

mRNA fusion constructs serve in a general cell-based assay to profile oligonucleotide activity

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ABSTRACT

A cellular assay has been developed to allow measurement of the inhibitory activity of large numbers of oligonucleotides at the protein level. The assay is centred on an mRNA fusion transcript construct comprising of a full-length reporter gene with a target region of interest inserted into the 3'-untranslated region. Luciferase and fluorescent reporter genes were used in the constructs. The insert can be from multiple sources (uncharacterised ESTs, partial or full-length genes, genes from alternate species, etc.). Large numbers of oligonucleotides were screened for antisense activity against a number of such constructs bearing different reporters, in different cell lines and the inhibitory profiles obtained were compared with those observed through screening the oligonucleotides against the corresponding endogenous genes assayed at the mRNA level. A high degree of similarity in the profiles was obtained indicating that the fusion constructs are suitable surrogates for the endogenous messages for characterisation of antisense oligonucleotides (ASOs). Furthermore, the results support the hypothesis that the secondary structure of mRNAs are divided into domains, the nature of which is determined by primary nucleotide sequence. Oligonucleotides whose activity is dependent on the local structure of their target mRNAs (e.g. ASOs, short interfering RNAs) can be characterised via such fusion RNA constructs.

INTRODUCTION

In post-genomic times pharmaceutical research often begins with the identification of a gene product which is dysfunctional or mis-regulated in a disease or in an experimental model of a disease. In cellular assays, protein over-expression or knock-down of expression are two readily accessible molecular biology techniques commonly used to link a specific protein causally to a phenotype or pathway thought

to play a role in the disease. These assays are followed with increasingly more detailed experiments in more sophisticated models which may lead to the point where the protein is 'validated' as a molecular target, to a sufficient extent to proceed to the next step, the identification and development of small molecule inhibitors, some of which will serve as starting points or leads for medicinal chemistry programmes (1–3). Gene knock-down is one of the most powerful techniques for validation of pharmaceutical targets and is also applicable on the genomic scale.

In 1977, Paterson *et al.* (4) demonstrated for the first time that gene expression could be inhibited sequence specifically using exogenously delivered nucleic acids. A new field, known as antisense, emerged and various experimental methods using the same principle were developed. The most robust of these exploits is the use of synthetically prepared, chemically modified, short oligonucleotides (5), delivered into cells, often with the aid of delivery reagents. This field has continued to develop, with the introduction of the RNA interference mechanism (RNAi) (6–9).

One of the most persistent problems with the antisense technique is the wide variability in biological potency often observed among a set of oligonucleotide reagents against a given mRNA target. Some are very potent, but many reagents are only weakly biologically active. This phenomenon is reported for most types of mRNA-targeting (oligonucleotide) reagents: DNA-like antisense oligonucleotides (ASOs) (10), artificial ribozymes (11), short interfering RNAs (siRNAs) (12) and RNA molecules expressed from transfected artificial DNA vectors (13). Furthermore, examples of sudden jumps in activity have been reported when the position of ASOs is shifted relative to the target sequence, sometimes by only a single nucleotide (14) or in the case of siRNAs by 3 nt (12). So far, robust rules to predict whether a given oligonucleotide will have a high inhibitory power have not been reported.

Thus, prior to initiation of detailed biological experiments against a given gene, some type of screening process is necessary for the identification of effective ASOs. Several reports show that such optimisation of the target sequence by pre-screening leads to antisense reagents with much higher potency than the initial, arbitrarily selected sequences (15,16). While this may be an effective method to identify the best reagent prior to beginning detailed biological investigations of

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single genes, this approach is, however, no longer practical to identify new targets on the genomics scale. On the level of hundreds and thousands of target genes, an efficient selection process must be in place to reliably identify highly potent and highly specific ASOs against each individual target gene. Multiple factors, many of which are interdependent, influence the biological activity of oligonucleotides in cell culture and *in vivo* (17). On the RNA target itself, this includes the folded structure, the proximity of the ribosomal binding site (i.e. the translational start site or the CAP site) and the degree of protein binding. For the oligonucleotides, chemical modification is an important element which influences a number of secondary factors including the nature of lipoplex formation, cellular uptake, interactions with RNA-cleaving enzymes (RNase H, RNase P, RISC), the secondary structure of the oligonucleotide, its metabolic stability and even the type of cell being targeted (18). All of these factors together are influenced by the primary sequence of the oligonucleotide and therefore also by the sequence of the binding site on the target. The nucleotide sequence not only influences the interaction with the target RNA, but also the affinity for other sequence-related RNAs and cellular factors through non-Watson-Crick binding interactions.

A prerequisite for an ASO strand to be an effective inhibitor is high affinity duplex formation between target and the ASO. Formation of this duplex should not be impeded by the target self-structure (19–21) or RNA-binding molecules, such as proteins (22) and the binding site should ideally be pre-organised to allow duplex formation, resulting in fast association and slow dissociation (23). Various methods of pre-screening ASOs for biological potency are known. Several cell-free screening methods using *in vitro*-transcribed RNAs have been described. Some of these use synthetic oligodeoxyribonucleotide (DNA) libraries and RNase H (24–27) in an *in vitro* translation screening (28, 29). Also, hybridisation of mRNA to immobilised oligonucleotides on arrays has been used (30). These approaches were designed to highlight those ASOs which bind most strongly to the target mRNA, based on the assumption that the strongest binders are likely to represent the most potent inhibitors in cells. These methods are in fact effective techniques for identifying potent inhibitors of gene expression. They work very well in cases where the opportunity exists to fully characterise an ASO before initiation of more complex biological experiments.

However, such a rigorous and often tedious characterisation is not possible in the case of experiments carried out on hundreds or thousands of genes, i.e. at the genomics scale. Theoretical approaches to predict 'good' target regions for ASOs using thermodynamic calculations have been reported to offer better results than a completely random selection of ASOs (31,32) but these often led to the identification of regions in which individual sequences within the region demonstrated sharp variations between high and low biological activity (14). Evidently, the most reliable way to identify the most potent, specific, ASO is to synthesise all possible candidate ASOs against a target sequence and test them in the cellular environment by monitoring their effect on target protein levels. While the technology for this approach, i.e. high-throughput oligonucleotide synthesis for preparation of very large numbers of oligonucleotides, is available today, the means to monitor target protein levels from large numbers

of transfections, preferably in real time, requires development of suitable assays. Once an assay is established, a process involving the synthesis of all possible candidate oligonucleotides against a target sequence and their testing in the cellular environment becomes feasible.

