GENETICALLY TARGETED RESEARCH & THERAPEUTICS: ANTISENSE & GENE THERAPY

Organizers: James W. Hawkins and W. French Anderson

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Plenary Sessions *Page*

Poster Sessions

Therapeutic Protocols-Cancer-I

S 001 GENE THERAPY PROGRAM AT UCLA, J. Economou [1], A. Miller[1], W. McBride[2], A. Shaked[1], A. Belldegrun[1], J. Rosenblatt [3], J. Glaspy[3], G. Schiller[3], P. Koeffler[3], B. Schuck[1], K. Rhoades[1], G. Dougherty[5], D. Thacker [5], M. Prager[4], M. VanBree[1], K. Drazen[1], D. Kohn[6], I. Bahner[6], Departments of [1]Surgery, [2]Radiation Oncology, [3]Medicine and [4]Anethesiology, UCLA, Los Angeles, [5]The Terry Fox Laboratory, Vancouver, B.C. and [6] Department of Research Immunology and Pediatrics, Children's Hospital of Los Angeles, USC.

The UCLA Gene Therapy Program is a multidepartmental effort with interests in cancer immunotherapy, transplantation immunology and fetal gene transfer.

Transduction of tumors with cytokine genes to augment immunity have been explored with several genes including interleukin-7 (IL-7). A murine fibrosarcoma tumor cell line, transduced with a retroviral vector (Jzen IL-7)/tkneo) to produce IL-7, was less tumorigenic in syngenic mice. Three log greater numbers of IL-7 producing cells were required to achieve tumor take; one third of these IL-7 producing tumors grew and regressed. Regressing tumors were densely infiltrated with CD8 and CD4 T lymphocytes and mice acquired specific systemic immunity. Several human tumor cell lines (melanoma, breast, ovarian) were transduced with the IL-7 vector and produce up to 10^5 pg IL-7/10^6 cells/24 hrs. There was no difference in in vitro proliferation or MAGE melanoma antigen expression. However, IL-7 producing human tumors had retarded growth in nude mice. IL-7 tumor transduction appears to be superior to TNF, IL-6 and IL-3.

Retroviral transduction of human tumor-infiltrating lymphocytes (TIL) using the same amphotropic IL-7 vector results in stable integration and poor expression. LTR-driven cytokine genes do not express protein well and may be transcriptionally silenced within several weeks. Adeno-associated virus (AAV) vectors may provide for improved gene transfer. A TIL marking trial is in progress.

The liver gene therapy program has focused on the use of retroviral and adenovirus (Ad) transduction of rat liver transplants and cultured human hepatocytes. Retroviral vectors can successfully transduce regenerating rat liver either by direct parenchymal injection or portal vein perfusion. Stable integration and expression of proviral sequences have been noted for as long as 35 days. Reduced hepatic grafts (50% hepatectomy followed by cold ex vivo portal vein perfusion and orthotopic transplantation) could also be successfully transduced. Adenovirus vectors are also useful in liver and hepatocyte gene transfer.

S 002 **APPLICATION OF GENE THERAPY IN ONCOLOGY: RESULTS OF A FRENCH STUDY.**

Marie C Favrot, Yacine Merrouche, Evelyne Goillot, Alain Mercatello, Robert Moen*, Jean-François Moskovtchenko, Paul Tolstoshev*, Sylvie Negrier and Thierry Philip

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Biotherapy represents a new field of investigation and treatment in oncology. In this context, in vitro or in vivo modifications of immunocompetent and/or malignant cell functions by gene transfer are one of the major advances.

We will summarize our own experience and results obtained in the treatment of patients with advanced melanoma or renal cell carcinoma, using TIL after retroviral transduction of the gene of resistance to neomycin (NeoR). The aims of our study were first to evaluate, in patients who have escaped any other efficient therapy, the toxicity and efficiency of a systemic infusion of interleukin 2 (IL2) associated with NeoR gene-modified TIL (NeoR-TIL); second to analyze, particularly in RCC, the preferential localization and survival of NeoR-TIL in blood and at tumor sites by detection of the marker gene. Our study differs from that previously reported by Rosenberg in the schedule of IL2 administration : we introduced a cycle of IV IL2 before and 6 weeks SC IL2 post NeoR-TIL reinfusion in order to improve the intratumoral homing and survival of TIL. The LNL6 retroviral vector was used to introduce the NeoR gene into TIL.

Fifteen patients were elligible for this study ; 6 received non transduced TIL, whereas only 4 (1 RCC, 3 melanoma) received marked TIL. TIL were transduced 13 to 27 days after initiating the cultures ; the number of transduced TIL ranged from 1.25 to $13.2x10^8$. They were then grown from 34 to 47 days in medium containing IL-2; the number of NeoR-TIL reinfused to the patient ranged from 0.62 to $100x10^8$. Using PCR techniques, NeoR-TIL were identified in culture and blood samples after TIL reinjection to the patient. No toxicity was seen due to the gene modification. Safety studies showed that no replicant virus was present at the time of cell transduction and cell transfer. The immunological follow up demonstrated the specific lysis of autologous tumor cells in vitro for 1 patient with melanoma.

This study confirms the feasibility of NeoR-TIL protocol for cancer therapy, but we will clearly discuss the practical limitations of such an approach. Finally, we will discuss our current prospectives.

S 003 **GENE THERAPY OF CANCER - IMMUNOLOGICAL APPROACHES.** Michael T. Lotze, Quan Cai, Elaine Elder, Joshua Rubin, Barbara Pippin, William Jacob*, Yawen Chen*, Kiyoshi Nishihara, Jill Siegfried, Walter Storkus, Howard Edington, Maury Rosenstein, Chet Nastala, Itzhak Pappo, Laurence Zitvogel, Paul Robbins, and Hideaki Tahara. From the Departments of Surgery, Molecular Genetics and Biochemistry, and Pharmacology and the Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15261 and *Genetic Therapy Incorporated, Gaithersburg, MD 20878.

The modern evolution of biologic therapies for cancer has led to improved strategies for either eliciting a cellular response to cancer (tumor vaccines) or adoptively transferring lymphocytes which have antitumor reactivity (1). We have identified tumor infiltrating lymphocytes (TILs), expanded *in vitro* in the presence of IL-2 and IL-4 (2), which have substantial specific reactivity for the autologous tumor. We have previously reported (3-5) on the use of retroviral vectors encoding the neomycin phosphotransferase gene to mark IL-2 expanded TILs given *in vivo* so that 1) trafficking could be assessed and 2) studies performed to monitor the effectiveness of cytokine administration to the host to both maintain and expand the cells following adoptive transfer. In those studies TILs could be identified in the circulation for as long as 189 days and at the tumor site up to 64 days following infusion. We infused our first patient with IL-2 and IL-4 expanded TILs at the PCI on 3/3/92. A *second infusion* was given 3/20/92. The second patient's treatment began on 3/4/92.

Patient Infusion	CD4/CD8 %	LU/10^7 Cells Daudi/Autologous		Latest Day Detected Blood	Latest Day Detected Tumor
JW/1	80/17	8	45	---	---
JW/2	98/2	1	11	6D	Not detected
MJ/1	58/39	9	61	---	---
MJ/2	78/10	0	9	83D	69D

Our experience was curtailed with this combination because of the limited availability of IL-4 for these studies (Sterling Pharmaceutical) Cells could be labeled and identified in the periphery for long periods following adoptive transfer. Patient JW had no evidence of a major response and ultimately succumbed to his disease. MJ had a minor response and has recently been able to be made disease free after surgical resection of her remaining disease. We plan to reinitiate these studies with Schering derived IL-4 in the near future.

Our other major area of interest in gene therapy involves the use of cytokine gene therapy for cancer, predominantly as tumor vaccines. We have recently confirmed that retroviral transduction of murine IL-4 can lead to regression of murine tumors *in vivo* and have been able to observe regression of the Meth-A sarcoma with delivery of IL-4 using transfected fibroblasts. We have chosen this method of delivery because 1) local production of the cytokine is sufficient to elicit a response, 2) they are readily available from the autologous host to culture, transfect and select and 3) tumor cells from patients may be difficult to culture and selection may alter expression of tumor antigens. We can now routinely introduce the IL-4 gene using these retroviral vectors and obtain levels of production as high as 10^5U/10^6cells/24 hrs even following delivery of 5000 rads. Transfection of human cultured melanoma and lung cancer lines as well as fibroblasts has been successfully carried out. A protocol for this purpose has been approved by the RAC/NIH.

The antitumor potential of IL-12 was suggested by the potent augmentation of gamma interferon production and enhancement of NK activity as well as the specific antigen-dependent proliferation of activated T-lymphocytes (7). We have shown that local administration of IL-12 blocks the *in vivo* progression of a murine adenocarcinoma and sarcoma. We have also shown that IL-12 can enhance LAK activity that is also (like that induced by IL-2) inhibited by coincubation with TGFβ. We have been able to successfully introduce the genes for murine IL-12 (p35,p40) into NIH 3T3 fibroblasts and use them to deliver IL-12 at the site of tumor and delay growth of a variety of murine tumors. To date this has not been associated with the development of an immune response but we are testing such cytokine gene therapies with subsequent systemic administration of IL-2. Recent construction of retroviral constructs containing **both** chains with an intervening internal ribosomal entry site (IRES) should allow continued progress along the path of ultimately beginning human clinical trials.

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S 004 DIRECT GENE TRANSFER AND MOLECULAR INTERVENTIONS FOR AIDS AND CANCER, G.J. Nabel[1], E.G. Nabel[1], G.E. Plautz[1], J. Liu[1], Z. Yang[1], C. Woffendin[1], X. Gao[2], L. Huang[2], D. Gordon[1], B. Fox[1], S. Shu[1], M. Malim[3], B. Cullen[3], H.K. Lyerly[3], T. Boyle[3], and A. Chang[1], [1]University of Michigan Medical Center, Ann Arbor, MI, [2]University of Pittsburgh, Pittsburgh, PA, [3]Duke University Medical Center, Durham, NC.

