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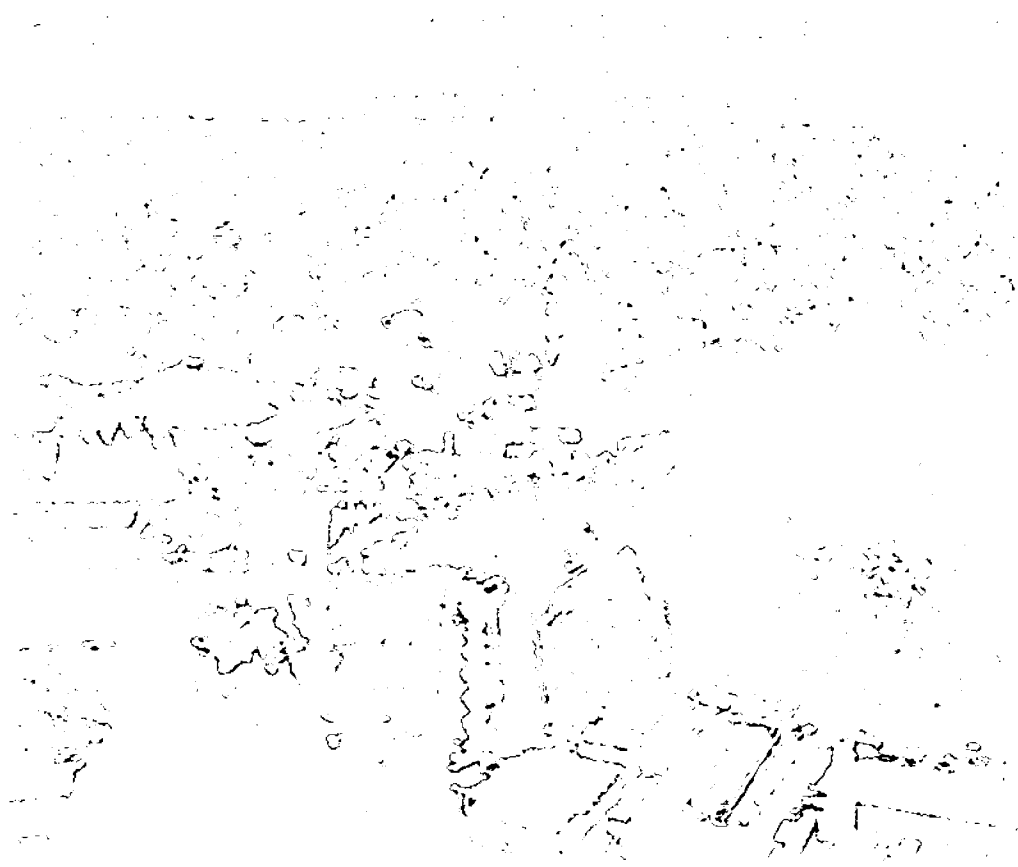
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## Prospective on the Potential of Imaging Gene Expression

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**Life Sciences Division**

June 2000



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# **Prospective on the Potential of Imaging Gene Expression**

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June 2000

# **Prospective on the Potential of Imaging Gene Expression**

## **Introduction**

Recently, much interest and speculation has been directed towards the use of non-invasive nuclear medicine imaging techniques to monitor gene expression. Many of the ideas expressed represent a shift in the strategy for nuclear imaging, with the suggested target being the actual messenger ribonucleic acid (mRNA) of specific genes, instead of the more classical approach of targeting the final products of gene expression, receptor, structural or enzymatic proteins, for imaging.

The application of antisense technologies, in which oligonucleotides can be easily generate that would have high specificity for targeted mRNAs, is a tempting, new approach to understanding changes in gene expression that occur in human diseases. Some see this as the logical next step in the utilization of the enormous information provided by the Human Genome Project. But the inherent difficulties of targeting mRNA, it being an intracellular target, its transitory nature, and its commonly extremely low cellular concentration (as few as 1-100 copies per cell) (Zhang et al. 1997) as compared to the levels of the final gene products, must be understood and overcome before we can seriously consider imaging gene expression with PET or SPECT at the level of gene transcription.