Furthermore, such methods will allow a head-to-head comparison of the inhibitory properties of hundreds to thousands of different oligonucleotide sequences in their cellular environment. Consequently, the possibility exists to search using, for example, statistical methods or neural networks (33), for the presence of short nucleotide motifs, which contribute not only to biological potency, but also to many other properties of oligonucleotides. Eventually such techniques may lead to methods which allow accurate prediction of the inhibitory activity of any given oligonucleotide for its target.

Unfolding and refolding studies of single RNA molecules have revealed that, in contrast to the secondary structural elements of proteins, those of RNA are independently stable (34). There is strong evidence suggesting that the structure of short RNA segments, for example, that targeted by an ASO, is primarily constrained by local secondary structure and that this has a direct impact on biological activity of the oligonucleotide (35). Thus, when a segment of mRNA is taken from its natural endogenous mRNA and inserted into another mRNA, it should maintain the same fundamental features of its original structure with respect to accessibility to oligonucleotides and, therefore, ASOs complementary to a sequence within the segment should demonstrate similar binding affinities, and therefore similar levels of antisense activity, to both the wild-type mRNA and the corresponding construct mRNA. This hypothesis formed the foundation for the development of a generally applicable, automated, cell-based, high-throughput assay of oligonucleotide activity. The method makes use of plasmid constructs which leads to transcription of a fusion mRNA, comprising the coding sequence of a reporter gene. The principle of these gene fusions was first demonstrated during experiments carried out on plants (36). A region of selected cDNA sequence representing a part of the target gene is inserted into the 3'-untranslated region (3'-UTR). The insert may originate from a full-length gene or a partial gene; it may be derived from coding or UTRs, and it may be from uncharacterised genes and also may even derive from a gene of another species. Each construct tested makes use of the same promoter and produces the same protein product with similar levels of expression. Therefore, this approach compares very favourably to one in which the insert is included as part of the coding region, from which potentially toxic, or even incorrectly folded fusion proteins result. Moreover, use of the 3'-UTR as opposed to the 5'-UTR for the location of the insert ensures that the reporter expression is not adversely affected by inclusion of undesired inadvertent upstream translation initiation. A modification in the 3'-UTR of an mRNA might potentially modify the RNA stability, since stability determinants often lie within this region. However, in any correlation between a reporter fusion mRNA versus an endogenous mRNA one would not expect a change in the relative potency ranking across a set of ASOs. Furthermore, using reporter genes of no physiological relevance results in a de-coupling of possible biological effects caused by inhibition of functional mRNA targets and helps

ensure that changes in target (reporter) protein levels result solely from the antisense mechanism.

Most types of ASOs used today act by specific hybridisation to their target mRNA, followed by induction of mRNA rapid degradation via a RNase H-dependent mechanism (37–39), resulting in lowered levels of protein. The fusion construct principle has been tested in this study with MOE-modified gapmer ASOs (40) which derive their biological activity from RNase H-induced degradation of mRNA. The inhibitory activity of a large number of oligonucleotides targeting a set of fusion constructs was correlated with their antisense activity against their natural wild-type targets to test the hypothesis that indeed the local mRNA structure is retained when the segment is present in different mRNAs: in the wild-type sequence and as part of the 3'-UTR of the reporter gene mRNA.

MATERIALS AND METHODS

Oligonucleotide synthesis

Synthesis and purification of 2'-*O*-methoxyethyl phosphodiester or phosphorothioate and unmodified deoxyphosphorothioate oligonucleotides were performed using an automated DNA synthesiser with phosphoramidite chemistry, as previously described (41). The chemical format of the oligonucleotides was NsNsNs NsNsNs NsNsNs nsnsns nsnsns nsnsn (Fig. 3) or npnpnp npnpNs NsNsNs NsNsNs Nsnpnp npnpn (Figs 4–7) where N indicates DNA; n is a 2'-*O*-methoxyethyl ribose modification; s and p are phosphorothioate and phosphodiester linkages, respectively. The sequences used in this study are listed in Supplementary Material.

Two unrelated control ASOs were used: CsCsTs TsAsCs CsTsGs cstsas gscsts gsgsc (4535) and cct taCs CsTsGs CsTsAs Gsct ggc (5596). Oligonucleotides were purified by HPLC. Quantity and purity were monitored by electrospray mass spectrometry and capillary gel electrophoresis, respectively.

Construction of reporter expression clones

The firefly luciferase reporter-based vector pNAS-020 (Fig. 2 and Table 1) was constructed to contain a multiple cloning site after the luciferase stop codon for inserting the appropriate cDNAs or ESTs of interest. The minimal CMV promoter containing seven repeats of the tetracycline operator was included for inducible expression studies known as the tet-off system (42). Plasmid pSFhCMVT7neo1 (F. Asselbergs, unpublished results) served as the starting vector for construction of vector pNAS-020. The vector experienced no changes at the cloning site (EcoRV, EcoRI, BglII), the splicing acceptor, splicing donor and poly adenylation sites of beta globin, pBR ori, the ampicillin resistance marker and the SV40 ori. Subsequent cloning steps resulting in further intermediate plasmids and finally the vector pNAS-20 was obtained after inserting the firefly luciferase gene (pGL3 control vector; Promega) at the NcoI/XbaI site.

The enhanced yellow fluorescent protein (eYFP) reporter-based vector pNAS-055 (Fig. 2 and Table 1) was constructed to contain a multiple cloning site after the stop codon of the eYFP for inserting the cDNAs. pNAS-055 was generated by inserting the BamHI/NotI fragment derived from the

peYFP-N1 vector (Clontech) into the BamHI/NotI site of pcDNA/TO (Invitrogen) bearing the tet-operator.

The enhanced cyan fluorescent protein (eCFP) and eYFP dual reporter-based vector pNAS-092 (Fig. 2 and Table 1) was constructed to contain a multiple cloning site after the stop codon of the eYFP for inserting the cDNAs. The eCFP reporter serves for normalisation measurements driven under the elongation factor 1 alpha (EF-1 α) promoter and the eYFP reporter was used to monitor antisense activity of ASOs driven under the CMV promoter. The origin of the vector was a plasmid pBudCE4 (Invitrogen) which contains a hCMV and a EF-1 α promoter. pNAS-092 was generated by inserting the eCFP gene derived from peCFP-N1 (Clontech) at the depicted restriction sites and by transferring the eYFP gene from pNAS-055 together with a synthetic DNA fragment bearing the cloning site (EcoRV, NotI, HindIII, KpnI, XbaI). All used synthetic DNAs (for pNAS-020 and pNAS-092) were confirmed by sequencing. All plasmids used for the final reporter assay were constructed by inserting into the cloning site the c-DNA fragment of the EST clone, respectively. The synthetic DNA sequences and the clone ID number of the used source plasmids are listed in Supplementary Material.

