenecyanol) precooled on ice. Single-stranded antisense RNA and target RNA/antisense RNA duplexes are separated by native agarose gel electrophoresis (0.8-1.2%, 89 mM Tris/HCl pH 8,3, 89 mM boric acid, 2 mM EDTA). Single-stranded and duplex RNA is excised from the gel and recovered by centrifugation of the excised gel slices which have been frozen in liquid nitrogen. After precipitation with ethanol, RNA is re-dissolved with stop buffer and analyzed by polyacrylamide gel electrophoresis under denaturing

conditions (10-15% polyacrylamide gels containing 7 M urea in 89 mM Tris-borate buffer pH 8.3). Gels are dried and exposed to X-ray film. Annealing rates for individual antisense RNA species are derived from the composition of single-stranded and double-stranded fractions: for quantitative analysis of band intensities, dried polyacrylamide gels can be scanned by a PhosphorImager (Molecular Dynamics), band intensities of single-stranded and double-stranded fractions can be measured, and be plotted against the time axis. A curve for an exponential decay can be fitted by non linear regression.

4. RESULTS AND DISCUSSION

4.1. Target RNA structures are important for efficacy of antisense oligonucleotides

The large observed differences of annealing rates between long-chain target RNA and complementary nucleic acids, even in cases with wide sequence overlaps among related antisense species (3,4,11,12), is usually explained by the complex relationship between RNA structure, the annealing of complementary nucleic acids *in vitro*, and their effectiveness *in vivo*. The structure/function relationship of asODN is not trivial since very few base exchanges may convert an efficiently annealing antisense species into an inefficient one and *vice versa* in cases in which neither the base composition nor the thermodynamic DNA/RNA duplex stability are significantly affected (e.g. 4,7,10,20).

One way to shed light on the structure/function relationship of asODN is to analyzed their sequences and to search for motives that are common among efficient asODN species and their local target sequences, respectively. Based on this approach, Tu *et al*. suggested that a GGGA motif in the target sequences of the tumor necrosis factor-α (TNF-α) and TCCC in the asODN, respectively, occurs at a statistically increased frequency in case of effective antisense ODN and concluded that antisense species containing the TCCC motif have an over 50% probability of being effective (21). Ratmeyer *et al*. suggested that stretches of ribopurines within the target can stabilize DNA-RNA heteroduplexes (22) and concluded that these represent favorable local targets for asODN. Meanwhile, there is a number of unpublished hypotheses on that matter. Some of which speculate that short stretches of three to four nucleotides with a specific base composition of the asODN are related to a statistically significant increase of efficacy. This kind of thinking does not necessarily regard directly structural features of the target RNA. A correlation between sequence motives of effective asODN, local primary target sequences, and efficacy may only exist in as far as short stretches of specific nucleotides may monitor on a statistical basis the presence of specific local secondary or tertiary structures which are accessible to asODN. Studies on *ICAM-1*-directed asODN in our own laboratory, however, do not support either of the above observations (20) though it is important to note that the base composition of asODN or specific sequence motives may influence characteristics other than annealing. If, for example, the biological half life, the cellular uptake, the interactions with cellular components, or the intracellular localization of asODN is influenced by specific sequence motives this might result in increased apparent efficacy in living cells though this may not have anything to do with increased annealing.

