GENE REGULATION BY ANTISENSE RNA AND DNA

Organizers: Robert P. Erickson and Jonathan Izant

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Naturally Occurring Antisense Regulation

CD 001 micRNA IMMUNE SYSTEM AGAINST VIRAL INFECTIONS, Masayori Inouye, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School at Rutgers, Piscataway, NJ 08854-5635.

Following the initial discoveries of natural antisense RNAs in prokaryotes, numerous applications of antisense RNA-mediated regulation have been demonstrated in both prokaryotic and eukaryotic systems. These non-translated RNAs inhibit gene expression by hybridizing to a target RNA and rendering it functionally inactive [see a review by K.M. Takayama and M. Inouye (1990) Crit. Rev. Biochem. Mol. Biol. 25, 155-182].

I will discuss new attempts and approaches towards constructing more effective antisense RNAs and DNAs, and present data describing a micRNA-immune system against *mouse* hepatitis virus.

CD 002 THE XENOPUS bFGF GENE: A NATURALLY OCCURING ANTISENSE TRANSCRIPT WITH A POSSIBLE REGULATORY ROLE, David Kimelman, Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195

The Xenopus oocyte contains three maternal basic fibroblast growth factor (bFGF) transcripts, the largest of which (4.5 kb) encodes the complete bFGF polypeptide (Kimelman et al., Science,1988). The smallest transcript (1.5 kb), which is 20-fold more abundant than the other two, is transcribed in the opposite direction of the largest transcript and overlaps part of the coding sequence of bFGF. This anti-sense transcript also encodes a 25 kd polypeptide which is highly conserved in cows and humans.

The anti-sense transcript causes modification of the large mRNA encoding bFGF during maturation of the oocyte, converting half of the adenine residues to inosine in the region of overlap between the sense and anti-sense transcripts. As this activity has been shown to act only on double-stranded RNA, the two mRNAs must be hybridized in the oocyte. This suggests a possible role for the anti-sense transcript in regulating the stability of the 4.5 kb bFGF mRNA, which is degraded as part of the process of oocyte maturation. We are currently exploring this idea using synthetic mRNAs produced in vitro with bacterial phage polymerases.

CD 003 IS*10* ANTISENSE RNA CONTROL: A MODEL SYSTEM FOR THE DESIGN OF EFFICIENT AND SPECIFIC ARTIFICIAL ANTISENSE RNAs, Robert W. Simons, Charles Ma, Casey Case and Juan González, Department of Microbiology and Molecular Genetics and the Molecular Biology Institute, University of California, Los Angeles, CA 90024.

Antisense RNAs are small, diffusible, untranslated transcripts that pair to target RNAs at specific regions of complementarity, thereby controlling target RNA function or expression. Antisense RNAs have been shown or proposed to control expression of a number of prokaryotic genes by mechanisms that include premature transcription termination, facilitated message decay and translation inhibition. In all but two of these cases, the antisense and target RNAs are transcribed from opposite strands of the same DNA template by opposing promoters, although many probably act efficiently in *trans*. Antisense control has received considerable attention in recent years, partly because of a general interest in the control mechanisms involved, including the mechanisms of RNA/RNA pairing, and partly because of a keen interest in the appropriate design of efficient and specific artificial antisense control. Studies on the naturally occurring prokaryotic systems suggested, early on, that artificial antisense control could be achieved, and continue to suggest useful "rules" for the design of such RNAs. One of the best characterized antisense systems is that of the prokaryotic mobile genetic element, IS*10*, where an antisense RNA inhibits expression of the IS*10* transposase. IS*10* specifies two transcripts: the 70 nt antisense RNA, RNA-OUT, and the longer transposase message, RNA-IN. The 5' ends of RNA-OUT and RNA-IN are complementary to one another across a 35 bp region that includes the ribosome binding site for the transposase gene. Indeed, RNA-OUT/RNA-IN pairing inhibits transposase translation *in vivo* and specifically blocks ribosome binding *in vitro*. In addition, pairing leads to rapid RNaseIII-dependent destabilization of RNA-IN *in vivo*, showing that control occurs at two levels. RNA-OUT/RNA-IN pairing occurs efficiently and specifically *in vitro* and *in vivo*. Genetic and physical studies identify critical regions for initiation of pairing, suggest the process of pairing, and show that the secondary structures of both RNA-OUT and RNA-IN are important. RNA-OUT has a simple stem-loop secondary structure. The loop domain contains critical pairing determinants and the stem domain is essential for RNA-OUT's unusual stability. RNA-IN secondary structure is more complicated. When nascent, the 5' end (which also contains critical pairing determinants) is single-stranded and thus available for pairing to RNA-OUT. However, full-length RNA-IN forms a secondary structure in which the 5' end is sequestered, thus preventing pairing to RNA-OUT. These features, which have biological importance for IS*10*, emphasize the importance of target RNA secondary structure in antisense control. The basic features of IS*10* antisense control will be compared and contrasted with other well-characterized systems, and several "rules" for the design of efficient and specific artificial antisense RNAs will be proposed.

Factors that Modulate RNA Interactions in Vivo

CD 004CHARACTERIZATION OF A BIOLOGICAL ACTIVITY THAT MODIFIES ADENOSINES WITHIN DOUBLE-STRANDED RNA, Brenda L. Bass, Ron Hough, Michael Paul and Andy Polson, Department of Biochemistry, University of Utah, 50 North Medical Drive, Salt Lake City, Utah 84132

We are studying a biological activity that converts adenosines to inosines within double-stranded RNA. The activity seems to be ubiquitous among the various phyla of the animal kingdom, but very little is known about the substrates or functions of the activity in vivo. We are studying the activity in Xenopus laevis oocytes, eggs and early embryos and at present have three goals: 1) to purify the activity, 2) to identify cellular RNAs that serve as substrates for the activity in vivo, 3) to determine the substrate specificity of the activity. Recent progress towards these goals will be discussed. In addition, the relevance of the activity to antisense RNA techniques will be considered.

CD 005 EXPRESSION AND SUBSTRATE SPECIFICITY OF THE dsRNA UNWINDING/ MODIFYING ACTIVITY, Kazuko Nishikura, The Wistar Institute, Philadelphia, PA 19104 A novel double-stranded RNA unwinding/modifying activity, originally found in *Xenopus* eggs, converts up to 50% of adenosine residues to inosine (1,2). Although neither its physiological function nor its biological substrate RNAs are yet known, the activity may be involved in the RNA turn-over mechanism. One such example, the degradation of maternal bFGF mRNAs mediated by antisense transcripts and the unwinding/modifying activity during *Xenopus* oocyte maturation, has been reported (3). In addition, the activity may be the cause of base specific mutations which occur in several viral gene systems (2,4). The most striking of these cases is that of defective measles virus genes derived from the brain tissues of patients with measles inclusion body encephalitis (2,5). In hopes of determining the specific localization of the activity, we have surveyed different primary tissues and cell lines. Regardless of the differentiated, mitotic or transformed state of the cell, the activity, localized in the nucleus, was detected ubiquitously in all of the many cells and tissues we tested, including CNS where the persistent infection of defective measles viruses occurs. Thus, the unwinding/modifying activity appears to be one of the typical housekeeping genes (6). In addition, we have also tested various artificial dsRNAs in order to better understand the biological substrate RNAs for the activity. We found that not only the intermolecular dsRNA but also intramolecular dsRNA of human α-globin RNAs can be modified by the unwinding/modifying activity. Our results indicate that a variety of hairpin or stem and loop-type intramolecular dsRNAs commonly present in eukaryotic and viral RNAs can also be potential substrates for the activity. Finally, our progress on biochemical purification of the unwinding/modifying activity will be reported.

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6. R.W. Wagner , C.Yoo, L. Wrabetz, J. Kamholz, J. Buchhalters, N.F. Hassan, K. Khalili, S.U. Kim, B. Perussia, F.A. McMorris, and K. Nishikura (1990) *Mol. Cell. Biol.* 10; in press.

Ribozyme and Other Antisense Chemistry

CD 006 ANTISENSE RNA AND TRANSLATIONAL CONTROL OF CREATINE KINASE-B; INSIGHTS TO DESIGNING AN EFFECTIVE ANTISENSE STRATEGY, E.W. Holmes, J.L.C. Ch'ng, R. Cirullo, and L.E. Clark, Departments of Medicine and Biochemistry, Duke University Medical School, Durham, NC, 27710.

Creatine Kinase-B (CK-B) is being utilized as a model system to study antisense RNA. The 3' untranslated region (UTR) of CK-B has been the focus of these studies because this region has been conserved over 340 m.y. of evolution. In vivo studies have been performed with human cells transformed with a retroviral vector that produces antisense RNA complementary to the 3' terminal 370 bases of coding and all 185 bases of 3' non-coding sequences of CK-B. By comparing the ratio of mRNA to antisense RNA with the percentage inhibition of CK-B activity we estimate that one antisense transcript blocks the expression of one mRNA. CK-B mRNA abundance is unchanged in these cells and >90% of the mRNA is present in the cytoplasm, suggesting the antisense RNA may block translation directly. To test this hypothesis antisense transcripts have been added to in vitro translation reactions containing CK-B transcripts, as well as other mRNAs. In this in vitro system antisense RNA inhibits CK-B translation specifically and with an efficiency comparable to that observed in vivo, i.e. >90% inhibition at a 1:1 molar ratio of sense:antisense RNA. Mapping studies indicate that only 60 bases of 3' coding and 50 bases of 3' non-coding sequence are as effective as the larger antisense RNA tested in vivo. The fraction of radiolabeled CK-B mRNA in duplex with antisense RNA is not altered following in vitro translation, even in the presence of excess unlabeled CK-B mRNA, suggesting this duplex is resistant to helicase activity of the translational apparatus. Another property of CK-B mRNA may be relevant to the antisense results described above. Related studies have shown that CK-B mRNA is present in some cells yet no CK-B protein is detectable. CK-B expression can be induced in these cells without altering the abundance of CK-B mRNA by overexpressing in trans the 3' UTR (sense orientation) of CK-B. This induction of CK-B translation is associated with the disappearance of a cytoplasmic factor that binds specifically to the 3' UTR of CK-B. The region of the 3' UTR that mediates translational repression is near or overlapping the region targeted by the most effective antisense transcripts. Based on these results we hypothesize that conserved, but non-translated regions of an mRNA like CK-B that interact with cellular factors may be more accessible for duplex formation with antisense RNA and duplexes that encompass 3' UTR sequences may be more stable because of inaccessibility to helicase activity of the ribosome. Assuming accessibility of target sequences and duplex stability are important determinants of antisense effectiveness, these observations suggest a rationale for designing an antisense strategy for a special class of mRNAs.

CD 007 INTRAMOLECULAR DUPLEXES IN EUCARYOTIC mRNA SUPPRESS TRANSLATION IN A POSITION-DEPENDENT MANNER, Stephen A. Liebhaber, J. Eric Russell, Faith Cash, and Susan S. Eshleman, Howard Hughes Medical Institute, Departments of Human Genetics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6145

Formation of a duplex in an mRNA transcript may affect one or more steps in gene expression. In the present study we have investigated the impact of intramolecular mRNA duplexes on translation. By introducing antisense segments into the 3' nontranslated region of the human α-globin mRNA we are able to synthesize a series of transcripts with identical primary, and site-specific secondary structures within the 5' nontranslated and coding regions. Stems close to or including the initiation codon (AUG) severely inhibit translation. In contrast, mRNAs with extensive stems restricted to downstream coding regions translate at levels approximately 70% of native mRNA. The persistence of substantial double-stranded segments in the former set of mRNAs is supported by their specific ability to trigger a general suppression of translation in microinjected Xenopus oocytes. The proximity of the duplexed region to the AUG and not its size or proximity to the cap is the main determinant of translational inhibition. The observed position-dependent translational arrest by the mRNA duplexes is paralleled by an inhibition of 80S assembly at the initiation AUG. These data demonstrate the substantial potential of duplexes to affect translation. The position-dependency of this effect predicts a model in which specific positioning of duplexes at the appropriate positions within the mRNA may mediate significant alterations in gene expression.

CD 008 FACTORS INFLUENCING THE ROUTE OF SELF-CLEAVAGE OF RNA VIA SINGLE OR DOUBLE HAMMERHEAD STRUCTURES, Robert H. Symons, Christopher Davies and Candice C. Sheldon, Department of Biochemistry, University of Adelaide, Adelaide, S.A. 5000, Australia.

The plus and minus RNAs of the 247 nt avocado sunblotch viroid (ASBV) and RNAs of four encapsidated, circular, viroid-like, satellite RNAs or virusoids (324 - 388 nt) of plant viruses undergo site-specific self-cleavage reactions *in vitro*. We have characterized these reactions and defined the basic properties of the hammerhead secondary structure which is common to the self-cleavage sites of these RNAs (1-4). In the case of ASBV, we showed that, during transcription of a dimeric *plus* RNA, a double hammerhead structure formed and was necessary for cleavage (4), whereas the other RNAs self-cleaved by a single hammerhead, both during transcription and after purification.

We have now extended this work to characterize what happens during the transcription of *dimeric minus* ASBV RNA, *plus* and *minus monomeric* ASBV RNAs, and with all full length transcripts after gel purification. *Dimeric plus* ASBV transcripts after purification still cleaved via a double hammerhead structure, while *monomeric plus* transcripts did not self-cleave at all, either during transcription or after isolation. By contrast, *dimeric minus* RNA self-cleaved via a double hammerhead during transcription but by a single hammerhead after purification. Further, *monomeric minus* RNAs cleaved via a single hammerhead structure but only after purification. The results illustrate the importance of the pathway of folding of the various RNAs in determining which active self-cleavage structure, if any, is formed.

All evidence indicates that the stability of stem III of the hammerhead structure is the basic factor which determines single or double hammerhead self-cleavage (2-4). Further support is shown by mutagenesis of the normal hairpin loop of stem III of *monomeric plus* ASBV RNA which does not self-cleave either during transcription or after isolation. The stem III of this RNA consists of two base pairs with a three nucleotide hairpin loop. A simple increase of the size of the hairpin loop to four nucleotides allowed very efficient single hammerhead self-cleavage during transcription of the *monomeric plus* RNA.

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Ribozymes Continued; Oligonucleotide-Mediated Antisense - I

CD 009 SEQUENCE-SPECIFIC RNA CLEAVAGE BY TWO CLASSES OF RIBOZYMES, Arthur Zaug, Daniel Herschlag and Thomas Cech, Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309-0215

Cleavage of target RNAs *in vivo* is being investigated using the hammerhead ribozyme (1,2). A hammerhead catalytic domain has been inserted near the 5' end of the U1 snRNA from yeast, with the purpose of delivering a ribozyme directly to the actin pre-mRNA 5' splice site using the U1 snRNP as a vector. The ribozyme has been expressed from a plasmid vector (kindly provided by B. Seraphin & M. Rosbash). The resulting hammerhead-U1 snRNA appears to be as stable as the natural U1 snRNA and is properly processed (trimethylG cap) in yeast. This chimeric ribozyme cleaves its target RNA with reasonable efficiency *in vitro*, and there is evidence for cleavage with lower efficiency *in vivo*. A single-base change in the catalytic domain abolishes the effect of the hammerhead *in vivo*.

Detailed studies of ribozyme-catalyzed RNA cleavage *in vitro* have been carried out with the L - 21 Sca I RNA, a ribozyme engineered from the self-splicing intervening sequence RNA of *Tetrahymena* (3). Because steady-state kinetic parameters (K_m and k_{cat}) have been largely uninformative and even misleading with respect to understanding the mechanism of action of this ribozyme, we have recently combined pre-steady-state and steady-state kinetic measurements to determine rate constants for individual steps (4). This new kinetic framework has guided efforts to improve both the activity and specificity of the ribozyme. One principle that emerges from this work, which is generally applicable, is that "too much recognition" leads to decreased specificity.

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CD 010 CONTROL OF GENE EXPRESSION BY ANTISENSE OLIGONUCLEOSIDE METHYLPHOSPHONATES, Paul S. Miller, Purshotam Bhan, Cynthia Cushman, Joanne Kean and Esther Chang', Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD 21205, #Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD, 20889

Antisense oligonucleoside methylphosphonates (ONMPs), which contain nuclease resistant, nonionic internucleotide linkages are effective inhibitors of gene expression in mammalian cells. The effectiveness of inhibition can be increased considerably by derivatization with the photoreactive crosslinking group, 4'-N-(aminoethyl)aminomethyl-4,5',8-trimethylpsoralen [(ae)AMT]. (ae)AMT-ONMPs cross-link to complementary sequences on single-stranded DNA and RNA and to single-stranded regions of stem/loop structures when triggered by irradiation with long wavelength (365 nm) ultraviolet light. Cross-linking is complete within 15 min and the kinetics of cross-linking are essentially the same for both DNA and RNA targets. (ae)AMT-ONMPs have relatively long half lives in serum-containing media and are taken up intact by mammalian cells in culture. An (ae)AMT-derivatized 11-mer directed against the splice junction of the first acceptor splice site of *ras* p21 precursor mRNA inhibits p21 synthesis >95% when cells are irradiated with 15 μM oligomer. The effectiveness of inhibition is 10-fold greater than that of an underivatized octamer which inhibits p21 synthesis approximately 90% at 150 μM. (ae)AMT-ONMPs (12-mers) directed against either a normal *ras* p21 mRNA or *ras* p21 mRNA that is mutated at a single base in codon 61 effectively inhibit p21 in a sequence specific manner. Each (ae)AMT-ONMP, at moderate concentration (12 μM), inhibited expression of only the form of *ras* p21 to which it was completely complementary and left the other form of p21 virtually unaffected when mixed cultures of cells expressing both forms of p21 were irradiated in the presence of the oligomer. The enhanced efficacy, the sequence specificity and the ability to trigger the inhibitory activity of (ae)AMT-ONMPs suggests that they will be useful antisense agents for studying temporal expression of genes in cells. (Supported by grants from NIH GM 39127 and CA 42762)

CD 011 STRATEGIES FOR THE USE OF ANTI-SENSE RIBOZYMES AS ANTI-HIV-1 THERAPEUTIC AGENTS, John J. Rossi[1], Nerida Taylor, David Elkins, Sean Sullivan, Nava Sarver, Edouard Cantin and John Zaia1. Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, CA 91010

The autocleavage "hammerhead" domain common to plant viroids and virousoids represents a simple structure for incorporation into anti-sense molecules. Anti-sense RNAs harboring this cleavage domain become endowed with enzymatic activity and can specifically cleave heterologous target RNAs. We have exploited this catalytic motiff for the design and application of catalytic anti-sense RNAs targeted to the HIV-1 genome, with the goal of developing an anti-viral ribozyme therapy. Our studies have dealt with both a gene therapy approach as well as an exogenous delivery approach. Like any emerging technology, a number of problems must be overcome for the successful application of this technology. We have been systematically investigating strategies to deal with these problems which include: optimizing target choices, enhancing the intracellular stability of ribozymes, intracellular geography versus activity, endogenous expression and exogenous delivery systems, and the large scale fermentation of ribozymes for exogenous delivery. The rationale for, exprimental designs and results of these investigations will be discussed.