Cell lines and cell culture

The Chinese hamster ovary (CHO) cell line SSF-3 was derived from the dihydrofolate reductase (dhfr)-deficient CHO line DUKXB11. It can be grown in a basal medium completely devoid of proteins (43). A recombinant line of SSF-3 bearing the tetracycline responsive transactivator protein (tTA) and the mutant hamster dhfr as selection marker (methotrexate resistance) was used. tTA is compatible with the reporter vector pNAS-020 for constitutive luciferase expression. SSF-3 cells were grown as adherent cells in 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES) buffered Chomaster medium (Messi Cell Culture Technology, Zürich, Switzerland) containing 10% bovine calf serum (BCS) (Life Technologies) in a 5% humidified CO₂ atmosphere at 37°C. Stable cells expressing the red-shifted enhanced green fluorescent protein [pd2eGFP-N1 (Clontech) Lipofectamine-Plus transfection according to the manufacturer (Life Technologies)] were selected as *neo*⁺ clones by the addition of 1 mg/ml geneticin and further designated as SSF-3-G.

eGFP-expressing cells were used for accurate measurement of cell numbers. This allows monitoring of each physical manipulation of the cells during the different medium replacement steps in the assay, and, in addition, the fluorescent measurement serves for the normalisation of the luciferase activity value per cell number equivalent.

The African green monkey kidney cell line COS-1 (CRL 1650) and the human non-small cell lung carcinoma cell line H-1299 (CRL-5803) were purchased from ATCC (Rockville, MD). COS-1 cells were grown in DMEM (Life Technologies) containing 10% serum fetal bovine serum (FBS) (Life Technologies). The H-1299 cell line was maintained in a 5% humidified CO₂ atmosphere at 37°C in RPMI 1640 medium (Life Technologies) containing 10% FBS. Stable cells of H-1299 expressing the eCFP and the tetracycline repressor (TR) protein [peCFP-N1 (Clontech), pcDNA6/TR (Invitrogen); Lipofectamine-Plus co-transfection according to the manufacturer (Life Technologies)] were selected as *neo*⁺ clones by the addition of 1 mg/ml geneticin and further

designated as H-1299-C-TR. The purpose for using eCFP-expressing cells was a practical measurement of the cell number which serves for the normalisation of the eYFP activity value per cell. TR is compatible with the reporter vector pNAS-055 for constitutive eYFP expression by adding 1 $\mu\text{g}/\text{ml}$ tetracycline.

Luciferase reporter assays

SSF-3-G cells were split 48 h prior to transfection, reaching an 80% subconfluent stage. Cells were trypsinised, washed and resuspended in OptiMEM-I (Life Technologies) and transfected with plasmids directly afterwards in polypropylene tubes and distributed in Costar 96-well assay plates (white, clear bottom) using Lipofectamine-Plus reagent (final concentration: 1 $\text{ng}/\mu\text{l}$ plasmid, 2 $\mu\text{l}/\text{ml}$ Lipofectamine). After 2 h, oligonucleotides were added in the presence of Lipofectin (4 $\mu\text{g}/\text{ml}/100$ nM oligonucleotide) which was then further incubated for 2 h. The medium was removed and replaced with 100 μl of standard Cho-master medium containing 10% BCS and incubated overnight at 37°C. eGFP expression, serving as the cell number equivalent, was measured (an excitation filter of 485/15 nm and an emission filter of 510/10 nm on a Victor²TM; Berthold Technologies) after 22 h. The medium was aspirated and cells treated with 50 μl of passive lysis buffer (Promega). Luminescence was measured immediately [in relative light units (RLU)] after a 100 μl luciferase substrate (Promega) injection. The quotient RLU/eGFP fluorescence counts express the luciferase activity per cell number equivalent. The cell number versus fluorescence intensity for the experiments described here was in the linear range. For the correlation between the luciferase reporter assay and the quantitative real-time reverse transcription PCR (q-PCR) assay all oligonucleotide treatments were done in triplicate. The luciferase mismatch control sequence 4535 was set as 100%. The relative standard deviations were calculated from the means of at least three independent oligonucleotide treatments and found to be on average 13.3% (Fig. 3).

Fluorescent protein reporter assays (intercellular normalisation)

H-1299-C-TR cells were transfected with a similar procedure using culture medium containing 1 $\mu\text{g}/\text{ml}$ tetracycline and the cells were seeded in black 96-well assay plates (Costar, clear bottom). Fluorescence was measured at 24 h intervals for 3 days. The fluorescence of eCFP and eYFP was measured with an excitation filter of 436/20 nm and an emission filter of 480/30 nm and an excitation filter of 500/25 nm and an emission filter of 535/30 nm, respectively. The quotient of eYFP/eCFP fluorescence counts expresses the eYFP activity per cell number equivalent. For the correlation between the eYFP reporter assay and the luciferase reporter assay all oligonucleotide treatments were done in triplicate. The mean values are presented as percent of unrelated control 5596. The standard deviations from the mean of at least three independent oligonucleotide treatments were calculated and found to be on average 15.8% and for the luciferase reporter assay 15.3% (Fig. 4). The average of mismatch control sequences was set as 100% in Figure 5. The error bars represent the standard deviation from the mean of at least three independent oligonucleotide treatments.

Fluorescent protein assays with dual reporter construct carrying a reference gene (intracellular normalisation)

COS-1 cells were transfected with a similar procedure in black 96-well assay plates using the Fugene (Roche Molecular Biochemicals) reagent for plasmid transfection (final concentration: 1 $\text{ng}/\mu\text{l}$ plasmid, 2 $\mu\text{l}/\text{ml}$ Fugene). After removing the transfection mix, the medium without phenol red was added and incubated for 3 days. All read-outs were measured as described in section Fluorescent protein reporter assays (intercellular normalisation). The quotient of eYFP/eCFP fluorescence counts expresses the relative eYFP activity of transfected cells. The results are presented as percent of unrelated control 5596 in Figure 6. Error bars represent the standard deviation from the mean of three independent oligonucleotide treatments. An optimised procedure was established to achieve a relative standard derivation of <13% in the 96-well assay format which allows a single well screening mode to identify highly active oligonucleotides (Fig. 7A and B). A improvement was observed when the cell suspension was carefully and equally dispensed (50 μl) into 96-well assay plates before adding the plasmid Fugene mixture to the cells. In addition, the final concentration of Lipofectin was 1.5 $\mu\text{g}/\text{ml}/100$ nM oligonucleotide and the oligonucleotides were pre-diluted to 1.8 μM in 0.05 mM HEPES buffer at pH 6.5.

mRNA analysis with q-PCR

H-1299 cells were plated in 6-well assay plates 1 day prior to the transfection.