## **Factors to Consider in Imaging Gene Expression**

The potential of noninvasive detection of gene expression by direct measurement of mRNA expression rather than through protein detection is determined by several factors:

1. Specific activity of the radiopharmaceutical probe;
2. Affinity of the radioligand for the target mRNA
3. Number of mRNA copies per cell;

4. Concentration of cells expressing the specific mRNA;
5. Sensitivity of the imaging instrumentation;
6. Background or nonspecific accumulation of the radiopharmaceutical;
7. Ability of the radiopharmaceutical to reach the intercellular target;
8. Prevention of degradation of mRNA after hybridization; and,
9. Potential for amplification of the radiolabel.

All of these factors have major importance in the development of new technologies directed towards imaging gene expression utilizing antisense oligonucleotides as radioligands. They must all be understood to fully answer the two important questions that may limit the applicability of this approach: are there enough copies for the known specific activities to allow reliable quantitation in a reasonable time, and secondly, what will be the background?

### **Direct Imaging of mRNA**

One likely candidate as a ligand for the direct imaging of mRNA would be radiolabeled antisense with a complementary sequence to the mRNA. Numerous antisense molecules have been developed to bind to specific mRNAs, and modified oligomer backbones have been created to increase *the in vivo* stability of these oligonucleotides. All of this knowledge has been applied to the development of antisense-based radiolabeled oligonucleotide tracers with limited results (Dewanjee 1993, Hnatowich et al. 1995) but further questions still arise. The mechanisms and kinetics of transport of antisense to their intracellular mRNA targets are not fully understood, and could limit the use of short-lived positron emitting isotopes. The subcellular compartmentation of the antisense may severely limit the delivery of the radiopharmaceutical to its target, and could also increase the levels of non-specific binding if these compartments are slow to clear. Other pools for non-specific binding include extracellular peptides, especially albumin and thrombin, and cell surface proteins. If the antisense reaches the nucleus, it may bind (non-specifically) either DNA or protein instead of the targeted mRNA. The nuclear protein pool may be very problematic, as it has been estimated to have a concentration of antisense binding sites at above 1  $\mu\text{M}$  and could totally mask the binding by mRNA. The therapeutic mode of action of the antisense may also cause problems, as the binding of the

antisense to the mRNA is thought to cause the mRNA to be degraded by dimer-seeking RNAses (see Lebleu et al. 1997 for review).

Taking these factors into consideration, one can estimate the feasibility of using antisense as an imaging agent. The strategy for this calculation was to incorporate into a set of operational equations all of the parameters above and the sensitivity of the instrument to arrive at estimates of the reliability of a quantitative value derived from PET or SPECT studies. The parameter definitions for these formulas are:

- S:** specific activity (Curies per mole)
- n:** concentration of cells (cells per ml)
- m:** mRNA copies per cell
- A:** Avagadro's number ( $6.022 \times 10^{23}$  )
- Ci:** Curie ( $3.7 \times 10^{10}$  disintegrations per second)
- C:** contrast or target to background ratio
- rms:** root-mean-square uncertainty (%)
- t:** time (seconds)
- z:** activity per ml (dps/ml))
- f:** sensitivity of instrument (events.second per dps.ml in tissue)
- r:** radius of the transverse section (cm)
- r<sub>2</sub>:** radius of the target (cm)

The two basic equations that govern this estimate are:

$$z = S \times Ci \times (n \times m / A) \quad (1)$$

$$rms = 120 \times (T)^{1/4} / (T/W)^{3/4} \quad (2)$$

Where **T** is the total counts detected:

$$T = f \times (z \times [(\pi r^2) - (\pi r_2^2)] / C) + z \times (\pi r_2^2)$$

and **W** is the total number of resolution elements (assumed to be 1 cm x 1 cm in this calculation):

$$W = 2\pi r^2$$

The minimum activity per ml can be calculated based on the number of mRNA copies (assume 10 per cell), the specific activity of the radiopharmaceutical (assume  $10^6$  curies per mole), and the concentration of cells (assume  $2 \times 10^8$  per ml). Under these conditions, one can expect 7400 disintegrations per min per ml of cells. With a modern 3D instrument this translates to a detection event rate of about 240 events per minute for each target ml. With the assumption of a target to background ratio of 2, this level of tissue activity could be quantitatively evaluated by a PET instrument with 7 mm resolution with 10 min of data collection and with an uncertainty of 20 percent.

