Antisense oligonucleotides: an experimental strategy to advance a causal analysis of development

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ABSTRACT A variety of modified and unmodified oligonucleotides (ODNs) have been examined as antisense inhibitors of gene expression. Of particular interest has been the application of antisense inhibitory experimental strategies to advance a suggested causal relationship between signal transduction and inductive epithelial-mesenchymal interactions during mandibular morphogenesis, early tooth development, tooth enamel formation, lung branching morphogenesis, kidney, muscle and heart development. Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and a number of transforming growth factor beta (TGF-beta) isotype mediated signal transductions have been demonstrated to regulate inductive processes associated with significant processes in development including mouse molar tooth morphogenesis. Antisense strategies have also been useful in studies designed to associate a specific morphogen signal with homeobox (HOX) gene regulation in several embryonal carcinoma cell lines. The application and results from a number of antisense inhibitory strategies serve to support the utility of this experimental paradigm for future investigations of tooth development. This review discusses the experimental strategy, a number of technical issues and the rationale for future investigations of tooth development.

KEY WORDS: *growth factors (EGF, TGF-beta isotypes), growth factor receptors, transcription factors (Msx-1, LEF-1), mouse tooth and lung development, in vitro, serumless chemically-defined medium*

Introduction

Loss-of-function mutations that disrupt specific biochemical processes can define a set of genes that are required for a specific event during development. Loss of function mutations are readily apparent within "experiments of nature" (congenital structural and metabolic birth defects) and within transgenic animals generated by homologous recombination to produce null mutations or "knock-outs" of specific genes of interest. The mouse null mutation can be extremely informative or may result in lethal effects which prevent easy access to a specific developmental process. In contrast, the antisense oligonucleotide (ODN) inhibition can provide an alternative experimental strategy which enables examination of translation arrest or under-expression of specific gene products in cell lines, primary cell culture, tissue culture or organ explant models *in vitro* at any stage during development. The mechanism of action of antisense inhibition is either to block biochemical processes that require physical access to the sense sequence of nucleic acids, to promote cleavage of the sense strand by the action of the antisense oligonucleotide itself (e.g. ribozymes), or by stimulating intracellular enzymes (e.g. RNAse-H) (see Table 1).

In this review, the application of antisense inhibition strategies to investigate the function of growth factor-mediated signal transduction during inductive epithelial-mesenchymal interactions will be evaluated and a number of experimental caveats are presented. Since a number of transgenic studies using null mutations of growth factors and/or their cognate receptors can be lethal during early stages of craniofacial-oral-dental development, antisense inhibition strategies offer potentially significant alternatives for studies designed to analyze when, where and how translation arrest of one or more specific gene products functions during tooth development.

Signal transduction during tooth development

During the last decade there has been a remarkable increase in the discovery of growth factors and their cognate receptors associated with developmental processes (see reviews edited by Rosenblum and Heyner, 1989; Waterfield, 1990). The time and position of growth factor and cognate receptor gene expressions have been carefully documented using *in situ* hybridization to define transcription and employing immunocytochemistry to define the location(s) of specific translation products; the "when" and "where" questions. Molecular microdissection of precise developmental processes have been further advanced using small tissue samples to extract RNA for reverse transcription-polymerase chain reactions (RT-PCR) or "mRNA phenotyping" (Rappolee *et al.*, 1988, 1992) in combination with microscale nucleic acid sequence

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TABLE 1

POTENTIAL SITES FOR REGULATION BY ANTISENSE OLIGONUCLEOTIDES DURING GENE EXPRESSION FROM GENOMIC DNA (PRE-TRANSCRIPTION) TO FUNCTIONAL PROTEIN (TRANSLATION)

Block genomic DNA transcription
Block alternative splicing (amelogenins)
Block subsequent transcript processing (e.g. ribozymes)
Block export of functional transcripts from nucleus
Enhance attach of nascent transcript by ribozymes
Enhance cytoplasmic nuclease degradation of transcript
Block ribosome assembly
Block initiation of translation
Block ribosome migration
Block protein functions

analyses to confirm the authenticity of the specified amplified PCR products (Tracy and Mulcahy, 1991; see discussion in Chai *et al.*, 1994); the "what" question.