Oligonucleotide was added in the presence of Lipofectin reagent (final concentration: 1.5 $\mu\text{g}/\text{ml}/100$ nM oligonucleotide) for 4 h. The OptiMEM I lipoplex mix was removed, replaced with fresh medium and incubated overnight. Total RNA was extracted using the RNeasy 96 kit (Qiagen) and quantified fluorometrically with ribogreen (Molecular Probes). For quantitative mRNA analysis, the TaqMan technology with the q-PCR was performed on the ABI PRISM 7700 (Applied Biosystems) according to the manufacturer's instructions for one-tube q-PCR.

The relative quantitation of gene expression was calculated as described in the ABI PRISM 7700 user bulletin no. 2 (Applied Biosystems). Each oligonucleotide was analysed in at least two independent assays with duplicate samples. The q-PCR was run in triplicate. The results are presented as percent of unrelated control sequence 4535. Samples with a standard deviation from the mean >25% were excluded in Figure 3.

RESULTS

Reporter gene constructs for mRNA fusion transcripts and measurement of ASO activity

A reporter gene system for a cell-based screening of ASO activity has been developed. The assay is based upon a series of gene cassettes in which a cDNA segment is inserted such that it becomes part of the 3'-UTR of the expressed reporter gene (Fig. 1A). The reporter protein expressed is identical for each fusion RNA independent of the size of the insert (Fig. 1B), although plasmids bearing different inserts yield mRNAs which may exhibit different expression levels of the reporter gene. The plasmid expressing the fusion mRNA and

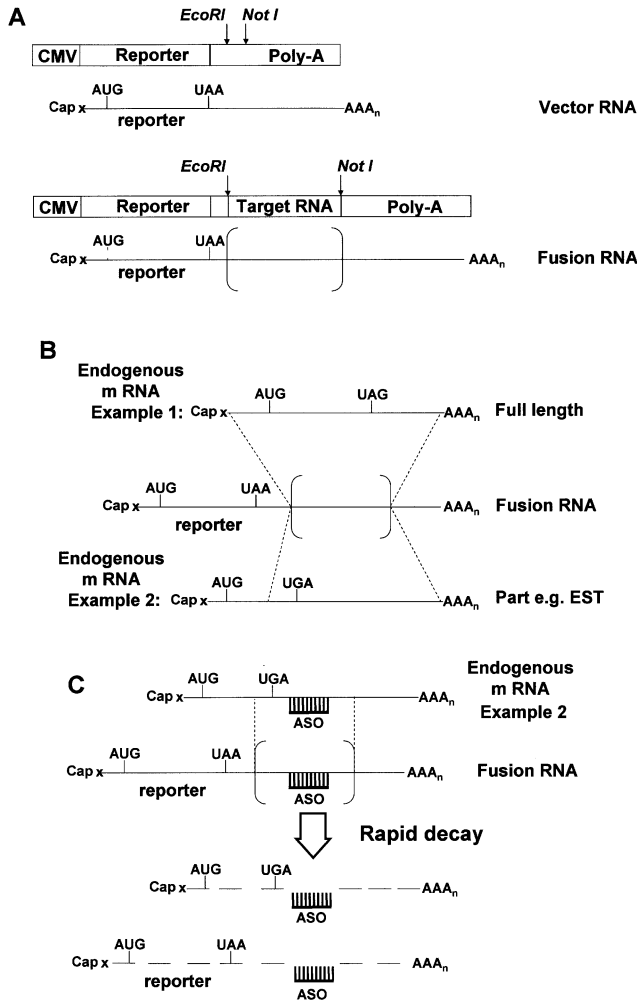


Figure 1. Schematic drawing of the construction of a fusion mRNA and the mechanism of action of an oligonucleotide targeting the fusion mRNA. (A) A fusion mRNA is generated from a plasmid in which an *EcoRI/NotI* cDNA fragment is inserted into the reporter gene. Thus, a target RNA region is created in the 3'-UTR of the mRNA encoding the reporter protein (luciferase or fluorescent protein). (B) Two examples of reporter constructs produced from endogenous mRNAs are shown. In example 1, a full-length cDNA is cloned into the vector to produce the fusion mRNA, whereas in example 2, only a part of a cDNA, e.g. EST, is cloned into the vector. Both types of constructs have been used as target sequences. (C) The ASO binds to the endogenous mRNA as well as to the fusion RNA in the same local environment, leading to message degradation due to RNase H cleavage.

ASOs complementary to the cDNA insert, which bears all of its own local structural elements, are sequentially transfected into cells. The activity of the ASOs is scored as the degree of inhibition of the expression of the reporter protein, either luciferase or fluorescent protein (Fig. 1C).

Different reporter plasmids

Plasmids expressing different reporter proteins with multiple cloning sites suitable for inserting any cDNA after the stop codon were constructed. The plasmid maps are shown in Figure 2 and the applications of these constructs are summarised in Table 1.