It is important to note that even if the copy number is much higher than a few per cell, the background activity is likely to overwhelm the image as the mRNA will be saturated at a local concentration of only  $0.0033 \mu\text{Ci/ml}$ . Note that 1 mCi distributed uniformly throughout the body would be an average concentration of about  $0.015 \mu\text{Ci/ml}$ . It may be possible to reduce the background by either allowing for clearance of non-specific by delaying the time after injection before acquiring images, or reducing the specific/non specific signal by greatly reducing the dose injected (possibly as low as a 0.3 mCi injection). This requires optimization of the imaging times, as a balance has to be struck between the biological washout rate of the background activity, the washout rate of the mRNA-bound activity, and the radiological decay of the tracer bound to both the target and background material. If these approaches aren't successful, biochemical methods of to amplify the signal will need to be developed to overcome this problem.

## **Amplification of the mRNA Molecule's Signal**

One approach to overcome the limitations on imaging imposed by low copy number is to find a means of amplifying the signal within the cell. One potential method would be the use of engineered biomolecules that would react with specific mRNA, resulting in the activation of a process that would modify radioligands to facilitate their retention within the cell. Such a change in retention can easily be detected by both dynamic and static nuclear imaging techniques

Application of such an approach is currently used in numerous systems that track by in vivo imaging expression of specific genes introduced by gene therapy by the co-introduction of "reporter genes". Examples of such applications include incorporation of genes coding for  $\beta$ -



galactosidase (Mohler and Blau 1996), Herpes-simplex virus type I thymidine kinase (Tjuvajev et al. 1995; Gambhir et al. 1998) and cytosine deaminase (Stegman et al. 1999). Such enzymes are useful in imaging because they can modify chromophores or radioligands via hydrolysis, phosphorylation or deamination so that the cell retains these imaging agents. Another approach is to incorporate a gene coding for a specific receptor, such as a dopamine receptor. Expression of this receptor within a cell would result in enhanced retention of an exogenously applied dopamine-like radioligand (MacLaren et al. 1999). All of these methods are biologically invasive, as they require the modification of the existing genome by the introduction of these new genes.

This methodology may be adaptable to a system where specific mRNA can be imaged without the incorporation of new genes in the targeted tissues. Instead, enzymatic complexes could be modified so that they would not become active until they interacted with a specific mRNA. If they were delivered to tissue containing that specific mRNA, their activation could be then exploited to modify an applied radioligand to increase its cellular retention.

One example of such a procedure would be the adaptation of the complementary  $\beta$ -galactosidase system developed by Blau and her colleagues (Mohler and Blau 1996, Rossi et al. 1997). They have constructed deletion mutants of the enzyme from the *E. coli lacZ* gene, in which selected regions of the protein are missing, resulting in loss of enzymatic activity. Activity can be restored by the combination of two different mutants that have together a complete set of all of the essential domains of the enzyme. This technique has been used to follow dimerization of cellular peptides, receptor molecules, and complementary DNA. In this procedure one inactive  $\beta$ -Gal deletion mutant is attached to a peptide (or DNA), and a different inactive mutant  $\beta$ -Gal is attached to the second peptide (or DNA). When the two peptides or DNA molecules dimerize, the interaction of the two  $\beta$ -Gal deletion mutants results in the formation of a stable hetero-octameric enzyme with  $\beta$ -galactosidase activity (Mohler and Blau 1996). This activity can then be detected with a fluorogenic or chromogenic substrate for the enzyme (e.g. 5-Br-4-Cl-3-indolyl- $\beta$ -galactopyranoside).

