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PROSPECTS

Imaging Gene Expression Using Oligonucleotides and Peptide Nucleic Acids

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Abstract The development of methods for non-invasive, real-time imaging of gene expression would provide powerful tools for biomedical research and medical diagnostics. A broadly applicable strategy for achieving this goal is the use of complementary oligonucleotide probes for recognition of mRNA. The major challenge for molecular imaging is the development of specific and efficient transducers for signaling probe–target interaction. This review summarizes the strengths and limitations of reported molecular approaches for imaging of mRNA expression and discusses the challenges to development of in vivo methods. J. Cell. Biochem. 90: 437–442, 2003. © 2003 Wiley-Liss, Inc.

Key words: antisense; molecular beacons; molecular imaging; molecular probes; PNA; mRNA

The ability to monitor intracellular events in vivo provides important opportunities for basic researchers and clinicians. The main advantage of molecular imaging is the ability to study cellular events in real-time and in their in vivo context. As non-invasive imaging allows repeated analyses of animals, such experiments provide highly valuable longitudinal data.

Clinically, imaging of gene expression is of interest particularly in view of advances towards pharmacogenomic classification of disease. Imaging gene expression would reduce the need for invasive procedures such as explorative surgery and biopsy sampling and increase efficiency and speed of diagnostic testing. Moreover, as rationally designed and target specific pharmaceutical agents are developed, non-invasive monitoring of genomic activity could provide early signs of patient response to treatment.

Until now, however, molecular imaging has been largely confined to detection of proteins and reporter constructs. Detection of proteins as a general tool is problematic because of the need to develop suitable ligands on a case by case basis, a challenge at least as great as that for therapeutic drug development. Although reporter genes are of considerable value the need for engineered cell lines and animals is an obstacle for widespread use. Thus, the development of broadly applicable methods to detect endogenous gene expression would be of significant interest.

OLIGONUCLEOTIDES AS AGENTS FOR RECOGNIZING mRNA

Recognition of intracellular nucleic acids can be achieved with complementary oligonucleotides via Watson–Crick base pairing. Therapeutic antisense oligonucleotides bind to mRNA preventing post-transcriptional processing. As a class of molecule, oligonucleotides are well tolerated by animals and patients. Low toxicity and relatively low cost make it reasonable to envision that oligonucleotides can be applied to molecular imaging in vivo.

There are two key issues to consider in oligonucleotide design [reviewed in Braasch and Corey, 2002]. The first is oligonucleotide stability. As native phosphodiester nucleic acids are

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highly susceptible to nuclease mediated degradation, such constructs are of limited value for cell culture and in vivo use. The second matter involves the effect of oligonucleotide-target hybridization. DNA based oligonucleotides, when bound to RNA, are substrates for RNase H and cause degradation of the mRNA target. While this may be desirable for manipulating gene expression such action would be detrimental for imaging applications. Thus, nuclease resistant and non-nuclease inducing RNA oligonucleotides modified at the 2′-position (2′-*O*-methyl RNA) and the nucleic acid mimic, peptide nucleic acids (PNA), are of particular value in developing oligonucleotide based imaging probes.

A key advantage of oligonucleotide and PNA based probes is that ligands for mRNA are readily obtained through knowledge of mRNA sequence. When designed appropriately, high fidelity ligand–target binding can be achieved. Thus, oligonucleotides and PNAs are versatile tools for recognition of nucleic acids. As such, strategies to monitor gene expression have been predominantly oligonucleotide based. Here, we present a review of the principle, milestones, and limitations of reported molecular approaches for real-time, non-invasive imaging of endogenous nucleic acids.

Labeled Antisense Oligonucleotides

In vitro, mRNA levels can be detected by northern analysis using radiolabeled oligonucleotides. This commonly used technique has led to the hypothesis that radiolabeled antisense oligonucleotides can be used as probes for non-invasive imaging of gene expression in vivo. This straightforward strategy utilizes the high affinity of antisense oligonucleotides for their complementary targets (Fig. 1A). The problem with the approach is that intracellular entry of oligonucleotides is not dependent on the presence of target. Thus, detection of signal may not be tightly associated with expression of target genes.