An alternative view on the structure/function relationship of asODN is based on the finding that the 3' and 5' ends, regardless of their base composition, are important for annealing and efficacy of asODN. Systematic analyzeds of annealing by using arrays of oligonucleotides (4) or mixtures of related antisense RNA species in solution (3,11) show that terminal nucleotides crucially influence annealing. Thus, one might hypothesize that terminal rather than internal nucleotides are important for annealing which seems to be valid for asODN whereas in case of asRNA terminal nucleotides might also influence annealing via effects on its folding. This hypothesis has several consequences: firstly, single terminal nucleotides may play a central role in the process of duplex formation between the target RNA and the asODN. Unfortunately, almost nothing is known about the underlying mechanisms, so one has to define this issue as a major field of future research on the design of asODN. Secondly, when considering the above search for sequence motives that are common among effective asODN, focus may be given to terminal portions of asODN rather than central ones. Thirdly, it seems to be reasonable to „scan" along the target RNA by sets of related antisense RNA species that differ at either their 3´ end or their 5´ end successively by one complementary nucleotide in order to identify sites at which duplex formation can be efficiently initiated as described recently (19; see also: ´Materials and methods´ section). In principle, such studies on RNA-RNA annealing can provide direct information for the design of asODN which has been successfully undertaken in case of *bcr-abl* sequences (11). In this case, the sequence of an effective antisense 23mer was derived from RNA-RNA annealing kinetics with sets of successively elongated antisense RNA *in vitro*. According to a similar approach in which sequences of asODN were derived from probing studies with complementary RNA, one of the most effective HPV 16 E6/E7-directed asODN was designed (12).

Finally, one should mention that the accessibility of a target RNA sequences can be monitored by completely different approaches, e.g. by measuring the reactivity for chemicals or enzymes like in conventional structural probing. The difficulty to interpret such results, however, is the same as before: as long as rules governing the structure/function relationship of asODN are not known it is difficult to derive reliable general rules for the design of effective asODN.

4.2. Theoretical versus experimental approaches: advantages and disadvantages

The need for guidelines to design effective asODN is reflected by the large number of original and different approaches to find effective asODN species out of the space of all possible species (figure 1**)**. Some of these are experimental approaches and others are theoretical or solely computer-based in nature. At the first glance, one might intuitively favor experimental

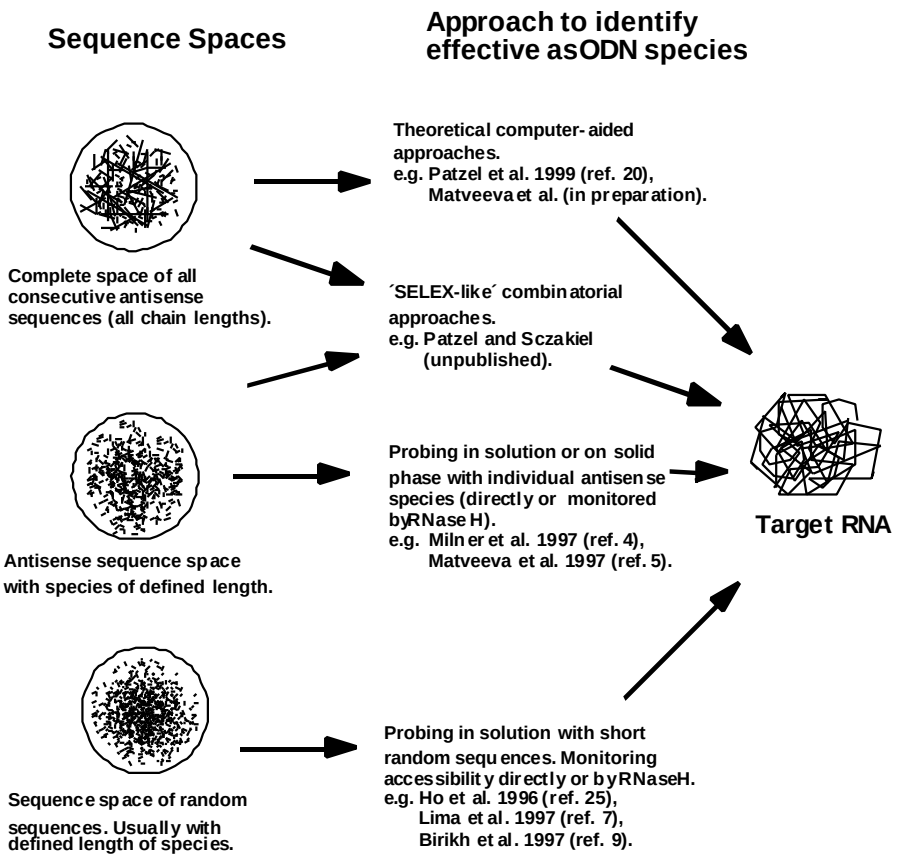

Figure 1. Examples for theoretical and experimental approaches to identify accessible local target sites for asODN and, thereby, to select and to design effective asODN species. Note that the pools of species of complementary nucleic acids used for probing may differ substantially.