The immune system confers protection against a variety of pathogens and contributes to the surveillance and destruction of neoplastic cells. Several cell types participate in the recognition and lysis of viruses and tumors, and appropriate immune stimulation provides therapeutic effects for these diseases. We have developed several molecular genetic interventions which alter the immune response to viruses and cancer. For example, foreign major histocompatibility complex (MHC) proteins serve as a potent stimulus to the immune system. We have introduced a foreign MHC gene directly into malignant tumors in vivo in an effort to stimulate tumor rejection. In contrast to previous attempts to induce tumor immunity by cell-mediated gene transfer, the recombinant gene was introduced directly into tumors in vivo with retroviral vectors or DNA liposome complexes. Expression of the foreign class I gene induced a cytotoxic T cell response to H-2 and, more importantly, to other antigens present on unmodified tumor cells. This immune response attenuated tumor growth and caused complete tumor regression in many cases. Direct gene transfer in vivo can therefore induce cell-mediated immunity against specific gene products which provides effective immunotherapy for malignancy and can be applied to the treatment of cancer and infectious diseases in man. Based on these and other studies, a phase I human trial using direct gene transfer for patients with melanoma has begun.

Additional studies have been undertaken to develop gene therapy protocols for AIDS utilizing a transdominant inhibitory form of Rev. HIV infection can be inhibited in susceptible T leukemia cells stably expressing a transdominant Rev protein, in the absence of any deleterious effect on T cell function. Such a strategy could provide a therapeutic effect in the T lymphocytes of AIDS patients.

S 005 GENE THERAPY OF CANCER, S.A. Rosenberg, National Cancer Institute, Bethesda, Maryland 20892

Attempts are underway to develop new immuno- therapeutic approaches to the treatment of patients with advanced cancer. Early efforts of adoptive immunotherapy using lymphokine activated killer (LAK) cells and interleukin-2 (IL-2) resulted in 10% incidence of complete regression and 10 to 25% incidence of partial regression in patients with advanced kidney cancer and melanoma. In subsequent studies, tumor infiltrating lymphocytes (TIL) were shown to recognize unique cancer antigens on murine and human cancers including melanoma, breast cancer, colon cancer and lymphoma. The MHC restricted recognition of human cancer antigens was detected by assaying panels of HLA typed target cells and by transfection into target cells of genes coding for appropriate HLA specificities. In animal studies therapy with TIL was 50 times more potent than with LAK cells and in pilot trials of TIL in melanoma patients 40% underwent objective cancer remission. Indium-111 labelled TIL trafficked to and accumulated in cancer deposits.

In initial studies to genetically modify TIL to improve their therapeutic effectiveness, TIL retrovirally transduced with the gene for NeoR, were administered to 10 patients with advanced cancer and were detected in the circulation 189 days and in the tumor 64 days later. In subsequent studies TIL transduced with the tumor necrosis factor (TNF) gene, secreting over 100 ugTNF/10^6cells/24 hrs have been used to treat seven patients with advanced melanoma. Genes coding for chimeric T cell receptors (TcR) composed of the constant region of the TcR and the variable region of a monoclonal antibody have been transduced into TIL and can redirect TIL lysis to cancer recognized by the monoclonal antibody.

Genetic modification of tumors is also being studied. Antigen processing defects in selected murine and human tumors can be corrected by exposure to interferon-gamma (IFN-gamma) or transduction with the gene for IFN-gamma. Transduction of murine tumors with cytokine genes can increase tumor immunogenicity. These studies led to clinical protocols in which human tumors transduced with the gene for TNF or IL-2 were used to immunize five autologous patients with advanced cancer. Efforts to clone the gene for tumor antigens that are recognized by murine and human TIL are underway.

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Therapeutic Protocols-Cancer-II

S 006 TRANSFERRED GENE EXPRESSION AFTER AUTOLOGOUS BONE MARROW TRANSPLANTATION IN MAN, Malcolm K. Brenner,[1*] Donna R. Rill,[1] Michael J. Buschle,[1] Martha S. Holladay,[1] Helen E. Heslop,[1] Robert C. Moen,[2] Robert A. Krance,[1] Joseph Mirro, Jr.,[1] W. French Anderson,[3] James N. Ihle[1], [1]Department of Hematology/ Oncology, St. Jude Children's Research Hospital, Memphis, TN, 38105, and the Departments of Pediatrics and Medicine, University of Tennessee, Memphis, College of Medicine, Memphis, TN, 38163, [2]Genetic Therapy Incorporated, Clinical Labs, Gaithersburg MD, 20878, [3]National Institutes of Health, National Heart, Lung, and Blood Institute, Bethesda, MD, 20892

Autologous bone marrow transplantation (ABMT) allows cancer patients to be rescued from the effects of supralethal chemotherapy. The incidence of relapse remains high and it has been unclear whether recurrence occurs from residual disease in the patient or in the infused marrow. The concern that patients may have been cured by intensive treatment, only to have their tumor returned by the ABMT, has lead to the adoption of marrow purging. Unfortunately there are no data to show that this treatment is necessary or effective - but ample evidence to show that such marrow treatment slows engraftment and increases the risks of ABMT. We have used retroviral mediated gene transfer (RMGT) of the neomycin resistance gene to mark putative residual malignant cells in the marrow of children receiving ABMT for neuroblastoma and acute myeloid leukemia (AML). Since normal progenitor cells are also marked, this approach can provide information about the efficiency of gene transfer in man and the persistence of these modified cells in vivo.

Since September 1991 we have treated 17 patients. Gene expression in hemopoietic and T and B cell lineages has been at a higher level than predicted from most animal models (2-15%) and has persisted for more than one year. The marrows used for transfer are obtained shortly after multiple courses of chemotherapy and are highly proliferative; this may render them more susceptible to RMGT. Two patients with AML have relapsed. In both, a proportion of the relapse blasts were marker gene positive, showing the infused marrow contributed to disease recurrence. One neuroblastoma patient has relapsed and the marker gene is being sought. Modifications of this marker approach with two distinguishable vectors are now being used to compare the efficacy of available purging techniques. Prolonged gene expression in hemopoietic and lymphoid lineages offers encouragement for gene therapy protocols in which marrow derived cells are the targets.

S 007 RETROVIRAL GENE TRANSFER IN ADULT AUTOLOGOUS BONE MARROW TRANSPLANTATION, Ken Cornetta[1], Ann Moore[1], Edward Srour[1], Robert C. Moen[2], E. Randolph Broun[1], Isabel Cunningham[1], Ann Hedderman[1], Robert Hromas[1], Richard A. Morgan[3], W. French Anderson[4], Ronald Hoffman[1], and Guido Tricot[1]. Department of Medicine, Indiana University, Indianapolis, IN 46202, Genetic Therapy, Inc., Gaithersburg, Molecular Hematology Branch, NHLBI, NIH, Bethesda, and Norris Cancer Center, USC School of Medicine, Los Angeles.

Autologous bone marrow transplantation (AuBMT) is becoming an increasingly common procedure in the treatment of acute leukemia. Using the patients own marrow for transplantation overcomes the obstacles of donor identification and graft-versus-host disease. Unfortuntely, the major type of treatment failure, disease relapse, is poorly understood. Specifically, does disease relapse come from the transplanted marrow, leukemic cells that survive the AuBMT chemotherapy, or both. We hope to better understand disease relape by marking transplanted marrow used in AuBMT for acute myelogenous leukemia and acute lymphocytic leukemia. Marrow is harvested in complete remission (no evidence of leukemia) and a portion (10-30%) of marrow mononuclear cells are exposed to the neomycine resistance gene vector GIN (supplied by Genetic Therapy Inc.). A portion of the marked marrow is evaluated for vector integration and tested for contaminating replication-competent retrovirus prior to infusion. Patients receive cyclophosphamide and busulfan chemotherapy as the AuBMT preparative regimen followed by infusion of the marked and unmarked bone marrow after chemotherapy. Patients are monitored for evidence of vector in blood and bone marrow samples after AuBMT and at disease relapse.

To date 5 patients have been enrolled on the study, all with the diagnosis of AML. One patient was not marked because of inadequate number of cells obtained at bone marrow harvest. Three patients have been marked and are awaiting transplantation. One patient has been transplanted. Immediately after gene marking the transduction efficiency for marrow GFU-GM and BFU-E was 2.0% $\pm$ 0.6 and 1.3% $\pm$ 0.5, respectively. The course of our first transplanted patients was complicated by slow engraftment and multiple infections. The patient died of sepsis on day +146 post-AuBMT. No evidence of vector integration was noted in bone or bone marrow after transplantation. The patient was in a morphologic and cytogenetic remission at the time of death. No evidence of contaminating replication-competent retrovirus has detected in pre or post AuBMT samples from this patient or any of the marked marrow in patients awaiting transplantation.

Therapeutic Protocols-Viruses and AIDS-I

S 008 OLIGONUCLEOTIDE THERAPEUTICS, Stanley T. Crooke, Isis Pharmaceuticals, Carlsbad, CA

Progress in oligonucleotide drug discovery and development will be discussed. Specifically, progress in core technologies, combinatorial methods and progress in the development of oligonucleotides as drugs will be reviewed.

S 009 IN VIVO GENE TRANSFER FOR THE TREATMENT OF SOLID TUMORS, Kenneth W. Culver[1], Charles J. Link[2], Nicholas Akdemir[1], Donald Moorman[3], Daniel Stanley[3], Daniel Butler[3], Mark Ackermann[4], Carol Jacobson[4], Jay Schnitzer[5] and R. Michael Blaese[2], [1]Genetic Therapy Inc., Gaithersburg, MD 20878, [2]National Cancer Institute, Bethesda, Md, [3]Iowa Methodist Medical Center, Des Moines, IA, [4]Iowa State University, Ames, IA and [5]Massachusetts General Hospital, Boston, MA

The direct injection of retroviral vector-producer cells into growing tumors in situ results in the transduction of a variety of tumor cell types. This in vivo gene transfer method has several advantages over the direct injection of DNA, liposomes and viral particles. First, the vector particles are produced continuously within the tumor for several days to maximize gene transfer efficiency. This is particularly important for tumors with longer doubling times. Second, retroviral vectors require cellular proliferation for integration and expression. In many organs, the majority of cells are cycling at much slower rates than the tumor cells (e.g. brain. liver) allowing for some selectivity for tumor cell transduction.