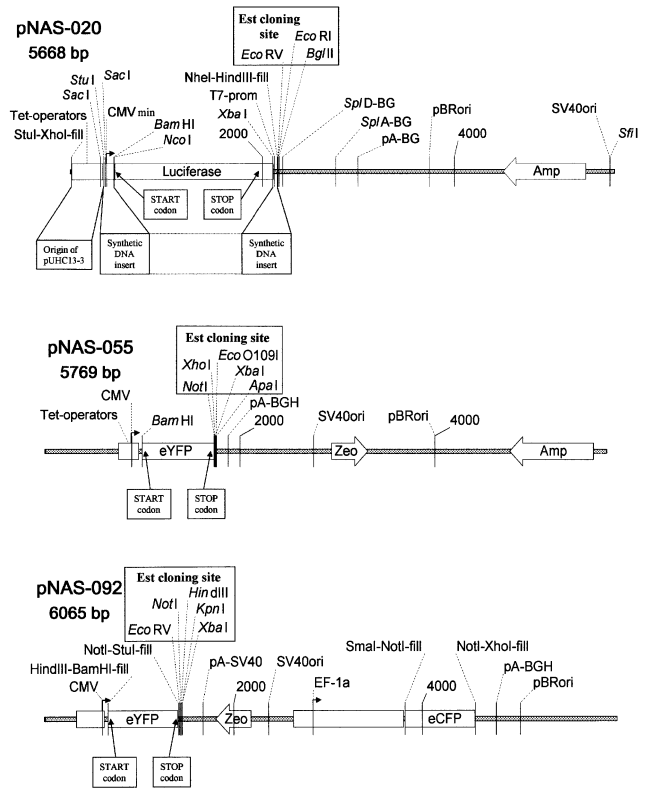


Figure 2. Detached map of the reporter plasmids pNAS-020, pNAS-055 and pNAS-092 (see also Table 1). The cloning site adjacent to the STOP codon of the firefly luciferase (pNAS-020) and the eYFP (pNAS-055 and pNAS-092) for cDNA or EST inserts is illustrated in the separate box. Amp, ampicillin selection marker; Zeo, Zeocin selection marker; CMVmin, minimal human cytomegalovirus promoter; CMV, human cytomegalovirus promoter; EF-1a, elongation factor 1 alpha promoter; T7-prom, bacterial T7 promoter; Sp/D-BG, splicing donor site of rabbit β -globin; Sp/A-BG, splicing acceptor site of rabbit β -globin; pA-BG, polyadenylation site of rabbit β -globin; pA-SV40, polyadenylation site of simian virus 40; pA-BGH, polyadenylation site of bovine growth hormone; pBRori, origin of replication of pBR322; SV40ori, SV40 origin of replication. *StuI*-XhoI-fill, NheI-HindIII-fill, HindIII-BamHI-fill, NotI-*StuI*-fill, SmaI-NotI-fill, NotI-XhoI-fill, filled in Klenow-blunted ligation sites used for vector constructions.

Validation of the use of the reporter assay

A series of transfection experiments using eight luciferase reporter fusion RNAs were performed. The set of eight plasmids was constructed containing cDNA inserts of different lengths derived from eight human genes all expressed in a human non-small lung cancer (H-1299) cell line. For each plasmid, a series of 5–10 ASOs spread across the length of each insert region were designed and synthesised. The ASOs had a GC content between 28 and 72% (average of 51%). A BLAST search (GenBank) was performed against the whole genome to ensure that the oligonucleotides had full complementarity to the target and no significant cross-matching to other sequences. Only ASOs containing more than two mismatches against a cross target were selected. ASOs were deliberately not selected within 40–60 nt of fusion junctions, i.e. in regions where differences in local mRNA structure between the two RNAs might be expected. The reporter

Table 1. Reporter constructs used in the study

Reporter construct	Reporter protein (fusion RNA)	Read-out ratio	Normalisation	Cells
pNAS-020	Firefly luciferase	Luminescence/fluorescence ^a	Cell number equivalent	SSF-3-G ^b
pNAS-055	Fluorescent protein ^c	Fluorescence ^d /fluorescence ^e	Cell number equivalent	H-1299-C-TR ^f
pNAS-092	Fluorescent protein ^c	Fluorescence ^d /fluorescence ^e	Intracellular reference	COS-1

^aFilter: excitation, 485/15 nm; emission, 510/10 nm.

^bStable expression of eGFPd2.

^ceYFP.

^dFilter: excitation, 500/25 nm; emission, 535/30 nm.

^eFilter: excitation, 436/20 nm; emission, 480/30 nm.

^fStable expression of eCFP.

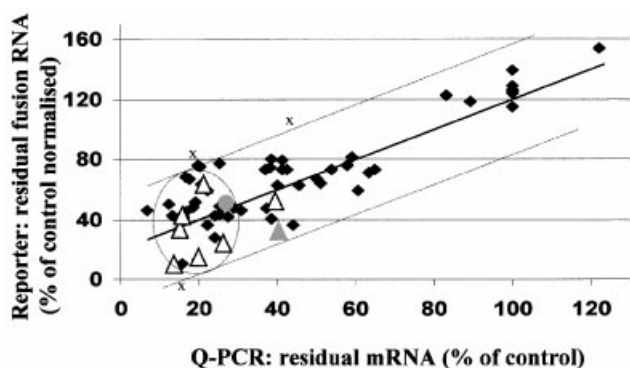


Figure 3. Correlation plot comparing the effect of 58 transfected ASOs on endogenous mRNA levels in human H-1299 cells and the effect on fusion mRNA of interest expressing the reporter in SSF-3-G cells. A series of eight targets were compared derived from ESTs. Correlation coefficient $r^2 = 0.73$ (linear least-squares regression). Dashed lines indicate the 95% confidence level for the prediction. The circled area indicates the region of highly active ASOs. (Triangles) Most potent hits in the reporter assay from each target. (Grey circle) Second-best ASO in the reporter assay corresponding to the grey triangle. The white triangle outside of the circled area belongs to a target against which no better ASO was found in the q-PCR. (Crosses) Three values outside the 95% confidence level.

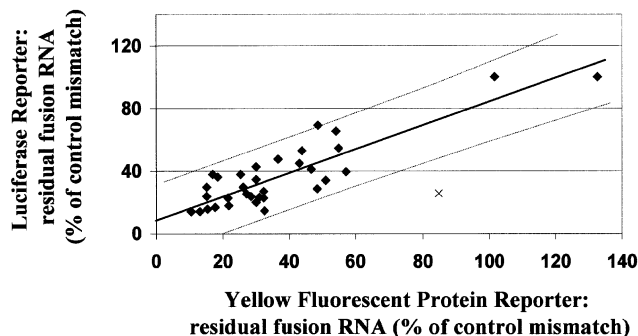


Figure 4. Correlation plot comparing the effect of 32 transfected ASOs on eYFP reporter constructs (construct type pNAS-055) bearing the fusion RNA of interest in human H-1299-C-TR cells versus the effect on luciferase reporter constructs (construct type pNAS-020) bearing the fusion RNA of interest in SSF-3-G cells. Two EST targets were compared. Correlation coefficient $r^2 = 0.75$ (linear least-squares regression). Dashed lines show the 95% confidence level for the prediction. The cross indicates one value outside the confidence level.

plasmids were transfected into a CHO cell line (SSF-3) stably expressing eGFP. After 2 h, the relevant ASOs were transfected into the cells complexed with Lipofectin reagent (see Materials and Methods). After an incubation period of 2 h