approaches and neglect theoretical ones. However, a closer look at the advantages and disadvantages shows that both strategies are worth being explored (table 1). The major disadvantage of theoretical approaches is related to the extent or lack of reliability of secondary and tertiary structure predictions for RNA and the lack of mechanistic insights into structural requirements for efficient RNA-DNA annealing. Conversely, there are also basic advantages which favor computational approaches over experimental ones. These include the possibility of generating and investigating the complete relevant antisense sequence space, the possibility to analyzed a single target structure rather than a possible mixture of isoforms, and the possibility to exclude interference between different antisense species with respect to annealing. In particular the latter point is important since a facilitator function or negative interference of asODN may lead to false positive antisense species or, even worse, to overlooking positive ones in experimental approaches.

It might well be that at the current state of the design of effective asODN, a combination of experimental and theoretical strategies could be of greatest benefit to progress in the field. For this reason, specific approaches from each of both strategies will be described in the following.

4.3.Current experimental protocols to design asODN

The first and one of the most critical steps of the action of antisense inhibitors is their efficient invasion into the structure formed by target RNA followed by double-strand formation. This step is usually related to what is called ´local target accessibility´ of the target RNA of interest. To directly measure sites of the target RNA that are accessible to complementary nucleic acids, one has developed a number of protocols that make use of pools often containing oligomeric species and sometimes pools containing longer species complementary to the target sequence. It is reasonable to assume that probing strategies to identify accessible local target sites bypass the uncertainty involved in the otherwise necessary analysis or prediction of RNA structures. Thus, probing of target RNA by pools of fully or partially complementary species provides one in principle with direct functional data of interest, i.e. the identification of sites suitable for the binding of asODN. Further, many probing protocols

Table 1. Advantages and disadvantages of theoretical and experimental approaches to design effective asODN.

ADVANTAGES	DISADVANTAGES
Experimental approaches	
• (Real) annealing in solution	• Incomplete sequence space
• (Real) RNA structures	• Interference of as ODN species
• Positive image	• Expensive, time consuming
Theoretical approaches	
• High throughput	• Largely unknown structure-function relationship
• Little expense	• Image: unreliable results
• Complete relevant Sequence space	

include a step in which the complex between local target RNA and asODN is cleaved by RNase H, thereby adding a step subsequent of binding which is thought to be important in living cells as well. The specific composition of the pools used, however, is one of the major differences among these protocols. Some make use of pools of oligomeric antisense sequences of a defined length, others use pools of completely or partially random sequences which, in principle, also include all possible antisense species of a defined length, and some make use of pools of antisense RNA species of varying length between 15 to 25 nucleotides and up to several hundred nucleotides. It should be mentioned that protocols have been established solely for the design of efficient hammerhead ribozymes (e.g. 23). They are elegant but not described here because they are restricted to ribozyme species and seem to be of limited value for the design of asODN. In the following, examples for the most common approaches will be summarized and their advantages and disadvantages will be discussed in the light of antisense design rather than aspects related to the specific biological test system, technical details, or chemical modifications.