Oligonucleotide-Mediated Antisense - II

CD 012 RATIONAL DESIGN OF SEQUENCE-SPECIFIC REGULATORS OF GENE EXPRESSION BASED ON DERIVATIZED OLIGONUCLEOTIDES

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INSERM, Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, 43, Rue Cuvier, 75OO5 Paris and +Centre de Biophysique Moléculaire, 45071 Orleans Cedex (France).

Oligodeoxynucleotides covalently linked to intercalating agents were used to control gene expression at the translational level (antisense strategy)and at the transcriptional level (antigene strategy).

Oligonucleotide-intercalator conjugates were shown to inhibit activated Ha-ras mRNA translation both *in vitro* and in T24 tumor cells. A strong discrimination between the mutated and the wild-type sequences was observed when the mRNA-oligodeoxynucleotide hybrid was used as a substrate for RNaseH.

Oligonucleotides can bind to the major groove of the DNA double helix. Triplex structures are strongly stabilized when an intercalating agent is covalently attached to the 5'-end of the triple helix-forming oligonucleotide. The intercalating agent can be converted into a cleaving reagent (phenanthroline-copper chelate) or into a photo-cross-linking reagent (psoralen). Triple helix formation inhibited restriction enzyme cleavage and binding of transcription factors to eukaryotic genes. Transcription of the β-lactamase gene by *E. coli* RNA polymerase was inhibited by a triple-helix-forming oligonucleotide targeted downstream of the transcription initiation site.

Oligonucleotides are made resistant to nucleases when the anomeric configuration of the nucleoside units is changed. In α-anomers the base is on the same side as the 3'-OH with respect to the deoxyribose (5'-OH in the natural β-anomers). An oligo-[α]-deoxynucleotide binds to a complementary sequence in an opposite orientation as compared to the corresponding natural oligo-[β]-deoxynucleotide. α-oligomers bind to the DNA double helix and can be used to control transcription by triplex formation. They can be substituted by intercalating agents or reagents that induce irreversible reactions in their target sequence.

CD 013 STEREOCHEMISTRY OF UNCHARGED ANTI-ONCOGENE DNA DERIVATIVES

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Antisense DNA inhibition has worked against several human and viral genes in cell culture in a dose dependent and sequence specific manner. For therapeutic applications, DNA derivatives which survive in the bloodstream, penetrate cells easily, and display high efficacy must be prepared. Oligodeoxynucleoside methylphosphonates are uncharged and nuclease resistant, enter animal cells and accomplish hybrid arrest, but are limited in their efficacy by racemization at each phosphorus. Preliminary experiments in mice are encouraging, but very high doses are necessary. Stronger hybridization by all-R oligodeoxynucleoside methylphosphonates is likely to increase efficacy substantially.

As a first step in development of an automatable stereospecific pentavalent route, the diastereomers of 5'-DMT-thymidyl-3'-(cyanoethyl)methylphosphonothioate were separated by chromatography on silica. Coupling of each diastereomer to 5'-iodo-thymidyl-3'-acetate, followed over time by ^{31}P NMR, indicated complete reaction without epimerization. This was confirmed by liquid chromatographic analysis of the two diastereomers of dithymidine methylphosphonothioate prepared from each monomeric diastereomer. At 50°C, the coupling reaction was complete within 6 hours. Efforts are being made to allow coupling which eliminates 5'-sulfur in the products, without losing stereospecificity.

In an attempt to develop a trivalent route as well, the diastereomers of 5'-DMT-thymidyl-3'-methylphosphon(N,N-diisopropyl)amidite were separated anhydrously on silica. A 5'-activated monomer, 5'-trifluoroacetyl-thymidyl-3'-acetate, was prepared in order to allow coupling in the absence of tetrazole, which would otherwise epimerize the activated monomer. ^{31}P NMR confirmed that the activated monomer retained its configuration during the coupling reaction; however, the resulting dimers were racemic. Efforts are being made to circumvent epimerization of the transition state in order to allow stereospecific trivalent coupling.

This work was supported by grants to E. W. from the US National Cancer Institute, the Florida High Technology and Industry Council, and Genta, Inc.

Antisense Applications in Lower Eukaryotes and Plants

CD 014 INHIBITION OF GENE EXPRESSION IN PLANTS USING ANTISENSE AND SENSE DNA CONSTRUCTS, William R. Hiatt, Raymond E. Sheehy, Julie Pear and Matthew Kramer, Calgene, Inc., 1920 Fifth Street, Davis, California, 95616. *Agrobacterium tumefaciens* transformation provides a means for stable integration of DNA constructs into the plant genome. We have used this method to achieve stable down-regulation of several endogenous plant genes in whole plants of various species. The enzyme polygalacturonase (PG) degrades pectin and becomes extremely abundant during tomato fruit ripening. Transgenic plants containing a full-length PG cDNA in the antisense orientation to a high-level, constitutive promoter produced fruit with PG enzyme and mRNA levels reduced by greater than 99%. Similar results were obtained with antisense constructs to pectinmethylesterase (PME), another abundant cell-wall degrading enzyme in tomato fruit. A cDNA from tomato (pTOM13) has recently been shown by antisense experiments to encode an enzyme involved in ethylene biosynthesis (Hamilton et al. [1990] Nature 346:284). We have obtained similar results in that antisense constructs with the tomato pTOM13 cDNA inhibited ethylene production and flower wilting when introduced into petunia. Interestingly, sense constructs utilizing PME or pTOM13 cDNAs were also effective in reducing expression of the respective gene product. Thus, the mechanism by which antisense constructs are functioning to inhibit gene expression in plants is unclear.

CD 015 KNOCKING OUT DEVELOPMENTAL GENES IN DICTYOSTELIUM, William F. Loomis and Danny L. Fuller, Center for Molecular Genetics, Department of Biology, University of California San Diego, La Jolla, CA 92093

The physiological role of specific gene products can be clearly assessed by impairing the expression of the genes and comparing the resulting phenotypes to wild-type. Genes can be inactivated by a variety of stable hereditary events including deletions, insertions and missense mutations. Antisense inactivation has the advantage that a gene can be left active at one stage and inactivated at another stage since the regulation of the construct that generates antisense RNA need not be related to that of the target gene. The conditional aspect of antisense inactivation is particularly useful for vital genes.
A half dozen genes of interest have been analyzed in transformed cell lines of *Dictyostelium discoideum* carrying antisense constructs specific to each. These include discoidin I, myosin II heavy chain, the chemotactic cAMP receptor, a specific esterase, clathrin, and a component of the early adhesion mechanism, gp24, that is essential for cell-cell aggregation. Various aspects of development in this organism have been recognized by detailed analyses of the resulting phenotypes.
Null mutants lacking gp24 could not be isolated by classical genetics because the gene is duplicated in the genome. However, the products of these genes are almost identical and so antisense RNA to one also inactivates the other. When the gp24 genes are inactivated, the cells do not cohere and morphogenesis is blocked. We have recently isolated a mutant strain altered in the regulation of the gp24 genes. The phenotype of these cells indicates that multicellularity is necessary for many of the events in early development.
This work was supported by a grant from the National Institutes of Health (GM 36400).

Use of Antisense Technology in Animal Development

CD 016 THE USE OF ANTISENSE RNA IN MAMMALIAN EMBRYOS, Robert P. Erickson, Departments of Pediatrics and Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85724. Our laboratory has studied the use of antisense RNA, both in vitro synthesized and driven from DNA constructs, and oligodeoxynucleotides in the pre-implantation mouse embryo. This material affords an opportunity to study antisense regulation in mammalian zygotes. Most of our work has used the lysosomal enzyme, beta-glucuronidase, as a target. We chose this enzyme because it undergoes a dramatic, 2 log increase in activity during pre-implantation development which we had previously shown is secondary to a large increase in amount of messenger RNA. In addition, very sensitive fluorometric assays are available which allow one to measure the enzyme in single embryos and thus be able to look at events which might only occur in a small number of embryos, eg. transgenic integration. Our work with cytoplasmic injections of antisense RNA showed that a 350 base pair fragment that covered the start codon was more effective than 1400 base pairs of coding sequence not including the start codon. On the other hand, when the same RNAs were made from DNA constructs which have been injected into the pronucleus, the effectiveness was reversed: an antisense transgene for the 1400 base pair coding region inhibited beta-glucuronidase expression in a significant proportion of injected embryos, whereas the antisense transgene "covering" the start codon did not result in significant inhibition of beta-glucuronidase expression. In contrast to the success with the cytoplasmic injections of RNA and the expression from transgenes, we were not able to find an effect of oligodeoxynucleotides in pre-implantation embryos. We tried 20 mer.s directed across a start codon or directed to coding sequences from exon 7, alone or in combination, injected or added to the culture media. In fact, very low uptakes of radiolabelled oligodeoxynucleotide occurred and no significant inhibition was achieved. Having gained this preliminary information on the effectiveness of various modes of antisense regulation of genes in pre-implantation embryos, we applied them to the study of gap junction communication in the pre-implantation embryo. Our results showed clearly that antisense RNA directed at connexin 32 can inhibit the formation of gap junctions in the early embryo, delaying and inhibiting compaction and blastulation. Currently we are using similar approaches to study E-cadherin, another molecule thought to be important for this early differentiation step in the pre-implantation mammalian embryo.

CD 017 *N-MYC* ANTISENSE RESTRICTS THE DIFFERENTIATION POTENTIAL OF NEUROECTODERMAL CELL LINES, Leonard M. Neckers, Angelo Rosolen and Luke Whitesell, NCI, NIH, Bethesda, MD 20892

The nuclear protein encoded by the protooncogene *N-myc* is a DNA-binding phosphoprotein of unknown function. Unlike its homolog *c-myc*, *N-myc* expression is limited to immature B- and T-lymphocytes, fetal tissues and various tumors of neuroendocrine origin, such as neuroblastoma, retinoblastoma, and small cell lung carcinoma. Its transient and localized expression during early stages of organogenesis, together with the presence of conserved motifs identified previously in transcription factors, suggest that *N-myc* may regulate gene expression in the developing organism.

To investigate this possibility, we studied the role of *N-myc* in the differentiation of primitive neuroectodermal tumors by utilizing both exogenous addition of *N-myc* antisense oligonucleotides as well as cells stably transfected with an episomally replicating vector expressing large amounts of *N-myc* antisense RNA. Both neuroblastoma and neuroepithelioma cell lines demonstrate at least two distinct morphologies which can apparently interconvert or "transdifferentiate" between each other. Alternatively, a low abundance stem cell present in the cultures may give rise to both cell types. One morphologic type consists of flat cells with an epithelial appearance, which are contact inhibited, do not clone in soft agar and are not tumorigenic in nude mice. The other type consists of round cells with a neuronal appearance, which do not display contact inhibition, do clone in soft agar and are tumorigenic in nude mice. While the neuronal-type cells express *N-myc* protein, the epithelial-type cells do not. Since wild-type cultures maintain this mixed morphology over many passages, we reasoned that the process responsible for this heterogeneity must be well regulated. In addition, maintenance of such cellular heterogeneity must supply a growth advantage to wild-type cultures.

Results obtained with either oligonucleotide antisense or the antisense episomal vector suggest that both epithelial and neuronal cell types arise *in vitro* from a low abundance stem cell. *N-myc* suppressed cells, while resembling neither neuronal nor epithelial cells morphologically or phenotypically, are able to give rise to both cell types upon release from *N-myc* suppression. These results raise the intriguing possibility that transient *N-myc* expression in neuroendocrine stem cells is required to generate both *N-myc*-negative, non-tumorigenic epithelial-type cells and *N-myc*-positive, tumorigenic neuronal-type cells, and that both cell types are required for full immortalization and tumorigenicity.

Control of Viral Function by Antisense Nucleotides

CD 018 INHIBITION OF HIV REPLICATION AND EXPRESSION BY SYNTHETIC OLIGONUCLEOTIDES, Sudhir Agrawal*, Prem S. Sarin** and Paul C. Zamecnik*; *Worcester Foundation for Experimental Biology, Maple Avenue, Shrewsbury, MA 01545; **Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD 20892.

The use of antisense oligonucleotides in control of gene regulation and inhibition of virus replication is a unique approach designed to interfere with cell and virus replication, transcription, and translation machinery at the molecular level. Extensive studies have been carried out to inhibit human immunodeficiency virus by synthetic oligonucleotides and their several phosphate backbone modified analogues. Thus far, studies have been carried out in tissue culture, and inhibition of HIV expression have been monitored by several parameters. To date, the most effective oligonucleotides are antisense phosphorothioate oligodeoxynucleotides, showing ID_{50} of 1×10^{-8} to 1×10^{-7}M.[1] Antisense oligonucleotide phosphoramidates have likewise been effective inhibitors, and are currently being studied by us. We have also employed mixed backbone antisense oligomers, consisting of peripheral methyl phosphonates and a central section of phosphorothioates or unmodified internucleoside phosphates. These have the property of activating RNase H only in the central segment of the oligomer[2]. Antisense oligoribonucleotide and its phosphorothioate analogue were not as effective as their oligodeoxynucleotide counterpart in inhibiting HIV replication. Preliminary mouse and rat toxicity studies showed that these analogues are non-toxic up to a dose of 100 mg/kg bodyweight if given intraperitoneally or subcutaneously, daily for 14 days. Thus, there may be an exploitable therapeutic window for "oligonucleotide therapy".

(1) Agrawal S. *et al.*, (1989). *Proc. Natl. Acad. Sci. USA.*, **86**, 7790-7794.
(2) Agrawal S. *et al.*, (1989). *Proc. Natl. Acad. Sci. USA.*, **87**, 1401-1405.

CD 019 CHEMICALLY MODIFIED OLIGODEOXYNUCLEOTIDE ANALOGS AS REGULATORS OF VIRAL AND CELLULAR GENE EXPRESSION, Jack S. Cohen, Pharmacology Department, Georgetown University Medical Center, Rockville MD 20850

Watson-Crick base pairing enables an oligodeoxynucleotide to act in principle as an antisense complement to the target sequence of a mRNA in order to bring about translation arrest, and selectively regulate gene expression. However, natural oligodeoxynucleotides are susceptible to degradation by nucleases *in vivo*. Chemically modified analogs that are nuclease-resistant enable the antisense strategy to be utilized in practice. There are two main types of oligodeoxynucleotide analog that have been tested for this approach, those that are neutral, and those that retain the charge of the phosphodiester group. We have chosen to use the water-soluble phosphorothioates, in which one non-bridging oxygen is replaced by a sulfur. Studies in cell free and model systems indicate that these are more effective inhibitors of β-globin expression than the natural congeners. They also enter cells, albeit more slowly than the natural compounds, apparently by interacting with a specific cellular receptor. These analogs have been found to regulate *rev* gene expression in HIV in a chronically infected H9 cell assay, and to inhibit bcl-2 gene expression in 697 leukemic cells. However, they also inhibit HIV and HSV-2 in a sequence non-specific manner, that can be traced to their selective inhibition of viral polymerases. In general, a chemically modified oligodeoxynucleotide analog can be considered to be a special case of a novel form of *informational drug*, in which an oligomer has increased specificity for a target site by virtue of the information encoded in its sequence, and is protected against endogenous enzyme activity that might degrade it.

CD 020 INHIBITION OF REPLICATION OF AVIAN RETROVIRUSES BY ANTISENSE RNA, Richard To and Paul Neiman, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, 98104. We have observed that cell clones expressing *neo*^{r} RNA are resistant to infection by avian retroviral constructs bearing antisense *neo*^{r} inserts adjacent to the polypurine tract near the 3' end of the viral RNA genome(1). In addition we have shown that the degree of resistance is proportional to the level of *neo*^{r} RNA in the host cell and that the block occurs early in the viral replication cycle before integration of vDNA into the host chromosome (1). More recent analysis has employed antisense RNA introduced into polyclonal cultures by retroviral vectors. We observed that the replication of target viruses on such cultures can be reduced by as much 3 to 6 logs of titer of infectious progeny. Analysis of the early stages of replication of target viruses with antisense *neo*^{r} inserts showed that linear vDNA intermediates were formed in the presence of complementary *neo*^{r} RNA, but closed circular vDNA was not detected and stable integration of vDNA did not occur. The structure of the termini of the vDNA linear intermediates appears to be abnormal may explain the failure of integration. Although not completely resolved at present these results are consistent with a testable model in which antisense RNA inhibits the formation and/or function of the RNA primer for second strand synthesis of vDNA. These findings also illustrate an intracellular stage in the retroviral relication cycle which may be vulnerable to biologically significant inhibition.

1. To, R. Y-L., Booth, S.B. and Neiman, P.E. Inhibition of retroviral replication by antisense RNA.(1986) Mol. Cell. Biol. *6*, 4758-4762.

Gene Regulation by Antisense RNA and DNA

Antisense Regulation of Neoplastic Cell Proliferation

CD 021 **USE OF ANTISENSE OLIGONUCLEOTIDES TO DISSECT THE V-SIS-DEPENDENT TRANSFORMATION PATHWAY,** John Westwick, Michael Karin and Dan Mercola, Departments of Pathology and Pharmacology, University of California at San Diego, La Jolla, CA 92093.

Clonal lines of simian sarcoma virus transformed NIH-3T3 (SSV-3T3) cells continuously over-express c-fos and the role of c-fos was tested by transfection with antisense fos RNA expressing plasmids (SSVnsof cells) which markedly reduces steady state transcript levels[1]. SSVnsof cells exhibit flat morphology, reduced growth rate and restored contact inhibition in vitro but remain tumorigenic in athymic mice albeit with a slow growth rate. Thus over-expressed c-fos appears to contribute to but is not essential for the transformed phenotype of SSV-3T3 cells[1]. In order to identify additional targets for antisense intervention of transformation a model system was developed using NIH-3T3 cells bearing a metalothionein-v-sis construct (NSVnMTsis cells). Multiple clones of these cells exhibit rapid, complete and reversible transformation including reversible tumorigenesis as evidenced by reversion of subcultured cells to normal morphology, growth rate, saturation density and zinc-dependent anchorage-independent growth[2]. Thus in this system induction of v-sis-dependent pathway alone is sufficient for transformation. As a first step to identifying downstream events, synthetic (18 mers) antisense oligonucleotides to c-fos, fos-B, fra-I, c-jun, jun-B, and jun-D were applied at 50 μM to NIH-3T3 or SSVnMTsis cells in the presence and absence of zinc. For example in preliminary studies anti-c-fos or anti-c-jun reduced zinc-stimulated growth as judged by uptake of ^{3}H-T by over 50% suggesting a role of the AP-1 complex. This is supported by transient transfection assays using a thymidine kinase-CAT fusion reporter construct containing three AP-1 regulatory elements. Zinc stimulation led to 2-13 X increase in CAT synthesis which was inhibited by 50-90% by either anti-c-fos or anti-c-jun but not by a random sequence oligonucleotide. Thus this approach may be useful in identifying other gene products which mediate sis-induced transformation.