It is possible, however, that oligonucleotides pass reversibly through membranes, and that the presence of target mRNA will result in a detectable increase in intracellular probe retention. Using an established tumor mouse model Dewanjee et al. [1994] observed greater tumor content of ^{111}In-labeled antisense molecules against c-myc mRNA relative to sense controls. Unfortunately, the study employed a

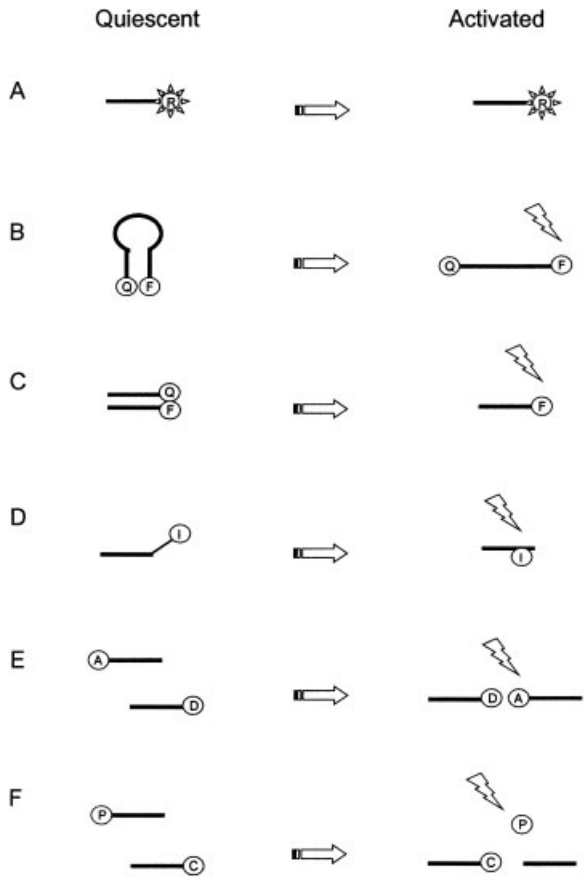

Fig. 1. Schematic representation of molecular approaches for detecting intracellular nucleic acids (**A**–**F**). A: Radiolabeled (R) oligonucleotide probes emit signal in the presence and absence of target sequences. Experimentally, the cause of higher signal detection in the presence of target transcripts has been inferred as greater intracellular retention of probes. B: Molecular beacons. The molecular beacon unfolds and hybridizes with target nucleic acids, increasing the distance between the quencher and fluorophore and increasing fluorescence. C: Duplex oligonucleotides. A target nucleic acid competitively hybridizes with either strand disrupting the quencher–fluorophore complex. D: Oligonucleotide probes chemically tethered to an intercalating optical moiety (I) produce low levels of light emission. Following binding of the oligonucleotide probe to the target, the signaling molecule intercalates the newly formed duplex to produce light of significantly greater magnitude. E: Oligonucleotide probes designed against adjacent target sequences, when simultaneously hybridized, approximate a 5′-donor (D) molecule of one oligonucleotide with a 3′-acceptor (A) molecule of an adjacent oligonucleotide. In this application, excitation of the donor moiety results in fluorescence resonance energy transfer (FRET) to the acceptor resulting in light emission of the acceptor wavelength. F: ''Prodrug'' strategy involving covalent attachment of a 5′-catalytic (C) molecule to one oligonucleotide and a 3′-prodrug (P) to an adjacent oligonucleotide. When linked to the oligonucleotide the prodrug is maintained in an inactive, non-fluorescent state. In the presence of a target sequence, the catalyst causes cleavage of the prodrug and increases fluorescence.

phosphodiester DNA oligonucleotide that would be expected to degrade rapidly and recruit RNase H, degrading target transcripts. Urbain et al. [1995] obtained similar results in cell culture studies and proposed that the greater signal observed with labeled probes relative to sense controls is due to higher levels of cellular retention of antisense oligonucleotides following hybridization to target mRNA.