4.3.1. Protocols in the use of pools of oligodeoxyribonucleotides

Early studies on the systematic probing of target sequences made use of a solid phase onto which nucleic acids were stably fixed. Two ways were shown to be feasible. First, immobilizing the target sequence and probing with libraries of oligonucleotides and, secondly, immobilizing arrays of oligonucleotides and probing with target sequences. One of the first tools to perform and to monitor systematically the annealing of antisense oligonucleotides to a given target RNA was described by Southern et al.(24) who synthesized sets of octapurine sequences covalently attached to glass plates and hybridized them with oligopyrimidines. Several years later the same group showed a combinatorial approach to select asODN *in vitro* by using an array of 1938 glass-bound oligonucleotides. Most of these were effective inhibitors of the translation of rabbit β-globin mRNA in an *in vitro* translation system (4). This method makes use of parts of the possible antisense sequence space and might be affected by the fact that hybridization is measured close to the equilibrium of binding of antisense species.

A combinatorial approach described by Lima et al. (7) used liquid phase annealing to probe folded hepatitis C virus (HCV) RNA with pools of fully randomized oligonucleotide 10mers and subsequently monitored binding by using RNase H-mediated cleavage of the annealing products. This method was used to measure annealing kinetics as well as equilibrium characteristics of oligonucleotide binding to the target RNA which provides a large set of parameters potentially related to the understanding of efficacy of asODN in living cells. This work showed a way to identify asODN species with a good correlation to efficacy against production of HCV core protein in an *in vitro* translation assay and in tissue culture. In a similar approach, Ho et al. (25) made use of a sequence space comprising oligomer 11mers with 10 nucleotides randomized to probe the human multidrug resistance-1 (MDR-1) mRNA for accessibility. Binding of oligonucleotides was monitored either directly by RNase H activity and gel analysis or after primer extension analysis of cleavage products. Efficacy in tissue culture cells was related to the identification of effective species in the *in vitro* probing assay in a statistically significant way. In an attempt to identify favorable binding sites for hammerhead ribozymes directed against the human acetylcholinesterase mRNA Birikh *et al.* (9) used a pool of completely randomised decadeoxyribonucleotides and RNase H. From this probing experiment Birikh *et al.* derived target sites for ribozymes species that turned out to be very active *in vitro* and that would not have been predicted according to computational secondary structure prediction. One should add, however, that in case of hammerhead ribozymes stable intramolecular structures can be formed which are thought to affect annealing and efficacy.

Individual antisenseODN species have been used by Scherr & Rossi (26) to probe the accessibility of the structure adopted by the native mRNA of murine DNA methyl-transferase. Probing in this case was performed in the presence of cellular extracts including endogenous RNase H which might be closer to the situation in living cells than probing studies performed in buffer alone. A target sequence-specific space of oligomeric DNA species was used by Matveeva *et al.* (5) to search for accessible sites along a set of RNA targets with known structures or established structural models including the yeast $tRNA^{Asp}$, the P4-P6 domain of the group I intron, and rat 18S rRNA. This work described an alternative though not completely new way to identify promising local target sites for asODN. More importantly, however, it provides interesting information of the complex relationship between local RNA structure and annealing. All three protocols represent ways to experimentally identify accessible local target sequences and to design effective asODN species. They share the advantage of including an RNAse H activity into the assay, thereby considering an enzymatic activity that is thought to play a crucial role in asODN mechanism in living cells including mammalian ones. The protocols by Birikh *et al.* (9), Lima *et al*. (7), Matveeva *et al*. (5), and, probably also the one by Ho *et al*. (25) used *E.coli*

RNase H which seems to display properties different from the human enzyme(s) (27) and most likely also those of other higher organisms. Thus, probing results obtained in the use of *E. coli* RNAse H could be somewhat misleading when concluding to the efficacy of asODN in cells from different species origin inclduing human cells.

4.3.2. Experimental kinetic probing of target structures with pools of RNA species

Measurements of the annealing kinetics of sets of successively 3´-shortened long-chain antisense RNA and long-chain target RNA derived from the human immunodeficiency virus type 1 (HIV-1) showed that association rate constants were increased or decreased by up to two orders of magnitude by extending or shortening of an antisense species by as few as two to three complementary nucleotides (3). This method can also be used with complementary RNA species that share a common 3´-end and are successively elongated at their 5´-end (6). It stresses the importance of the 3´- or 5´-terminal nucleotides of antisense strands and indicates that this kind of kinetic probing provides one, in principle, with information on the local accessibility of the target strand for complementary nucleic acids including asODN.