[1]Mercola, D. et al. (1988) Gene, 72, 253.
[2]Carpenter, P. et al. (1989) J. Cell. Biochem., 13B, 119.

CD 022 ANTISENSE-MEDIATED DEPLETION OF C-MYC CAUSES REVERSION OF RAS-TRANSFORMED NIH 3T3 CELLS, Marshall Sklar, Elizabeth Thompson, Monica Liebert, H. Barton Grossman, Michael Welsh, Michael Smith, and Edward V. Prochownik, University of Michigan Medical School, Ann Arbor, MI 48109.

Transformation by oncogenes whose protein products are localized extranuclearly requires that their signals be transmitted to nuclear effectors which ultimately mediate the stable genetic changes associated with the neoplastic phenotype. We tested the hypothesis that certain "nuclear" proto-oncogenes such as c-myc, c-jun, and c-fos serve this effector role in transformation by ras oncogenes. Plasmids were constructed which allowed for the conditional expression of antisense transcripts of these genes from a MMTV promoter. These plasmids were transfected into the highly transformed DT cell line which contains two copies of a v-K-ras oncogene and stably transfected clones were selected. We found that as many as 85% of clones transfected with the MMTV-c-myc construct assumed a flat, non-transformed morphology within 24 hours of adding dexamethasone (DM). (DM) had no effect on the morphology of untransfected DT cells or of cells transfected with various control MMTV sense and antisense plasmids. Molecular analyses of several c-myc antisense clones showed that they contained unrearranged plasmid DNA sequences and that the induction of antisense transcripts coincided with 80-90% reductions in the level of c-myc protein. All c-myc antisense clones examined showed a greatly reduced capacity for soft agar colony formation and for tumorigenesis in nude mice. These results indicate that the c-myc protein may be one component of the ras signal transduction pathway. The approach described here may be generally useful for defining a role for other nuclear proto-oncogene products in transformation by "extranuclear" oncoproteins.

Gene Regulation by Antisense RNA and DNA

Natural Antisense Activities and Mediators

CD 100 **CHARACTERIZATION OF A NATURALLY OCCURRING ANTISENSE RNA TRANSCRIPT WITH HOMOLOGY TO THE SECOND INTRON OF THE HUMAN C-MYC GENE**, Paul Celano, Craig M. Berchtold and Robert A. Casero, Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231

The regulation of the transcription of c-myc and other growth associated genes are subject to a complex set of poorly understood regulatory mechanisms. Our studies have identified an antisense RNA transcript with homology to the second intron of the human c-myc gene using strand specific probes. After growth stimulation of normal human peripheral blood lymphocytes with phytohemmaglutinin, steady state levels of this antisense RNA species increase 10-20 fold which parallels the expression of c-myc. Northern blot analysis of this antisense RNA transcript during the differentiation of human leukemia cell line HL-60 with the phorbol ester 12-0-tetradecanoyl-phorbol-13-acetate (TPA) revealed that a somewhat different pattern of expression from c-myc. Steady state levels of c-myc mRNA increase 2 to 4 fold 15 to 60 minutes after TPA treatment and with levels decreasing to near undetectable levels by 8 hours, corresponding to cellular differentiation. In contrast, the antisense RNA levels decline dramatically by 60 minutes post TPA treatment. Further analysis with RNAase protection studies suggest that this RNA may arise from a different gene locus. These studies have identified a naturally occurring antisense RNA transcript with homology to c-myc that can be modulated during cell growth and differentiation and suggest a potential regulatory role of this transcript in the expression of the c-myc gene.

CD 101 CO-EXPRESSION OF ENDOGENOUS OVERLAPPING HUMAN GENES, Stephen E. Gitelman, James Bristow, Synthia Mellon, Walter L. Miller, Departments of Pediatrics and OB-GYN, University of California, San Francisco, CA 94143

We have discovered a gene in the Class III region of the HLA locus that may offer insights into the co-expression of endogenous overlapping genes. This novel gene, operationally termed Gene X, is part of a tandemly duplicated cluster of genes including complement component C4 and adrenal steroid 21-hydroxylase (21OH). These 6 genes lie in the order C4A-21OHA-XA-C4B-21OHB-XB. 21OHA is the only pseudogene in this gene cluster. Gene X is encoded on the opposite strand of DNA from C4 and 21OH, and overlaps the last exon of the 21OH gene (PNAS 86: 6582, 1989). We have cloned and sequenced both X genes, and their corresponding cDNAs. The XB gene spans more than 20 kb, with 21 exons identified to date. The deduced XB protein has 13 peptide repeats resembling the type III fibronectin domain, and has a carboxy terminal fibrinogen-like domain; a similar juxtaposition of these domains is found in the extracellular matrix protein tenascin. The 5' end of the XA gene is truncated, and it spans only 7 kb with only 13 exons. It also has a 121 bp deletion eliminating 91 bp of exon 4. Thus, the deduced XA protein codes for a protein with only two and a half type III fibronectin repeats. Solution hybridization RNase protection assays demonstrate the presence of XB mRNA in almost all fetal tissues tested, including the adrenal gland; however, XA expression is restricted to the adrenal gland. Similarly, 21OHB mRNA is detected only in the adrenal gland, in similar abundance to X. To determine if these genes are expressed in the same cell at the same time, we performed *in situ* hybridization assays for X and 21OH mRNAs. The antisense riboprobes showed similar patterns of diffuse hybridization throughout the fetal adrenal cortex in adjacent tissue slices, indicating co-expression of these genes. The co-expression of complementary transcripts in the same cell raises intriguing topological questions about the regulation of expression of overlapping genes.

CD 102 Involvment of an endogenous antisense RNA and a ds RNase in the regulation of mRNA stability of a *Dictyostelium* gene.

Martin Hildebrandt, Axel Möhrle, Petra Kröger and Wolfgang Nellen

Max Planck Institute for Biochemistry, Department of Cell Biology, D-8033 Martinsried, FRG

The Dictyostelium prespore gene EB4 is constitutively transcribed throughout development, however, the corresponding mRNA is accumulated only in aggregating cells. From the same gene locus, an endogenous antisense RNA is transcribed in vegetative and mechanically disaggregated cells. We present evidence, that mRNA and antisense RNA may form in vivo hybrids which are rapidly degraded by an endonucleolytic double strand specific RNAse.

CD 103 NATURALLY OCCURRING ANTI-SENSE TRANSCRIPTS FROM NORMAL AND MUTANT BURKITT LYMPHOMA C-MYC GENES Vinay Jain and Ian Magrath, Pediatric Branch, National Cancer Institute, Bethesda, MD 20892

We have detected approximately equal sense (S) and anti-sense (AS) transcriptional activity from a fragment encompassing the first exon and intron of c-MYC in transient transfection assays using luciferase as a reporter gene. To mimic breakpoints found in Burkitt's lymphoma, we made deletion constructs of c-MYC coupled to luciferase in both S and AS orientation. When transcriptional activity from various MYC fragments was compared, location of breakpoint affected relative abundance of S and AS transcripts. Almost 70% of AS transcriptional activity originated upstream of the P2 promoter. Constructs deleted immediately downstream of exon 1 had only 10-20% of AS transcriptional activity seen in the intact fragment. When the MYC Intron Factor binding sites (MIF-1 and MIF-2) were also deleted, there was a significant increase in AS transcription.

Dual reporter constructs (luciferase in S and beta-galactosidase in AS orientation) made with normal and mutant MYC genes obtained from Burkitt lymphoma cell lines (NAB and PA682) showed a difference in the ratio of S to AS transcripts. Thus, both the location of the chromosomal breakpoint and the presence of mutations in the MYC oncogene in Burkitt's lymphoma could alter the ratio of S to AS transcripts. Alteration in the ratio of AS to S transcripts may be a mechanism of deregulation of c-MYC in Burkitt's lymphoma.

CD 104 BIOCHEMICAL PURIFICATION OF THE DOUBLE-STRAND RNA UNWINDING/ MODIFYING ACTIVITY, Unkyu Kim and Kazuko Nishikura, The Wistar Institute, Philadelphia, PA 19104

The double-strand RNA(dsRNA) unwinding/modifying activity converts up to 50% adenosine residues to inosine creating I-U base pair mismatches in the dsRNA substrate. The unstable I-U base pairing introduced by the activity is proposed to be responsible for the unwinding of the dsRNA. The dsRNA unwinding/modifying activity is present in the nuclei of most of the mammalian cells as well as in the lower vertebrates and invertebrates such as *Xenopus laevis* , *Spisula solidissima* (the surf clam) and *Drosophila*. In order to eventually determine its physiological function in somatic cells, we have begun biochemical purification of the dsRNA unwinding/modifying activity. When the nuclear extract prepared from the bovine liver was passed through several biochemical columns followed by a dsRNA affinity column, the activity was enriched at least two thousand fold. We are in the process of scaling up the purification procedure in order to obtain the purified activity in enough quantities for peptide sequencing and antibody production.

CD 105 DEGRADATIVE CLEAVAGE OF ANTISENSE RNAI IS THE RATE-LIMITING STEP IN REGULATING RNAI CONCENTRATION AND CONTROLLING COPY NUMBER OF ColE1-TYPE PLASMIDS IN VIVO. Sue Lin-Chao[1,2], W.T. Chen[1] and Stanley N. Cohen[2,3], Institute of Molecular Biology[1], Academia Sinica, Nankang, Taipei 11529, R.O.C., and Department of Genetics[2] and Medicine[3], Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

We report here experiments that investigate the role of antisense RNAI stability on the in vivo regulation of replication and copy number of ColE1 and related plasmids. Using an E. Coli mutant that conditionally inactivates RNAseE, a ribonuclease implicated initially in the processing of 9S ribosomal RNA, we show that cleavage of RNAI by RNAseE is the rate-limiting step in regulating RNAI half-life in vivo, and consequently RNAI concentration and plasmid copy number. Mutational replacement of three nucleatides near the 5'end of pBR322 RNAI with those of the related p15A-derived plasmid, pACYC184, increased pBR322 RNAI half-life and decreased plasmid copy number to levels characteristic for pACYC184. Modification of the sequence at the 5'end of RNAI to make it insusceptible to cleavage by RNAseE prolonged RNAI half life and consequently inhibited plasmid replication. While antisense RNA lacking the five nucleotides removed by RNAseE (i.e. $RNAI^{-5}$) interacts poorly with its wild type target (i.e.RNAII), it could regulate plasmid copy number when the complementary five nucleotides were deleted from RNAII. Both mutants were compatible with otherwise identical replicons encoding the full length RNAI/RNAII sequence. Effects similar to those seen in the RNAseE mutant strain were observed in bacteria mutated in the ams gene, which maps in the vicinity of the RNAseE locus and is known to have generalized effects on messenger RNA stability.

CD 106 IDENTIFICATION OF POTENTIAL ANTISENSE REGULATION OF eIF-2α GENE EXPRESSION, Toby Silverman, Tom Boal, Roger Cohen, Masayuki Noguchi, Suzanne Miyamoto and Brian Safer, Molecular Hematology Branch, NHLBI, NIH, Bethesda, MD 20892

Human Go T lymphocytes contain very low levels of eIF-2α mRNA. Following mitogenic activation, expression of mRNA encoding this rate-limiting translation factor increases > 50-fold and translational activity increases 10-fold. The mechanism responsible for increased eIF-2α mRNA expression was explored by nuclear run-on transcription analysis and actinomycin D pulse chase experiments. Neither an increased rate of eIF-2α transcription nor a decreased rate of α mRNA degradation could account for the large increase in the level of α mRNA. Using an intron specific probe, the increased expression of eIF-2α mRNA was correlated with stabilization and/or more efficient processing and transport of the eIF-2α primary transcript. We have now identified a divergent transcript which overlaps the eIF-2α primary transcript for approximately 450 nucleotides. Since the antisense RNA spans the first intron/exon junction and mutations which inhibit its expression increase eIF-2α promoter activity in vivo, a role in the regulation of eIF-2α gene expression is indicated.

CD 107 A SEARCH FOR NATURALLY OCCURRING ANTISENSE RNA CONTROL SYSTEMS,

F. Söderbom and E.G.H. Wagner, Department of Microbiology, Biomedical Center, Uppsala University, Box 581, S-75123 Uppsala, Sweden.

Natural, *bona fide*, antisense RNA control systems have been identified and characterized in some detail almost exclusively in prokaryotes, and more specifically in accessory elements such as phages, transposons and plasmids. As *bona fide* antisense RNAs we define the cases where both antisense and target RNAs are transcribed from the same DNA segment, but with opposite polarity, such that the RNAs are completely complementary. To our knowledge, all antisense RNA systems have so far been found "by accident", i.e. by identifying a control element for a gene under investigation as an antisense RNA. We have initiated a systematic search for antisense RNAs with the objective to test for the occurance of control systems in bacteria and , subsequently, in eukaryotes.

This strategy is based on the expected stability of the (perfect) duplex between antisense and target RNA, and is briefly outlined here: Total RNA is extracted from strains with and without plasmid R1. The R1-containing strains are used as positive controls, since this plasmid regulates its copy number by a well-characterized antisense RNA (see poster Wagner et al) . An aliquot of this RNA is run on gels and blotted onto membranes. Another aliquot of RNA is polyadenylated *in vitro* by *E.coli* poly(A) polymerase, then hybridized to the membrane, stringently washed, and reeluted. This fraction - which is presumed to consist of antisense/target RNAs that are complementary to RNAs immobilized on the filter - is then converted into cDNA, with the help of a primer complementary to the poly-A tail. Linkers are ligated to the ends, and after PCR-amplification, the DNA-fragments are cloned in a plasmid between two phage promoters. Transcription from those plasmids will yield probes for screening of an *E.coli* genomic library.

If this method can detect new antisense RNA-regulated systems in *E.coli*, it will be of interest to screen other prokaryotic or eukaryotic organisms for such control systems.

CD 108 ANTISENSE RNA IN REPLICATION CONTROL OF PLASMID R1: ANALYSIS OF STRUCTURE/SEQUENCE REQUIREMENTS FOR CopA/CopT INTERACTION AND THE KINETICS OF BINDING,

E.G.H. Wagner, C. Persson, and K. Nordström, Department of Microbiology, Biomedical Center, Uppsala University, Box 581, S-75123 Uppsala, Sweden.

The main replication control element in plasmid R1 is an antisense RNA called CopA. CopA post-transcriptionally inhibits the synthesis of the initiator protein RepA by binding to the leader region (called CopT for "target") in the RepA mRNA. The mechanism of inhibition is not clearly understood, but we have shown that the CopA/CopT duplex is cleaved by RNase III, and that this cleavage affects *repA* expression (Blomberg, Wagner and Nordström [1990] *EMBO J.* **9**, 2331).

The kinetics of duplex formation between the two RNAs has been studied *in vitro* (Persson, Wagner and Nordström [1988] *EMBO J.* **7**, 3279). We report here on the structural requirements for the two main steps in duplex formation, recognition ("kissing") between the main 6-nt loops, and hybridization to form the complete duplex (Persson, Wagner and Nordström [1990a] *EMBO J.* in press). The results can be summarized as follows: i) the major stem-loop is required but not sufficient for binding; ii) a single-stranded stretch of nucleotides distal to the loop is involved in the hybridization step; iii) the major stem-loop alone is inactive in forming the duplex but forms the kissing complex; iv) formation of the kissing complex involves nucleotides in the upper stem.

We have also studied the kinetic scheme for the reaction from the free CopA and CopT RNAs to the complete duplex, and esimated the relevant rate constants (Persson, Wagner and Nordström [1990b] *EMBO J.* in press). The reaction is a true second-order reaction with the binding rate constant k_1 determining the copy number of the plasmid. The off-rate constant k_{-1} is very small compared to the rate constant representing the conversion of the "kissing" intermediate to the duplex, k_2. The values determined for the rate constants suggest that the reaction is far from equilibrium in the cell, and in practice its rate will be determined almost exclusively by the binding rate constant k_1. Our data therefore argue strongly for a reaction scheme for CopA/CopT interaction that formally resembles Briggs-Haldane kinetics.

Gene Regulation by Antisense RNA and DNA

Ribozymes and Modified Oligdeoxynucleotide Approaches to Anitsense Control

CD 200 DEGRADATION AND MEMBRANE TRANSPORT CHARACTERISTICS OF MODIFIED ANTISENSE OLIGONUCLEOTIDES, Saghir Akhtar, Yoko Shoji, Ryszard Kole and Rudy Juliano. Department of Pharmacology and Lineberger Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599.

This laboratory has interests in the delivery and transport characteristics of modified antisense oligonucleotides into cells in order to evaluate their potential as therapeutic agents. To this end we have investigated the degradation of a series of modified oligodeoxynucleotides in cellular extracts and sera. Degradation of 5' end [^{32}P] labelled oligodeoxynucleotides (14 mer) containing methylphosphonate (MP-oligo), alternating methylphosphonate and phosphodiester (Alt-MP-oligo), phosphorothioate (S-oligo) and unmodified phosphodiester (D-oligo) internucleoside linkages was studied in Hela cell nuclear extract, S100 cytoplasmic extract, normal human serum and calf bovine serum at 37°C. For the oligodeoxynucleotides studied, increasing degradation was observed in the order: S100 cytoplasmic extract < nuclear extract < normal human serum < calf bovine serum. In any given degradation medium, the D-oligo was the least stable oligodeoxynucleotide whereas the Alt-MP, MP and S-oligos were of comparable stability and all relatively more stable than D-oligo. In a study involving α-MEM cell culture medium containing 10% heat inactivated fetal calf serum (heated to 56°C for 1 hour), the D-oligo was found to be rapidly degraded (degradation evident within 10 mins) whereas degradation products for the S-oligo were observed within 1 hour. In contrast, the Alt-MP oligo remained stable throughout the 3 hour degradation experiment. These results indicated that in cell culture medium containing heat inactivated serum Alt-MP oligo was more stable than D- and S-oligos. Transport studies investigating the mechanism of uptake with modified oligodeoxynucleotides have been carried out with liposomes, as model membranes, and in cell culture.

