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Introduction: Antisense Oligonucleotides: Strategies and Successes

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New approaches to examining the nervous system are applicable to studies of the chemical senses. Various technical interventions have been developed to alter or 'delete' a gene product to provide insight into the role of a particular macromolecule during development, during cell–cell signaling or in a behavioral context. Antisense oligodeoxynucleotide (ODN) delivery to the cell nucleus prevents translation of an mRNA of interest, thus eliminating the protein product from the cell. This approach offers certain advantages over other technologies that modify gene products. In theory, expression of proteins of interest may be decreased at will at any time by introduction of an antisense ODN that interferes with synthesis of the protein from its mRNA. In its optimal embodiment, antisense 'knockout' of a protein through eliminating its cognate mRNA is reversible, and the strategy could be used in a range of species at any time during the development or life of the animal. At the desired time point, gene blockade could be tested for its effects on development, on responses to particular signals, or on learning or behavior before or after a training paradigm. This approach circumvents some of the disadvantages of other technologies that modify protein levels. For example, transgenic techniques have been widely utilized to introduce new genes that result in a 'knockout' or 'knock-in' animal. Genes are deleted or replaced by swapping new genetic material for an endogenous gene through homologous recombination events (van der Neut, 1997). The new construct may delete the reading sequence of the target gene or contain a stop signal to eliminate translation into protein, or produce a truncated, non-functional protein product. However, production of 'knockouts' is technically demanding, time-consuming and expensive. Results are sometimes disappointing, yielding deficiencies that are lethal to the embryo so that no viable animals are born. Further, gene deletion sometimes appears to have no apparent consequences, perhaps because the protein product is redundant or because alternative pathways activated during development replace the function of the missing gene or increase expression of a compensatory gene(s).

'Antisense' technology potentially is inexpensive, straightforward, and readily adaptable to almost any gene and any animal species. ODN sequences complementary to regions of a particular mRNA (or 'antisense') are synthesized and introduced into the cell. These form duplexes with native mRNA of interest, suppressing translation through mechanisms that are still not entirely clear but probably involve degradation by nucleases. Advantages of this approach are that it is relatively simple to synthesize appropriate antisense sequences. Further, antisense ODNs can be used both *in vitro* in cell cultures or tissue slices and at chosen times *in vivo* to lower levels of gene expression under defined trial conditions to test the role of the gene. Difficulties raised by trying to suppress gene products that are essential for development can be bypassed by intervening at later time points to test the role of these proteins in various biological processes. Compensatory biological responses and mechanisms that may confound the interpretation of 'knockouts' are less of an issue under these circumstances.

However, realization of the promise of antisense technology in research and therapy has not been rapid. The approach requires a carefully controlled, systematic examination of ODN design and presentation as well as extensive controls. Nonetheless, recent advances have led to increased optimism about its use (Leslie and Jones, 1997; Roush, 1997; Wagner and Flanagan, 1997).

The pay-off for developing successful ways of inhibiting expression of particular genes is in discovering the biological role of specific products in development, for example, signaling, behavior, developing therapies for treatment of cancer, viral or bacterial infections, or counteracting undesirable autoimmune reactions. A number of reviews have considered various aspects of this technology (Wagner *et al.*, 1993; Stein and Narayanan, 1994; Wagner, 1995a,b; Gewirtz *et al.*, 1996; Wagner and Flanagan, 1997).

No hard-and-fast guidelines exist for designing effective antisense ODNs. A number of articles deal with the development of first- and second-generation compounds. These were engineered to deal with problems associated with non-specific binding of ODNs to nucleotides other than target mRNA or to proteins. Synthetic modifications have been introduced to enhance ODN stability, and to facilitate ODN entry into the cell and nuclear compartments as well

as avoid toxicity (e.g. Altmann *et al.*, 1996; Akhtar and Agrawal, 1997; Crooke, 1997).

In designing experiments, the following recommendations apply (compiled from Dean *et al.*, 1996; Crooke, 1997):

1. Directly measure target mRNA or protein levels to determine ODN effectiveness.
2. Screen several ODNs spanning the mRNA, not just regions around the start site. Perform dose–response curves on a model system (e.g. a cell line expressing the gene of interest) to select among ODNs targeting several regions of the gene and to choose the best dose, decide on the optimal delivery method and evaluate the time course of the effect.
3. Compare rank-order potency of the effect on the phenotype (or pathway or biological parameter of interest) with that required to knock down gene expression to insure that ODN action is through an antisense mechanism.
4. Evaluate specificity by comparing effects on closely related gene products and by using control ODNs (e.g. sense, scrambled and mismatch sequences).
5. If more than one active ODN is identified, show that these give consistent functional effects.