Transfer of the Herpes Simplex-thymidine kinase (HS-tk) gene into tumor cells confers a sensitivity to the anti-herpes drug, ganciclovir (GCV). The HS-tk/GCV system has successfully eliminated carcinomas, sarcomas, adenocarcinomas, and melanomas in situ in subcutaneous, brain and hepatic tumor models. One of the unexpected advantages of the HS-tk/GCV system, is that only a portion of the tumor needs to contain the HS-tk gene for complete eradication of the tumor. This "bystander" effect destroys rodent and human tumor cells in vitro and in vivo with as few as 1-10% of the tumor cells containing the HS-tk gene. Toxicity studies involving the direct injection of vector-producer cells IV or IP into mice has shown no evidence of illness with or without GCV therapy for >12 months. The biochemical basis for this selective toxicity to surrounding HS-tk negative tumor cells is unknown.

We have also evaluated the survival and toxicity of the direct injection of murine HS-tk vector-producer cells into immune-competent rat liver. Histologic evaluation showed no evidence of hepatic toxicity with or without GCV with survival of the xenogeneic cells for 7 days. In each animal model, there has been no evidence of significant damage to the surrounding normal tissues as a result of the bystander effect. These findings suggest that the injection of vector-producer cells into a growing tumor may be sufficiently selective to the growing tumor to permit clinical applications of this in vivo gene delivery system in visceral organs. The first step toward the development of therapeutic applications of this HS-tk/GCV in vivo gene delivery system has begun for the treatment of human brain tumors .

S 010 RECONSTITUTION OF VIRAL IMMUNITY BY THE ADOPTIVE TRANSFER OF T CELL CLONES MODIFIED BY GENE INSERTION. Phil Greenberg, Käthe Watanabe, Mark Gilbert, Brad Nelson, and Stan Riddell, University of Washington and Fred Hutchinson Cancer Research Center, Seattle, WA 98195.

Restoring or augmenting immunity by the adoptive transfer of *in vitro* cultured antigen-specific T cells has proven therapeutically effective for viral infections in animal models. Our laboratory has applied insights from studies in these animal models to the treatment of human viral disease in immunocompromised hosts. Our initial studies have focused on reconstitution of CMV-specific host $CD8^+$ cytotoxic T cell responses in CMV-seropositive patients immunosuppressed by the preparative regimen given prior to bone marrow transplantation (BMT) from an HLA-matched sibling. The essential role of $CD8^+$ Tc for protection from CMV disease is supported by studies in which we demonstrated that patients who endogenously reconstitute their CMV-specific $CD8^+$ Tc response are protected from the development of CMV disease, whereas patients lacking this response are at high risk for disease. Five patients have been entered on our initial adoptive T cell therapy protocol. $CD8^+$ CMV-specific T cell clones were derived from HLA-matched seropositive sibling bone marrow donors and infused in escalating cell doses over 4 consecutive weeks. Infusion of $>2.2 \times 10^9$ $CD8^+$ CMV-specific T cell clones was non-toxic and performed safely in an out-patient setting. Transfer of immunity was detectable following infusion of even the lowest cell dose (3.3×10^7 T cells/m^2) and responses greater than those present in healthy donors with protective immunity were detectable following transfer of higher cell doses. Transferred $CD8^+$ Tc immunity was detectable for >6 weeks, and long-term persistence of transferred clones is being analyzed by PCR using amplimers specific for the TCR of the transferred clones. None of the patients who received adoptive T cell therapy developed subsequent evidence of CMV disease.

Our laboratory is now evaluating methods to improve the efficacy and safety of adoptive T cell transfer by the introduction of genes into T cell clones. To improve safety, a retroviral vector containing an inducible suicide gene has been constructed by Steve Lupton and Bob Overell (Targeted Genetics Corporation). The herpes virus thymidine kinase (TK) gene has been fused in frame with the *hph* gene, resulting in a gene encoding a single bifunctional protein (HyTK) conferring hygromycin resistance and *in vitro* sensitivity to gancyclovir. Studies in mice have demonstrated that T cell clones expressing this gene can be readily eliminated *in vivo* by the administration of non-toxic doses of gancyclovir. A clinical trial employing T cells modified with this gene is now underway in HIV seropositive patients undergoing allogeneic BMT for the treatment of HIV-related lymphomas. These patients are receiving high-dose chemoradiotherapy pretransplant to eliminate the lymphoma cells and ablate their lymphohematopoietic system, which represents the major reservoir of HIV-infected cells, and are receiving AZT drug therapy and adoptively-transferred HyTK-modified $CD8^+$ T cell clones post-transplant in an effort to prevent or minimize infection of donor bone marrow derived cells. If patients develop toxicity at sites of infection potentially related to exuberant immune responses mediated by transferred T cell clones, such as encephalitis or pulmonary alveolitis, gancyclovir will be administered to ablate the T cells. The persistence and/or elimination of the transferred T cells will be monitored by PCR.

The therapeutic efficacy of transferred $CD8^+$ T cell clones in murine models is limited by the inability of the clones to proliferate and survive long-term *in vivo* in the absence of either exogenous IL-2 or a concurrent $CD4^+$ helper T cell response. Therefore, we are attempting to modify $CD8^+$ T cells to render them independent of exogenous growth factors and capable of proliferating in response to TCR ligation. Several types of gene constructs are being evaluated. The first is designed to provide the additional signals necessary to result in endogenous IL-2 production following T cell activation. Based on previous studies of bifunctional $CD8^+$ T cells isolated from mice, $CD8^+$ Tc clones were transduced with a vector constructed by Bob Overell containing the gene for the IL-1 receptor. Such T cells proliferate in response to binding of ligands to both the TCR and IL-1R, but fail to proliferate in response to either signal alone. A second type of construct being developed with Steve Lupton involves the generation of a hybrid gene, in which the IL-2 coding sequence is under control of a promoter normally activated by TCR ligation. Preliminary studies are being performed with a vector containing the IFN-γ promoter driving an IL-2 cDNA. Finally, a third type of construct containing chimeric cytokine receptors potentially capable of providing an autocrine loop and delivering to a T cell the signal normally provided by the binding of IL-2 to its receptor is being evaluated. Our initial studies are with a vector containing the extracellular domain of the TNF receptor fused in frame to the transmembrane and intracytoplasmic domains of the IL-2 receptor β chain.

Therapeutic Protocols-Viruses and AIDS-II

S 011 THE ROLE OF PERIPHERAL BLOOD LYMPHOCYTES AND BONE MARROW CELLS IN THE DEVELOPMENT OF A FUNCTIONAL IMMUNE SYSTEM AFTER GENE THERAPY FOR ADA DEFICIENT SCID.

Giuliana Ferrari[1], Luigi Notarangelo[2], Paolo Servida[1], Giulia Casorati[1], Silvano Rossini[1], Alberto Ugazio[2], Fulvio Mavilio[1], and Claudio Bordignon[1]. [1]Gene Therapy Program, Istituto Scientifico H. S. Raffaele, Milano, Italy; and [2]Clinica Pediatrica, Università di Brescia, Brescia, Italy.

Deficiency of the enzyme adenosine deaminase (ADA) results in a variant of severe combined immunodeficiency (SCID). This lethal disease is the first genetic disorder considered for human somatic cell gene therapy. Previously, we reported a murine model in which successful ADA gene transfer and expression could restore immune functions in human ADA deficient peripheral blood lymphocytes *in vivo* (1,2). Based on these results, the clinical application of gene therapy for the treatment of ADA$^-$ SCID patients who previously failed replacement treatment therapy with bovine pegilated enzyme was approved by our institutional Ethical Committee and by the Italian National Committee for Bioethics. Aim of the study is to evaluate safety and efficacy of this procedure, and to identify the relative role of peripheral blood lymphocytes, and hematopoietic stem cells and progenitor cells in the long term reconstitution of immune functions after retroviral vector-mediated ADA gene transfer. For this purpose, two vectors have been utilized for gene transfer into peripheral blood lymphocytes (PBL) and bone marrow cells (BM), DCAl and DCAm respectively. The two vectors are identical in the construction design, the packaging cell line utilized, and viral titer ($1\text{-}2 \times 10^5$). However, they differ for a restriction site in the viral LTR that allows to distinguish the progeny of cells transduced with the two vectors.
On March 1992, the first ADA deficient patient entered the study. Approximately 10^8 T-cell depleted BM cells were transduced utilizing the ADA vector DCAm, approximately 10^8 PBL were transduced utilizing the ADA vector DCAl, and gene-modified cells were injected into the patient in three monthly intervals. Five months after the beginning of in vivo gene therapy, ADA vector-transduced cells were detected among both peripheral blood and bone marrow cells. Vector-derived ADA activity was detected in cell lysates of unselected bone marrow cells and peripheral blood lymphocytes at concentrations ranging from 1-10% of normal controls. By PCR analysis and utilization of vector-specific restriction enzymes, it was possible to define the origin of corrected marrow cells and peripheral blood lymphocytes. Five months after the first administration of gene modified cells, PBL appeared to be predominantly derived from transduced PBL, while BM cells were predominantly derived from the transduced marrow progenitors. The long term follow up of the origin and survival of vector - transduced circulating lymphocytes is utilized to determine the relative role of peripheral blood lymphocytes, and hematopoietic stem cells and progenitor cells in the long term reconstitution of immune functions after retroviral vector-mediated ADA gene transfer.