CD 201 CRITICAL ASSESSMENT OF THE USE OF HAMMERHEAD RIBOZYMES FOR REVERSE GENETIC ANALYSIS IN MAMMALIAN CELLS: LACK OF EFFICIENCY AND SPECIFICITY ? Edouard Bertrand, Thierry Grange, J Roux and Raymond Pictet, Inst. J. Monod, 2 pl. Jussieu, 75251 PARIS Cedex 05 (FRANCE)

We have analysed the *in vitro* activity (at 37°C) of hammerhead ribozymes directed against the mRNA coding for the rat pituitary-specific transcriptional activator PIT1/GHF1.

-First, we have found that the affinity of a ribozyme (R3) for a slightly truncated Pit1 mRNA (1 Kb long) was 10 fold lower than for a shorter RNA sequence (60 b long) containing the same target sequence (GAAGTAGTA↓AGAGTGTGG). Moreover, the affinity of 4 other ribozymes for the Pit1 mRNA was of the same order than the one of R3. Thus, the reduction of the ribozyme affinity for long substrates (as mRNAs) could be a general phenomenon. This reduction of affinity is probably due to secondary structure determinants.

-Second, the length of the hybridized region (H) in the R3/Substrate complex has been symetrically reduced. We show that: -A. R3 is inactive when H is 8bp long, is not catalytic when H is 17 bp long, and has similar catalytic efficiency when H is 14, 12 and 10 bp long. -B. R3 has the same affinity for its substrate, when H is 17, 14, 12 and 10 bp long. From this, we infere that (*i*) the optimal efficiency of hammerhead ribozymes is when H is betwen 10 and 14 bp; (*ii*) because 10 bp of H is rather short to discriminate a single mRNA in the complex mRNA population of an eucaryotic cell, hammerhead ribozymes may be not specific enough to be of general use for reverse genetic analysis.

CD 202 FUNCTIONAL ANALYSIS OF SYNTHETIC RIBOZYMES CONTAINING MODIFIED OLIGONUCLEOTIDES, Marta Blumenfeld, Franck Lescure, Gilbert Thill, Elizabeth Petit-Koskas and Marc Vasseur, GENSET SA and Laboratoire de Virologie Moléculaire, UFR de Biochimie, Hall des Biotechnologies, Université Paris 7, 2 place Jussieu, 75251 PARIS Cedex 05.

Ribozymes and antisense oligonucleotides provide a unique tool for the rational development of molecules design to control the expression of selected genes, and thus offer a promising approach in specific antiviral therapy. Since one of the major problems of a ribozyme-based therapy concerns the stability of these molecules *in vivo* , we were interested in developping modified ribozyles that display increased stabilities and/or better catalytic properties than the "classical" ribozymes. Using firefly luciferase as the target mRNA, we have synthesized ribozymes containg different modified oligonucleotides and we have tested them in an *in vitro* assay with both synthetic substrates and luciferase mRNA. We have also studied their *in vivo* effect on several luciferase-expressing cell lines. Comparison of the kinetic constants of the modified ribozymes as well as their relative efficiencies in inhibiting luciferase expression in cultured cells will be presented and discussed.

CD 203 INTRACELLULAR DISTRIBUTION OF MICROINJECTED ANTISENSE OLIGONUCLEOTIDES.Geneviève Degols, Jean-Paul Leonetti, Nadir Mechti and Bernard Lebleu, Laboratoire de Biochimie des Protéines, URA CNRS 1191, Université Montpellier II, Sciences et Techniques de Languedoc, Place E. Bataillon C012, 34095 Montpellier cedex 5, France

The mechanism(s) of action, and the intracellular distribution of antisense oligonucleotides (oligomers) remain poorly understood. To ascertain their fate and their intracellular location, antisense oligomers conjugated with various fluorochromes or with BrdUrd were microinjected in the cytoplasm of somatic cells and their cellular distribution was followed by fluorescence microscopy in fixed and non-fixed cells. A fast translocation in the nuclei and a concentration on nuclear structures was observed whatever probe was used. This transport occurs by diffusion since it is not affected by depletion of the intracellular ATP pool, temperature or excess of unlabeled oligomer. Accumulation of the oligomers takes place on a set of nuclear proteins ranging from 36 kD to 50 kD as revealed by cross-linking of photosensitive oligomers. Present data provide interesting prospects in the study of antisense oligomer mechanism of action.

CD 204 RIBOZYME MEDIATED CLEAVAGE OF THE ALZHEIMER AMYLOID PEPTIDE PRECURSOR

Robert Denman, Benjamin Purow and David L. Miller NYS Institute for Basic Research in Developmental Disabilities 1050 Forest Hill Road, Staten Island, NY 10314 USA.

A versatile synthetic oligonucleotide cassette was synthesized which contained a 23 bp consensus sequence of a classic hammerhead ribozyme interspersed between a 14 bp sequence complementary to bases 133-139 and 142-148 of the Alzheimer amyloid peptide precursor (AAPP) mRNA. The 42 base ribozyme RNA synthesized from a T7 promoter within the cassette was shown to cleave AAPP mRNA at three sites within the conserved GUC sequence *in vitro*. The specificity and efficiency of cleavage exhibited by this ribozyme was found to be inversely dependent upon the Mg^{+2} concentration using two different buffer systems. The cassette was ligated into the *SalI/XhoI* sites of the eucaryotic expression vector pMAM-neo and transfected into COS-7 cells. Steady-state AAPP mRNA levels determined by a quantitative PCR assay before and after dexamethasone induction will be presented showing the cleavage efficiency of the ribozyme *in vivo*.

CD 205 ANALYSIS OF PROTOPLASTS AND TRANSGENIC TOBACCO PLANTS CONTAINING A RIBOZYME GENE TARGETED TO THE "REPLICASE" REGION OF TMV POSITIVE STRAND GENOMIC RNA. Brent V. Edington, Reyne Mathewson, Frank A. Coker, Richard A. Dixon, and Richard S. Nelson, Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402

An RNA enzyme (ribozyme) capable of bisecting TMV genomic RNA at nucleotide position 2467 has been synthesized. The catalytic region, identical to the catalytic sequences in the satellite of tobacco ringspot virus, is targeted to TMV by forty nucleotides of sequence complementary to the RNA-dependent RNA polymerase gene(s) of TMV. This ribozyme cleaves purified positive strand TMV producing fragments of the expected size. Binary vectors (pKYLX71) containing ribozyme or antisense constructs driven by the 35S promoter of CaMV were introduced into tobacco protoplasts concurrent with TMV. Accumulation of TMV coat protein in leaf mesophyll protoplasts containing the ribozyme gene was 10% that of the levels found in protoplasts containing TMV and vectors with the antisense construct, TMV and vectors or TMV only. Nicotiana tabacum cv. Xanthi and Xanthi "nc" were both transformed with vectors containing the ribozyme construct, antisense construct, or pKYLX71. Analysis of these primary transformants, including resistance to TMV infection, will be presented.

CD 206 EFFECTS OF NOVEL NUCLEOSIDE DERIVATIVES ON STABILITY AND SPECIFICITY OF NUCLEIC ACID DUPLEXES. Susan M. Freier, Walt F. Lima, David J. Ecker, Oscar L. Acevedo, Françoise Debart, Charles J. Guinosso, Andrew M. Kawasaki, Kanda Ramasamy, Yogesh S. Sanghvi, Jean-Jacques Vasseur, Maryann Zounes, and P. Dan Cook, ISIS Pharmaceuticals, 2280 Faraday Ave, Carlsbad, CA 92008

Nucleoside derivatives are often incorporated into antisense oligonucleotides to increase cellular uptake and/or nuclease resistance. It is critical, however, that these modifications do not reduce the affinity or specificity of traditional Watson•Crick pairs. To determine the effects of nucleotide modifications on duplex stability and specificity, the thermodynamic stability of nucleic acid duplexes containing novel substitutions at complementary and non-complementary sites were compared to their wild-type DNA analogs. Effects of certain modifications at the 5 and 6 positions of pyrimidines, the 2 and 3 positions of purines, the 2′ sugar position and the phosphodiester linkage on both stability and specificity will be reported.

CD 207 USE OF A PHOTOACTIVATABLE OLIGONUCLEOTIDE/CROSSLINKER COMPLEX TO IDENTIFY EXTRACELLULAR AND INTRACELLULAR OLIGONUCLEOTIDE BINDING SPECIES, Daniel A. Geselowitz and Leonard M. Neckers, NCI, NIH, Bethesda, MD 20892

Understanding the mechanism by which oligonucleotides penetrate cells and reach their targets is important for the design of more effective antisense and antigene agents. We have previously ascertained that oligonucleotides are internalized via endocytosis and have preliminarily identified a membrane protein involved in this process. Here we continue these studies using an oligonucleotide coupled to a photoactivatable, iodinated crosslinking reagent. This enables us to take a "snapshot" at any given time of the cellular components with which an oligonucleotide has become associated. In these experiments, intact HL60 cells, or their subcellular fractions, were incubated with the reagent and then exposed to UV light. When a labeled 15-mer or 39-mer phosphodiester oligonucleotide was incubated with live cells for 2 h, the primary labeled product was a protein of Mr = 75 kDa. Several fainter bands were identified as nuclear proteins, as they were also plainly seen when whole nuclei were incubated with the reagent. When a 27-mer phosphorothioate oligonucleotide was used, the main protein observed was approximately 50 kDa, and when incubated with whole nuclei, the protein labeling was also different than that seen with phosphodiester oligos. This technique may also be used to demonstrate the intracellular association of an oligonucleotide with its putative target: either mRNA or DNA. Studies using an antisense sequence to label mRNA and an antigene sequence to label genomic DNA will be presented.

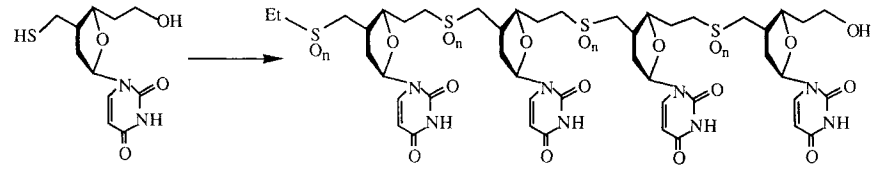
CD 208 TRIPLE HELIX FORMATION UNDER PHYSIOLOGICAL CONDITIONS, Jeffery C. Hanvey, Ellen M. Williams, and Jeffrey M. Besterman, Chemotherapy Division, Glaxo Inc., 5 Moore Dr., Research Triangle Park, N.C. 27709

A triple helix can form at homopurine sequences in DNA with either homopyrimidine or homopurine (G-rich) oligonucleotides as the third strand. Hoogsteen hydrogen bonds form between the purines of the duplex and the oligomer (A with T, G with CH^+, or a G•G bond for a purine oligomer). Pyrimidine-oligomer triplexes are more stable at acidic pH due to protonation of cytosine. We investigated the potential of pyrimidine oligomers to form triplexes at physiological pH values under a variety of conditions (temperature, ionic strength, multivalent ions). Triplex formation was monitored by band-shifts, inhibition of restriction enzyme cleavage, and DMS footprinting. Results indicate that triplexes form readily at pH 7.5 under appropriate conditions; both Mg and spermine stabilize triplex formation while KCl destabilizes it. The effects of pyrimidines within the purine strand of the duplex on triplex formation was examined. The number and the position of the interruptions critically affect the ability of the oligomers to form triplexes. Footprinting studies indicate a localized melting ("bubble") in the triplex around the interruption. Finally, the effect of oligomer length was examined; increasing the length of the oligomer stabilizes triplex formation.

CD 209 NON-IONIC ANTISENSE OLIGONUCLEOTIDES CONTAINING SULFIDE AND SULFONE LINKAGES IN PLACE OF PHOSPHODIESTER GROUPS IN NATURAL OLIGONUCLEOTIDES

Zhen Huang, Steven A. Benner, Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule, 8092 Zürich, Switzerland

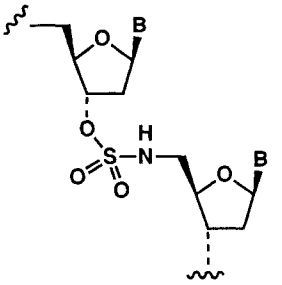
We have synthesized sulfide and sulfone analogues of DNA as antisense compounds. The linkers are stable to alkaline and biological hydrolysis, and lack an asymmetric center, meaning that the analogs are not mixtures of diastereomers with different binding constants. In both respects, the analogs are substantially improved over other non-ionic analogs. Further, sulfone analogs are both water and lipid soluble, and sulfones assist in the penetration of cell membranes of natural oligonucleotides. Sulfone analogs as short as tetramers form stable duplexes with natural oligonucleotides. The enhanced binding is presumably due to the lack of interstrand charge repulsion.



n = 0, 1 or 2

CD 210 **NOVEL OLIGONUCLEOTIDE ANALOGUES WITH A SULFUR-BASED LINKAGE.** M. R. Kirshenbaum, E. M. Huie, and G. L. Trainor, Central Research and Development Department, E. I. du Pont de Nemours and Co., Inc., Experimental Station, PO Box 80328, Wilmington DE 19880-0328.

A novel class of oligonucleotide analogues has been developed as research tools and potential anti-sense reagents. In these analogues, one or more of the internucleotide phosphodiester linkages has been replaced by the isoelectronic, isostructural sulfamate linkage. This linkage is synthetically accessible, chemically stable, uncharged, achiral, nuclease resistant, and capable of supporting duplex formation. This presentation will focus on aspects of the *in vitro* biochemistry of these analogues. The substrate properties of duplex oligonucleotides containing a restriction endonuclease (*Eco* RI, *Nsi* 1) recognition sequence with the sulfamate moiety at the position of strand scission, will also be detailed. The effects of sulfamate modification on hybridization, endo- and exo-nuclease susceptibility and polymerase activity will be described along with implications for anti-sense function.



CD 211 INTERACTION OF PSORALEN DERIVATIZED OLIGONUCLEOSIDE METHYLPHOSPHONATES WITH VSV MESSENGER RNA IN VITRO AND IN CELLS. Joel T. Levis, Cynthia D. Cushman and Paul S. Miller, Department of Biochemistry, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland, USA.

Oligodeoxyribonucleoside methylphosphonates(ONMPs) inhibit gene expression at the level of mRNA translation in cells. Psoralen derivatization of ONMPs enhances their antisense properties in vitro. ONMPs derivatized at their 5'-ends with 4'-[[N-(aminoethyl)amino] methyl]-4,5',8-trimethylpsoralen,(ae)AMT, form covalent adducts with complementary RNA targets when irradiated at 365nm. (ae)AMT-derivatized ONMPs(12-mer, 16-mer and 21-mer) complementary to a putative loop/stem in the coding region of vesicular stomatitis virus(VSV) M protein mRNA(M-mRNA) each specifically crosslink to M-mRNA when a mixture of the oligomer(1μM) and poly adenylated RNA(1.3μg) from VSV-infected mouse L cells are irradiated at 365nm in TMK buffer at 22°C. The 12-mer does not crosslink to poly adenylated RNA from uninfected L cells. A (ae)AMT-12-mer complementary to a sequence in the coding region of VSV N-mRNA did not crosslink to any of the VSV mRNAs under similar conditions. The binding site for this oligomer appears to be a stem structure as predicted by computer folding of the N-mRNA. A second oligomer(5μM) complementary to VSV-M mRNA specifically inhibits synthesis of M-protein in VSV-infected L cells(37% inhibition), with little or no inhibitory effect on synthesis of the other four VSV proteins. These results show that psoralen derivatization enhances the inhibitory activity of ONMPs in cells and suggests that such oligomers may be useful in elucidating the mechanisms of translation-inhibition by this class of antisense agents.

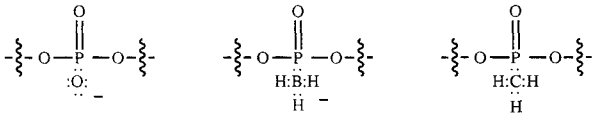
(Supported by NIH GM39127, CA42762).

CD 212 BACKBONE BORONATED OLIGONUCLEOTIDES: SYNTHESIS AND PHYSICAL STUDIES

Barbara Ramsay Shaw*, Anup Sood*, Jon M. Madison*, and Bernard F. Spielvogel‡, *Department of Chemistry, Duke University, Durham, NC 27706, ‡Boron Biologicals, Inc., 2811 O'Berry Street, Raleigh, NC 27607

A novel class of backbone modified oligonucleotides, in which one of the non-bridging oxygen atoms has been replaced with an isoelectronic borane group, has been synthesized. This modified internucleotide linkage, i.e., the boranophosphate linkage, is isoelectronic as well as isosteric to the methylphosphonate linkage. The boranophosphate linkage is remarkably stable in acid and base and the boronated oligonucleotides are resistant to exonucleases.

Boronated Oligonucleotides: Comparison with normal nucleotides and methylphosphonates



Phosphate Boranophosphate Methylphosphonate

CD 213 COMPUTER ASSISTED SELECTION OF ANTISENSE OLIGONUCLEOTIDE TARGET SEQUENCES. Robert A. Stull, and Francis C. Szoka, Jr., Dept. of Pharmacy, University of California, San Francisco , CA 94134.

Antisense oligonucleotides have been shown to be a useful tool in suppressing gene expression. We have developed a protocol using existing data bases and software packages to assist in the selection of antisense targets. Four main constraints are used in selecting optimal target sequences. 1) The target region should be single-stranded to afford hybridization with the antisense oligo. 2) The target should be at least 15 nt in length. 3) The oligo should not possess its own secondary structure. 4) The oligo should not cross react with non-targeted genes. The last constraint is verified by computer homology searches of all gene sequences residing in the GenBank data base. Constraints one and three are tested using an RNA workbench tool (H. Martinez, Nucleic Acids Res. (1988) 16:1789) which predicts multiple RNA secondary structures. The data from the structures is summarized in a histogram, where the individual bases in the sequence are plotted versus the frequency at which they appear in some form of secondary structure. Bases that tend to appear in single-stranded regions will have low bar heights on this plot-- hence quick inspection for "Taylor valleys" that satisfy constraint two reveals potential target sequences. We are currently testing this protocol in a murine interleukin-1 alpha system. Supported by NIH GM 30163.

CD 214 Rapid Nuclear Accumulation of Oligonucleotides Microinjected into Mammalian Cells.