Experimental constraints on the use of antisense include the fact that in practice even effective antisense ODNs often only reduce mRNA translation or targeted protein levels, rather than eliminate them completely. Thus antisense technology sometimes is called a 'knockdown' method. The amount of 'knockdown' achieved is often in the range of 30–70%, as in the examples given below. Nonetheless, as noted in the examples, this may be sufficient to alter biological events.

Targeting

Targeting ODNs to the desired cell or tissue and delivering sufficient amounts to the appropriate site in the cell often have proved difficult. An important consideration in deciding to use antisense in any given system is whether the extent and period of suppression of gene expression are meaningful within the time frame of the response or effect to be monitored. If protein turnover is very slow, blockade of mRNA translation will not rapidly down-regulate the protein in that cell. This can be monitored experimentally.

Antisense methods have been used both *in vitro* and *in vivo*, but because of potential problems in delivery they have been used most widely and successfully in cell cultures or slice systems. A variety of delivery systems have been used, including direct injection into cells or nuclei (Wagner *et al.*, 1993), addition of naked ODNs, transfection (Goetzl *et al.*, 1994), packaging of ODNs in liposomal vesicles (Akhtar and Agrawal, 1997), retroviruses (Galileo *et al.*, 1992) and transgenic vectors (Katsuki *et al.*, 1988; Khillan *et al.*, 1994; Leslie and Jones, 1997). The limitation of the direct injection approach, which provides specificity, is that relatively few cells can be evaluated. Addition of free antisense ODNs relies on uptake across the cell membrane to generate concentrations inside the cell sufficient to bind to all of the targeted cellular mRNA. In the intact organism, effective antisense methodology requires getting the ODN into the cells of interest in the context of a complex environment. Particular questions about *in vivo* delivery focus on whether the ODN will make it to the desired site at concentrations high enough to be effective. Pfaff's experiments provide one approach to this problem, restricting delivery to a local region of the brain (see Ogawa and Pfaff, 1998). To some degree, targeting can be achieved by small injections of an ODN which has limited access to cells beyond a certain diffusion range or by delivery to axon terminal areas where it is directed to the cell bodies by retrograde transport (Ji *et al.*, 1994). Other targeting strategies have encapsulated ODNs in cationic liposomes (Dean *et al.*, 1996; Gewirtz *et al.*, 1996) or used other delivery vehicles that recognize targeted cells (Rensen *et al.*, 1997), providing more selectivity for systemically administered agents (Akhtar and Agrawal, 1997).

Stability

In addition to delivery of the ODN, there are questions of stability and temporal availability. Will the reagent be stable in culture medium, body fluids, blood or CSF for a period sufficient to provide adequate uptake, will it be scavenged by other tissues and will it resist degradation in the cell (Crooke, 1997; Summerton and Weller, 1997)? Breakdown of the DNA (RNA is not stable enough to consider, as RNases are everywhere) must be considered, or design of analogues more resistant to degradation. Analogue evaluation must include screening for possible alterations or decreases in hybridization affinity or specificity of any modified forms of DNA.

Drug design

Many antisense ODNs are designed to bind to the mRNA initiation site for translation, with the thought that they will block the access to appropriate enzymes and factors and perhaps trigger degradation. But other elements may be at work, including altered susceptibility of the mRNA to degradation when it is in different configurations induced by antisense ODN binding. Modifications of ODN structure have been designed to optimize interactions with RNA rather than DNA or protein and to resist elimination through breakdown by nucleases or scavenger mechanisms (Buhr *et al.*, 1996; Bhan *et al.*, 1997; Bijsterbosch *et al.*, 1997).

Magnitude and duration of effect

Considerations about the temporal properties of the process under consideration may indicate whether antisense methods are appropriate. For achieving effects that are slow to develop, it may be necessary to suppress protein

concentrations for an extended period. Turnover rates vary widely among proteins, and the depression of protein to levels sufficient to alter the biological outcome is presumably related to the availability of that particular protein and whether it is rate limiting. Some receptors, for example, are present in great excess over what is needed to activate signal transduction. However, down-regulating the receptor protein by 50% may be sufficient to alter cellular responses (e.g. Mattson *et al.*, 1993). Pathways activated by neurotransmitter (or odorant or tastant) can be probed by interfering with levels of a particular receptor or G protein, an ion channel, or hydrolase or kinase. Depressing the levels of each presumptive component might give insight into the contribution of the macromolecule in question. On the other hand, if induction of a new molecule must take place or the model involves regeneration of neurons or taste buds, or formation of new synapses, prolonged down-regulation of the suspected regulatory protein is required.

Experimental applications of antisense ODNs

Antisense ODN approaches have been applied to both *in vivo* and *in vitro* systems. Antisense effects are most easily validated *in vitro*, as in cell-free systems and in cell culture. Some examples are described below.