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S 012 FIBROBLAST GENE THERAPY FOR HEMOPHILIA B, Jerry L.Hsueh,Jiemin Zhou,Daru Lu,Xingfang Qiu,Jianmin Peilin Meng,Fenglei Han,Bihu Ming,Xiaopang Wang,Jianpo Wang,Jiaqian Liang and Zhoushu Jiang, Institute of Genetics,Fudan University, Department of Hematology,Changhai Hospital and Department of Biology,East China Medical University, Shanghai 200433,China

Two brothers with apparently moderately severe hemophilia B were selected for the gene transfer therapy. Patient 1 (Liu Dou)aged 9,weighed 28kgand Patient 2(Liu Wei) aged 13,weighed 44kg. The retroviral vector XL-IX was constructed by the insertion of a 1.58kb fragment of Factor IX cDNA into the unique BamHl site upstream from Neo gene in pZIPNeoSV(X). The double copy retroviral vector N2CMVIX was constructed by the insertion of a 2.3kb BglII/HpaI fragment containing FIX cDNA driven ;by hCMV promotor into the BglII/SnaBI sites in the U3 region in 3' end of LTR in pN2A. Highly expressed recombinant retroviral vectors XL-IX and N2CMVIX were transduced to the primary skin fibroblast of the patients in vitro. After selection with G418, identification with PCR, Southern blot, ELISA and safety assesments, the autologous skin fibroblasts were embedded with rat collagen matrix, injected to the dosal subcutaneous space of patients. A total of 1.1 and 0.61×10^9 cells were injected over a period of 4 months respectively to the Patient 1 and 2. The blood from LD was samped for ELISA 2 weeks later and the concentration of human FIX protein gradually increased from 71ng/ml to the maximium lavel of 245ng/ml,maintained at the level of 220ng/ml from the baseline levels that are approximately 3% of normal for ten months at the time of writing. A doubling of clotting activity was also detected in the asme patient.In Patient 2, a doubling from baseline levels of FIX antigen was observed that are approximately 1% of normal while the clotting activity did not show a steady increase.FIX inhibitor in plasma of the brothers were showed negative for 10 months after injection.The clinical symtoms for LD have been alleviate obviously. The patient did not rereceive any transfusion since the ex-vivo cell were implanted. There is no deleterous effect to be found in two patients for 10 months after treatment. The patients appear to have tolerated the procedure well. The patients are now under follow-up investigation. The regular evaluation seems promising, though the current results are still too priliminary to make conclusions regarding the efficacy of this Protocol. (Human Gene Therapy 3(5)1992.

Supported from State High-Tech Program.

S 013 ISSUES IN THE CLINICAL APPLICATION OF HEPATIC GENE THERAPY. Fred D. Ledley, Departments of Cell Biology and Pediatrics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

Various methods have been described for targeting gene therapy to the liver including both ex vivo and in vivo strategies. The goals of these two strategies are somewhat different, with ex vivo strategies intended to provide permanent expression of a therapeutic product, and current in vivo strategies aimed at using genes as medicines which can be administered repetitively with variations of the dose and schedule to meet the patient's therapeutic need. The problems inherent in the clinical application of these two methods are different; the clinical application of ex vivo gene therapy with the attendent need for surgery and cell transplantation requires confidence that long-term gene expression can be achieved, and the application of genes as medicines requires information about the pharmacokinetics and feasibility of repetitive administration of the gene. We have performed pre-clinical studies with both methods to assess the feasibility and safety of these approaches. In anticipation of ex vivo strategies for gene therapy we have demonstrated that human hepatocytes can be harvested from liver segments preserved in Belzar solution, cultivated in hormonally defined, media, and transplanted into SCID mice. These data suggest that 0.1-1% of hepatocytes in the recipient may contain the recombinant gene, a fraction which is sufficient for some, but not all, clinical indications of gene therapy. These preclinical studies, as well as those of other laboratories, also indicate that this procedure can be performed safely. Studies with asialoglycoprotein (ASO) mediated gene transfer and a gene for methylmalonyl CoA mutase deficiency demonstrate that recombinant gene expression can be achieved reproducibly at therapeutic levels for >24 hours in the liver after IV administration. With repetitive administration, however, high titer ($>10^4$) antibodies were formed against the ASO-PL complex. While these antibodies may not block action of the complex, antibodies may complicate the pharmacology of administration and safety. A novel method of gene therapy using a natural, DNA-binding protein to effect specific receptor mediated DNA-uptake and expression is described. This method is shown effect receptor mediated uptake in hepG2 cells and primary hepatocytes and produce marker gene expression are achieved which are equivalent to those reported with ASO/PL/DNA complexes. The presence of adenovirus in the media further enhances expression of a CAT marker gene. Experiments are underway to assess the utility of this method for in vivo gene transfer and the feasibility of repetitive administration.

S 014 STEM CELL GENE THERAPY FOR ADENOSINE DEAMINASE DEFICIENCY, Dinko Valerio[1], Victor W. van Beusechem[1], Mark P.W. Einerhand[1], Peter M. Hoogerbrugge[1,2], Alexandra, A.J. Migchielsen[1], and Leonie Kaptein[1], [1]Gene Therapy Department of the Institute for Applied Radiobiology and Immunology TNO, P.O.Box 5815, 2280 HV, Rijswijk, The Netherlands, [2]Dept. of Pediatrics, Univ. Hospital, Leiden, The Netherlands.

The preferred target cell in gene therapy for blood disorders is the pluripotent hemopoietic stem cell (PHSC) because of its selfrenewing potential combined with a capacity to contribute to the production of all blood cells over extended periods of time (probably lifelong). To date, the typical example for gene therapy as a whole and for bone marrow gene therapy in particular remains the putative treatment of an inherited form of immunodeficiency caused by a defect in the gene encoding the enzyme adenosine deaminase (ADA). The aim would be to genetically correct blood forming cells from such deficient patients which, following reinfusion into the patient, could regenerate a functional immune system. For the efficient transduction of PHSC we have generated an amphotropic retrovirus carrying the human ADA gene. In vitro experiments with our ADA virus indicated that hemopoietic progenitors from both rhesus monkeys and man can be transduced with high efficiency (up to 40 %) as demonstrated by ADA overexpression in cultured colonies. To assess which factors determine the efficiency of retrovirus-mediated gene transfer into PHSC, murine bone marrow was exposed to the virus-producing cells under varying culture conditions. Large variations in gene transfer efficiency became manifest, mainly dependent on the added growth factors. Under optimal conditions we reproducibly infect 15-20% of the PHSC that contribute to a stably regenerated hemopoietic system (> 6 month post transplantation). In order to extend these studies towards the clinical situation we performed autologous bone marrow transplantations in lethally irradiated rhesus monkeys using retrovirus-infected hemopoietic stem cells. Variables tested to achieve efficient and reproducible gene transfer ex vivo included: growth factor addition, virus titer, virus backbone, the presence of stromal cells and stem cell purity. Upon transplantation of the modified bone marrow, regeneration rates of peripheral blood cells were monitored and indicated that in all instances regenerative capacity of the graft could be conserved. All 14 monkeys transplanted sofar were shown to express the functional hADA enzyme in their peripheral blood cells, as detected by zymogram analysis. The frequency of provirus containing cells in the peripheral blood was determined using a semiquantitative PCR analysis. Depending on the infection procedure used, 8 out of 13 monkeys carried the provirus in their peripheral blood cells for the duration of the experiment (currently 3 monkeys > one year and one monkey > two years after transplantation). Moreover, in all peripheral blood mononuclear cells tested hADA expression could be detected. Genetic modification could also be demonstrated in BMC of various densities, in spleen, in lymph nodes and in cultured T lymphocytes. Based on our data we conclude that safe stem cell gene therapy procedures were derived which reproducibly yield long term genetic modification of the hemopoietic system of non-human primates. The efficacy of our technology can be tested in the clinic, since a stem cell gene therapy protocol for ADA deficient patients has been approved by the Dutch government and the local ethical committee of the University Hospital in Leiden. As a long term goal we plan to develop gene targeting procedures to be used in stem cell gene therapy. Such procedures will need to be tested and validated in a relevant animal model. Therefore, we are also making an effort to generate ADA deficient mice using ES cell technology. See also the abstracts by: Van Beusechem et al., Hoogerbrugge et al., Migchielsen et al., and Kaptein et al.

Cell Biology: Delivery Mechanisms-I

S 015 DEVELOPMENT OF HERPES SIMPLEX VIRUS AS A GENE TRANSFER VECTOR FOR THE NERVOUS SYSTEM

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Herpes simplex virus (HSV) displays a natural propensity to establish lifelong latent infections within neurons characterized by the exclusive production of latency associated transcripts (LATs) in the complete absence of lytic gene expression. In order to exploit these natural features for gene transfer to CNS two fundamental problems must be overcome: (i) A mutant virus must be engineered which does not destroy brain tissue yet is capable of establishing latency in a substantial number of targeted neurons without the possibility of reactivation and (ii) a means of expressing the transferred gene of interest during latency must be devised which involves the identification of a latency active promoter/enhancer sequence and an appropriate genome location for insertion of the promoter-gene cassette.

For the efficient delivery of genes into target get cells, different HSV mutant backgrounds are being considered for use as vectors. The conditions for the consideration of mutants background are (a) that the mutant virus is nonreplicating and can only be produced in specifically engineered cell line, (b) that there be no detectable background of wild-type virus in the mutant vector stock, and (c) that the cytotoxic effects of infection with the mutant virus be minimized. The first two conditions have been met by deleting the two essential immediate early regulatory proteins of the virus, ICP4 and ICP27. This virus expresses an extremely limited subset of HSV genes and can only be propagated on cell lines expressing complementing levels of ICP4 and ICP27. While this virus still retains some cytotoxic properties, persistence of significant quantities of functional viral genomes in infected cells could be demonstrated. Elimination of the remaining cytotoxic potential is being explored by deleting other nonessential immediate early genes and genes encoding metabolically active virion components.

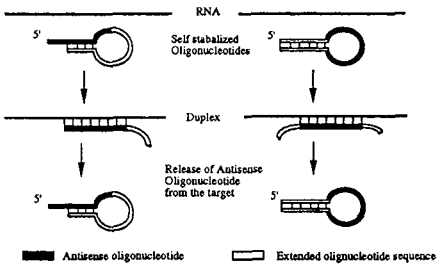
To address the second problem, studies were carried out to identify the natural latency promoters within the LAT region in order to exploit their latency active feature to drive foreign gene expression. In addition to a TATA box containing promoter reported by others (LAP1), a second TATAless LAT promoter (LAP2) was uncovered. Because LAT expression can be detected in latently infected brain neurons long-term, attempts were made to exploit these promoters for transgene expression *in vivo*. However, insertion of the *lacZ* reporter gene behind LAP1 or LAP2 provided only transient expression of ß-galactosidase in brain. Experiments are now in progress to identify specific cis-acting LAP elements for introduction into the highly active, albeit short lived, human cytomegalovirus immediate early gene promoter. These recombinant promoters will be tested for sustained high level expression from the latent viral genomes in brain.