Knowing when and where a specific growth factor and its cognate receptor are expressed provides opportunities to determine the function(s) of the growth factor by employing antisense ODN inhibition or translation arrest during a specific developmental process (see discussions by Slavkin *et al.*, 1992). For example, after demonstrating that epidermal growth factor (EGF) and its cognate receptor EGFR were expressed in E9 embryonic mouse mandibular processes, Kronmiller *et al.* (1991) used antisense EGF ODN inhibition in E9 mandibular explants cultures and demonstrated adontia. Shum *et al.* (1993) used antisense EGF ODN inhibition in E10 mandibular explants cultured in serumless medium and demonstrated cartilage and tooth dysmorphology; EGF abrogation induced *fusilli*-form dysmorphogenesis of Meckelís cartilage and hypodontia under these experimental conditions. The EGF abrogation was rescued by exogenous EGF. Hu *et al.* (1992) used antisense EGF ODN inhibition in E15 cap stage mouse molar explants in serumless medium and demonstrated tooth dysmorphogenesis and hypodontia which were rescued by exogenous EGF. These three complementary studies support the hypothesis that EGF functions through signal transduction to mediate several developmental processes associated with tooth morphogenesis, extending from the initiation of the dental lamina through bell stages of tooth formation.

Further questions need to be addressed to advance the hypothesis that EGF-mediated signal transduction is in fact the basis for the developmental aberrations (see discussion by Slavkin, 1993). For example, acknowledging that endogenous EGF is inhibited or down-regulated, perhaps endogenous TGF-alpha is expressed and provides a redundancy in function to substitute for the altered EGF. RT-PCR studies demonstrated that both EGF and TGF-alpha transcripts and translation products were expressed in E10 mandibular explants (Slavkin *et al.*, 1990). Further, since both EGF and TGF-alpha share the same cognate EGFR, inhibition of the EGFR should provide the same developmental aberrations as selective loss-of-function for either EGF or TGF-alpha (using the same model at the same stage of development). This experimental strategy to inhibit EGFR and compare the results with inhibition of endogenous ligands has been utilized in studies of early tooth formation and lung branching morphogenesis; both investigations have reported similar results. Antisense EGF ODN inhibition inhibits tooth development (hypodontia and dysmorphogenesis) and lung branching morphogenesis, and both models can be rescued using exogenous EGF (Hu *et al.*, 1992; Seth *et al.*, 1993; Shum *et al.*, 1993). Moreover, in both models pharmacologic inhibition of EGFR produces comparable abnormal phenotypes in tooth and lung development which can not be rescued by exogenous EGF (Hu *et al.*, 1992; Seth *et al.*, 1993; Shum *et al.*, 1993). Collectively, these results strongly support the hypothesis that EGF ligand binding to its cognate receptor provides for several levels of control for development. However, these studies did not quantitate the EGF translation arrest nor the EGF mRNA concentrations in the presence and absence of antisense EGF ODNs.

Another antisense ODN inhibition study of tooth development provides some important lessons for controls for growth factor specificity. Chai *et al.* (1994) demonstrated that endogenous TGF-beta isotypes regulate embryonic mouse molar tooth development using serumless medium. Antisense TGF-beta$_1$, -beta$_2$ and -beta$_3$ subtypes were each used as 15 Mer synthetic ODNs (sense versus antisense ODNs) at concentrations of 20 uM in E10 mandibular explant cultures using serumless, chemically-defined medium. All three subtypes were expressed as transcripts and translation products in this model during 9 days of culture *in vitro*. In addition to no treatment, sense and antisense ODN treatments, additional groups were treated with either exogenous TGF-beta ligand or antisense ODN plus exogenous TGF-beta ligand for a rescue. In separate studies, different combinations of individual TGF-beta ligands were added to either complementary antigens DON treated cultures or to non-complementary antigens DON treated cultures to test for the specificity of the recovery. Under these experimental conditions, Chai *et al.* (1994) discovered that antisense TGF-beta$_1$ or TGF-beta$_3$ ODNs showed little or no effect on tooth development (controls versus treated tooth size and shape), whereas antisense TGF-beta$_2$ ODN induced a four-fold increase in tooth size and precocious tooth development (e.g. cap stage rather than control bud stage). Neither exogenous TGF-beta$_1$ or TGF-beta$_3$ ligands rescued the effects; only the addition of the TGF-beta$_2$ ligand reduced tooth size to that of controls, and similarly inhibited tooth morphogenesis to that attained by controls (Chai *et al.*, 1994).