More recently, Pardridge et al. have employed nuclease resistant antisense PNAs that do not recruit RNase H upon binding to mRNA. These studies reported that conjugates between PNAs labeled with ^{125}I and peptides that bind to the transferrin receptor showed a threefold higher level of signal in mouse brains expressing the target gene [Lee et al., 2002]. These findings are encouraging and emphasize the need for careful design of probes. However, more data will be needed on the trafficking of oligonucleotides in and out of cells and additional studies are required to substantiate the sensitivity of this approach.

Molecular Beacons

To increase signal to noise ratio, it would be ideal if hybridization was a prerequisite for production of signal. The approaches described below have been designed to this end. Of these, molecular beacons are the most advanced strategy. First described by Tyagi and Kramer [1996], molecular beacons consist of a hairpin oligonucleotide labeled with a fluorophore at one terminus and a quencher at the other. In the unbound or inactive folded state the proximity of the quencher to the fluorophore prevents light emission in the presence of an excitation light source. Upon hybridization to the target sequence the bound and active unfolded oligonucleotide results in released sequestration of the quencher on the fluorophore permitting light emission following excitation (Fig. 1B).

The advantage of molecular beacons is that observation of signal requires that the probe hybridize to the target mRNA. Probes entering cells that lack target expression or control probes should remain quiescent. Two laboratories micro-injected molecular beacons and complementary RNA and cDNAs into single mammalian cells and used fluorescence microscopy to demonstrate binding of target sequences [Sokol et al., 1998; Perlette and Tan, 2001]. In the study by Gewirtz et al., the predicted sensitivity of detection was as few as ten molecules of mRNA per cell. Problems with the probes used in these studies, however, are that they are susceptible to nuclease degradation and form DNA–RNA hybrids that can lead to RNase H mediated degradation of target nucleic acids. To avoid these problems the synthesis of functional 2′-*O*-methyl RNA (a chemistry that resists nuclease degradation and does not support RNase H cleavage) molecular beacons has been reported [Tsourkas et al., 2002]. However, the ability of quiescent probes to retain their stem loop configuration may be compromised inside cells [Molenaar et al., 2001]. Molecular beacons composed of PNA bases may be of value in this regard [Kuhn et al., 2002].

Duplex Oligonucleotide Probes

A concept that is closely related to molecular beacons involves the use of complementary DNA oligonucleotides, one strand covalently attached to a 5′-fluorophore and the other to a 3′-quencher (Fig. 1C). While duplexed, in the quiescent state, the optical moieties are in close proximity, emitting low levels of light. Similar to the above mentioned hairpin beacons, the duplex undergoes a conformational change in the presence of nucleic acids complementary to either probe strand. This competitive hybridization disrupts the interaction between the fluorophore and the quencher and increases light emission. In solution, rapid hybridization of the duplex probe to target DNA in the low picomolar range was observed [Morrison et al., 1989]. Using a similar design corresponding results were reported by Sixou et al. [1994] who also demonstrated detection of intracellular fluorescence following microinjection and dissociation of duplex oligonucleotide probes. It is not obvious if this approach confers any advantages relative to molecular beacons, but it does provide an additional option for optimizing probes for in vivo use.