Cell Biology: Delivery Mechanisms-II

S 016 SELF-STABILIZED ANTISENSE OLIGONUCLEOTIDE PHOSPHOROTHIOATES: SYNTHESIS, PROPERTIES AND ANTI-VIRAL ACTIVITY, Sudhir Agrawal* and JinYan Tang, Hybridon, Inc., One Innovation Drive,Worcester, MA 01605.

Antisense oligonucleotides and modified analogs may be used to regulate the expression of genes including ones implicated in cancer or in the replication of viruses[1]. This provides an opportunity for rational drug design. A major limitation in the use of antisense oligonucleotides as drugs is their nuclease susceptibility. Unmodified oligodeoxynucleotides in particular have limited half lives *in vivo*. Oligodeoxynucleotides containing phosphorothioate backbones have increased nuclease resistance but are still eventually degraded. Degradation of oligodeoxynucleotides is primarily from the 3'-end. Various modifications of oligonucleotides have been carried out to avoid this problem. These include incorporation of a few nuclease resistant internucleoside linkages at 3'-end[3], incorporation of various chemical constituents at the 3' hydroxyl[4,5] and circularizing the oligonucleotides by joining the 3' and 5' end[6].

We have designed and studied oligonucleotides in which 3' segment of oligonucleotide sequence is involved in hydrogen bonding with part of its own oligonucleotide sequence called here 'self stabilized' oligonucleotides. Two examples of 'self stabilized' oligonucleotides are shown here (a) an oligonucleotide which has hairpin loop structure at 3' end and (b) an oligonucleotide in which 3' segment of oligonucleotide sequence is hydrogen bonded with 5' segment of oligonucleotide sequence. These self stabilized oligonucleotides have increased nuclease resistance, hybridize effectively to the complementary nucleic acids and show increased antiviral activity.



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S 017 ADENO-ASSOCIATED VIRUS VECTORS FOR GENE TRANSFER, Barrie Carter, Terry Flotte, Sandra Afione, Rikki Solow, Pamela Zeitlin, and Sharon McGrath, Targeted Genetics Corporation, Seattle WA, NIDDK, NIH, Bethesda, Md and Johns Hopkins Medical School, Baltimore, Md.

Adeno-associated virus (AAV) has several features which suggest that it will be useful as a delivery vector for human gene therapy. First, AAV is a replication defective virus which replicates only upon expression of the AAV rep gene and an additional helper function provided by a co-infecting helper virus, generally an adenovirus or herpesvirus. Second, in the absence of helper virus, the AAV genome integrates at high efficiency into the host cell genome. For wild type AAV this integration may occur about 60% of the time at a preferred site on human chromosome 19. Integration of AAV does not cause any gross alterations or morphological changes to cell growth and does not cause malignant transformation. The provirus can be rescued (i.e. excised and replicated) by superinfection with helper adenovirus. Third, AAV is not known to cause any human disease. The only regions of AAV DNA which are required in cis for replication of AAV genomes, encapsidation into AAV particles, integration into host cell chromosomes at high efficiency, and rescue of the integrated provirus are the inverted terminal repeats. All of the AAV coding functions for the rep and cap genes can be provided in trans.

AAV genomes inserted into bacterial plasmids and transfected into adenovirus infected permissive cells are infectious. AAV vectors can be generated by deleting the AAV coding regions and inserting the appropriate gene and regulatory elements such as transcription promoters and polyA signals. Transfection of this vector plasmid into adenovirus infected cells, together with a second plasmid to provide the complementing rep and cap functions, results in a population of AAV transducing particles containing only the vector genomes. Thus the AAV vector contains no AAV genes and no wild type AAV genomes and is easily purified and concentrated free of any helper virus by a banding in CsCl gradients and heat inactivation of the helper.
AAV vectors containing reporter genes such as selectable markers can yield very high transduction frequencies and can transduce at least 70% of the cell population. Use of AAV vectors to develop a gene delivery system for the CFTR gene in in vitro and in vivo experiments will be described will be described.

S 018 STABILITY, CLEARANCE AND DISPOSITION OF INTRAVENTRICULARLY ADMINISTERED OLIGODEOXYNUCLEOTIDES: IMPLICATIONS FOR THERAPEUTIC APPLICATION WITHIN THE CENTRAL NERVOUS SYSTEM, Len Neckers, Christine Chavany, Daniel Geselowitz, Brigid Fahmy, Stuart Walbridge, Jeff Alger, and Luke Whitesell, NCI, NIH, Bethesda, MD 20892.

Antisense oligodeoxynucleotides (ODN's) have demonstrated efficacy in modulating the expression of various genes, thus providing important insights into their roles in tumorigenesis or normal growth and development. Although attention has been focused recently on the development of antisense ODN's as therapeutics for a variety of diseases, systemic application of ODN's has been hindered by three prominent obstacles: 1)rapid degradation of unmodified molecules in plasma, 2)extremely rapid clearance from the circulation of intravenously administered compounds and 3)ability of ODN's to concentrate in target tissue at therapeutic concentrations (particularly, if the target is a solid, metastatic tumor).To avoid some of the obstacles associated with systemic administration, we have focused on regional therapeutic strategies to evaluate ODN actions *in vivo*. We have previously reported that continuous local perfusion with an unmodified ODN can specifically modulate gene expression in a subcutaneous tumor model system. We now describe the stability, disposition and clearance of ODN's within a clinically important, physiologically well-defined biologic compartment, namely the CSF space of the rat. Although we find little intrinsic nuclease activity in cerebrospinal fluid (CSF), phosphodiester ODN's are rapidly degraded by brain-associated 3'-exonuclease activity both *in vitro* and following *in vivo* injection. Phosphorothioate ODN's, however, appear resistant to degradation in the CNS and after intraventricular administration, we find they are cleared in a manner consistent with CSF bulk flow. Continuous infusion of ODN at 1.5 nmole/hr by mini-osmotic pump can maintain micromolar concentrations of intact phosphorothioate ODN in CSF for at least one week without obvious neurologic or systemic toxicity. Following infusion, extensive brain penetration and marked cellular uptake, especially by astrocytic cells, is demonstrated. Our findings clearly indicates that although CSF itself is low in nuclease activity, ODN modifications which protect at least the 3' base linkage from exonuclease attack are required to stabilize ODN in the CSF space and achieve tissue penetration of intact material. The penetration into brain we now report suggests the possibility of targeting both leptomeningeal and intraparenchymal disease processes. In addition, the reversible modulation of normal brain function by continuous infusion of antisense ODN's becomes practical.

S 019 CELLULAR PHARMACOLOGY OF OLIGODEOXYNUCLEOTIDES IN HL60 CELLS C.A. Stein, J. Tonkinson, Z. Khaled, L.-M Zhang, and N. Nelson[1], Columbia University, New York, NY 10032, [1]Roche Institute, Nutley, NJ 07110

The dynamics of the cellular internalization (Int) of phosphodiester (PO) and phosphorothioate (PS) oligos are imperfectly understood. We have shown, in HL60 cells, that the rate of internalization of a 15-mer homopolymer of thymidine phosphate linked to a 5'-fluorescein (F) moiety (F-POdT15) appears to be dependent of the activity of protein kinase C (PKC). Inhibitors of PKC activity (e.g., H4, staurosporine) decrease Int, while phorbol-12, 13-dibutyrate acetate, an activator of PKC, increases Int. However, PO, and especially PS oligos are also inhibitors of PKC activity: SdC28 (1-5 uM) decreases Int of FOdT15, and also inhibits purified PKC-β1 (IC50 = 1-5 uM). The kinetics of in-vitro substrate (EGFR) phosphorylation demonstrate that inhibition of PKC-β1 is not competitive with respect to ATP, but is competitive with respect to substrate (K_i = 5.4 uM). Examination of the rate of efflux of FOdT15 in HL60 cells can be best fit to a three-compartment model, where $C_T = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$. Determination of the kinetic efflux parameters have $t_{1/2\alpha}$ = 16 min; $t_{1/2\beta}$ = 33 min; $t_{1/2\gamma}$ = 136 min. 90% of the fluorescent signal was lost in the α + β phases, while only 10% was lost in the γ phase. Probenecid (5 mM) significantly blocked the α, but not the β + γ phases, but exhibited significant cytotoxicity. By employing F-^{32}POdT15, and loading the HL60 cells with it for five hours, we could demonstrate that significant endonucleolytic degradation had occurred in the β + γ phases, with little occurring in the α phase. However, chain-extended oligos were observed in all three efflux phases. The pH-sensitive (F) signal from internalized F-POdT15, unlike F-albumin, did not increase in the presence of the ionophor monensin, indicating that the oligo did not occupy an acidic vesicle. Preliminary experiments with reconstituted endosomes indicated that oligos may non sequence specifically inhibit the function of the vacuolar H^+-ATP pump.

Design, Modification, and Production of Nucleic Acids-Chemistry-I

S 020 SYNTHESIS, BIOCHEMISTRY, AND BIOLOGICAL APPLICATIONS OF NEW POLYNUCLEOTIDE ANALOGS, Marvin H. Caruthers[1], Mary D. Hall[1], Darla Graff[1], Charles Greef[1], Yixiang Zeng[1], Lendell Cummins[2], William S. Marshall[3], and William T. Wiesler[4], [1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, [2]Isis Pharmaceuticals, Carlsbad, CA 92008, [3]Amgen Inc., Boulder, CO 80301, [4]Cadus Pharmaceutical Corp., New York, NY 10014.

The synthesis of a new class of DNA analogs called dithioate DNA will be described. This analog, which has a deoxynucleoside-OPS$_2$O-deoxynucleoside linkage, is isosteric and isopolar with a normal phosphodiester, nuclease stable, and activates RNase H toward RNA degradation. Synthesis begins with deoxynucleoside 3′-(N,N-tetramethylene)phosphorothioamidites. These synthons are condensed with silica linked deoxynucleosides and oxidized with sulfur to yield the dithioate linkage. When used in combination with deoxynucleoside 3′-phosphoramidites, deoxyoligonucleotides having dithioate and phosphate linkages in any predetermined sequence can be prepared on standard DNA synthesis machines. Preliminary studies demonstrate that sequence specific dithioate oligomers are competitive inhibitors, relative to primed template, for HIV-1 reverse transcriptase (K_i = 0.5 nM) and block the expression of HIV-1 antigens from chronically infected cells. (Supported by NIH GM25680 and GM21120).