Oligonucleotide synthesis, purication and analysis

A variety of modified and unmodified oligonucleotides (ODNs) have been examined as antisense inhibitors of gene expression in a wide variety of procaryotic and eucaryotic (plants and animals) cell and tissue models. A relatively new application for the antisense inhibition strategy has been to investigate loss of function mutations in a number of developing systems (see review by Melton, 1988). Although a great deal of progress has been made in antigen inhibition strategies during development (e.g. Kronmiller *et al.*, 1991; Potts *et al.*, 1991; Sariola *et al.*, 1991; Biro *et al.*, 1993; Diekwisch *et al.*, 1993; Seth *et al.*, 1993; Shum *et al.*, 1993; Chai *et al.*, 1994; Faiella *et al.*, 1994; Souza *et al.*, 1994), certain pharmacokinetic factors may limit the use of unmodified antisense ODNs *in vivo* and possibly *in vitro* (Tidd, 1992). The major concerns include: (i) degradation of exogenous antisense ODNs by ubiquitous nucleases that could render unmodified ODNs unsuitable as antisense inhibitors, or that (ii) reduced intracellular half-life for antisense ODNs would minimize the efficiency of their biological activity.

To address these concerns, a number of chemical approaches have been advanced, designed to modify the phosphorous atom of the phosphodiester linkage (e.g. phosphothioates, methylphos-

phonates and phosphoramidates) (see review by Sanghvi *et al.*, 1993). These modified ODNs have been synthesized, purified and tested under a variety of experimental conditions including (i) stability in 10% heat inactivated fetal calf serum, (ii) binding affinities to RNA and DNA complements, and (iii) ability to support RNAse-H degradation of targeted RNA in DNA/RNA heteroduplexes. In general, these studies suggest that 22 Mer ODN with as many as 5 modified pyrimidines (thymidine/cytosine bases) within its sequence (e.g. 6-Azathymidine capped ODNs) demonstrated a 7- to 12-fold increase in stability in serum over unmodified ODN, yet maintains hybridization properties similar to the unmodified ODNs (Sanghvi *et al.*, 1993). A major caveat for the use of such synthetic modified ODNs resides in their ultimate purification. Failure to remove minute traces of organic chemicals introduces confounding toxic effects into studies of early embryonic stages of development. At the time of writing, the state-of-the-art is currently to use reverse phase high pressure liquid chromatography (HPLC) to remove trace contaminants from either unmodified or modified antisense and sense ODNs (see discussion in Chai *et al.*, 1994).

Experimental caveats

A number of experimental caveats need to be considered when evaluating the results from antisense inhibition investigations using complex organ culture model systems. Whereas antisense oligonucleotide inhibition studies can provide significant loss-of function or under-expression mutations to enhance a molecular understanding of developmental processes (Melton, 1988; Moffat, 1991; Malcolm, 1992; Murray and Crockett, 1992), the precise mechanisms of action, putative side effects, and/or specificities of action are as yet not fully understood (Boiziau *et al.*, 1991) (Fig. 1).

A number of previous investigations have ascertained that the most efficient translation arrest effects were produced when antisense ODNs were designed to hybridize with the 5' codons of the precursor targeted mRNA, and in particular with sequences associated with the AUG initiation codon (Minshull and Hunt, 1986; Shakin-Eshleman and Liebhaber, 1988) (Fig. 1a,b). In addition, the design of the antisense ODN must consider that the synthetic oligonucleotide must be of sufficient length to provide specificity for hybridization, yet small enough to optimize diffusion through the organ explants and incorporation into target cells. Moreover, the concentration of the ODN incorporated into target cells must be sufficient to provide a suitable half-life to produce translation arrest (e.g. the percentage of translation arrest of the translation product). Several investigations have recommended that ODN of 15 base pairs in length (15 Mer) is ideal to meet these criteria (see Marcus-Sekura, 1988; Rappolee *et al.*, 1992; Diekwisch *et al.*, 1993; Seth *et al.*, 1993; Shum *et al.*, 1993; Chai *et al.*, 1994). Florini *et al.* (1991) demonstrated that a 15 Mer synthetic antisense IFG-II ODN designed to the 5' region of the sequence inhibited endogenous IFG-II translation and inhibited myogenic cell differentiation. Diekwisch and colleagues (1993) demonstrated that a 15 Mer synthetic antisense amelogenin ODN at a concentration of 20 uM diffused into all of the cells within a cap stage molar tooth explant within 24 h, and produced a 30% inhibition of amelogenin translation. With respect to half-life of antisense ODN, approximately 2% of exogenously added anti-c-myc ODN was taken up by HL-60 cells and remained stable for 24 h (Wickstrom *et al.*, 1988); initial concentrations of exogeneously added ODN (15 Mer) of 20 uM in culture medium are often required to produce a 2% incorporation of antisense ODN into target cells (see discussion by Diekwisch *et al.*, 1993). Therefore, it is essential that each investigation establish the optimal conditions for that particular system.