F. C. Szoka, Jr.[1], G. G. Green[1], G. Zon[2], R. M. Straubinger[3], D.J. Chin[4], [1] School of Pharmacy, University of California, San Francisco, Ca 94143, [2] Applied Biosystems, Foster City, CA, [3] School of Pharmacy, SUNY/AB, Amherst, NY, [4] Agouron Institute, La Jolla, CA 92037.

The intracellular transport of nucleic acids and fate of cytoplasmic nucleic acids is poorly understood. To study this process we injected fluorescent oligodeoxynucleotides (oligos) into the cytoplasm of monkey epithelial cells and human fibroblasts. Rapid nuclear accumulation was found with phosphodiester(PD) , phosphorothioate(PT) and methylphosphonate (MP) forms of the oligo. Nuclear accumulation was temperature but not energy dependent. The intranuclear distribution of the oligos was influenced by the chemistry of the nucleoside linkages. PT and PD oligos colocalized with small nuclear ribonucleoproteins while the MP oligo concentrated regions of genomic DNA. These data have significant basic and therapeutic implications for the nuclear accumulation of exogenous nucleic acids into mammalian nuclei. (supported in part by NIH AI250992-F.C.S. and a Chicago Community trust award to D.J.C.)

CD 215 CATALYTIC ANTISENSE RNAS BY INCORPORATION OF SPECIFIC RIBOZYME CASSETTES INTO cDNA, Martin Tabler and Mina Tsagris, Institute of Molecular Biology and Biotechnology, P.O. Box 1527, GR-71110 Heraklion, GREECE

A technique was developed to convert a cDNA into a recombinant DNA construct that allows the synthesis of antisense RNA carrying the catalytic domain of the hammerhead ribozymes. As a result, these "catalytic antisense RNAs" ("antizymes"), cleave their target RNA in the manner as described by Haseloff and Gerlach (Nature **334**, 585-591). For such a conversion, a specific DNA cassette is incorporated into one of about 30 different restiction recognition sequences, which contain a cleavage motif for the hammerhead ribozymes. For example, each SalI-site (G'TCGAC) contains the GUC cleavage motif against which a ribozyme can be directed. After SalI digestion of the cDNA and removing the protruding ends, a SalI-specific ribozyme DNA cassette is inserted, containing the catalytic ribozyme domain plus three of the four nucleotides that had been removed by trimming. Transcription delivers the desired antizyme. This approach, which is appropriate for any SalI site in any cDNA, without requiring sequence data of the target, was successfully tested with a cDNA fragment of plum pox virus (PPV). It was also possible to develop selectable DNA cassettes, containing a removable marker gene allowing selection for antizyme constructs. Surprisingly, even such RNAs, carrying the tetracycline resistance gene inserted into the catalytic domain, showed ribozyme activity.

CD 216 CONSTRUCTION OF SEVERAL KINDS OF RIBOZYMES: REACTIVITIES AND UTILITIES, Kazunari Taira*, Hirotsugu Miyashiro, Masao Tanaka[a], Toshiaki Kimura[b], Hideaki Shinshi, Yuriko Yoshida, Naoyuki Yonemura, Eiko Kayano, Fumiko Nishikawa, and Satoshi Nishikawa*, *Fermentation Research Institute, Agency of Industrial Science & Technology, MITI, Tsukuba Science City 305, [a]Industrial Research Institute of Chiba Prefecture, Kasori, Chiba 280, [b]Applied Biosystems Japan, Kohtoh-ku, Minamisuna, Tokyo 136, Japan.*

Since the revolutionary discovery of the first RNA enzyme by Cech, the list of such ribozymes is still growing. In the list are typically the original ribozyme from *Tetrahymena* (Cech's ribozyme), hammerhead ribozymes, hairpin ribozymes, and the ribozymes from human hepatitis δ virus (HDV RNA). Among them we have been working with two types of ribozymes, namely, hammerhead-type and the HDV ribozymes. Utilizing the former ribozyme, we have constructed several types of RNA transcript releasing plasmids in which, in one case, 5'- and 3'-ends are trimmed by means of the *cis*-acting hammerhead ribozymes. This kind of plasmid is useful not only in elucidating the function of 5'-cap structure and/or polyA tails *in vivo* but also in testing the sequence-dependent stabilities of various RNA transcripts. *In vivo* studies of *trans*-acting ribozymes are also underway. The *trans*-acting ribozymes can either be transcribed *in vivo* from appropriate templates or supplied externally. One drawback of the external supply is that once introduced ribozymes have been degraded, one has to battle with misfortune. One way for the introduced ribozymes to survive from RNase activities is to coat them with DNA. For this purpose, we have chemically synthesized, utilizing DNA synthesizer, DNA/RNA mixed ribozymes where most of the stem regions have been replaced by DNA. Lastly, the gene of the HDV ribozyme has been chemically synthesized and introduced into pUC118 vector. Various mutants of the latter ribozyme furnish structure/reactivity relationships. We find that, contrary to the hammerhead-type ribozyme, the cleavage site U(688) can be replaced by all three other nucleotides, with the order in descending activity; U,C,A,G. The least active G(688) mutant still possesses 60% of the wild-type activity.

CD 217 **PRODUCTION OF ANTI-HIV-1 RIBOZYMES IN *E. COLI* USING A NOVEL GENE FUSION TECHNIQUE,** Nerida R. Taylor and John J. Rossi, Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, CA 91010

Exogenous delivery of ribozymes to cells for therapeutic use will require large scale syntheses of these molecules. At present, the best method available for this is a scaled up *in vitro* transcription process. We have developed a novel method for large scale, biological production of hammerhead type ribozymes in bacteria. The process involves production of a chimeric molecule, in which a hammerhead type ribozyme targeted to a *gag* HIV-I sequence is fused to a stable RNA which can be overproduced in the bacterial host. A 61 base pair DNA fragment harboring the ribozyme sequence was cloned into a restriction sight in the interior portion of the *E. Coli* 4.5S RNA gene. This fusion construct is transcribed by a strong bacterial promoter and transcription terminates within a ribosomal RNA termination signal. This construct led to the production of a 199 base 4.5S anti-gag ribozyme hybrid that could be produced at the rate of 5.6 ng RNA/ml (OD 0.6) of bacterial culture. The fusion ribozyme is catalytically active and cleaves the appropriate HIV gag sequence with a Km of 0.5 μM at 55C. This Km is comparable to a non-fusion, *in vitro* transcribed ribozyme of the same target sequence specificity (Km 0.2μM at 55C). In an effort to make this *E. Coli* produced ribozyme more active at 37C, we are cleaving off 4.5S sequences utilizing synthetic oligonucleotide directed RNase H cleavage to eliminate various secondary and tertiary structural elements of the chimera.

CD 218 MECHANISMS OF INHIBITION OF GENE EXPRESSSION BY ANTISENSE OLIGONUCLEOTIDES.

Jean-Jacques Toulmé[1], Claudine Boiziau[1], Christian Cazenave[2], S. Boutorine[3] & N.T. Thuong#, INSERM U201, Paris, France and #CBM, CNRS Orléans, France. Present addresses: [1]Biophysique Moléculaire, Université de Bordeaux, 146 rue Léo Saignat, 33076 Bordeaux, France, [2]University of Boulder, CO 80309 and [3]Institut de Chimie BioOrganique, Novossibirsk, URSS.

Alpha-oligos i) are more resistant to DNases than unmodified natural ones, ii) bind in a parallel orientation to their complementary RNA sequence, and iii) do not elicit RNase-H activity. We studied the effect of a series of alpha 17-mers on the translation of rabbit beta-globin mRNA. Only the oligo complementary to the cap site prevented globin synthesis in cell free-extracts and in micro-injected frog oocytes. The efficacy of the anti-cap alpha oligo is even higher than that of its unmodified counterpart. An oligo spanning the AUG initiation codon was inhibitory in cell-free systems but not in oocytes. The 17-mer complementary to the coding region was not active in any investigated system. Therefore, initiation of translation can be prevented by antisense oligos via an RNase-H independent mechanism whereas inhibition of elongation requires an irreversibly damaged mRNA. Indeed, the alpha-oligo targeted to the coding region was converted into an efficient molecule by covalent linking to an alkylating reagent which led to cross-linked oligo/mRNA hybrids. Moreoveer, in contrast to unmodified beta oligos, alphas oligos did not arrest cDNA synthesis by AMV reverse transcriptase supporting the idea that such a process involves the RNase-H activity of the retroviral enzyme.

CD 219 DETECTION OF TRIPLE-HELIX FORMATION BY GEL ELECTROPHORESIS: DIFFERENCES IN SENSITIVITY OF DETECTION BY SILVER-STAINING AND AUTORADIOGRAPHY, Kyonggeun Yoon, Cheryl A. Hobbs, John J. Malinowski and Alexander Weis, Departments of Molecular Biology and Medicinal Chemistry, Sterling Research Group, 25 Great Valley Parkway, Malvern, PA 19355

Currently little information is available on the physical chemical parameters of third strand binding to double-stranded DNA. The code for recognition of mixed Pu:Py DNA sequences and the influence of mismatches upon binding is unknown. To address this problem, we designed a series of oligonucleotides to quantitate triple-helix formation with a single base mismatch, separated single-, double- and triple-stranded DNAs by gel electrophoresis and measured the amount of triple-helix formation by silver-straining and autoradiography. The sequences of oligos used were $A_{10}NT_{10}$ and $T_{10}NT_{10}$ where N=A,T,G,C. The composition of the triple-helix was determined to be two pyrimidine strands and one purine strand for all combinations of sequences tested. The formation of Pu:Pu:Py triple-helix was not detected by this method for the sequences tested so far. The amount of triple-helix complex was not appreciably affected by mismatches at the middle of the sequence (N), detected by silver-staining. In contrast, quantitation of triple-helix formation by autoradiography demonstrated a marked sensitivity on the base substitution (N) in the center of the third strand. Detailed experimental results will be presented and a plausible mechanism for the differences in the sensitivities between the two detection methods will be discussed.

Antisense Approaches to Biological Studies

CD 300 CONTROL OF LYMPHOKINE GENE EXPRESSION IN T CELLS BY ANTISENSE RNA, Linda Andrus, Kenton S. Miller and Julie Rosenbaum, The Lindsley F. Kimball Research Institute of the New York Blood Center, New York, NY 10021 and The University of Tulsa, Tulsa, OK 74104.

Antisense RNA was used to suppress interferon-gamma (IFN-γ) gene expression in a transformed T cell line. A plasmid vector was constructed to express cloned sequences under transcriptional control of the cytomegalovirus enhancer/promoter (CMV E/P) and the splice and polyadenylation signals of the bovine growth hormone gene. Vector pCMV-IFN carries a 643bp fragment of the murine IFN-γ cDNA cloned in antisense orientation with respect to the CMV E/P. This plasmid, and a similar plasmid expressing the bacterial gene for neomycin phosphotransferase, were transfected into T cells by electroporation. Stable transformants were selected with the neomycin analog G418, and cloned lines were established by limiting dilution. About 25% of these co-transfectants expressed high but variable levels of antisense IFN-γ RNA. Southern blot analysis revealed that individual clones contained between one and thirty integrated copies of the anti-sense transcription unit. Using antigen-specific ELISA, levels of IFN-γ synthesis were found to vary from clone to clone. Several clones were isolated whose ability to produce IFN-γ was inhibited by >95% relative to the parent cell line. These results suggest that antisense RNA mutagenesis may be used to assess the role played by individual gene products in T cell effector function.

CD 301 INHIBITION OF INTERCELLULAR ADHESION MOLECULE-1 EXPRESSION BY ANTISENSE OLIGONUCLEOTIDES, C. Frank Bennett, Ming-Yi Chiang, Walt Lima, and Susan M. Freier, Department of Molecular and Cellular Biology, ISIS Pharmaceuticals, 2280 Faraday Ave. Carlsbad, CA 92008

Intercellular adhesion molecule-1 (ICAM-1) is a cytokine induced cell surface molecule expressed on numerous cell types, which is involved in emigration of leukocytes out of the vasculature, epidermal trafficking of T-lymphocytes, and activation of T-lymphocytes by antigen. Antisense oligonucleotides 18 to 20 bases in length were synthesized which hybridized to 15 different target sites on the ICAM-1 mRNA. Antisense oligonucleotides were tested for inhibition of ICAM-1 expression by an ELISA on two cell lines, human umbilical vein endothelial cells (HUVEC) and A549 cells. Antisense oligonucleotides containing phosphodiester linkages failed to inhibit IL-1 induced ICAM-1 expression, while oligonucleotides with the more stable phosphorothioate linkages did inhibit IL-1 induced ICAM-1 expression. The potency of the antisense oligonucleotides did not correlate with Tm, but did correlate with target site selection. Immunoprecipitation studies were performed demonstrating that the active antisense oligonucleotides selectively inhibited the synthesis of ICAM-1, having no effect on the synthesis of the class 1 MHC (HLA-A,B,C) antigen expression. Active antisense oligonucleotides inhibited adherence of DMSO differentiated HL-60 cells to TNF-α treated human umbilical vein endothelial cells, while control oligonucleotides failed to reduce adherence. Experiments are currently in progress to identify the mechanism of action of the active antisense oligonucleotides.

CD 302 REGULATION OF ANTISENSE SEQUENCES IN PLANTS UTILIZING tRNA PROMOTERS, June E. Bourque and William R. Folk, Department of Biochemistry, University of Missouri, Columbia, Missouri, 65211.

The major function of tRNA is its involvement in the translation of mRNA into protein of the ribosome. While the functions of translation are mediated by the ribosome, the fidelity of protein synthesis is dependent on the esterification of the correct amino acid to the cognate tRNA, as well as on the specificity of codon-anticodon interactions between mRNA and tRNA. The tRNA structure must contain all information necessary for specific interactions with components in the translation apparatus that interact with all tRNAs. The $tRNA^{met}_i$ gene encodes the initiator tRNA utilized for virtually all cytoplasmic protein synthesis in plants and as such is functional in every plant tissue making it ideal for use as a constitutively expressed promoter. We have isolated, cloned and sequenced a plant $tRNA^{met}_i$ gene from soybean and *Arabidopsis*. We have placed several regions from the chloramphenicol acetyltransferase (CAT) gene immediately downstream of the coding region. *In vitro* transcription assays, using human 293 whole cell extracts, indicated that the $tRNA^{met}_i$ gene correctly initiated transcription, reading through the antisense sequence, and terminated at a predicted stretch of 4-5 thymidine residues. We have achieved suppression of the target gene, with differential expression from the sequences of the 3' and 5' portions of the CAT gene, by electroporating DNA and RNA(transcribed using the T7 polymerase) into carrot plant cells. These results as well as the fate of the RNA within the cell will be discussed.

CD 303 ISOLATION AND ANTISENSE EXPRESSION OF A CHALCONE SYNTHASE cDNA FROM LISIANTHUS

Marie Bradley[1], Kevin Davies[1], Richard Gardner[2], Susan Ledger[2], Simon Deroles[1], Robyn Miller[1], Nigel Given[1]
1 Levin Horticultural Research Centre, MAFtechnology, Private Bag, Levin, New Zealand.
2 Dept. Cellular & Molecular Biology, University of Auckland, Auckland, New Zealand.

Chalcone synthase (CHS) is an enzyme involved in the early stages of the anthocyanin biosynthetic pathway in plants. A number of CHS cDNA sequences were compared and found to be highly conserved. An oligonucleotide with 32 fold degeneracy was designed for use in PCR (Polymerase Chain Reaction). Using the RACE method (Rapid Amplification of cDNA Ends), developed by Frohman et al, two DNA fragments of 900bp and 700bp were amplified from lisianthus (*Eustoma grandiflorum*) total RNA. These fragments have been cloned and their identity confirmed by sequencing. A transformation system for lisianthus has been developed, and binary vectors are being constructed carrying the CHS fragment in an antisense configuration. Similar work with other flavonoid biosynthetic genes is in progress.

CD 304 CK-B ANTISENSE RNA: MECHANISM OF ACTION, Ronald E. Cirullo and Edward W. Holmes, Departments of Medicine and Biochemistry, Duke University, Durham, NC 27710

Antisense RNA complementary to 3' coding and untranslated sequences of human creatine kinase B (CK-B) has been shown to be a potent inhibitor of CK-B translation *in vivo*. To define the mechanism of action of the antisense RNA, we have employed an *in vitro* transcription/translation system with the following results. 1) *In vitro* translation of CK-B mRNA is inhibited up to 90% by an equimolar amount of antisense RNA using the rabbit reticulocyte lysate system. The inhibition is specific and has no effect on translation of either rabbit β-globin or BMV control mRNAs as assessed by polyacrylamide gel electrophoresis (PAGE). 2) PAGE analysis of *in vitro* translation products of CK-B mRNA in the presence of antisense RNA shows a discrete band of decreased intensity corresponding to intact CK-B protein without obvious truncated peptide bands. 3) The most potent antisense RNA contains only sequences complementary to the last 60 nt of the 3' coding region and first 50 nt of the 3' UTR. Antisense RNA lacking either of these regions is less effective and lack of both sequences results in loss of the inhibitory effect on translation. 4) RNase protection assays have shown the magnitude of the translational inhibition to be directly proportional to the amount of duplex formed between the antisense RNA and CK-B message prior to translation. These duplex structures appear to be stable and are not dissociated during translation or by competition with excess unlabeled CK-B mRNA. The results indicate that antisense RNA complementary to the 3' end of CK-B mRNA is effective in arresting CK-B translation in this *in vitro* system and effects either translational initiation or a combination of initiation and elongation.

CD 305 SUPPRESSION BY ANTISENSE mRNA OF SYNTHESIS FROM HIGHLY EXPRESSED GENES IN ASPERGILLUS ORYZAE AND ASPERGILLUS NIGER. Ib G. Clausen, Tove Christensen, Frank Andreasen, Kirsten L. Jensen and Marianne Hansen, Department of Fungal Gene Technology, Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark.

The filamentous fungi Aspergillus oryzae and Aspergillus niger are used for transformation and expression of heterologous industrial enzymes. In addition to the desired product, however, the fungi produce other enzymes in high yields. Major products are the TAKA-amylase from A. oryzae and amyloglucosidase from A. niger. In order to decrease the content of these unwanted products in the enzyme preparation without introducing elaborate purifications, the expression of the two amylases were suppressed genetically.

In each strain, the antisense approach was used for lowering the expression of TAKA-amylase and amyloglucosidase, respectively, by transforming with integrative plasmids expressing partial antisense genes. In this way, the amount of unwanted product in the best case was reduced to less than 5% compared to the wild-type strain.