S 021 DESIGN AND BIOLOGICAL PROPERTIES OF ANTISENSE OLIGODEOXYNUCLEOTIDE ANALOGS, *Jack S. Cohen, Nasser Farschtschi, and Nikolai Polushin, Georgetown University Medical Center, Washington DC 20007.*

Previous work has focussed on phosphorothioate as a suitable analog for antisense oligodeoxynucleotide applications. Several successful antisense applications have been achieved with this analog in *in vitro* systems against viruses and mammalian genes. Phosphorothioates have also been applied in animal model systems, and pharmacokinetics and toxicity have been determined, and this analog is now being developed for therapeutic applications. However, this analog also has some undesirable properties, notably a tendency to bind to proteins, slow cellular uptake, and a lowered Tm relative to the natural phosphodiester. In a recent study we found that certain phosphorothioate oligomers containing four consecutive G's gave a non-sequence specific growth inhibition of certain cells. In order to overcome some of the disadvantages of phosphorothioates, we synthesized a series of chimeric co-polymers of PO and PS, that have properties that are a compromise between all-PO and all-PS. However, in order to bring about a greater improvement in antisense activity we have embarked on a program requiring synthesis of three different kinds of derivatives, (a) second generation, or improved backbone analogs that do not contain sulfur, (b) conjugates with groups attached to improve cellular uptake, and (c) conjugates that have RNase-like activity. For (a) we have chosen the 3'-methylenephosphonate analog, since this has no S present, is achiral, and can be expected to give both nuclease resistance and good hybridization with the target mRNA. To synthesize such analogs we prepare the 3'-methylenephosphonamidite synthons. (b) For improved cellular uptake we are preparing the 5'-esters of carboxylic acids with lipid chains, such as palmitoyl. This would have the advantage of a pro-drug, releasing the intact oligomer as a result of intracellular esterase activity. (c) In order to achieve RNase-like activity we have synthesized phosphoramidites with linked imidazoyl groups. The length of the linker was estimated by molecular modelling. We are testing these conjugates for the appropriate activity, and intend to combine these structural aspects into an overall improved antisense compound that will be suitable for therapeutic applications.

S 022 ANTI-GENE AND ANTI-SENSE ACTIVITY OF OLIGONUCLEOTIDE-INTERCALATOR CONJUGATES VIA TRIPLE HELIX FORMATION, Claude Hélène[1], Thérèse Garestier[1], Jian-Shen Sun[1], Carine Giovannangeli[1], Christophe Escudé[1] and Nguyen T. Thuong[2], [1]Laboratoire de Biophysique, INSERM U.201 - CNRS URA 481, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75005 Paris, [2]Centre de Biophysique Moléculaire, 45071 Orléans Cedex 02, France.

Oligonucleotides can bind to the major groove of double-helical DNA at polypurine.polypyrimidine sequences[1]. Triple helix stability can be increased by using base analogs. Oligoribonucleotides and their 2'-O-methyl derivatives bind more strongly than oligodeoxynucleotides[2]. The stability of triple-helical complexes can be further increased by covalent attachment of an intercalating agent at one of the extremities of the triplex-forming oligonucleotide[3]. Oligonucleotide-intercalator conjugates can be used to control gene transcription both *in vitro* and in cell cultures[4].

Triple helix formation on a single-stranded target can be achieved by chimeric oligonucleotides consisting of two parts : one part forms Watson-Crick base pairs, while the second part engages Hoogsteen hydrogen bonding interactions with the WC base pairs. The two parts are linked together by either a polyethyleneglycol tether or an oligonucleotide sequence. The two "jaws" can be closed when a reactive intercalator, such as psoralen, is attached to the 5'-end of the Hoogsteen portion. These oligonucleotide "clamps" can be used to block processing of single-stranded nucleic acids by enzymatic systems.

Oligonucleotides synthesized with the α-anomers of nucleotide units form triple helices which are nearly as stable as those formed by β-oligonucleotides[6]. These nuclease-resistant α-oligomers could be used to target DNA sequences *in vivo*.

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S 023 **RNASE H AND THE DEVELOPMENT OF THERAPEUTIC OLIGONUCLEOTIDES, Roxanne Y. Walder[1,2], Janel R. Hayes[2], Zijun Li[2], YuYing Wang[1] and Joseph A. Walder[1,2], [1]Department of Biochemistry, University of Iowa, Iowa City, IA, 52242, [2]Integrated DNA Technologies Inc., 1710 Commercial Park, Coralville, IA 52241.**

The arrest of translation by antisense oligonucleotides is greatly augmented by cleavage of the target mRNA by RNase H. This observation led us to study the RNase H activities of higher eukaryotic cells. The major human isoform of RNase H, RNase H1, was purified from K562 erythroleukemia cells and its substrate specificity characterized in detail.[1] Surprisingly, in addition to cleaving RNA:DNA duplexes which adopt A-form geometry the enzyme was also found to cleave duplex substrates with a single RNA residue embedded in one of the strands. High field NMR studies showed that such molecules assume the standard B-form conformation with only localized pertubations of the structure at the site of the RNA residue. Cleavage occurs at the 5'-phosphate of the ribose residue at what is formally a DNA internucleotide linkage lacking an adjacent 2'-OH. The observation of cleavage on the 5'-side of the RNA residue suggested to us that in addition to removal of RNA primers used to initiate DNA synthesis that RNase H1 may play a role in excision repair of RNA reisudes which become misincorporated into DNA. We have subsequently demonstrated the existence of this repair system in HeLa cell extracts.

In order to take advantage of RNase H for antisense inhibition we developed chimeric oligonucleotides in which the 5' and 3' flanking sequences are modified to completely block exonucleolytic attack and to provide partial protection against endonucleases, with an internal sequence left unmodified to afford a substrate recognition site for RNase H. Such analogs in which the flanking sequences are phosphoramidates can be readily prepared using the H-phosphonate method. These derivatives are very active when microinjected into Xenopus embryos and are now an important tool for studies of early development in this system. The are also able to pass through cell membranes and are active when incubated with intact cells. Introduction of the phosphoramidate group results in a mixture of diasteriomers. Di-t-butyl siloxy analogs will be described in which the phosphoramidate group is replaced by an achiral substituent.

The recognition properties of human RNase H are distinctly different from those of the RNase H of retroviral reverse transcriptases. This result provides the basis for the design of modified oligonucleotides with anti-HIV properties that selectively inhibit the retroviral RNase H.

[1]Eder, P.S. and Walder, J.A. (1991) J. Biol. Chem. 266, 6472-6479.

Design, Modification, and Production of Nucleic Acids-Chemistry-II

S 024 SYNTHESIS AND BINDING PROPERTIES OF PYRIMIDINE OLIGODEOXYNUCLEOSIDE ANALOGS CONTAINING NEUTRAL PHOSPHODIESTER REPLACEMENTS , Robert J. Jones, Kuei-Ying Lin, John F. Milligan, Shalini Wadwini, and Mark D. Matteucci, Gilead Sciences, Inc., 346 Lakeside Drive, Foster City, CA 94404.

The replacement of the phosphodiester linkage with neutral, achiral, nuclease resistant entities is desirable for the development of oligodeoxynucleotide (ODN) analogs as therapeutic agents in either the antisense or antigene modes. Described herein is the use of the formacetal and 3'-thioformacetal connections as phosphodiester backbone analogs. Pyrimidine dimer blocks containing these moieties were synthesized and incorporated into ODNs in an alternating array with phosphodiester bonds, such that the ODNs had seven acetal and seven phosphodiester linkages. The binding properties of the resulting chimeric ODNs to single-stranded (ss) RNA and double-stranded (ds) DNA were then determined. ssRNA binding properties were determined by thermal denaturation (Tm) analysis, and the 3'-thioformacetal ODN:ssRNA duplex showed a 5.5°C enhancement in Tm relative to the control phosphodiester ODN. The triple helix formation properties of the 3'-thioformacetal and formacetal ODNs were determined by footprint and restriction enzyme inhibition assays. The 3'-thioformacetal ODN binds to dsDNA with an affinity slightly less than the control ODN. The high affinity and specificity of an ODN containing the 3'-thioformacetal for the ssRNA target and dsDNA target suggest that this linkage is a promising analog for both antisense and triple helix therapeutic applications. Additionally, further modifications will be discussed.

S 025 PSORALEN-DERIVATIZED ANTISENSE OLIGONUCLEOSIDE METHYLPHOSPHONATES, Paul S. Miller, Cynthia D. Cushman, Joanne M. Kean, Joel T. Levis and John J. Thaden, Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD.

Nuclease resistant oligodeoxyribonucleoside methylphosphonates conjugated with the photoreactive cross-linking group, 4'-N-(aminoethyl)aminomethyl-4,5',8-tri-methylpsoralen effectively cross-link in vitro to synthetic, single-stranded DNA and RNA target molecules and with target molecules that contain stem/loop secondary structures, when triggered by irradiation with long wavelength (365 nm) ultraviolet light. The extent of cross-linking depends on the temperature of the reaction, the type of target, DNA or RNA, and the structure of the target, random coil or hairpin. Psoralen-conjugated oligomers derivatized with the fluorescent dye, tetramethylrhodamine also cross-link efficiently. Psoralen-conjugated oligomers complementary to vesicular stomatitis virus (VSV) mRNA specifically cross-link to their complementary mRNA targets in vitro. Reverse transcriptase-catalyzed primer extension assays show that cross-linking occurs at the oligomer binding site on the mRNA. In the case of an oligomer complementary to nucleotides 387 to 402 of VSV N-protein mRNA, cross-linking decreases from 88% to 50% as the temperature of the cross-linking reaction is increased from 4°C to 37°C. The extent, but not the specificity, of cross-linking by 16-mers of different sequences varies, which may reflect differences in the structure of the oligomer binding sites. Psoralen-conjugated oligomers are completely stable in serum-containing medium and are taken up intact by mouse L-cells in culture. Examination of cell lysates by polyacrylamide gel electrophoresis shows that the single phosphodiester linkage at the 5'end of the oligomer is slowly hydrolyzed by a nuclease activity within the cells, resulting in the formation of a psoralen-conjugated mononucleotide, which is found almost exclusively in the cell culture medium. The stability of the 5'-phosphodiester linkage is considerably increased when the 5'-deoxynucleoside is replaced with a 2'-O-methylribonucleoside. Psoralen-conjugated oligomers show sequence-specific inhibitory effects in cells infected with VSV or HSV and in ras-transformed NIH 3T3 cells in culture. For example, an oligomer complementary to the splice junction of HSV immediate early mRNA 4&5 gave maximum inhibition of viral replication, 98%, when HSV-infected cells where irradiated 1-3 hrs postinfection. This same oligomer also showed inhibitory activity in mice infected with HSV. These results suggest that psoralen-conjugated oligonucleoside methylphosphonates will be useful antisense tools for studying gene expression in cells. In addition these oligomers provide a means to study the interaction of oligonucleoside methylphosphonates with complementary target molecules and can potentially be used to study the mechanism of action of antisense oligonucleoside methylphosphonates in cells in culture. (This research was supported by NCI, CA 42762, and by DOE, DE-FG02-88ER60636.)