This procedure could be adapted for use in PET imaging to target the expression of a specific gene. In this model, two different antisense molecules that are complementary to two distinct regions of the targeted mRNA would be attached to two of the  $\beta$ -Gal deletion mutations,  $\Delta\alpha$   $\beta$ -Gal and  $\Delta\omega$   $\beta$ -Gal. These would have to be constructed in such a manner that both (inactive) antisense- $\beta$ -Gal complexes bind to the targeted mRNA in a manner that would allow for the

creation of an active  $\beta$ -Gal complex, as illustrated below in figures 1 and 2. Thus the formation of even a single mRNA  $\beta$ -Gal complex could result in the accumulation of multiple radiotracer molecules, and an amplification of the signal.

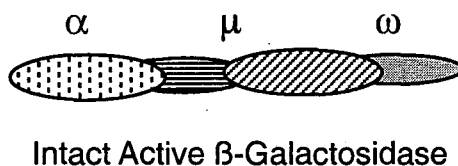


Figure 1. Diagram of the intact  $\beta$ -galactosidase protein, showing the three regions ( $\alpha$ ,  $\mu$ ,  $\omega$ ) that are sites for deletion mutations.

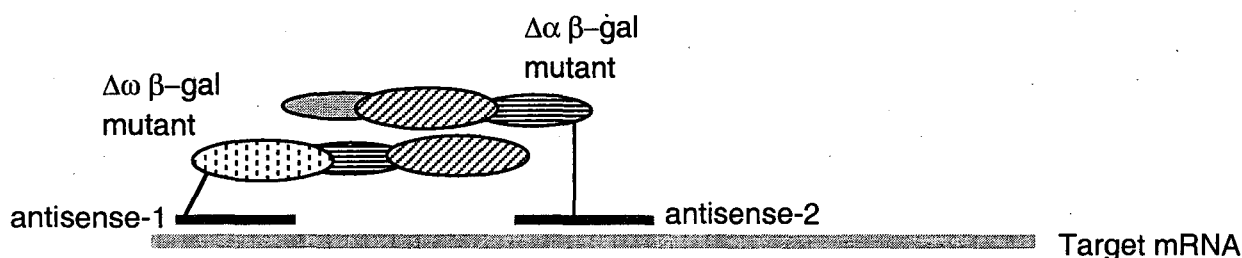


Figure 2. Conceptual depiction of oligonucleotide antisense molecules joined with  $\beta$ -galactosidase deletion mutants to amplify the signal obtained from a specific mRNA. The antisense molecules target two separate sites on the targeted mRNA, and will be used to recognize the specific mRNA and to anchor the deletion mutant enzyme peptides in place. When the two  $\beta$ -Gal peptides interact, providing a complete set of domains necessary for enzymatic activity,  $\beta$ -galactosidase activity will be restored. Introduction of the proper radiotracer will then allow for the entrapment of the radioisotope via interaction with the  $\beta$ -galactosidase

Two additional components of this procedure need to be determined: the delivery system for the transport of the antisense- $\beta$ -galactosidase complex into cells, and the proper radiotracer that would accumulate in cells following interaction with active  $\beta$ -galactosidase.

Delivery of the antisense- $\beta$ -galactosidase complex may be possible to via liposomes, or by the attachment of another component that would be recognized by a cellular uptake system (e.g. LDL, folate, polyamines, biotin) to facilitate their transfer across the plasma membrane. This later approach may prove successful with the incorporation of a HIV-1 Tat protein fragment into the amplification molecule. The basic domain of this peptide has recently been shown to enhance

translocation of exogenous Tat into certain cells. In addition, this Tat-derived peptide, when attached to other proteins, has been found to facilitate the uptake of these proteins into cells (Vives et. al 1997).

A new radiotracer that takes advantage of the  $\beta$ -Gal activity will also have to be developed. This new imaging agent will be a substrate for the  $\beta$ -galactosidase, and upon hydrolysis by the enzyme will need to undergo a modification that will result in its retention within the cell. Similar, nonradioactive  $\beta$ -galactosidase substrates are currently available that are residualized within a cell following hydrolysis by the enzyme. These compounds would be the starting point for the development of new radiolabeled  $\beta$ -galactosidase substrates that could be used for *in vivo* imaging.

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