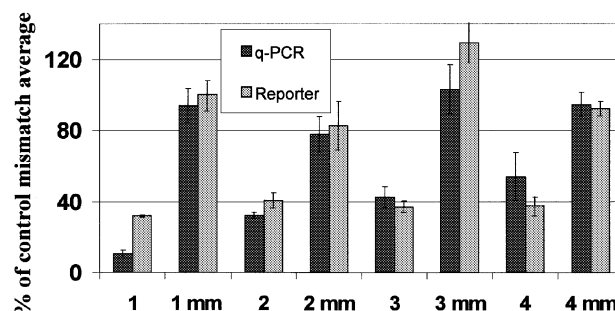


Figure 5. Out of 20 ASOs, the four most potent were identified in a fluorescent protein reporter screen using a derivative of pNAS-055. Subsequently, these four ASOs and their corresponding mismatches (mm) were re-tested at 400 nM concentration using either activity of the reporter construct in H-1299-C-TR cells (activity measured 48 h after transfection) or in H-1299 cells, by measuring the expression level of the endogenous mRNA activity assayed by q-PCR (RNA isolated 24 h after transfection). Pairwise correlation was found between both RNA targets, the model fusion mRNA and the endogenous mRNA. From a correlation plot of the various activities a correlation coefficient of $r^2 = 0.86$ was calculated (linear least-squares regression).

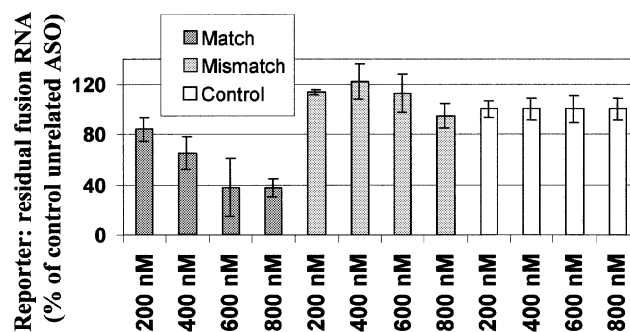


Figure 6. Dose-response effect of three transfected ASOs tested on reporter construct type pNAS-092. COS-1 cells were transfected and the read-outs after 72 h are shown; plasmid transfection using FugeneTM reagent.

for the ASO, the medium was changed and the cells were lysed on the following day to quantitate luciferase activity. The experiments were performed using the pNAS-020 construct (Table 1) and the ratio activity/cell number was determined using the luciferase/fluorescence counts, respectively. Assays were performed in triplicate in a 96-well format. The value observed for the control ASO (4535) was set to 100%. The ASOs were then tested for their inhibitory activity of the endogenous mRNAs in H-1299 cells using q-PCR. Transfection involved the same but separately prepared

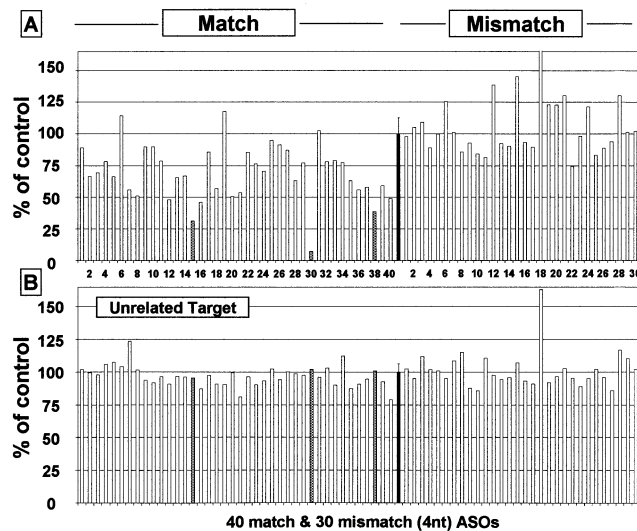


Figure 7. Ninety six single well screening of match and mismatch ASO pairs on reporter construct type pNAS-092 with COS-1 cells after 48 h of ASO transfection (150 nM); plasmid transfection with Fugene. Match ASOs are on the left side of the figure. Four nucleotide mismatch controls to the first 30 ASOs are shown on the right side. From left to right, the first depicted mismatch sequences correspond to the first match sequences, etc. Grey bars represent the most potent oligonucleotides. The 100% of control black bar from each plate represents the average of 26 untreated plasmid wells with the indicated relative error bar of 12.7 and 6.3%, respectively. (A) Treatment of the reporter bearing the fusion RNA of interest with ASOs. (B) Treatment of a control plasmid of an unrelated RNA fusion with ASOs.

ASO-lipoplex and incubation was for 4 h. After 16 h, total RNA was isolated and analysed by q-PCR. The two inhibitory activities for each of the 58 ASOs and the control ASO were plotted and are shown in Figure 3. Approximately half of the ASOs reduced mRNA to 10–30% of control values (circled area in Fig. 3). Only a few of the ASOs yielded <50% of target reduction. The most potent ASOs against the reporter constructs from the eight series are depicted as triangles in Figure 3. Two data points fall outside of the circled area representing highly active ASOs, although one of them was also the most potent ASO in a transfection experiment in which the endogenous target mRNA was measured by q-PCR analysis. One ASO, represented by the triangle shaded in grey, was found not to be highly potent against its endogenous target as measured by q-PCR, compared with its high activity against the reporter fusion. However, for this target mRNA the second most potent ASO against the luciferase fusion mRNA was found to be the most potent one against the endogenous gene (grey circle in Fig. 3).

The results show an excellent correlation ($r^2 = 0.73$), demonstrating that the rank-order potency of each ASO series is similar for both the reporter RNA and the natural endogenous mRNA counterpart.

Comparison of luciferase and fluorescent protein reporter systems

'Fingerprint' profiles were generated representing the inhibitory activity of 32 ASOs targeting a luciferase reporter derived from luciferase construct NAS-020 and the comparable eYFP construct derived from NAS-055 (Table 1). A correlation

coefficient of $r^2 = 0.75$ (Fig. 4) was obtained. The two reporter assays were performed in different cell lines, a non-human line SSF-3-G and a human line H-1299-C. This result, therefore, demonstrates that the two reporting systems yield comparable results.