S 026 STRUCTURE-FUNCTION RELATIONSHIPS OF OLIGONUCLEOTIDE DRUGS: PHARMACOLOGICAL AND PHARMACOKINETIC IMPACT, Christopher K. Mirabelli, Isis Pharmaceuticals, Carlsbad, California

Oligonucleotides represent a class of chemicals who's pharmacological potential has only recently been recognized. Receptors for these ligands include nucleic acids, proteins, lipids, and other biological macromolecules. Targeting oligonucleotides to work at the level of RNA and blockade of expression of specific proteins, requires that various pharmacologic and pharmacokinetic parameters of a compound be optimized. These parameters must be considered at the molecular, cellular, and whole body level. Naturally occurring phosphodiester oligonucleotides have limited utility due to their rapid degradation by nucleases in biological fluids and tissues. Therefore, chemical modifications must be introduced into the structure to stabilize the compounds. A broad array of modifications have been shown to provide stability against certain types of nuclease activities. These modifications include changes in the heterocycle, sugar, and backbone structure of oligonucleotides. The impact of these and other novel types of modifications on in vitro and in vivo pharmacodynamics and pharmacokinetics will be reviewed. These data suggest that optimization of the pharmacologic properties of an oligonucleotide drug for a specific target on a RNA requiresx consideration of a complex set of determinants and the combination of multiple types of chemical modifications within the oligonucleotide. Specific examples of oligonucleotides with intended use as antiviral, antiinflammatory and anticancer drugs will be used in the presentation.

S 027 PHYSICOCHEMICAL STUDIES IN DUPLEX AND TRIPLEX FORMATION INVOLVING OLIGONUCLEOTIDE ANALOGS (MATAGEN), Paul O.P. Ts'o, and Tina L. Trapane. Johns Hopkins University, School of Hygiene, Baltimore, MD 21205

In the design of oligonucleotide analogs for anticode properties, two major considerations are essential. 1) Oligomers which bypass or circumvent barrier and defense systems of living cells and tissues, such as the cellular membranes and nucleases. 2) Oligomers which have absolute sequence specificity coupled with high affinity for the cellular target sequence so that high potency and reduced side effects can be achieved. For the past 20 years, we have considered the nonionic backbone to be applicable toward achieving the above two purposes, and we have adapted the methylphosphonate backbone as a replacement for the natural phosphodiester backbone. The two requirements of single-base sequence specificity and high affinity are theoretically contradictory. The oligomer-nucleic acid interaction must have high on- and off-rates in order for the oligomer to search for its precise target sequence in living cells, thereby ensuring high specificity. As a first approximation, the on-rate is primarily dependent upon diffusion. Thus, the association constant or affinity can be increased by reducing the off-rate. Additional components such as positive charges or intercalators not related to sequence specificity may significantly reduce the off-rate. This increase in affinity is achieved at the sacrifice of sequence selectivity at the highest level. A way to have both high affinity and selectivity would be to reduce the off-rate after the perfectly matched duplex is formed between the oligomer and the target sequence. These considerations demand a two-step, "search and seal" approach. Previously, the Hopkins group has used photo-induced cross-linking of oligomers derivatized with psoralen as such an approach. This cross-linking takes place when the oligomer is perfectly matched with the target sequence, with the psoralen facing a pyrimidine. Currently, we are adopting a physico-chemical approach to this problem through triplex formation. First, a duplex is formed between the single-strand target and oligomer, and subsequently, a second oligomer is used to recognize the duplex, resulting in formation of a triplex with two oligomers and one single-strand target sequence. This scheme is facilitated by non-ionic oligomers which contain no charges, and, therefore, no charge repulsion for complex formation. In the process of duplex-triplex formation, off-rates of complexes may be reduced as a result of increased affinity constants for formation of the triplex. Specificity is not sacrificed as the second oligomer reads the duplex with a stringent base-pairing mechanism. In the past, triplex formation was achievable only with a homopurine or homopyrimidine target sequence. We have designed a series of analogs of C-nucleosides so that additional hydrogen bonding sites at the base moiety become available. Utilizing oligomers consisting of these C-nucleoside analogs we may be able to form triplexes with any sequence of single strand target nucleic acid. In addition, new electrophoresis methods are being developed which can determine the association constants for duplex and triplex formation between charged nucleic acid target strands and nonionic oligomer probes at isothermal conditions.

S 028 RECENT ADVANCES WITH PHOSPHOROTHIOATE OLIGONUCLEOTIDES, Gerald Zon, Lynx Therapeutics, Inc. 465 Lincoln Centre Drive, Foster City, CA 94404

Following the initial reports in 1984 of general methods for automated synthesis and purification of nuclease-resistant oligodeoxynucleotides with either single, alternating, end-capped or poly-phosphorothioate linkages, these procedures were soon extended, refined, and commercialized by various investigators to provide such compounds for antisense studies. Most of the work to date with antisense phosphorothioates has been carried out by using the original approach with fully-modified oligodeoxynucleotides, which have become the leading class of antisense agents based on more than forty independently reported examples of sequence-specific effects *in vitro* and, importantly, 4 cases of efficacy *in vivo*. Among the latter are Lynx's collaborative investigations of different mouse models of human leukemia. Gewirtz and coworkers injected SCID mice with K562 cells derived from a patient with chronic mylegenous leukemia (CML) and, after the onset of measurable disease, used an osmotic pump to deliver for 2 weeks 100 μg/day of a fully-phosphorothioated 24-mer antisense to mRNA corresponding to codons 2-9 of the protooncogene c-*myb*, which encodes a protein critical for hematopoietic cell proliferation. Calabretta and coworkers injected mice with Philadelphia-1-positive human leukemia cells expressing the *bcr-abl* gene as a fusion protein derived from a translocation of the second exon of c-*abl*, with the second exon of *bcr*. Treatment given after the onset of measurable disease utilized a fully-phosphorothioated 26-mer administered i.v. for 9 days. Biodistribution was studied by use of either ^{35}S-labeled oligomer or a ^{32}P-labeled probe. To enable further experimentation with these and other antisense phosphorothioate oligodeoxynucleotides in animal models for pharmakokinetics, biodistribution, and safety, extensive process development has been conducted on a 1-g scale of automated synthesis in concert with the development of new analytical methods to establish product identity and quality. In particular, a remarkably selective and highly accurate strong-anion exchange HPLC method is used to fractionate full-length products into populations that differ by having either 0, 1, 2, or etc. phosphodiester defects anywhere in the chain. Absolute base composition is measured by enzymatic digestion and then HPLC using an internal standard for highly accurate measurement of nucleoside components in mole-units. Scale-up of the synthesis technology to 10-20 g/run is proceeding well.

Cell Biology: Activity and Fate of Nucleic Acids

S 029 ANTISENSE STRATEGIES FOR GENE TRANSPLANTATION: COMBINED INHIBITION AND GENE REPLACEMENT, Jeffrey T. Holt, Cheryl Robinson-Benion, Yin-Xiong Li. Department of Cell Biology, Vanderbilt University, Nashville, TN 37232

Optimal gene replacement protocols would include both inhibition of the endogenous gene and overexpression of the preferred (or mutant) gene. We developed a novel antisense method to test whether antisense-resistant genes (designed by deletion of antisense RNA target sequences) can replace c-fos expression during serum-induced DNA synthesis. Immunoprecipitation studies demonstrated that inducible anti-fos RNA reduces endogenous c-fos expression by 90%, but does not affect the transfected antisense-resistant mutant c-fos genes. Nuclear labelling studies indicate that C-terminally truncated Fos mutants including FBR v-fos cannot rescue endogenous Fos, but that full-length and minimally truncated c-fos expression vectors could restore serum-induced DNA synthesis in fibroblasts expressing inducible anti-fos RNA.These differences between antisense induction and controls are not merely non-specific effects of steroid because "sense" controls (which do not produce antisense RNA) do not influence DNA synthesis as measured by nuclear labelling. Transcriptional studies of surrogate target gene expression in these clones expressing antisense-resistant mutants and inducible anti-fos RNA demonstrate that the endogenous c-fos protein contributes to AP-1 activity and normally suppresses regulated SRE (serum response element) activity. Overexpression of c-jun protein could not restore serum-induced DNA synthesis to fibroblasts which also expressed inducible anti-fos RNA despite similar trans-activation of an AP-1 target gene. This "gene transplant" method for inhibition of endogenous genes and replacement with preferred genes has implications for gene therapy of hereditary diseases and for the correction or "repair" of oncogene or tumor suppressor gene mutations in cancer.

S 030 CELLULAR UPTAKE AND BIOLOGIC EFFICACY OF PHOSPHODIESTER, PHOSPHOROTHIOATE, AND CHIMERIC OLIGODEOXY-NUCLEOTIDES. Arthur M. Krieg[1], Qiuyan Zhao[1], Sara Matson[1], Charles J. Herrara[2] and Eric Fisher[2]. [1]University of Iowa, Iowa City, IA 52242, [2]Amgen Boulder Inc., Boulder, CO 80301.