Intercalating Oligonucleotide Probes

Another potentially discriminating approach for optical imaging combines the use of a single stranded oligonucleotide probe with a covalently attached molecule that undergoes a fluorescent change following binding to mRNA (Fig. 1D). Cyanine dyes such as thiazole orange are relatively poor optical transducers but produce increased fluorescence of up to 50-fold following intercalation of double stranded

nucleic acids. Using nuclease and protease resistant and non-nuclease inducing PNAs, chemically tethered to thiazole orange, PNA-target hybridization was shown to result in sequence specific enhancement of light emission relative to free dye [Svanvik et al., 2000]. As flexibility of the tether is required the neutral charge of the PNA backbone is a particularly useful characteristic in this application minimizing the potential for probe-dye binding due to electrochemical affinity of the cationic dye for negatively charged phosphate backbone oligonucleotides [Svanvik et al., 2001].

Using modified DNA oligomers with an alkyl linkage chain to thiazole orange, Privat et al. [2001] localized poly adenine nucleic acids in the cytoplasm and nucleus of fixed osteosarcoma cells. The availability of intercalating fluorophores with greater nucleic acid affinity, more desirable optical emission properties and more intricate chemical approaches for integrating dyes with oligonucleotides are likely to further advance this approach.

Adjacent Linear Oligonucleotide Probes

Other approaches for detecting gene expression employ two oligonucleotides each complementary to adjacent sequences on target nucleic acids. The advantage of these strategies is that signal emission requires that both oligomers bind the target, greatly increasing the stringency of target recognition and output specificity. The requirement that two different oligomers must enter cells and bind mRNA may complicate recognition, but the advent of efficient hybridization options like PNA and 2′-modified RNA reduces this concern.

The first report of this strategy was described by Cardullo et al. [1988] who labeled two oligonucleotides complementary to contiguous target sequences, one with a 5′-donor and the other with a 3′-acceptor chromophore (Fig. 1E). Upon hybridization, light emission characteristic of the acceptor optical molecule was observed following excitation of the donor. In this fluorescence resonance energy transfer (FRET) the quantum energy resulting from excitation of the donor molecule by an external light source is propagated to an acceptor fluorophore up to 10 nm removed [for review see Didenko, 2001]. In this non-photon emitting interaction, the donor molecule is quenched and the acceptor moiety is excited resulting in emission of light of the acceptor wavelength.

Although acceptor and donor chromophore positioning on oligonucleotide termini were interchangeable, FRET was highly dependent on the intervening space between probes. Too great a distance resulted in loss of energy transfer and inadequate separation resulted in quenching of fluorophores [Cardullo et al., 1988; Okamura et al., 2000]. While probe separation of a few nucleotides was generally viable the optimal distance varied with donor and acceptor pairs as did efficiency of FRET [Okamura et al., 2000].

A modification to enhance probe signal was demonstrated by labeling of the donor probe with two fluorophore molecules [Okamura et al., 2000]. Double labeling of the acceptor probe yielded reduced FRET efficiency. In further attempts to improve signal to noise fluorescence, minimizing the effects of non-specific photon emission from biological autofluorescence and light emanating from non-hybridized probes, Tsuji et al. [2001] reported the use of time-resolved fluorescence decays in determining hybridization events. The principle requires use of donor chromophores with longer fluorescence half-lives than that of acceptors such that specific donor–acceptor FRET interaction results in a longer excitation of the acceptor molecule relative to direct excitation of the acceptor and specimen autofluorescence. Such efforts were prompted following demonstration of FRET-based adjacent oligonucleotide detection of intracellular c-fos mRNA in a human cell line [Tsuji et al., 2000].

Nucleic Acid-Triggered Catalytic Drug Release

Another elegant strategy for using adjacent oligonucleotides employs catalytic activation of a prodrug in the presence of a target nucleic acid sequence [Ma and Taylor, 2000]. The catalytic moiety comprised of an imidazole, a catalyst for hydrolysis, at the 5′-end of one oligonucleotide and the prodrug component consisting of a *p*-nitrophenol ester at the 3′-end of a downstream binding oligonucleotide (Fig. 1F). Sequence-specific hybridization of the oligonucleotide probes approximated the prodrug and catalytic constituents, causing release of *p*-nitrophenol.

