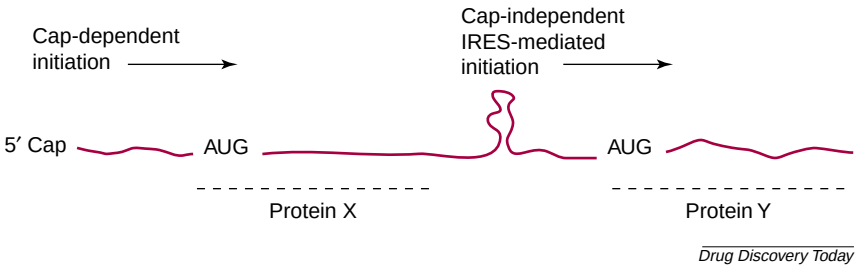

Figure 2. A dicistronic reporter gene contains two open reading frames, typically encoding two different reporter proteins (X and Y). The translation of one of these proteins is initiated by a cap-dependent mechanism. An internal ribosomal entry segment (IRES) could be inserted upstream of the start codon of the second reading frame, that is, in the intercistronic spacer region. Accordingly, the translation of this protein can be mediated by the IRES, that is, be initiated by a cap-independent mechanism.

Therefore, targeting IRES structures would enable the modulation of translation of distinct mRNAs in specific cell types without affecting protein expression in other tissues. This provides an additional level of drug specificity that is generally not achieved by targeting proteins.

For many years it has been known that aminoglycosides and other antibiotics act by binding to microbial RNA. However, it was not until recently that the concept of screening for small molecules with high affinity for a given RNA structure has been recognized by the pharmaceutical industry. Antisense oligonucleotides provide a valid concept for RNA modulation. However, such macromolecules possess several disadvantages as therapeutic drugs, not least in terms of bioavailability. Today, several pharmaceutical and biopharmaceutical companies are developing strategies to perform HTS for mRNA modulators. Several companies have already reached the stage of clinical trials with drugs that interfere with the translation of viral transcripts, for example, HIV and hepatitis C.

In a conventional HTS campaign, the resource-consuming procedures typically involve the production and purification of functional target protein and/or assay development. Translation reporter assays represent a generic concept and the assay set-up can be completed in a few days. A large number of reporter genes are available on the market, for example, growth hormone, luciferase and β-galactosidase. Transiently transfected cells can readily be used for HTS of low molecular-weight compounds and it is possible to generate highly reproducible data (J. Ekblom *et al.*, unpublished).

Dicistronic reporter genes typically encode a transcript that can be started at two different sites – a classical cap-dependent start site in addition to a cap-independent site initiated by an IRES (Fig. 2). The transcript will thus yield two recombinant proteins. Accordingly, the level of IRES-mediated translation can be expressed as a ratio to the cap-dependent translation. Dicistronic plasmid vectors encoding, for example, the enhanced blue and green fluorescent proteins (EBFP and EGFP, respectively), can be delivered into mammalian cells by transient or stable transfection and used as a translation reporter system. The cap-dependent translation will provide an excellent 'internal control signal', that is, it will detect non-specific inhibitors of translation. Translation reporter assays could, for instance, be used to screen for small ligands that specifically interfere with IRES-mediated c-myc translation in IRES-mutants in multiple myeloma cells, without affecting expression of this multi-functional protein in other cells.

It is likely that a large number of IRESes will be discovered and characterized in the next few years because of the development of powerful experimental tools for the identification of IRESes and the publication of the entire human genome sequence. Moreover, it can be assumed that the accumulating data on genetic variability will reveal mutations that cause disease in humans by interfering with translational regulation, such as in the case with c-myc. These RNA sub-domains could prove to be of great value as pharmaceutical drug targets.

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Antisense technology: inaccessibility and non-specificity ▼

Despite the simplicity and the elegance of the concept, antisense methods remain largely empirical. In principle, only the gene sequence is required for targeting with antisense reagents. In practice, it is far more demanding than this and almost every step in the application of these methods poses a problem.

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In the recent *Genomics* supplement to *Drug Discovery Today*, Taylor presented an excellent summary of the possible applications of antisense technology to the analysis of gene function and drug target validation[1]. I would like to draw further attention to two important challenges that we still face when using antisense methods, which demand considerable input before the full potential of this technology is realized.

One important problem is finding accessible sites in mRNAs, and this directly determines success or failure of an antisense experiment. Considerable evidence suggests that a major determinant of accessibility is intramolecular folding in mRNAs (of course, secondary structure in antisense oligonucleotides can also affect binding). Messenger RNAs, being single-stranded, can form intramolecular base pairs between complementary sequences, and there might be further tertiary interactions. This results in ~90% of the molecule becoming inaccessible. Finding the ~10% accessible sites is not easy and some sites might also be more or less accessible than others. Our understanding of RNA folding is limited, and the theoretical tools currently available are not sufficient to predict the folding of RNAs as large as mRNA. Furthermore, the mechanisms of heteroduplex formation between RNA and oligonucleotides are not well understood.

Several methods have been developed to find the accessible sites empirically. Those of particular interest employ the use of oligonucleotide libraries[2] and oligonucleotide arrays[3]. Whereas the first method is easy to set up in a laboratory with basic molecular biology expertise, it has several limitations. In principle, the most accessible site on an mRNA molecule would be the best target. However, it is not possible to find out the most accessible site using oligonucleotide libraries. The use of oligonucleotide arrays appears more successful, but requires considerable technical skill and resources for setting up and is mainly limited to the pioneering laboratory[4]. More work is, therefore, still needed to develop approaches that are easy to use and that also produce desired results.

Another big challenge is the unwanted side effects of antisense reagents. Although some of these effects can be related to the type of chemistries used to produce antisense reagents (e.g. phosphorothioates can bind non-specifically with cellular proteins), there are others that are of a more intrinsic nature. The ability of antisense reagents to bind only with target mRNA has never been proven because it is difficult to analyze the global effects within a cell, and these reagents can bind with the wrong mRNA resulting in unpleasant side effects. For example, the activity of most antisense oligonucleotides is mediated by RNase H (an enzyme with a natural role in DNA replication), which cleaves the RNA part of the RNA–DNA heteroduplex. It requires only short regions of homology (~7 bp) whereas most antisense oligonucleotides are ~20 bp in length. Thus, there are 14 shorter 7mer sequences in a 20mer oligonucleotide, with each one potentially capable of recruiting RNase H at a complementary accessible site on a non-target mRNA. However, the use of shorter oligonucleotides could compromise affinity for the target. Considerable research is being conducted into the use of reagents with chemistries that do not recruit RNase H but work by blocking translation, and also into those with mixed backbone and mixed bases[5]. However, there is still much to be done in this area to improve specificity and avoid the side effects associated with antisense technologies.

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Structural genomics: lessons to be learnt ▼

Over the past decade the pharmaceutical industry has embraced large-scale automation in perhaps two key areas: making and testing compounds. Indeed, the introduction of combinatorial chemistry and HTS into lead discovery allowed pharmaceutical company CEOs to boast to their shareholders about the numbers of compounds their companies could synthesize and test. In the 1990s, we became seduced by numbers and the profound belief that the 'factory' mentality could increase productivity in research. The ethos was: if we could make the compound cheaply and quickly then why not make and test it? There was less discussion on whether the compound was the right compound to make. It was assumed that the probability of finding an active compound was linear; if you made and screened 50-fold more compounds you would find 50-fold more hits. It is now questionable whether this approach improved productivity in finding new drugs, and many would argue that it did not. In the more far-sighted companies,

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