In vitro

Role and colleagues used antisense ODNs to various nicotinic acetylcholine receptor subunits to define how these contribute to nicotinic ion channel properties in neurons cultured from chick embryo. Blockade of expression of particular subunits identified which subunits contribute to functional ion channels (Yu *et al.*, 1993; Brussaard *et al.*, 1994).

Specific G proteins that are involved in coupling to heptahelical transmembrane receptors were identified in oocyte expression systems or cell lines (Kleuss *et al.*, 1993; Goetzl *et al.*, 1994; Quick *et al.*, 1994; Dippel *et al.*, 1996). Down-regulation of endogenous G protein alpha subunits or gamma subunits showed which G protein subunits participate in coupling particular receptors to a cellular effector, namely an ion channel or phospholipase C-β. Other transduction steps, involving particular isoforms of phospholipase C (Dippel *et al.*, 1996), or kinases (Shih and Malbon, 1994) have also been targeted in this way.

The function of extracellular matrix elements or receptors for cell adhesion in cell attachment and neurite outgrowth has been investigated. For example, antisense experiments showed that both the extracellular matrix receptor integrins (Lallier and Bronner-Fraser, 1993) and the amyloid precursor protein (Kibbey *et al.*, 1993) are involved in cell attachment or neurite outgrowth in culture systems.

In the neurotrophin field, the controversial role of the p75 receptor (the low-affinity neurotrophin receptor once described as the 'nerve growth factor receptor') has been explored with antisense methods in cultures of developing sensory neurons. Results suggest that p75 switches roles during the perinatal period, initially mediating survival and later activating a constitutive death signal in the absence of NGF (Barrett and Bartlett, 1994).

In vivo

A variety of approaches illustrate different modes of delivering antisense ODNs *in vivo*.

Transgenic animals expressing antisense sequences have been constructed, and the introduced sequences appear to be effective both in down-regulating protein levels and in altering behavior (Katsuki *et al.*, 1988; Khillan *et al.*, 1994).

Many other *in vivo* experiments utilized injection paradigms. ODNs have been injected into intraperitoneal, intravenous, intraventricular or intrathecal sites, or to particular loci in the brain, including areas targeting axon terminals. For example, retrograde transport was used to deliver antisense ODN for the peptide galanin. Galanin is present in rat dorsal root ganglia, and is thought to be involved in suppressing autotomy or self-mutilation behavior that is triggered by transection of the sciatic nerve. Application of antisense ODN for galanin to the proximal stump of the transected nerve appears to down-regulate galanin immunoreactivity in the ganglion and exaggerate this mutilation behavior (Ji *et al.*, 1994).

Developmental organization of the nervous system has been explored using antisense ODN delivered by a retroviral vector. Injection of antisense retroviral constructs into the embryonic chick optic tectum demonstrated a role for β1-integrin in directing cell migration of developing neuroblasts (Galileo *et al.*, 1992). Intracerebroventricular (i.c.v.) or intrathecal injection routes for free ODN have also been widely used. Tseng *et al.* (1994) reported that intrathecal injection of antisense to the delta subtype of opioid receptor blocked the antinociceptive effects of delta but not mu or kappa opioid receptor agonists. It should be noted that repeated injections are sometimes required to observe behavioral modification. For example, grooming behavior induced by D_1 dopamine receptor agonist can be modified by repeated i.c.v. injections of antisense to the D_1 dopamine receptor in a manner related to the amount of the D_1 antisense and the length of time it was administered (Zhang *et al.*, 1994). Studies also were carried out in 6-hydroxydopamine-lesioned mice to probe which of the many dopamine receptors may be involved in rotational behavior induced by D_1 agonists or non-dopamine agonists. D_1 antisense blocked only D_1 agonist-induced stereotyped behavior, but not muscarinic or D_2 agonist-induced rotation (Zhang *et al.*, 1994).

The utility of direct injections of antisense into the brain to confine the ODN to the region of interest is illustrated by the following two studies. To test the hypothesis that c-fos mediates the induction of the neuroactive peptide neurotensin by neuroleptic (haloperidol), c-fos antisense was

injected into the rat caudate-putamen. C-fos antisense treatment produced a 50% blockade of the induction of neurotensin/neuromedin mRNA in dorsolateral striatum elicited by systemic treatment with haloperidol (Merchant, 1994). However, another study illustrates that results must be interpreted with caution. Injection of antisense to oxytocin into the supraoptic nucleus reduced the milk ejection response of the dam to suckling, but this may not involve regulation of oxytocin levels (Neumann *et al.*, 1994, 1995).

In spite of the challenges of using the antisense method, its relative flexibility and ease of use make it very attractive. While not applicable to all situations, it has been successful in a number of systems. Carefully designed antisense experiments and controls have provided insight into the genes involved in signaling pathways, cellular responses and behavioral events.

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