Match–mismatch pairs comparison

Mismatch oligonucleotides were selected as gene-specific negative controls. In our experience, the lipoplex of an inactive ASO is preferable as a negative control over the use of transfection reagent alone, whereas a 'silent' irrelevant oligonucleotide sequence serves as a generic negative control. As a general rule, four nucleotides at internal positions were permuted, generating mismatch control oligonucleotides very closely related to their fully matched counterparts, with care to avoid introduction of motifs with 4 consecutively identical nucleotides. Mismatches were chosen after consideration of the nearest neighbour free energy coefficients (<http://rna.chem.rochester.edu>) by choosing the strongest disrupting mismatch pairs. In 96% of the mismatch cases examined with the reporter assay, inhibitory activity of <25% was observed. None of the mismatches was found to be more active than the fully matched ASO. Using one reporter construct derived from a pNAS-055 vector in a representative experiment, the four most potent matched ASOs were re-tested together with their mismatch controls (Fig. 5) against the reporter transcript and the endogenous mRNA in the same H-1299 cell line. The results were compared and, once again, the most inhibitory ASO in the reporter assay was also found to be the most potent one against the endogenous gene. Mismatch oligonucleotides in both assays were found to be considerably less active than their matched counterparts in both assays. Mismatched and potent matched ASOs characterised with such reporter constructs subsequently proved to be highly inhibitory on function level read-outs in biological follow-up experiments (data not shown).

Dual reporter construct carrying a reference gene—use in single well screens

The performance of the reporter assays could be systematically improved by normalisation for the efficiency of plasmid transfection. In the dual reporter plasmid NAS-092, a second fluorescent protein, eCFP was introduced adjacent to the eYFP (Fig. 2). eCFP expression thus serves as a means to normalise for plasmid transfection efficiency, whereas the fusion mRNA reporter (eYFP) bears the RNA target region of interest. Experience gained over a large number of experiments performed with this normalisation has shown that in transient transfection assays this leads to a very high level of reproducibility, similar to that level obtained in experiments performed in a stable cell line. An example of a dose–response titration in a non-human cell line (COS-1) using a match–mismatch pair of oligonucleotides targeting this dual reporter vector is shown in Figure 6. The vector was then used to screen 70 ASOs in single wells of a 96-well plate. The results of a typical experiment using two 96-well plates are shown in Figure 7A and B. Each plate carries 40 matched ASOs and 30 corresponding 4 nt mismatch ASOs. The microtitre plate oligonucleotides were transfected into cell lines previously transfected with either a dual reporter construct bearing the target insert (Fig. 7A) or one carrying an unrelated insert

(Fig. 7B). The use of a relatively low dose of ASOs (150 nM) ensured a wide dynamic range from which discrimination of ASO potency can be accurately discerned. In each plate, 26 randomly distributed wells were used for plasmid transfected controls in order to calculate standard deviations. The match-mismatch changeover at oligonucleotide no. 40 is clearly observable. The use of an unrelated target reporter provides an additional control for the specificity of the ASOs for their target mRNAs (Fig. 7B). This type of control is useful to assess the toxicity of ASOs, caused, for example, by non-specific binding to proteins. For example, ASO no. 40 showed a significant level (~25%) of non-specific inhibition.

DISCUSSION

Oligonucleotides are powerful biological tools commonly used to help determine the function of genes. The potency of the oligonucleotide varies widely according to a large variety of interdependent parameters most of which are determined by the primary sequence of the oligonucleotide and the structure around the complementary binding site on the mRNA.

The purpose of this study was to develop a cell-based reporter assay suitable for measuring in high throughput the inhibitory properties of a whole series of ASOs, complementary to sets of genes. In the first instance, such an assay enables a rapid identification of the most potent oligonucleotide against its target as a tool for biological experiments. However, the assay also permits head-to-head comparisons of the inhibitory properties of very large numbers of oligonucleotide sequences under identical experimental conditions. The analysis of such data sets using pattern searching tools is expected to help reveal the role of primary sequence, in particular sequence motifs, in the mechanism of gene knock-down by antisense or other oligonucleotide reagents.

To build a general homogeneous cellular assay suitable for mRNA targets from any origin, it was first necessary to develop a strategy to rapidly clone cDNA inserts from target sequences into a suitable plasmid cassette, carrying a reporter gene for which target protein levels could be monitored. To validate the use of this assay, it was then necessary to demonstrate that ASOs targeting the insert sequence would show similar levels of inhibition of the reporter protein as for the endogenous wild-type target.

All of the constructs developed for this study involved insertion of target cDNAs, or parts of target cDNAs, into the 3'-UTR of the reporter. When working with large numbers of different cDNA inserts from multiple sources, this cloning strategy offers significant practical advantages over an insertion into the 5'-UTR, for example, as noted earlier. Transcription of the plasmid results in different fusion mRNAs of the reporter gene, all extended in the 3'-UTR region, but with a constant open reading frame and therefore with expression of a common reporter protein. This approach helps to render the assay general with respect to a homogeneous expression of a reporter protein for inserted targets from any location on a given gene, from any species. A key step in the plasmid design was the inclusion of attR1 and attR2 cloning sites in the most advanced version of the cassette. These render the vector suitable as a destination vector in the Gateway (Invitrogen) cloning system. Clones isolated from cDNA libraries constructed in Gateway entry vectors can be

transferred in high throughput in a single reaction (recombination) with high efficiency.

A wide range of experiments were performed in order to validate the concept of the reporter fusion mRNA as a surrogate for the endogenous message. The relative rank-order potencies of 58 oligonucleotides targeting eight pairs of fusion reporter and endogenous mRNAs across their common regions were compared (Fig. 3). With the exception of a relatively short common domain region, the reporter and the endogenous mRNA pairs shared no sequence homology; two different cell lines from two different species were used to screen the two RNAs with the ASOs (hamster and human, respectively). The reporter assay requires two transfections, a plasmid transfection, followed by oligonucleotide transfection, compared with a single ASO transfection required for a test against endogenous mRNAs. Furthermore, inhibition of gene expression was assayed at two different levels, the endogenous mRNA at the mRNA level using q-PCR and the reporter fusion at the functional level of the protein. In spite of these differences, a very high correlation, $r^2 = 0.73$, was obtained. Furthermore, close analysis of the data revealed that if samples of the q-PCR measurements which showed relatively high standard deviations ($>\pm 25\%$) from the mean of at least three independent transfections were included, the correlation dropped to $r^2 = 0.68$, indicating that the noise of the individual assay method probably limits the maximum correlation of the two methods. The results proved conclusively that the eight domains examined in this study exhibit identical accessibility to multiple ASOs from their corresponding groups. With such a strong correlation between the methods, it is easily possible to select potent ASOs against an endogenous mRNA using the reporter fusion as a surrogate target, and thus the primary goal of the study was achieved.