In order to exploit the potential information of kinetic probing for the design of asODN, one may choose the following strategy: (i) perform kinetic probing with groups of homologous antisense RNA species with one constant end and a successively shortened opposite end such that the complete stretch of target sequences of interest is covered, (ii) identify sites showing increased kinetic accessibility, and (iii) analyzed local targets with sets of overlaping asODN. Recently this method has been used for the design of effective and selective antisense oligonucleotides. In the following, two examples for this kind of developing effective asODN as well as obtaining information on the relationship between annealing kinetics, RNA structure and efficacy in living cells will be described.

4.3.2.1.HPV 16-directed asODN: kinetic probing and the design of an effective antisense 20mer

The expression of E6/E7 open reading frames of HPV 16 is thought to be related to malignant cell proliferation (28). One strategy to suppress the malignant phenotype is the use of antisense nucleic acids against E6/E7 target sequences. According to the above three-step strategy to design effective asODN, we first performed kinetic probing of E6/E7 target sequences by various sets of 3´-elongated complementary RNA and identified only one site within the downstream portion of E6 (pos. 437 – 447) that showed significant annealing (12). Subsequently, a set of 17 antisense 20mers each overlapping by two nucleotides with its neighbouring species was tested for binding to an un-spliced and a spliced version of E6/E7 target RNA *in vitro*. The fastest annealing asODN species directed against this local target annealed *in vitro* at physiological ionic strength and temperature at rate constants in the order of 10^4 $m^{-1}s^{-1}$. When tested for gene suppression and for anti-proliferative effects in tissue culture, only the identified fast-annealing asODN species showed efficacy which was even stronger in the same experiment than the best asODN described in the literaure so far. Conversely, a number of asODN directed against the E6/E7 open reading frames at different sites had no effect. Even though HPV 16 E6/E7 target sequences do not seem to be particularly favorable for an antisense approach, this individual example indicates the feasibility and success of this stepwise approach to design effective asODN.

4.3.2.2. Sequence-selective asODN against *bcr-abl* targets: kinetic probing and efficacy in human cells

Expression of the *bcr-abl* fusion gene is a result of the chromosomal t14/21 translocation in human hematopoietic cells of the myeloid lineage and is causavtively related to the development of chronic myeloid leukemia (CML). In normal cells, however, the controlled expression of the *bcr* and *abl* wild type genes is required for normal cell proliferation. Thus, efficacy as well as selectivity are the two major criteria for the design of *bcr-abl*-directed asODN. In the following, experiments will be described that led to the design of asODN directed against the b3/a2 fusion which is one major fusion points of the gene rearrangement occuring in case of the t14/21 translocation.

First, the annealing between sets of 5´-labelled and successively 3´-shortened antisense RNA species covering the fusion point region b3/a2 was used to determine annealing kinetics with either a *bcr-abl* fusion target RNA, a *bcr* target RNA, or an *abl* target RNA. Antisense RNA species were identified that annealed relatively fast with the *bcr-abl* fusion sequences and slowly or not even measurably with both wild type sequences. From these annealing studies with complementary RNA, asODN species were derived which shared the 3´-end with that of effectively and selectively annealing antisense RNA species. Annealing studies with asODN and three possible traget RNA in solution in the presence of RNase H showed selective cleavage of the *bcr-abl* RNA indicating selective binding of the asODN used (11). When considering the differences between RNA-RNA annealing and DNA-RNA annealing, it is somewhat surprising that the asODN species showed properties similar to what one would naively expect from this kind of RNA probing study.