CD 306 MODULATION OF β-GLUCURONIDASE (GUS) TRANSIENT EXPRESSION IN *PETUNIA HYBRIDA* PROTOPLASTS USING ANTISENSE GUS, Pieter de Lange, Jan Kooter and Jos N.M. Mol, Department of Genetics, Free University, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

The expression of several genes involved in the anthocyanin biosynthesis in petunia can be inhibited by introducing extra gene copies in the antisense orientation (1). Since the inhibition is gene-specific and is only observed if part or the entire mRNA-coding region in its antisense position is used, the simplest explanation is that the block in expression is due to the formation of a dsRNA between the sense and antisense transcripts. Although dsRNAs have not directly been detected, it is conceivable that such a complex may prevent normal processing and translation of the mRNA. However, recent evidence suggests that additional factors affect the inhibition efficiency, like the position of the trans-gene in the genome and environmental conditions. In an attempt to determine the role of dsRNAs in this process, we first set up a transient expression assay with leaf protoplasts using the β-glucuronidase (GUS) as a reporter enzyme. Co-expression of a gus gene driven by a CaMV-35S promoter was inhibited by an antisense RNA producing gus gene transcribed from the same promoter. Promoter-less antisense gus genes had no effect. Similar results were obtained with the nopaline synthase promoter and a chalcone synthase promoter. Since these results resemble those obtained in plants transformed with sense and antisense gus genes (2), this approach is a rapid alternative to study the mechanism of 'antisense' inhibition in detail. Results will be presented on the fate of the sense and antisense gus transcripts.

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CD 307 AN ANTISENSE OLIGONUCLEOTIDE DESTROYS AN ENTIRE SUPERFAMILY OF ZINC FINGER TRANSCRIPTS IN XENOPUS OOCYTES AND EMBRYO'S. T. El-Baradi; T. Bouwmeester and T. Pieler Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, 1000 Berlin 33, FRG

We have used antisense oligonucleotides to analyse the biological function *in vivo* of a superfamily of zinc finger genes in *Xenopus* which we have isolated by virtue of their homology with the *Drosophila* developmental control gene *Krüppel*. The superfamily of *Krüppel*-like genes in *Xenopus* comprises at least 100 different members which are all maternally expressed. Like the *Krüppel* gene product itself, all the *Xenopus* homologues contain multiple conserved sequence elements, which join consecutive finger units (the H/C link).

The successful application of antisense oligonucleoitde technology in *Xenopus* has been hampered by the high toxicity of the amounts of oligonucleotide (≈ 20 ng) necessary to eliminate transcripts in oocytes and embryo's. However, using subtoxic, subnanogram amounts of a very potent unmodified antisense oligonucleotide directed against the H/C link regions we were able to destroy the *entire* family of more than 100 different zinc finger transcripts in oocytes and embryo's. We describe the developmental fates of these finger transcript minus embryo's and speculate on the reasons for the high potency of the H/C antisense oligonucleotide.

CD 308 IDENTIFICATION OF GENE FUNCTION USING ANTISENSE-RNA, Andrew J. Hamilton, Grantley W. Lycett and Donald Grierson, AFRC Research Group in Plant Gene Regulation, Department of Physiology and Environmental Science, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, LE12 5RD, U.K.

Antisense techniques have previously been used only to reduce the expression of plant genes of known function. The cDNA, pTOM13, has been characterised by sequencing (1) but although we have previously shown that its synthesis is correlated with that of the phytohormone ethylene (2) the exact role of the protein is unknown. We show here that the antisense RNA strategy when applied to pTOM13, reduces ethylene synthesis in a gene dosage dependent manner confirming that this gene is involved in the production of this growth regulator. Furthermore, biochemical analysis of these novel mutants suggests pTOM13 encodes at least part of ACC-oxidase (the ethylene forming enzyme) which catalyses the last step in ethylene synthesis (3).

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CD 309 STUDY ON THE 5' NONTRANSLATED REGION OF THE POTATO VIRUS Y.
Caroline Levis, Maurice Tronchet and Suzanne Astier,I.N.R.A. Versailles, Department of plant pathology, 78026 Versailles Cedex.

The RNA genome of potato virus Y (PVY), the type member of the potyvirus group, contains 9704 nucleotides of which 184 belong to the 5' nontranslated region (NTR). Potyvirus RNA has a genome-linked protein (VPg). In order to understand the mechanisms of its translation initiation, the strategy of hybrid-arrest translation was used. The 5' NTR was fused to the ß-glucuronidase (GUS) reporter gene. Six antisense oligodeoxynucleotides were used for hybridization. We have found that the efficiency of the translation in reticulocyte lysate of the hybridized mRNA was modified upon the position of the oligodeoxynucleotide used. The greatest inhibition was obtained with an oligodeoxynucleotide hybridized to the 5' end. However, translation of GUS mRNA containing the PVY 5' NTR was strongly enhanced when this mRNA was capped.We have also created a biscistronique gene by inserting the PVY 5'NTR-GUS gene downstream the PVY capside gene. Even when the 5'NTR is internal, a GUS gene translation was observed. Thus, an internal initiation seems likely in reticulocyte lysate.

CD 310 OLIGONUCLEOTIDES ANTISENSE TO mRNA FOR INTERLEUKIN 1 RECEPTORS BLOCK THE EFFECTS OF INTERLEUKIN 1 IN CELL CULTURE AND IN VIVO. Lawrence C. Mahan and Ronald M. Burch, Laboratory of Cell Biology, NIMH, Bethesda, MD, 20892 and Nova Pharmaceutical Corp., Baltimore, MD, 21224-2788.

Oligodeoxynucleotides (O-oligos) and their phosphorothioate analogs (S-oligos) antisense to mRNA for murine or human interleukin 1 (IL-1) receptors specifically inhibited the expression of IL-1 receptors and the biological effects of IL-1 in Swiss 3T3 fibroblasts or human dermal fibroblasts. Incubation of cells for up to five days in the presence of antisense oligos (10-30 µM) reduced IL-1 receptor expression by ~50% and inhibited IL-1 stimulated PGE_2 synthesis 45-70%. O-oligos were more acute in effect whereas the effect of S-oligos was sustained over a longer period of time. Under conditions of increased turnover of IL-1 receptors, a >65% reduction of receptor number and a complete loss of IL-1 stimulated PGE_2 synthesis could be achieved by short (8 hr) incubations with S-oligos that had been encapsulated into liposomes. We then determined if results observed with oligos antisense to IL-1 receptors in vitro could be extended to an in vivo model of IL-1 mediated response. IL-1 subcutaneously injected in mice causes vascular endothelial cells to express adhesion molecules for neutrophils resulting in neutrophil infiltration at the injection site by 4 hrs. Prior injection of the site with up to 3 nmoles of S-oligo over 3 days caused a marked reduction (~65%) in the level of neutrophil infiltration in response to the injection of IL-1 . Thus antisense oligodeoxynucleotides present an attractive therapeutic alternative for the treatment of chronic inflammatory diseases once considerations of cost, delivery and metabolism can be achieved.

CD 311 THE ELUCIDATION OF RECEPTOR-EFFECTOR COUPLING IN RAT PITUITARY (GH_3) CELLS BY THE USE OF ANTISENSE TECHNIQUES, Ruth H. Paulssen, Eyvind J. Paulssen, Jan O. Gordeladze and Kaare M. Gautvik, Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112 Blindern, 0317 Oslo 3, Norway

The regulation of prolactin (PRL) secretion and synthesis in rat pituitary (GH_3) cells involve receptor interaction and coupling to G proteins that activate or inhibit second messenger systems. The thyroliberin (TRH) receptor in GH_3 cells is coupled to phospholipase C (PLC) via the G_p-complex, and is believed also to be coupled to adenylyl cyclase (AC) possibly via G_s. To test the hypothesis that TRH couples to multiple signalling systems, we constructed an antisense RNA expression vector that carries part of the $G_s\alpha$ cDNA in inverted orientation relative to an inducible metallothionein promotor ($pMTG_s\alpha as$). Transient expression of $G_s\alpha$ antisense RNA in transfected GH_3 cells resulted in specific inhibition of $G_s\alpha$ mRNA expression and was followed by decreased protein levels after a 24 hours incubation period. Measurements of TRH-elicited adenylyl cyclase (AC) activity in transfected cells resulted in a decrease of about 90% of it's activity. Phospholipase C (PLC) activity remained unaffected under the same conditions. We therefore conclude that the TRH receptor is also coupled to a G_s or a G_s-like protein. Further investigations using antisense RNA inhibition of G protein expression and function will help delineate intracellular signal systems involved in TRH-induced PRL secretion and synthesis.

CD 312 *FES* PROTO-ONCOGENE EXPRESSION IS REQUIRED FOR PROLIFERATIVE RESPONSE OF HL60 CELLS TO GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR, Peter T. Rowley, Shane C. Weber*, Patricia H. Lalik*, John A. Jaeger*, Douglas H. Turner*, Barbara A. Farley, and Gary R. Skuse, Department of Medicine and Division of Genetics, and *Department of Chemistry, University of Rochester, Rochester, NY 14642; and *Exploratory Sciences Division, Eastman Kodak Company, Rochester, NY 14650

Granulocyte-macrophage colony stimulating factor (GM-CSF) increases the number of colonies formed by HL60 acute promyelocytic leukemia cells. To determine whether expression of the *fes* proto-oncogene is necessary for this effect, cells were cultured with or without GM-CSF and with or without antisense (or nonsense) *fes*-specific synthetic oligodeoxyribonucleotides. Three antisense 15mers, selected for complementarity to either the initial coding region or to regions likely to be single-stranded, gave comparable results, viz. each had no effect by itself, but reduced colony number in the presence of GM-CSF. Since specific interference with *fes* gene expression blocks the observed GM-CSF induced increase in colony number, *fes* gene expression appears to be required for this GM-CSF stimulating effect. To determine whether a proliferative response to other myeloid growth factors requires *fes* expression, similar experiments were performed with suspension cultures of AML-193 acute myeloid leukemia cells, which require a hematopoietic growth factor to survive and to proliferate. The results suggest that *fes* gene expression may be required for factors acting on multipotent progenitors (GM-CSF, interleukin 3, and interleukin 6) but not for factors acting on progenitors committed to a single lineage (M-CSF and G-CSF).

CD 313 THE REGULATION OF ENDOGENOUS POLYGALACTURONASE GENE EXPRESSION IN TRANSGENIC TOMATOES BY CHIMAERIC SENSE AND ANTISENSE GENES,
Christopher J.S. Smith, Colin F. Watson, and Don Grierson, University of Nottingham, School of Agriculture, *Sutton* Bonington, Loughborough, LE12 5RD, UK. Colin R.Bird, John Ray, and Wolfgang Schuch, ICI Seeds, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, UK.

As tomato fruit ripen there is a substantial accumulation of the cell wall hydrolase polygalacturonase (PG), thought to be involved in pectin breakdown as fruit soften. This accumulation was largely prevented by transforming plants with a single antisense PG gene (1). In selfed progeny, believed to be homozygous with respect to the inserted antisense gene, PG accumulated during ripening to less than 1% of normal (2). The only other effect on ripening detected in these reduced PG plants was an inhibition of *in vivo* pectin depolymerisation (2). A similar reduction in PG activity has been observed in plants transformed with a chimaeric truncated PG sense gene (3).

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CD 314 ANTISENSE JUN-D RNA INHIBITS PROLIFERATION OF 3T3 CELLS, Paul Bui-Vinh Tran and Ramaswamy Narayanan, Department of Molecular Genetics, Hoffman La Roche Inc., Nutley, NJ 07110

The jun family of genes includes c-jun jun-B, and jun-D. In contrast to c-jun and jun-B, which are expressed at very low levels in unstimulated cells, jun-D is expressed at high basal levels in diverse cell types and is stimulated only marginally by serum or purified growth factors. To understand the role of jun-D in cell proliferation and in the growth factor-induced activation of various immediate early genes, we have established a stable 3T3 cell line expressing dexamethasone-inducible jun-D antisense RNA. Expression of antisense jun-D RNA resulted in pronounced growth inhibition of 3T3 cells, even in the absence of dexamethasone. The serum-induced activation of immediate early genes was not inhibited by antisense jun-D RNA. Stimulation of DNA synthesis by serum was significantly inhibited in the antisense jun-D-expressing quiescent cells, suggesting that jun-D expression is necessary for reentry of quiescent cells into the cell cycle.

CD 315 THE APPLICATION OF ANTISENSE RETROVIRAL CONSTRUCTS TO THE ANALYSIS OF G-PROTEIN FUNCTION, David C. Watkins, Nutan Sharma and Craig C. Malbon, Department of Pharmacological Sciences, State University of New York at Stony Brook, Health Sciences Center, Stony Brook, NY 11794.

GTP-binding regulatory proteins (G-proteins) transduce information from a populous class of receptors and photopigments to a much smaller group of effector molecules. G-protein-mediated effectors include adenylyl cyclase, phospholipases A_2 and C, and K^+ and Ca^{2+} channels. These G-proteins are heterotrimeric and molecular cloning has identified at least sixteen different α-subunits. In order to determine precisely the function of each of the separate G-proteins, we have prepared plasmid constructs harboring sequences antisense to those of each of the known G-protein subunits. The antisense sequences consist of the 5′ noncoding region (up to and including the initiation of translation codon) of the G-protein subunits under the control of the SV40 early promotor and polyadenylation sequences. When transfected into Chinese hamster lung cells, an antisense construct specific for $G_{i\alpha 2}$, decreased the expression of the $G_{i\alpha 2}$ subunit and attenuated inhibitory control of adenylyl cyclase. Antisense sequences have also been engineered into retroviral constructs under the control of the human cytomegalovirus immediate early promotor. Amphoteric retroviruses (generated by transfection of the ecotropic packaging cell line Ψ_{GP+E86}, followed by infection of PA317 packaging cells) were prepared as vehicles for infection of test cells. Transmembrane signaling is investigated using cells in which antisense-mRNA oligomers have rendered the cell deficient in a specific G-protein subunit. Supported by USPHS grants DK30111 and DK25410 from the NIH.

Gene Regulation by Antisense RNA and DNA

Antisense Approaches to Viral and Cancer Control

CD 400 TRANSFECTION OF CELLS WITH ANTISENSE OR SENSE RNA OF THE NUCLEAR MATRIX-ASSOCIATED REGION (MAR) OF C-MYC DECREASES THE STABILITY OF C-MYC TRANSCRIPTS. **Robin H. Chou[*+], Michael T. McIntosh[*], Marcella M. Flubacher[*], Department of Anatomy[+] and Molecular Biology and Biotechnology Program[*], Hahnemann University School of Medicine, Philadelphia. PA 19102**

A c-myc MAR and a nuclear protein (p25) which recognizes it have been identified at the 3'-end, DraI/DraI fragment of c-myc gene in human leukemia (HL-60) cells (Chou, et al. Cancer Res. 50:3199-3206, 1990). This 172 base pairs (bp) myc-MAR contains the first of two polyadenylation signals and a topo II box-like sequence. The myc-MAR location suggests a role in regulating c-myc transcript stability. To test this, the 172 bp myc-MAR was subcloned into pBluescript and sense and antisense myc-MAR RNAs were transcribed. HL-60 cells were transfected with either sense or antisense myc-MAR RNAs by electroporation. Purified total RNAs were analyzed for c-myc transcripts by Northern hybridization using cloned c-myc as probes. When compared to the cells electroporated without RNA, c-myc RNA was decreased in cells electroporated with either sense or antisense myc-MAR RNA. In addition, S1 protection assay using antisense c-myc DNA as probe on c-myc transcripts from nuclear run-on synthesis showed that the presence of p25 suppressed transcript cleavage between the two polyadenylation signals. These data suggest that antisense and sense myc-MARs may block the function of p25 in regulating the stability of c-myc gene transcripts.

CD 401 ANTISENSE OLIGONUCLEOTIDES AS INHIBITORS OF PAPILLOMAVIRUS, Lex M. Cowsert, Maureen C. Fox and David J. Ecker, Department of Molecular and Cellular Biology, ISIS Pharmaceuticals, Carlsbad, CA 92008

The products of the E2 open reading frame of bovine papillomavirus type 1 (BPV-1) are a family of site-specific DNA-binding proteins which regulate viral transcription by transactivation (E2-TA) and transrepression (E2-TR). At least 5 different species of E2 mRNA (3 E2-TA and 2 E2-TR) have been identified in BPV-1 transformed cells. The promoters P_{89}, responsible for transcription of BPV-1 transforming functions, and P_{2443}, responsible for transcription of E2-TA and viral transforming functions have been shown to be responsive to E2 transcriptional regulatory functions.

Antisense oligonucleotides complementary to mRNA for E2-TA and E2-TR have been synthesized with either a phosphodiester or phosphorothioate backbone. Oligonucleotides were initially screened for inhibition of E2 transactivation and relief of E2 transrepression in transient transfection assays. Antisense oligonucleotides were identified that could inhibit either E2 transactivation or E2 transrepression at nanomolar concentrations. Oligonucleotides that inhibited E2 transactivation also inhibited BPV-1 transformation of C127 cells at nanomolar concentrations as measured in a quantitative focus formation assay.

The effects of these antisense oligonucleotides on E2 mRNA translation both in vitro and in virally transformed cells are currently under investigation. In addition, the effects of E2-TA specific antisense oligonucleotides on E2 regulated transcription from the P_{89} and P_{2443} in BPV-1 transformed cells are being evaluated.

CD 402 ANTISENSE cDNA INHIBITS *neu* ONCOGENE EXPRESSION IN MAMMARY CELLS

Michele De Bortoli , *Daniela Taverna, *Nancy Hynes and Susanna Antoniotti. Laboratory of Molecular Cell Biology, Dept. of Animal Biology, University of Turin, Italy. * Friedrich Miescher Institute, Basel, Switzerland.