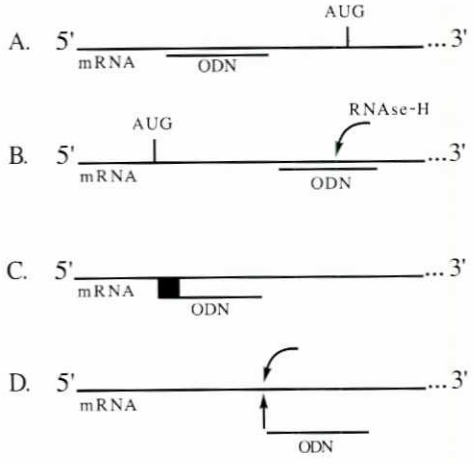

Fig. 1. Several mechanisms for inhibition of translation by antisense (ODN) inhibitory strategies. (a) *ODNs are designed to hybridize to sequences upstream on the initiation codon AUG producing a physical arrest of the initiation of the translation complex;* **(b)** *ODNs are designed to be complementary to the mRNA coding region, preventing polypeptide chain elongation when the mRNA is cleaved by RNase-H;* **(c)** *ODNs are designed to cross-link to sense strand of mRNA; and* **(d)** *ODNs are linked to active groups such as photsensitizers or metal complexes. In theory, each of these suggested mechanisms will produce translation arrest.*

Another key issue is putative hybridization arrest of translation. In one scenario, translation arrest by antisense ODN may involve RNAse-H, an RNAse which hydrolyzes the DNA part of the RNA/DNA hybrids (Haeuptle *et al.*, 1986). In addition, the RNA secondary structure may also play an important role in the efficiency of antisense ODN inhibition (Verspieren *et al.*, 1990) (Fig. 1).

Therefore, a number of significant experimentally-based questions need to be considered as one designs antisense inhibition for either *in vivo* or *in vitro* (Zamecnik, 1991; Woolf *et al.*, 1992) studies: (i) Is the length and specificity of the ODN sufficient to achieve translation arrest of the targeted mRNA translation product? (ii) Will the ODN enter the targeted cells? (iii) Is the intracellular concentration and half-life of the ODN sufficient to produce a significant translation arrest (e.g. percent inhibition of specific translation product per unit time), or will the ODN be rapidly degraded by intracellular enzymes? (iv) Is the nucleic acid sequence region selected for the synthesis of the ODN without confounding secondary and tertiary structures? (v) Does the synthetic ODN cross-hybridize with other RNA and/or DNA sequences homologous to that of the nucleic acid sequence within the targeted mRNA? and (vi) Can the loss-of-function mutation or more appropriately, the developmental abrogation be reversed by the addition of exogenous translation product, and is the reversal or recovery highly specific to the exogenous molecule? Critically designed control studies are essential to rule out these caveats and increase the validity of the results and their subsequent interpretations.

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References

BIRO, S., YA-MIN, F., ZU-XIN, Y. and EPSTEIN, S.E. (1993). Inhibitory effects of antigens oligonucleotides targetting c-myc mRNA on smooth muscle cell proliferation and migration. *Proc. Natl. Acad. Sci. USA 90:* 654-658.

BOIZIAU, C., KURFURST, R., CAZENAVE, C., ROIG, V., THUONG, N.T. and TOULME, J.J. (1991). Inhibition of translation initiation by antigens oligonucleotides via an RNAse-H independent mechanism. *Nucleic Acids Res. 19:* 1113-1119.

CHAI, Y., MAH, A., CROHIN, C., GROFF, S., BRINGAS, P., THUAN, L., SANTOS, V. and SLAVKIN, H.C. (1994). Specific transforming growth factor-beta subtypes regulate embryonic mouse Meckel's cartilage and tooth development. *Dev. Biol. 162:* 85-103.