Originally intended as a cell-specific drug delivery system, the authors recognized the potential of the model as an imaging tool. As such, Ma and Taylor [2001] employed their strategy using diacetyl fluorescein, non-fluor-

escent molecules in their native state which, following hydrolysis, exhibit light emitting properties. In the presence of target nucleic acids imidazole linked oligonucleotides were shown to activate fluorescein dipivalate to the excited optical state. A further advance was in establishing attachment of PNA and non-fluorescent ester-linked 7-hydroxycoumarin as the prodrug and an adjacent upstream target binding PNA attached to imidazole. In the presence of target sequence, the rate of 7-hydroxycoumarin release, now in its free fluorescent state, increased in a time-dependent manner [Ma and Taylor, 2003]. This rate of drug release decreased with increasing intervening nucleotides between the two oligonucleotide probes. Importantly, efficacy of catalytic drug release was demonstrated for linear DNA and folded RNA targets.

PROSPECTUS

An important goal for molecular imaging is the development of sensitive, real-time, non-invasive methods for assaying endogenous gene expression. The recognition of mRNA by oligonucleotides or PNAs is a tested practice for achieving this goal. Moreover, the generality of these molecules allows for the potential of monitoring the expression of any gene.

The challenge for the field is to transform this promise into practical tools for experimental science and diagnostics (Table I). Fundamental needs include understanding the mechanisms that regulate cellular uptake and subcellular distribution of oligonucleotides as well as improved pharmacokinetic properties, challenges also present in the therapeutic antisense field.

TABLE I. Challenges for Imaging Endogenous Gene Expression In Vivo

Pharmacokinetics
Understand mechanisms of cellular uptake of oligos
Development and use of oligos with appropriate subcellular localization
Development of oligos with favorable biodistribution characteristics
Signal detection
Advancing molecular approaches to improve signal to noise output
Development of optical moieties with advantageous excitation and emission properties
Design of internally activated probes
Establishing non-optical imaging moieties
Improving hardware sensitivity and spatial resolution
Chemistry
Synthesis/conjugation of oligonucleotides and signaling moieties
Development of biologically compatible probes to facilitate progress from in vitro to in vivo uses

There are also specific obstacles for oligonucleotide-based imaging probes. One is identifying signaling moieties robust enough for in vivo use. A number of technological developments may be of value in this. Among these are near-infrared fluorochromes, the use of which may circumvent absorption of chromophore light emission by tissues and blood components [Waddell et al., 2000]. Biocompatible, semiconductor nanocrystals, also known as quantum dots, are particularly attractive for their high quantum yield and resistance to photobleaching [Watson et al., 2003]. Moreover, advances in hardware may provide further augmenting of sensitivity and spatial resolution [Coe et al., 2002].

The greatest strides in this endeavor, however, may ultimately come from a movement away from optical imaging of gene expression to the use of modalities, which are not dependent on light penetration. Oligonucleotides have been covalently attached to superparamagnetic iron oxide crystals and sequence-specific signaling demonstrated through magnetic resonance imaging [Perez et al., 2002]. The iron oxide cores of these nanosensors increase spin relaxation times of surrounding water protons so as to serve as magnetic relaxation switches. As nontoxic labels of 3 nm in size such moieties may be ideally suited for biological applications.

Successful in vivo imaging of gene expression will necessitate interaction among diverse scientific disciplines. Chemistry will be needed for the synthesis of improved molecules and physics needed for their design. Physiology will be needed to guide in vivo studies, while molecular biology will govern selection of appropriate target genes. However, success demands more than clever concepts. Ultimately, it will require good experimental execution. In particular, stringent control experiments must be included to produce unambiguous results.

The challenges described above are significant. However, with rapid advances being made in molecular imaging and with in vivo application of oligonucleotides and PNAs, one may envision that molecular imaging will impact biology and experimental therapeutics in a manner similar to the impact of informatics on genomics.

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