The *neu* oncogene (c-*erb*-B2) plays a very important role in normal as well as in neoplastic mammary development. Its expression is inhibited by estrogens and high levels of the *neu*-encoded p185 protein are expressed by functionally differentiated mammary cells (Oncogene 5:1001, 1990). As a tool for studying the functions of *neu* in mammary cells, we have inserted a 2.2Kb fragment from the 5' end of the *neu* cDNA in antisense orientation in a SV40-based expression vector (pECE*aneu*). The efficiency of this construct has been checked using the normal mouse mammary cell line NOG8. These cells were first transfected with pSV2*neu*T, obtaining the fully transformed NOG8-3a1 clone. NOG8.3a1 cells were then transfected with pECE*aneu* together with a hygromycin-resistance plasmid, and 2 pools and 6 clones of hygromycin-resistant cells were selected and studied. NOG8.3a1 cells transfected with the hygromycin plasmid alone were used as control.The antisense-transfected cells were morphologically very similar to the normal NOG8 cells and showed a reduced capacity of growth in soft-agar as compared to NOG8-3a1 cells (58-92% reduction). In nude mice, we observed that the animals injected with antisense-transfected cells in some cases did not develop tumors at all, and in other cases they developed smaller tumors with longer latency times. Immunoblotting and Northern blot analysis showed that the antisense-transfected cells expressed definitely lower levels of *neu* mRNA and p185, as compared to NOG8.3a1 cells. However, it should be emphasized that the antisense mRNA could not be detected in transfected cells and, consequently, more studies are needed to conclude that the observed decrease of *neu* expression and tumorigenicity in antisense-transfected cells results from interaction of antisense RNA with the *neu* mRNA. Further studies are under way using different cell models and antisense constructs.

CD 403 ANTISENSE INHIBITION OF COLLAGENASE IV, Robert K. DeLong, Paul S. Miller and Sarah A. Bruce, Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD 21205.

The secretion of type IV collagenase(CL-IV) by tumor cells has been implicated in their invasive and metastatic abilities. To investigate further the role of CL-IV in metastasis, the expression of CL-IV in neoplastically transformed human bronchial epithelial cells(BZR-T33) [Ura et.al.,Can. Res.49:4615,1989] was inhibited with antisense oligonucleoside methylphosphonates(ONMPs) and the effect of this inhibition was monitored by the growth properties of the cells on an artificial basement membrane (matrigel). ONMPs complementary to the translation initiation codon region of CL-IV mRNA have been synthesized. Oligomer I (d-GpCGCCTCCATCGTAG) is complementary to a putative loop region which includes the initiation codon, whereas oligomer II(dGpCGCGCCCCGGGCCA) is complementary to a predicted stem region. d-TpGGCCCGGGGCGCGC(III) and d-Ap(Ap)$_{10}$C (IV) were prepared as controls. BZR-T33 cells were inoculated onto matrigel in the presence of 0, 100, or 200 uM oligomer. In the absence of any oligomer the BZR-T33 cells form a network on the matrigel surface, eventually degrading the matrigel layer. This is in contrast to the growth on matrigel of a less malignant type of these cells(BEAS-2B) which form cellular clumps at >24 hours. Treatment of the BZR-T33 cells with oligomer II resulted in their phenotypic reversion to a BEAS-2B type cell morphology. Experiments are underway to determine the effect of this treatment on the amount of CL-IV secreted by these cells. These experiments demonstrate the feasibility of using antisense ONMPs to:(a) study the role of CL-IV in metastasis and (b) control the expression of this enzyme in cell culture. Supported in part by NIH CA 42762 and ES03819.

CD 404 RNA SECONDARY STRUCTURE AS A TARGET FOR ANTISENSE BINDING, David J. Ecker, Tim Vickers, Brenda Baker, Tom Bruice, Susan M. Freier, Maryanne Zounes, P. Dan Cook; ISIS Pharmaceuticals, 2280 Faraday Ave, Carlsbad, CA 92008, Robert Buckheit; Southern Research Institute, 2000 Ninth Avenue South, Birmingham, Alabama 35255

Traditionally, investigators designing antisense oligonucleotides have tried to avoid regions of RNA secondary structure. We have begun to investigate the feasibility of deliberately targeting antisense oligonucleotides to RNA secondary structures delineate the rules for strand invasion. All human immunodeficiency virus mRNA's contain a secondary structure sequence known as TAR (trans-activating responsive sequence). The TAR element forms a stable RNA stem-loop structure which binds the HIV tat (trans-activator) protein and mediates increased viral gene expression. We have constructed a series of antisense oligonucleotide analogues which specifically bind to the HIV TAR element. Disruption of its natural secondary structure could be demonstrated by in vitro binding experiments with enzymatically synthesized TAR RNA. Several of these molecules inhibited trans-activation in a cell culture model and inhibited HIV in both acute and chronic model viral assays.

CD 405 TARGET SELECTION AND DELIVERY IN DEVELOPMENT OF ANTI-HIV RIBOZYMES. David Elkins[1], John J. Rossi, Sean Sullivan and John Zaia. Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, CA 91010.

It has been speculated that catalytic antisense RNAs (ribozymes) might offer a novel therapeutic weapon against viral infection, as well as other diseases. Two of the many challenges facing investigators who are developing this approach are target selection and exogenous delivery of the ribozyme. We are investigating the problem of target selection with a mixture of ribozymes containing two 5-nucleotide degenerate sequences harboring a common catalytic site targeted to an HIV-GAG site; these mixtures have been created by two different methods. We are also exploring the ribozyme-delivery possibilities of liposomes; preliminary results suggest that both exogenous ribozyme and exogenous target sequence (packaged in separate liposomes or in the same liposome) are satisfactorily delivered to cells in culture, and that specific cleavage takes place. An interesting observation from these studies is the apparent intracellular degradation of the 3' cleavage product. We anticipate that both lines of investigation will yield further results, to be discussed.

CD 406 PHOTOACTIVATABLE ANTISENSE DNA - SITE-SPECIFIC PSORALEN CROSSLINKS IN THE CONTROL OF GENE EXPRESSION. FP Gasparro, Department of Dermatology, Yale University, New Haven CT 06510

To make 8-methoxypsoralen (8-MOP) a sequence-specific drug for DNA, antisense DNA oligonucleotides containing 8-MOP monoadducts have been synthesized to facilitate covalent crosslinking to targeted DNA sequences within a particular gene. The sequence, 5'-TpApT-3', is a preferential site for 8-MOP monoadduct and crosslink formation. For example, an antisense sequence, 5'-GAGTATGAG-3', was chosen for two reasons. First, it contained the reactive triplet base sequence and second, it was complementary to a sequence within the initiation region of the β-lactamase gene contained within an E. coli plasmid. E. coli cells transformed with this plasmid are resistant to ampicillin. Treatment of the resistant bacteria with the monoadducted antisense oligonucleotide and UVA reduced colony formation. In this report studies are described in which the antisense strategy is extended to specifically suppress the expression of genes responsible for diseases of the most easily accessed human organ, the skin. The inducible expression of Intercellular Adhesion Molecule-1 (ICAM-1) in human keratinocytes offers a convenient system to test this new concept with subsequent potential clinical applications since these molecules may perform in vivo functions relevant to clinical disease. The use of antisense oligonucleotides to control gene expression offers the potential of developing highly specific and efficacious therapies for diseases known to result from the excessive production of specific gene products. Weak hydrogen bonding interactions between the antisense oligonucleotide and the target sequence may not be sufficient to prevent the eventual elimination of antisense strands. Psoralen crosslinks between the antisense DNA and its target sequence can prolong the antisense effect. The incorporation of a photoactivatable crosslinking agent within the antisense DNA may enhance its efficacy by covalently binding the oligonucleotide to the targeted gene segment.

CD 407 RETROVIRAL VECTORS EXPRESSING ANTISENSE SEQUENCES POSSIBLE APPROACH IN HUMAN ANTI-RETROVIRAL THERAPY

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One of possible therapeutic approaches to diseases induced by human retroviruses consists in chemo-/radiotherapy followed by bone marrow transplantation and repopulation of the hematopoietic system by hematopoietic stem cells. We are developing two systems which might be used for protection of the repopulating cells against the consequences of an incidental infection by virus having survived the chemo-/radiotherapy or being released from latently infected cells and provoke a relapse of the disease.

1/ GIV, growth inactivating vectors, have been designed for a selective elimination of the cells which produce the human T-cell leukemia virus (HTLV-I)-encoded transactivating protein, tax. Production of tax is known to trigger the development of aggressive human cancer, adult T-cell leukemia, ATL. GIV vectors, in which cDNA sequences encoding human dihydrofolate reductase, dhfr, are expressed in opposite transcriptional orientation from tax-inducible HTLV-I promoter, have been shown to efficiently reduce the production of the vital enzyme and to inhibit the growth of tax producing human T-cells in vitro.

2/ Retroviral vectors of double-copy type expressing, from two different strong constitutive promoters, sequences thought to be essential for expression of Human Immunodeficiency virus, HIV-I, and related Simian Immunodeficiency virus, SIV, are being tested in human T-cell lines infected with HIV/ SIV.

CD 408 THE REGULATION OF N-myc EXPRESSION BY ANTISENSE TRANSCRIPTION,

Geoffrey W. Krystal and Barbara C. Armstrong, Div. of Hematology/Oncology & Dept. of Microbiology & Immunology, Medical College of Virginia and The McGuire V.A. Medical Center, Richmond, VA 23298

Bidirectional transcription from the N-myc oncogene locus gives rise to naturally occurring sense and antisense transcripts which have complementary 5' ends. We have demonstrated, using a double RNase protection technique, that approximately 5% of the sense RNA forms an RNA:RNA duplex with nonpolyadenylated antisense RNA. The duplexes, which preferentially include certain of the multiple forms of sense mRNA, extend across exon 1 and intron 1 sequences, suggesting that they play a role in modulating the processing of specific N-myc transcripts. We are currently testing this hypothesis by transfection of expression constructs altered to decrease or enhance the amount of antisense transcription relative to sense transcription. Using this transfection system we can determine if primary sense transcription, mRNA processing, mRNA stability, or the ability to synthesize N-myc proteins is altered when antisense transcription is altered. We are also hoping to establish structural requirements for efficient duplex formation and to determine the effects of duplex unwinding activities in this system.

CD 409 EVALUATION OF ANTISENSE OLIGONUCLEOTIDES TARGETED TO ACTIVATED c-Ha-*ras* USING A *ras*-LUCIFERASE REPORTER GENE SYSTEM, Brett P. Monia, Joseph F. Johnston, Christopher K. Mirabelli, Stanley T. Crooke, David J. Ecker, ISIS Pharmaceuticals, 2280 Faraday Ave, Carlsbad, CA 92008

The enzymatic activity of firefly luciferase provides a sensitive and rapid means to assay transcriptional activity of DNA regulatory sequences when fused to the protein coding sequence of the luciferase gene. To establish a system for the rapid screening of antisense oligonucleotides targeted to the 5' protein-coding portion of activated c-Ha-*ras* mRNA, we have developed a *ras*-luciferase reporter gene assay in which a segment of the activated c-Ha-*ras* gene, encoding the first 22 amino acids of activated c-Ha-*ras*, is fused to the protein coding sequence of the firefly luciferase gene. Expression of this construction in cells produces a *ras*-luciferase fusion protein, the levels of which can be quantified through the activity of luciferase. This system is currently being employed for the identification of potential antisense inhibitors of activated c-Ha-*ras*.

CD 410 INHIBITION OF THE EGF RECEPTOR EXPRESSION BY ANTISENSE RNA TO REVERT THE TRANSFORMED PHENOTYPE OF HUMAN TUMORAL CELLS , M.Cristina Moroni and Laura Beguinot. , Institute of Microbiology,University of Copenhagen, Copenhagen, Denmark.

The human EGF receptor is a protooncogene and its overexpression can confer an EGF-dependent, fully transformed phenotype to normal fibroblasts in culture and induce tumors in nude mice. Overexpression of the normal EGF receptor has been also implicated in the development of some human neoplasia since the receptor has been found amplified in several human tumors of ectodermal origin. In order to address the question of the primary role of EGF-R overexpression in human transformed cells, we have used the tecnique of antisense RNA to specifically block the expression of the EGF receptor. KB cells, derived from a human epidermoid carcinoma, express 2x10^5 receptors/cell but do not show amplification of the gene. They have been transfected with antisense DNA, complementary only to the 5' or 3' or almost all the coding region of the EGF mRNA. The transfected cells express high levels of antisense RNA and show a reduced expression of the EGF-R protein, up to a 80-90% reduction. The levels of antisense RNA produced correlate with the reduction in EGF-R protein and mRNA expressed in the different clones. Furthermore, the most reduced clones show a reduced growth rate already in 10% serum containing medium, and their transforming ability (anchorage independent growth, plating efficiency and growth in defined medium) is severely impaired. We conclude therefore that antisense RNA efficiently blocks expression of the EGF receptor in human tumoral cells and that this induces a reversion of the transformed phenotype in vitro.

CD 411 INHIBITING *c-erbB*-2 OVEREXPRESSION IN SK-BR-3 HUMAN MAMMARY CARCINOMA CELLS WITH PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

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Amplification of the proto-oncogene c-erbB-2 occurs in 25 - 30% of human mammary carcinomas and is associated with a poor clinical prognosis. Its overexpression may thus be involved in the pathogenesis or malignant progression of mammary tumors. C-erbB-2 encodes a tyrosine kinase. We inhibited expression of c-erbB-2 in the overexpressing human mammary carcinoma cell line SK-Br-3 using a 14 mer antisense phosphorothioate oligodeoxynucleotide (S-ODN). The antisense technique was combined with sensitive biochemical assays as well as with assays of DNA synthesis and cell proliferation. Total cellular tyrosine kinase activity in cells treated with the S-ODN was reduced to 18.8%. Thus the major part of tyrosine kinase activity in this cell line can be attributed to erbB-2 protein expression.Phosphorylation of the S6 protein by S6 kinase plays a central role in controlling the initiation of protein synthesis and thus cell proliferation. A decrease in S6 kinase activity in our erbB-2 suppression experiments to 24% suggested that S6 kinase is regulated by erbB-2 tyrosine kinase. Repeated doses of the anti c-erbB-2 S-ODN completely inhibited cell proliferation. This shows that c-erbB-2 overexpression has an important impact on the biological behaviour of mammary carcinoma cells. Our results show that the antisense S-ODN technique is a valuable tool for studying the function of specific genes, their role in biochemical pathways and cell growth. Interference with gene expression can significantly alter the biological behaviour of tumor cells.

CD 412 ANTI-SENSE DNA OLIGONUCLEOTIDE APPROACH TO INHIBIT GROWTH OF MALIGNANT HUMAN B CELLS. Maureen Shuh, James Jackson, and Surendra Sharma. Department of Pathology, Roger Williams General Hospital-Brown University, Providence, RI 02908.

It is well understood that the T cell-derived lymphokine, B cell growth factor-12kD (BCGF-12kD), stimulates the proliferation of activated normal human B cells as well as EBV^+ and EBV^- transformed B cells. However, the pathogenetic role of BCGF-12kD in the growth of virally and non-virally transformed human B cells remains to be evaluated. Our results show that P3HR1, an $EBNA\text{-}2^-$ Burkitt's lymphoma cell line, produces and utilizes BCGF-12kD for its autocrine growth whereas EBV^+ lymphoma cell lines do not. In conjunction with other data in the laboratory, we suggest that BCGF-12kD, possibly with other stimulatory signals, supports the growth of EBV^- and $EBNA\text{-}2^-$ transformed B cell lines. It is pertinent to mention that resting and activated normal human B cells do not express the BCGF-12kD gene. To further delineate the role of BCGF-12kD in these cell lines, we are currently using the technique of modulating gene expression by targeting an 18bp specific anti-sense BCGF-12kD DNA oligonucleotides to BCGF-12kD mRNA. The preliminary experiments involve directly administering the non-modified DNA oligonucleotide to P3HR1 cells and monitoring its effects by 3-H thymidine incorporation and cell viability. Our results indicate that an anti-sense oligomer suppresses the growth of these cells, presumably due to the binding of the oligomer to the BCGF-12kD mRNA, thus inhibiting BCGF-12kD translation. The oligonucleotide will also be studied in several other cell lines available in our laboratory. More importantly, the experiments will be expanded to examine the effects of the oligomer at the RNA level to characterize its inhibitory mechanism(s).

CD 413 DEVELOPMENT OF NOVEL GENETICALLY ENGINEERED ANTISENSE INSECT VIRUSES AS IMPROVED VIRAL INSECTICIDES, **Natarajan Sivasubramanian and Robert H. Hice, Department of Entomology, University of California, Riverside, California 92521, USA.**

We have recently identified a naturally occurring antisense RNA inhibition mechanism for controlling a specific viral gene (gp64) expression in an insect virus, *Autographa californica Nuclear Polyhedrosis Virus* (AcNPV). One unusual feature of NPV is that two distinct phenotypes are observed in nature. One phenotype is the occluded form of NPV (OV) whereas the other form is the extracellular form (ECV) which buds from the plasma membrane of infected cells with the aid of viral coded surface glycoproteins (gp64). The ECVs mediate the intracellular spread of infection while the occluded virus is responsible for primary infection in the insect gut. Thus, these two forms of NPV have separate roles in the persistence of viruses in nature.The NPVs have developed a control mechanism whereby ECVs are produced early in the infection and are later shut off when the OVs are produced late in the infection. Recently, the gene coding for gp64 of AcNPV has been cloned and sequenced in our laboratory. Our results on the expression of this gene demonstrate the occurrence of RNA transcripts for both the DNA strands of the gp64 gene. The gp64 mRNAs (2.1 kb) started appearing around 2 hrs post-infection(p.i.), peaked at 24 hrs p.i. and later declined indicating a transcriptional regulation. However, antisense RNAs (RNAs complementary to the gp64 mRNAs) are also produced during later stages of viral development. These antisense RNAs (3.3, 7.3 and 10 kb) started appearing 12 hrs post infection, peaked at 24 hrs p.i. and later declined at 48 hrs p.i.. The concentration of these individual antisense RNAs were lower than gp64 mRNAs. However, the cumulative concentration of these antisense RNAs can inhibit the translation of gp64 mRNAs. In addition, our results indicate that the majority of these antisense RNAs do not have protein coding potential in an in *vitro* translation system. Also, during this same time period, the production of ECV (containing gp64) is reduced because of reduced amount of gp64 on the infected cell surface plasma membrane. One of the major criteria for the operation of the antisense RNA mechanism is the occurrence of both the RNAs (mRNAs and antisense RNAs) at the same time, at the same location. Our results show that, both the mRNAs and antisense RNAs of gp64 are made at the same time especially during 24 to 48 hrs post infection. Since these RNAs are transcribed at the same location (opposite strands of the DNA) and at the same time, there is a strong possibility of forming RNA-RNA duplexes in the nucleus itself and thereby preventing the transport of these RNAs to the cytoplasm for possible translation. Utilizing this antisense mechanism, NPVs can shut off the production of the gp64 instantly and thereby directing the host cell to use all its energy for making OV.