DIEKWISCH, T., DAVID, S., BRINGAS, P., SANTOS, V. and SLAVKIN, H.C. (1993). Antigens inhibition of AMEL translation demonstrates supramolecular controls for enamel HAP crystal growth during embryonic mouse molar development. *Development 117:* 471-482.

FAIELLA, A., ZAPPAVIGNA, V., MAVILIO, F. and BONCINELLI, E. (1994). Inhibition of retinoic acid-induced activation of 3' human HOXB genes by antisense oligonucleotides affects sequential activation of genes located upstream in the four HOX clusters. *Proc. Natl. Acad. Sci. USA 91:* 5335-5339.

FLORINI, J.R., MAGRI, K.A., EWTON, D.Z., JAMES, P.L., GRINDSTAFF, K. and ROTWEIN, P.S. (1991). "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor II. *J. Biol. Chem. 266:* 15917- 15923.

HAEUPTLE, M.T., FRANK, R. and DOBBERSTEIN, B. (1986). Translation arrest by oligodeoxynucleotides complimentary to mRNA coding sequences yields polypeptides of predetermined length. *Nucleic Acids Res. 14:* 1427-1445.

HU, C.C., SAKAKURA, Y., SASANO, Y., SHUM, L., BRINGAS, P., WERB, Z. and SLAVKIN, H.C. (1992). Endogenous epidermal growth factor regulates the timing and pattern of embryonic mouse molar tooth morphogenesis. *Int. J. Dev. Biol. 36:* 505- 516.

KRONMILLER, J.E., UPHOLT, W.B. and KOLLAR, E.J. (1991). EGF antisense oligodeoxynucleotides block murine odontogenesis *in vitro. Dev. Biol. 147:* 485-488.

MALCOLM, A.D.B. (1992). Uses and applications of antigens oligonucleotides: uses of antigens nucleic acids — an introduction. *Biochem. Soc. Trans. 20:* 745-746.

MARCUS-SEKURA, C.J. (1988). Techniques for using antisense oligonucleotides to study gene expression. *Anal. Biochem. 172:* 289-295.

MELTON, D.A. (Ed.) (1988). Summary. In *Current Communications in Molecular Biology: Antisense RNA and DNA.* Cold Spring Harbor Laboratory, New York, pp. 145-149.

MINSHULL, J. and HUNT, T. (1986). The use of single-stranded DNA and RNase H to promote quantitative "hybridization arrest of translation" of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. *Nucleic Acids Res. 14:* 6433-6451.

MOFFAT, A.S. (1991). Making sense of antisense. *Science 253:* 510-511.

MURRAY, J.A.H. and CROCKETT, N. (1992). Antisense techniques: an overview. *In Antisense RNA and DNA* (Ed. J.A.H. Murray). John Wiley and Sons, New York, pp. 1-49.

POTTS, J.D., DAGLE, J.M., WALDER, J.A., WEEKS, D.L. and RUNYAN, R.B. (1991). Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor β_3. *Proc. Natl. Acad. Sci. USA 88:* 1516-1520.

RAPPOLEE, D.A., MARK, D., BANDA, M.J. and WERB, Z. (1988). Wound macrophages express TGF-alpha and other growth factors *in vivo:* analysis by mRNA phenotyping. *Science 241:* 708-711.

RAPPOLEE, D.A., STURM, K.S., BEHRENDTSEN, O., SCHULTZ, G.A., PEDERSEN, R.A. and WERB, Z. (1992). Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. *Genes Dev. 6:* 939-952.

ROSENBLUM, I.Y. and HEYNER, S. (Eds.) (1989). *Growth Factors In Mammalian Development.* CRC Press, Boca Raton, Florida.

SANGHVI, Y., HOKE, G.D., FRIER, S.M., ZOUNES, M.C., GONZALEZ, C., CUMMINS, L., SASMOR, H. and COOK, P.D. (1993). Antisense oligonicleotides: synthesis, biophysical and biological evaluation of oligonucleotides containing modified pyrimidines. *Nucleic Acids Res. 21:* 3197-3203.

SARIOLA, H., SAARMAN, M., SAINIO, K., ARUMAE, U., PALGI, J., VAAHTOKARI, A., THESLEFF, I. and KARAVANOV, A. (1991). Dependence of kidney morphogenesis on the expression of nerve gowth factor receptor. *Science 254:* 571-573.