The second generation of reporter constructs uses instead of the luciferase enzyme the eYFP. The use of fluorescent proteins as reporters offers some significant advantages over the luciferase/luciferin system. The assay read-out is fluorescence which enables non-invasive measurement of the cellular state and thus assay conduction in real time. As antisense mechanisms have an important kinetic element, that of mRNA and protein half life, the technical ability to follow changes in reporter protein in real time is highly advantageous. In addition, there is no reagent cost associated with the read-out. This construct was tested in a set of experiments in order to determine whether the high correlation is a general phenomenon. A second set of transfections with four plasmids to determine the correlation between the inhibitory potency of 32 ASOs targeting a constant domain embedded in the 3'-UTRs of two different reporters and two cDNA targets, one luciferase and the other eYFP (Fig. 4), were carried out in an analogous fashion to the initial experiments described for the luciferase reporter/endogenous mRNA (Fig. 3). Once again, assays were performed in different cell lines and in this case at different time points: generation of a detectable luciferase activity over noise becomes significant as early as 24 h after transfection, whereas the signal from the eYFP is best measured after 48 h. The longer incubation time did not change the rank order of potency within the ASO series. In this experiment (Fig. 4), a very high correlation was observed, again showing that antisense activity within given domain regions is insensitive to the structure of the surrounding

sequence, therefore, suggesting that the local domain structure is identical in the two messages.

The results of reporter assays performed on recombinant gene constructs, endogenous genes, in various cell lines, and even in various species, correlate highly as shown in Figures 3 and 4. This finding can be interpreted to indicate that the folding is primarily determined by local target structure alone, that the effects of RNA-binding proteins from different species are very similar, or that these proteins have much lower affinities than the ASOs. Thus, our experiments confirm an assumption which underlies many published experimental designs, where ASOs found to be active in one cell type are used to inhibit the same gene in another cell type.

In order to draw meaningful conclusions from experiments using oligonucleotides, it is imperative to include good quality controls. Non-sequence-related effects from the use of oligonucleotides are well documented in the literature (44,45). Inverted, sense or scrambled ASO sequences have no relation to the parent compound and thus cannot be expected to have similar physicochemical properties. Thus, they are inferior controls. The commonly accepted best type of control is a closely related mismatch oligonucleotide. These oligonucleotides comprise of an almost identical sequence to that of the parent compound and therefore share most of the physicochemical properties of the match oligonucleotide. However, the permutation of a small number of nucleotides in the parent sequence is sufficient to ensure that it binds with much lower affinity to the complementary mRNA than the parent match compound. In this way, biological effects induced by the match oligonucleotide, but not by the mismatch control, are considered to be due to specific target gene inhibition. The 5' and 3' ends of the match and mismatch oligonucleotides are held identical to minimise any differences between match and mismatch in annealing properties (14, 23) and in susceptibility to exonuclease-derived degradation.

Typically, four bases in an 18mer match are permuted to generate the mismatch control, and the molecular weight and the base composition is maintained as closely as possible. Residual activity can sometimes be observed as shown with the corresponding mismatch of ASO no. 2 (Fig. 5), as one might expect in cases where the parent match oligonucleotide has a very high affinity for the target mRNA (46). In no cases were mismatches more active than their parent match sequences. Occasionally, a particular oligonucleotide treatment can lead to an increase in the induction of reporter protein (Fig. 7A and B, mismatch no. 18) for which no obvious explanation could be found.

The final refinement of the reporter assay was the incorporation of a cDNA coding for a second fluorescent protein, eCFP under the control of a EF1- α promoter. This leads to simultaneous transcription of two fluorescent proteins after transfection: eCFP and eYFP, which are measured in parallel. This permits an effective normalisation for the plasmid transfection efficiency and contributes to assay robustness.

The secondary structure of an RNA is the major determinant of its conformation and the ease by which it interacts with other biomolecules, including proteins, polynucleotides and also synthetic oligonucleotides. There are several lines of strong experimental evidence that the 'accessibility' of an

mRNA to binding and initiation of an antisense mechanism is determined by the secondary structure and not by sequence base composition alone (47 and references therein). This secondary structure is generally assumed to be formed in local domains, established as the RNA is transcribed, rather than folding after transcription is complete. These hypotheses were supported by the observations of Vickers *et al.* (48) during a study of single synthetically prepared DNAs transcribed as different locally structured RNAs of 44–54 nt in length bearing a complementary binding site to an ASO. Relocating the whole domain from the 3'- to the 5'-UTR of the luciferase reporter message did not affect the rank-order potency of the ASOs, showing that the accessibility of the binding site is conserved in that domain and is independent from the surrounding mRNA sequence. However, when the binding site was moved to different positions within the domain, for example, into a rigid hairpin structure, then the potency of the oligonucleotide was adversely affected.

In the experiments described here, a large number of cDNA inserts, originating from EST sequences, and present within the sequence of a series of natural genes expressed in the H-1299 cell line, were cloned into the 3'-UTR of a series of reporter genes. Sets of ASOs targeting these insert regions were designed and characterised for their inhibitory antisense activity, both against the endogenous genes in the H-1299 cell line and against the reporter mRNAs in a non-human cell line. A correlation plot of the inhibitory activity in both cell lines revealed a very high correlation for the inhibitory activity over a large number of examples and it may be concluded that the secondary structure of the target segments is highly similar in both the endogenous message and also the reporter fusion. In turn, the results of the experiment strongly imply that the segments share an unaltered structure whether embedded within their natural endogenous mRNAs or within the 3'-UTR of an unrelated mRNA.

These observations are in line with the view that mRNA folding indeed occurs in relatively independent domains during transcription, as it is unlikely that similar structures of a given segment, as measured by the biological activities of large sets of ASOs, would result from a global minimum free energy after mRNA synthesis is complete. Furthermore, the results also imply that sequence-specific RNA-binding proteins are also relatively unaffected by surrounding segments and that long range RNA-RNA interactions do not unduly influence the effects of local structural elements on antisense activity.

Development of this assay has delivered the means to screen very large numbers of oligonucleotides in a high-throughput manner for inhibitory activity. Coupled with a novel cloning strategy, the screening can be extended to oligonucleotides against multiple genes. The assay fulfils the original requirement for an assay to rapidly identify the optimal, i.e. the most potent and selective, oligonucleotides to be used as tools for biological experiments. Combining this type of assay with the high-throughput synthesis of oligonucleotides, experiments can be designed to help discover the most important factors which determine the biological potency of oligonucleotides. This assay was developed using ASOs, but it also can be used with oligonucleotides operating via other types of mRNA degradation or alternative inhibitory processes, e.g. siRNA. Finally, the development of methods to

study the action of large numbers of oligonucleotides in parallel, sets the scene for an eventual whole genome knock-down using such reagents.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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