This naturally occurring antisense mechanism in NPVs could be further exploited to generate highly improved viral insecticides (antisense insect viruses) by increasing the virulence, toxicity and host range of these viruses. Briefly, genetically engineered recombinant NPVs can be developed with "antisense host genes". The technique involves construction of a recombinant NPV with a gene coding for an antisense RNA against a vital host cellular mRNA. For example, the gene(s) coding for insect neural proteins can be introduced into NPVs in such a way that it will make antisense RNAs instead of mRNAs which code for neural proteins. Additionally, 'antisense-antiresistance insect viruses' could also be generated, in which, antisense RNA molecules can be directed against the cellular mRNAs of various detoxification enzymes. Such viruses can greatly reduce the production of these detoxification enzymes eventually making the host insect extremely susceptible to even low levels of chemical insecticides. Since insecticide resistance in the field is developed because of repeated application of chemical insecticides, these viruses can be used early in the insect control process, before the application of chemical insecticides. We are currently generating several 'antisense insect viruses'. The results on the pathogenicity, virulence and host range of these antisense insect viruses will be discussed.

CD 414 PROSPECTIVE GENE THERAPY FOR ORAL CANCER; Cherrilee Steele and Edward Shillitoe; University of Texas Health Science Center, Houston Texas 77225

Human papillomaviruses (HPV) have been shown to be responsible for squamous cell papillomas. Certain types of HPV have emerged as leading candidates in the etiology of squamous cell carcinoma. The majority of these oral and genital tumors contain HPV DNA. It has been shown that the E7 gene of HPV type 18 and 16 can contribute to cellular transformation and the maintenance of a malignant phenotype in vitro. To determine if the selective inhibition of E7 expression in oral cancer cells would alter their transformed phenotype, the entire E7 gene of HPV 18 was cloned in both orientation into an inducible mammalian expression vector so as to allow the expression of sense and antisense messages in vivo.

To test for toxicity, these plasmids were electroporated into Vero cells. Expression of sense and antisense RNA was induced by dexamethasone and confirmed by Northern analysis. Growth curves and saturation densities show there was no difference between the cell lines. Microscopic examination showed no morphological differences. Therefore, expression of HPV 18 antisense E7 is not toxic to cells that do not contain HPV. Preliminary experiments are underway to test the role of E7 expression in HPV-containing oral carcinoma cells for potential therapy of oral cancer.

CD 415 INHIBITION OF MOUSE HEPATITIS VIRUS REPLICATION BY ANTISENSE RNA, Kathy M. Takayama and Masayori Inouye, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854.

Antisense RNA has been extensively utilized to regulate gene expression in both prokaryotic and eukaryotic systems. A successful demonstration of the inhibition of phage SP replication in *E. coli* by producing antisense RNAs complementary to the phage genome led to studies which sought to disrupt viral functions in tissue culture cells stably transfected with antisense genes. Mouse L2 cells were transfected with vectors carrying the selectable neomycin-resistance marker and various regions (in the antisense orientation) derived from cDNA clones of the murine hepatitis virus (MHV) genome transcribed by the human cytomegalovirus promoter. Initial MHV production assays have shown significant reductions (nearly 90%) in viral titers at up to 24h post-infection for cells expressing antisense RNAs as compared to control L2 cells. Comparisons of inhibition of virus production by different antisense RNAs, which include those complementary to the 5' end of the MHV genome, the MHV leader sequence, and the gene 5/gene 6 region may provide significant information on effective targets for antisense suppression in the cytoplasm, as the MHV replication cycle is entirely cytoplasmic.

CD 416 INHIBITION OF FeLV GENE EXPRESSION BY ANTISENSE RNA, James R. Williams, David H. Roberts, Jean C. Gallo and Roger J. Avery, Department of Microbiology, Immunology and Parasitology, NYS College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

Retroviruses are responsible for a wide variety of diseases in man and other animals, but prevention and control of these diseases has historically been extremely difficult. Our long term goal is to develop targeted nucleic-acid based therapies for preventing and treating retrovirus infections. We are currently exploring the use of antisense RNA to inhibit feline leukemia virus (FeLV) gene expression. FeLV-infected cells and cats provide an excellent model system for developing and evaluating novel anti-retroviral therapies.

We have constructed retrovirus vectors that express antisense transcripts specific for various target sequences in the FeLV genome. Mink lung cells have been stably transfected with these antisense vectors, and we have evaluated the resistance of these stably-transected cell lines to infection by FeLV. FeLV replication in the antisense transfected cells is less than 20% of the level of virus replication in normal mink lung cells. We are currently determining the molecular basis for inhibition of FeLV gene expression by antisense RNA.

CD 417 INHIBITION OF RETINOBLASTOMA (RB) GENE EXPRESSION USING ANTISENSE RNA AND RIBOZYME, Klas G. Wiman, Floriana Bulic-Jakus, Laszlo Szekely, Gabor Dobos, and George Klein, Dept. of Tumor Biology, The Karolinska Institute, Box 60400, S-104 01 Stockholm, SWEDEN

Loss or inactivation of the retinoblastoma (Rb) gene is associated with the development of a variety of human neoplasms, including retinoblastoma, osteosarcoma, small cell lung cancer, and breast cancer. Available evidence indicates that the Rb gene negatively regulates normal cellular growth. The phenotypic effects of Rb loss alone are unknown. To address this question, we wished to specifically inhibit the expression of the Rb gene in non-malignant cells. A series of plasmids designed to express antisense RNA for the Rb mRNA have been constructed. Rb cDNA fragments of various lengths were inserted in antisense orientation downstream of strong, constitutive promoters, as well as the inducible MMTV promoter. The ability of the constructs to mediate a reduction in Rb gene expression is currently being tested in cells expressing Rb, including human fetal retinoblasts. As an alternative to the antisense RNA approach, we have constructed a ribozyme with specificity for the Rb mRNA. In vitro and in vivo analyses are being carried out to test the properties of this ribozyme.

Late Abstracts

C-*JUN* AND *JUN*-B HAVE OPPOSITE EFFECTS ON CELL PROLIFERATION - A STUDY WITH ANTISENSE PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

Wolfgang Brysch and Karl-Hermann Schlingensiepen, Department of Neurobiology, Max-Planck-Institut für biophysikalische Chemie, Am Faßberg, 3400 Göttingen, FRG

The c-*jun* and *jun*-B genes share high sequence homology with the v-*jun* gene. Their rapid induction in a variety of cell lines after stimulation with serum or growth factors is not dependent on prior protein synthesis. Thus they belong to the immediate early gene group. C-*jun* together with c-*fos* constitutes the DNA-binding factor AP-1. We inhibited expression of c-*jun* and *jun*-B in different cell lines using phosphorothioate oligodeoxynucleotides. C-*jun* inhibition strongly reduced ^{3}H-thymidine incorporation in two mammary carcinoma cell lines, in the rat phaeochromocytoma cell line PC12 and in NIH 3T3 mouse fibroblasts. The inhibition of c-*jun* expression and of c-*fos* expression had very similar effects.
In the same cell lines inhibition of *jun*-B expression drastically increased ^{3}H-thymidine uptake to more than 10fold. This was very similar to the effects resulting from supression of the anti-oncogene p53. We conclude that c-*jun* has the characteristics of a proto-oncogene but *jun*-B appears to be an anti-oncogene with strong anti-proliferative action, similar to that of p53. Our results show *Jun*-B and c-*jun* to be functional antagonists with regard to their effect on cell growth.

ALTERED TRANSACTIVATION OF CELLULAR GENE EXPRESSION IN HTLV-I INFECTION BY ANTISENSE DNA, Steven J. Greenberg, Craig Tendler, Jack Burton, and Thomas A. Waldmann. Department of Neurology, Roswell Park Cancer Institute and SUNY at Buffalo, Buffalo, NY 14263 and Metabolism Branch, National Cancer Institute, Bethesda, MD 20892

An approach toward abrogating the *ex vivo* expression of the human T-cell leukemia virus type I (HTLV-I) transactivator element *tax* was developed by using antisense oligodeoxyribonucleotide analogues that target various sequences within the retroviral coding regions. *In vitro* spontaneous proliferation, which uniquely characterizes PBMCs infected with HTLV-I in the pre- or non-leukemic states, served as a screening assay to test the efficacy of analogues. Certain *tax*-specific antisense probes were effective in inhibiting *in vitro* proliferation. It is postulated that expression of the *tax* transcript results in up-regulation of the components of the interleukin-2/ interleukin-2 receptor autocrine loop leading to T-cell activation. Thus, inhibition by *tax* antisense probes on mononuclear cell proliferation bridges the coordinate expression of the retroviral transactivator and the induction of certain host cellular genes.

CELLULAR UPTAKE AND LOCALIZATION OF FLUORESCEIN-LABELED, 15-MER PHOSPHOROTHIOATE AND PHOSPHODIESTER OLIGONUCLEOTIDES

K. Harewood, K. Pape, C. Gabel, R. Suleske and A. Cunningham, Molecular Genetics Research Department, Pfizer Inc., Central Research Division, Groton, CT 06340

Unmodified oligonucleotides have been reported to enter cells via an endocytic process facilitated by a putative 80 kD receptor. While phosphorothioate oligonucleotides appear to utilize a similar uptake mechanism, it has been hypothesized that these s-modified analogs are less-efficiently internalized. We are using 5' amino-linked phosphodiester and phosphorothioate 15-mers conjugated with FITC to study cellular uptake. Fluorescence microscopy studies carried out with living cells suggest that both types of oligonucleotides are transported into cells within 6 hours. Cells treated with the fluorescein-labeled oligonucleotides display a punctate pattern of intracellular fluorescence which is localized almost exclusively to the cytoplasm. Both fluorescence microscopy and flow cytometry analyses demonstrate that chloroquine enhances this cytosolic fluorescence suggesting that transit through an acidified endosomal compartment is involved in the internalization process. We are using flow cytometry to provide a more detailed analysis of the uptake mechanism. Data on the effects of temperature and sodium azide on oligonucleotide transport, as well as results from competitive binding studies will be presented.

RETROVIRAL VECTORS PRODUCING ANTISENSE RNA OR A HAMMERHEAD RIBOZYME AGAINST HIV, Donald Kohn, Ingrid Bahner, Chen Zhou, Pia Challita, John Rossi and John Zaia, Childrens Hospital, USC School of Medicine and City of Hope National Medical Center, Los Angeles, CA 90027
Initial applications of bone marrow transplantation for patients with AIDS have demonstrated substantial reduction in the detectable HIV burden. In this context, genetic modification of the donor marrow to confer cellular resistance to HIV may provide additional virus suppression. We have constructed a series of retroviral vectors which contain fragments from the 5' region of HIV in an antisense orientation. These vectors efficiently transfer and express the antisense expression units in human T lymphocytes and monocytic cells. A series of vectors have also been developed which transduce an anti-HIV hammerhead ribozyme. To quantitate the inhibitory effects of these vectors, we have developed a cell-based reporter construct which expresses a fusion protein of the HIV gag gene and the E.coli lacZ gene under control of the HIV LTR promoter. The relative efficacy of these vectors against HIV replication will also be assessed. Our studies seek to provide useful basic information about the ability of antisense RNA and ribozymes to inhibit HIV replication in the relevant human cell types.

Inhibition of HIV-1 by Deoxyribonucleotides Directed Against Regulatory Gene Messages and Response Elements. D.J. Looney, J.O. Ojwang, M.E. Harper, E. Wickstrom, and F. Wong-Staal. VA Medical Center, San Diego, CA; UC San Diego, La Jolla, CA; Genta Inc., San Diego, CA; University of South Florida, Tampa, FL.

We have investigated effects of modified (methyphosphonate & phosphorothioate) and unmodified oligodeoxyribonucleotides directed against HIV-1 RNA and DNA, including: (1) antisense oligomers targeting the HIV-1 tat message, starting at the CAP site, through the tar region, the major 5' splice donor site, and spanning the tat exon 1 initiation codon, (2) oligomers designed as antisense inhibitors and/or sense competitors of HIV-1 regulatory elements, including tar and individual loops of the RRE (regulatory response element), as well as (3) oligomers directed against conserved potential triplex-forming sites in duplex HIV-1 proviral DNA. Using assays including inhibition of virus expression following acute infection of H9 & SupT1 lymphocyte lines, p24 gag production from chronically infected cells following PMA stimulation, and inhibition of transactivation as judged by co-transfection of LTR-CAT and LTR-CAT-RRE with HXB2gpt (HIV-1 proviral) plasmid, some oligomers produced significant inhibition at concentrations below 1 μM.

REDUCTION IN TRANSFORMING GROWTH FACTOR BETA$_1$ ACTIVITY IN METASTATIC 10T1/2 FIBROBLASTS USING AN ANTISENSE VECTOR, Maureen A. Spearman[1], Dan Theodorescu[2], Robert S. Kerbel[2], Arnold H. Greenberg[1] and Jim A. Wright[1], Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba, Canada, R3E OV9[1], and Mount Sinai Hospital Research Inst., Toronto, Ontario, Canada, M5G 1X5[2].

Metastatic murine fibroblasts secrete elevated levels of transforming growth factor beta$_1$ (TGF-β_1) and exhibit altered cellular responses to the growth factor in DNA synthesis and collagen production (Schwarz et al., Cancer Res., 48, 6999-7003, 1988; Schwarz et al., Growth Factors, 3: 115-127, 1990). These results suggest that secretion and autocrine stimulation of metastatic cells by TGF-β_1 contribute to the malignant phenotype. To directly test the role of TGF-β in metastasis, highly metastatic ras-transformed 10T1/2 fibroblasts were transfected with a plasmid containing a portion of the TGF-β_1 gene in the antisense orientation, a DHFR locus and a neomycin resistance marker gene. Drug selection in G418 for neomycin resistance resulted in several colonies which were screened for a reduction in the normal TGF-β message. Further screening using a bioassay revealed that approximately half of these clones had reduced TGF-β secretion compared to the parental cell line. TGF-β antisense message has not been identified in these cells, however, amplification using methotrexate may allow detection of the antisense RNA and a further reduction in TGF-β secretion.
This work was funded by NCI Canada.

INHIBITION OF ADENOVIRUS (AD5H) AND BOVINE LEUKEMIA VIRUS (BLV) BY ANTISENSE RNA IN VITRO AND IN VIVO, Tikhonenko T.I., Miroshnichenko O.I., Ponomareva T.I., Borisenko A.S., Institute of Agricultural Biotechnology, 12 Pskovskaya St., Moscow 127253, USSR.

Several asRNA genes targeted at different sites of Ad5h and BLV have been designed and in some cases coupled with ribozyme sequences. The antiviral activity of different asRNA genes driven by several constitutive and inducible promoters has been tested either in the stable transformed cell lines or in cells cotransfected by the mixture of asRNA-plasmids and target viral genomes. The most efficient asRNAs provided for 80-95% of inhibiting viral infectivity, the presence of ribozyme in the asRNA gene enhanced the antiviral activity substantially.By pronuclear injection of asRNA genes into the rabbit fertilized zygotes with subsequent transmission into the oviduct of synchronized females transgenic animals with intracellular immunity to viral infection have been developed In spite of frequent rearrangements of the transgene in some trangenic animals and their offsprings the asRNA genes were expressed properly, inherited in accordance with Mendelian pattern and conferred upon rabbits the enhanced resistance to viral infection comparable with the degree of immunity observed in vitro.

MANIPULATION OF FLORAL PIGMENTATION BY SENSE AND ANTISENSE TECHNOLOGIES.Rik van Blokland, Nico van der Geest, Jan Kooter and Joseph Mol. Department of Genetics, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

Chalcone synthase (CHS) is one of the enzymes involved in floral pigmentation of *petunia*. The pigmentation can be reduced by introduction of an antisense chs cDNA (van der Krol et al (1988),Nature 333, 866-869,). This reduction corresponds with low levels of chs mRNA. Analysis of nascent RNA by nuclear run-ons indicates that the endogenous chs genes are transcribed at a normal rate. These results are consistent with a model in which antisense transcripts block the processing and/or translation of the sense mRNA by forming a sense-antisense RNA duplex which is rapidly degraded. An excess antisense RNA is probably not required for complete inhibition since the transcription rate of 'antisense' genes can be much lower than that of the endogenous gene . Similar phenotypes were obtained with a gene encoding an enzyme active later in the flavonoid biosynthesis route: the dihydroflavonol4-reductase (DFR).

To our surprise, transgenic petunias with extra (sense) chs or dfr genes also produce flowers with a reduced pigmentation (Van der Krol et al (1990) The Plant Cell 2,291-299). The mRNA levels from both the endogenous and the introduced genes were very low. This co-suppression takes place at a post-transcriptional level since nuclear run-on data indicate that the genes are transcribed at a normal rate. Although the mechanism of this extreme dosage-compensation of mRNA levels remains obscure, the genomic location of the transgene may play a role.

Interestingly, chs genes are normally transcribed in white flower sectors of the *petunia* mutant Red Star, whereas chs mRNA is absent. Therefore. Red Star may be a natural 'sense' mutant.

GROWTH INHIBITORY AND CYTOTOXIC EFFECT OF ANTISENSE OLIGONUCLEOTIDES IN CHRONIC PHASE OF CHRONIC MYELOGENOUS LEUKEMIA (CML), Tianying Yuan, Charles V. Herst, Hanchun Chen, Christopher Reading and Albert Deisseroth, Department of Hematology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

In CML, the formation of the bcr-abl chimeric mRNA and the p210 tyrosine-specific protein kinase emancipates late myeloid progenitor cells from dependence on extracellular growth factors. This results in an expansion of the late myeloid progenitor pool which leads to death of the patients through bleeding and infection. In order to develop autologous marrow transplantation therapy for this disease, we have established conditions under which antisense oligonucleotides inhibit the growth of and induce the death of CML cells in vitro. Antisense oligonucleotides of 17 and 28 nucleotides length were designed to bind to the region of the translation initiation codon of the bcr mRNA. These oligonucleotides were added at a concentration of 50 micromolar to cultures of the CML cell lines BV173, EM2, and K562. The in vitro growth of BV173 and EM2 were markedly inhibited by antisense but not sense oligonucleotides in the presence of serum-free as well as serum-containing cultures. The growth of the K562 cell was not inhibited by these antisense oligonucleotides. Growth of bcr-abl negative myeloid cell lines (Josk) were not inhibited by these oligonucleotides. Antisense oligonucleotides to p53 mRNA did not inhibit the growth of CML cells. 95% of the cells died in the presence of the bcr antisense oligonucleotide and in the absence of serum in chemically-defined culture conditions. Thus, the use of antisense oligonucleotides not only arrest cell growth but also results in CML cell death in serum-free conditions. The use of such antisense oligonucleotides in vitro to destroy CML cells under conditions which would permit the continued growth of normal cells have now been defined. These data are leading to in vitro methods of treating autologous marrow for autologous transplantation of CML.