SETH, R., SHUM, L., WU, F., WUENSCHELL, C., HALL, F.L., SLAVKIN, H.C. and WARBURTON, D. (1993). Role of epidermal growth factor expression in early mouse embryo lung branching morphogenesis in culture: antisense oligonucleotide inhibitory strategy. *Dev. Biol. 158:* 555-559.

SHAKIN-ESHLEMAN, S.H. and LIEBHABER, S.A. (1988). Influence of duplexes 3' to the mRNA initiation codon on the efficiency of monosome formation. *Biochemistry 27:* 3975-3982.

SHUM, L., SAKADURA, Y., BRINGAS, P., LUO, W., SNEAD, M.L., MAYO, M., CROHIN, C., MILLAR, S., WERB, Z., BUCKLEY, S., HALL, F.L., WARBURTON, D. and SLAVKIN, H.C. (1993). EGF abrogation induced fusilli-form dysmorphogenesis of Meckelis cartilage during embryonic mouse mandibular morphogenesis *in vitro. Development 118:* 903-917.

SLAVKIN, H.C. (1993). Rieger syndrome revisited: experimental approaches using pharmacologic and antisense strategies to abrogate EGF and TGF-alpha functions resulting in dysmorphogenesis during embryonic mouse craniofacial morphogenesis. *Am. J. Med. Genet. 47:* 689-697.

SLAVKIN, H.C., HU, C.C., SKAKURA, Y., DIEKWISCH, T., CHAI, Y., MAYO, M., BRINGAS, P., SIMMER, J., MAK, G., SASANO, Y. and DAVID, S. (1992). Gene expression, signal transduction and tissue-specific biomineralization during mammalian tooth development. *Crit. Rev. Eucaryotic Gene Expression 2:* 315-329.

SLAVKIN, H.C., SNEAD, M.L., LUO, W., BRINGAS, P., KIKUNAGA, S., SASANO, Y., BESSEM, C., MAYO, M., MacDOUGALL, M., RALL, L.B., RAPPOLLEE, D. and WERB, Z. (1990). Analysis of embryonic cartilage and bone induction in a defined culture system. In *Bone Regulatory Factors* (Eds. A. Pecile and B. de Bernard). Plenum Publishing Corp., New York, pp. 55-68.

SOUZA, P., SEDLACKOVA, L., KULISZEWSKI, M., WANG, J., LIU, J., YSEU, I., LIU, M., TANSWELL, A.K. and POST, M. (1994). Antisense oligonucleotides targeting PDGF-B mRNA inhibit cell proliferation during embryonic rat lung development. *Development 120:* 2163-2173.

TIDD, D.M. (1992). Uses and applications of antisense oligonucleotides: methylphosphonodiester/phosphodiester chimeric oligonucleotides. *Biochem. Soc. Trans. 20:* 746-749.

TRACY, T.E. and MULCAHY, L.S. (1991). A simple method for direct automated sequencing of PCR fragments. *BioTechniques 11:* 68-75.

VERSPIEREN, P., LOREAU, N., THUONG, N.T., SHIRE, D. and TOULME, J-J. (1990). Effect of RNA secondary structure and modified bases on the inhibition of trypanosomatid protein synthesis in cell-free extracts by antisense oligodeoxynucleotides. *Nucleic Acids Res. 18:* 4711-4717.

WATERFIELD, M.D. (Ed.) (1990). *Growth Factors In Cell And Developmental Biology.* J. Cell Sci. 13 Suppl. The Company of Biologists Limited, Cambridge.

WICKSTROM, E.L., BACON, T.A., GONZALEZ, A., FREEMAN, D.L., LYMAN, G.H. and WICKSTROM, E. (1988). Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA. *Proc. Natl. Acad. Sci. USA 85:* 1028-1032.

WOOLF, T.M., MELTON, D.A. and JENNINGS, C.G.B. (1992). Specificity of antisense oligonucleotides *in vivo. Proc. Natl. Acad. Sci. USA 89:* 7305-7309.

ZAMECNIK, P.C. (1991). Introduction: oligonucleotide base hybridization as a modulator of genetic message readout. In *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS* (Ed. E. Wickstrom). Wiley-Liss, New York, pp.1-6.