4.4. Computational approaches to design antisense oligonucleotides

Most theoretical approaches described so far are based on parameters of low resolution such as the ´local folding potential´ (13; see also: ´Materials and methods´ section), on individual secondary structure predictions of the target RNA, or calculations of the thermodynamic stabilities of intramolecular folding and of double strands formed by both complementary nucleic acids (29,30). The easiest theoretical approach to design asODN is to predict a secondary structure for a given target RNA or a segment thereof and to choose sequence elements that are thought to be favorable for annealing with asODN such as for example terminal loops. Often the asODN sequence is then chosen such that it is complementary to the unpaired stretch of target sequences. This method seems to be very naive and mainly suffers from the great unreliability of a single

secondary structure prediction, the lack of knowledge about what really is a favorable local target structure, and the lack of knowledge how to align effectively annealing antisense sequences with target sequence.

Based on the assumption that the efficacy of asODN in living cells is a function of many parameters including uptake, stability, subcellular localization,and also annealing characteristics it seems to be worth to search for sequence motives that are common among effective asODN. This kind of approach can be easiliy computerized and seems to be directly related to the successful use of asODN although it has not yet been worked out in much detail (Matveeva *et al*., manuscript in preparation).

By increasing the reliability of secondary structure prediction and by learning substantially more about the characteristics of target structures that favor the annealing of asODN, one might be able to increase the frequency of success to design asODN in the use of computational approaches. First evidence for the characteristics of favorable local target motives came from kinetic probing of pre-genomic HBV-derived target sequences with sets of successively elongated antisense RNA species (31). In this case, a conserved terminal stem/loop element containing 19 nucleotides predicted to occur in the loop turned out to represent a favorable target for asODN sequences: the asODN ASO1 which is directed against 14 loop nucleotides and downstream positions shows an annealing rate constant of appr. 10^5 $M^{-1}s^{-1}$ which is fastest annealing *in vitro* observed so far for HBV-directed asODN (31; Patzel & Sczakiel, unpublished) though faster rates for asODN-RNA annealing have been observed in other systems including TAR sequences of HIV-1 (32).

To further increase the potential usefulness of this method, large sets of secondary structure predictions were performed for ICAM-1-derived target sequences (20). For overlapping sequence segments of 1400 nucleotides in length sets of lowest energy structures were calculated and structural elements that were assumed to represent favorable local target sites were recorded. These included terminal and internal loops, joint sequences, bulges and terminal unpaired sequences of 10 or more consecutive nucleotides. Antisense ODN were directed against those predicted favorable target motives that occurred in the structure prediction of all sequence segments and for all low energy structures. For most target sequences analyzed so far, this was observed only once out of 1000 nucleotides. This strategy was then tested with target sequences derived from the *ICAM-1* gene which was chosen as a target because it had been shown earlier to be sensitive to asODN-mediated gene suppression. By applying the protocol described here a number of 34 promising asODN were synthesized and tested in tissue culture cells. A number of 10 ICAM-1-directed asODN species showed significant inhibition of target gene expression in the endothelial cell line ECV304. Further, 17 antisense species (50%) showed an extent of inhibition of *ICAM-1* expression which was up to 2.5fold increased when compared with the most effective asODN published so far (20). In this study 50% of all asODN selected by this theoretical approach showed an appr. 50% or greater inhibition of target gene expression which seems to represent an improvement over a selection of asODN on a random basis.

Regarding the reliability of the theoretical design, the stringency of the selection criteria seem to matter. For example, the approach by Patzel et al. (20) suggests one favorable local target site at a frequency of approximately one in one thousand nucleotides. Most alternative ways to identify promising local target motives tend to predict at higher frequencies which might decrease their probability of success. Increasing the knowledge on the structure-function relationship of asODN-RNA annealing could further increase this kind of stringency of selection and, thereby, further improve the theoretical design of asODN. An additional way to improve the theoretical target site selection is to define an ´accessibility score´ based on sets of annealing and/or inhibition data. With increasing numbers of examples one might be able to recognize distinct favorable structural target motives along a target RNA.

5. ACKNOWLEDGEMENT

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