Unlike phosphodiester oligodeoxynucleotides (O-oligos), phosphorothioate (S-) oligos, methylphosphonate (MP-) oligos, and chimeric (S-O- and MP-O-) oligos are nuclease resistant. To investigate the effects of such modifications on cellular uptake, we have used flow cytometry with fluorescein (F)-conjugated oligos and primary (untransformed) mouse spleen cells. The lowest level of surface binding was seen with the MP-O-backbone. Increasing fluorescence was seen with O-, S-O-, and S-(F)-oligos. To determine whether these oligos bind to the same cell surface receptors, and to determine their relative affinities, spleen cells were preincubated with competitor unconjugated O-, S-O-, and S-oligos, before addition of F-oligos. Competitor S-oligo completely blocked cell surface binding of F-O-oligo, even at a relative ratio of 0.1, while competitor O-oligo gave no significant decrease in F-S-oligo binding, even at a relative ratio of 100. Competitor S-O-oligo gave intermediate competition. Thus, S-oligos have increased affinity for cell surface binding and bind to the same cell surface molecules as do O-oligos.

To test the effect of these modifications on oligonucleotide efficacy, we studied different oligos bearing the same antisense sequence against a putative immunosuppressive protein. In principal, antisense to an immunoinhibitory protein should release suppression, leading to lymphocyte activation. Indeed, spleen cells treated with 20 μM antisense O-oligos had a 2-3 fold increase in total RNA synthesis (used as a simple measure of cellular activation). The sequence-specificity of this effect was demonstrated by the ineffectiveness of control oligonucleotides, the inhibition by sense oligos, the lack of hybridization on Northern analysis to other RNAs, and the lack of effect on rat cells (which do not contain the target sequence). S-oligos with the same sequences gave a 10-20 fold increase in RNA synthesis, even at just 1μM. Chimeric oligonucleotides bearing different numbers of terminal phosphorothioate linkages were studied. Empirically, 2 phosphorothioate modifications at the 5' end and 4-5 modifications at the 3' end of the oligo confer optimal nuclease resistance. Oligos containing methylphosphonate substitutions at the same positions have equal nuclease resistance but their antisense efficacy is no greater than that of O-oligos. Dithioates have been the most potent oligos, but control dithioates have more nonspecific stimulatory effects than control with other backbones.

S 031 TRANSFORMATION-SPECIFIC TARGETS OF INTERVENTION IN THE V-SIS SIGNAL TRANSDUCTION PATHWAY

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Recent studies have shown that posttranslational modification of c-Jun by phosphorylation of two N-terminal sites adjacent to the "activation" domain (serines 63 and 73) is required for oncogenic potentiation by several oncogenes including Ha-ras and v-*src*[1,2]. We have now examined the role of phosphorylation of c-Jun in model cells conditionally transformed by v-*sis*. Induction of v-*sis* in these cells leads to rapid, complete and reversible transformation including tumorigenicity and metal-dependent growth in soft agar[3]. This is accompanied by extensive phosphorylation of c-Jun ("hyperphosphorylation"). Two dimensional phosphopeptide analysis confirms that phosphorylation is predominantly at serines 63 and 73. Transient transfection assays using an AP-1 reporter construct show that induction of v-*sis* is accompanied by an increase in AP-1 activity also similar to the transforming pathway observed for Ha-*ras* and v-*src*. As for Ha-*ras*, transfection of the model cells with transdominant negative mutation expression vectors for Ha-*ras* and c-*raf*-1 nearly completely inhibited growth as colonies in soft agar whereas inclusion of an activated *ras* expression vector caused a greatly increased frequency. These observations suggest that a signal transduction pathway of v-*sis* utilizing c-Raf-1 and Ha-Ras may stimulate a kinase activity which leads to increased phosphorylation of c-Jun at serines 63 and 73 and oncogenic potentiation. N-terminal phosphorylation of c-Jun is barley detectable in untransformed cells growing in serum. These observations suggest that c-Jun kinase activity may be transformation-specific and a target for transformation-specific intervention.

1. Smeal, T., Binetruy, B., Mercola, D., Birrer, M. *et al. Nature* 1991;354:494;
2. Smeal, T., Binetruy, B., Mercola, D. *et al. Molec. Cell. Biol.*, 1992;12:3507;
3. Mercola, D. *et al. Onocogene*, 1992;7:1793.

Special Technologies

S 032 NEW STRATEGIES FOR RIBOZYME THERAPIES AGAINST HUMAN RETROVIRUSES, John J. Rossi[1], Garry P. Larson[1], Edouard Bertrand[1], Cecile Carbonelle[1], Chen Zhou[2], and Donald Kohn.[2] [1]Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, CA. 91010, [2] Division of Research Immunology and Bone Marrow Transplantation, Childrens Hospital, USC School of Medicine, Los Angeles, CA.

Ribozymes are informational molecules which possess enzymatic function. The hammerhead ribozyme motif derived from the self-cleavage domain of plant viroids and virusoids is perhaps the simplest catalytic domain, and is readily adaptable for incorporation into antisense RNAs. We have been developing ribozymes for their potential use as anti-viral agents in the treatment of HIV-1 infection. Our goal is the use of ribozymes in a gene therapy setting. To this end, we have focused our research efforts on targeting strategies and retroviral delivery. One novel strategy capitalizes upon the unique affinity of HIV (and HTLV and SIV) reverse transcriptase for cellular $tRNA^{LYS3}$ as a first strand primer molecule. We have synthesized and carried out *in vitro* and cell culture studies with a chimeric $tRNA^{LYS3}$-ribozyme molecule. Binding of this molecule to HIV reverse transcriptase occurs with the same efficiency as the tRNA itself. When bound in a ternary complex with the primer binding site RNA of HIV, the ribozyme moiety is positioned to cleave at a site just upstream of the primer binding site. This molecule is a self-contained Pol III transcription unit and is transcribed in tissue culture cell lines. The anti-HIV activity of this construct is currently under evaluation, and results will be reported upon. In preparation for *in vivo* studies in an SIV-1 animal model, we are targeting ribozymes to the SIV-1 TAR signal. *In Vitro* binding studies demonstrate that a symmetric ribozyme, an asymmetric ribozyme and an antisense RNA all bind with the same affinity to this TAR sequence. The asymmetric ribozyme has an advantage in that the cleavage product is readily released from the hybrid, thus *In vivo* mapping of *tat* protein-TAR interactions is also in progress, and will allow the direct correlation between *in vitro* ribozyme binding and activity and *in vivo* target susceptibility. We are also testing more conventional ribozymes for their antiviral activity when expressed from a retroviral vector. These include ribozymes targeted to the 5'LTR leader region, the *gag* initiation codon, *tat* and an exon common to *tat* and *rev*. These ribozymes have been inserted into MoMuLV-based retroviral vectors to allow their transfer and expression in human lymphoid and hematopoietic cells. The vectors were packaged into amphotropic virions and used to transduce human CEM T-lymphocytes. Northern blot analyses and RNAse protection assays consistently demonstrated that the highest levels of ribozyme RNAs were produced by simple vectors in which the ribozymes are part of a single MoMuLV-directed transcript. CEM cells transfected by vectors expressing ribozymes against the *tat* and *rev* regions are resistant to HIV-1 infection in comparison to cells harboing control vectors. Higherlevels of ribozyme RNA correlate with greater resistance to HIV-1.

Biological Expression and Genetically Targeted Molecules I

S 100 ANTISENSE p53 OLIGODEOXYRIBONUCLEOTIDE FOR TREATMENT OF HUMAN MALIGNANCY, Eliel Bayever, and Kathleen Haines, Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE 68198

A number of published reports have shown the effects of phosphorothioate antisense oligonucleotides (asODN) directed to a variety of cancer-associated genes such as c-myc, c-myb, bcl-2, ras, bcr-abl, MDR1, c-jun, and cell growth-associated genes, such as cyclin, on cell-lines and primary cells. One of the cancer-associated genes, the phosphoprotein p53, appears to play a role in cellular differentiation, proliferation and apoptosis in normal cells. It has been associated with a number of malignancies, and in its mutated form, is the most commonly associated gene with human cancer. We have initiated a Phase I study of the systemic use of an asODN to p53 in relapsed and refractory acute myeloid leukemia. This was based on in vitro studies showing cytotoxicity of the p53 asODN to primary human acute myeloid leukemia blasts from peripheral blood, with no adverse effects on normal bone marrow or intestinal cells. Systemic administration of the asODN by intravenous infusion to non-human primates and rats was safe and non-toxic. Preliminary studies have indicated that this asODN may also be useful in a variety of other human malignancies. Studies on the pancreatic carcinoma cell-lines, ASPD-1, MIACaTa2, and HS766T, show suppression of cell growth. In addition, some primary solid tumors, including renal cell, ovarian, breast, and testicular carcinoma, and osteogenic sarcoma, have been exposed to the p53 asODN in vitro resulting in suppression of cell growth. We are continuing to evaluate the response of other tumors to the p53 asODN as a basis for further clinical trials for systemic therapy, and for bone marrow and peripheral blood purging in autologous hematopoietic transplantation.

S 101 TRANSGENIC MICE HARBORING INTERPHOTORECEPTOR RETINOID-BINDING PROTEIN (IRBP) ANTISENSE GENE CONSTRUCT EXHIBIT ABNORMAL RETINAL MORPHOLOGY. Howard Y. Chen[1], Harris Ripps[2], Myrna E. Trumbauer[1], Suraporn Matragoon[3], Gregory I. Liou[3]. 1. Merck Research Laboratories, P.O. Box 2000, Rahway, NJ. 07065. 2. Department of Ophthalmology, University of Illinois College of Medicine, Chicago, IL. 60612. 3. Department of Ophthalmology, Medical College of Georgia, Augusta, Georgia 03912

Interphotoreceptor retinoid binding-protein (IRBP) is synthesized by the photoreceptor cells of the vertebrate retina. Several functions have been suggested for this fatty acid-conjugated glycoprotein: transportation or stabilization of the retinoids in the visual process, a carrier for fatty acids, and a buffering protein to protect visual cells. Since IRBP expression was detected during photoreceptor cell differentiation in the early embryo development, it may also play a role in the differentiation of photoreceptor cells and in the development of the retina. To examine the biological functions of the IRBP gene, an antisense approach was used to reduce IRBP expression in the transgenic mouse. The antisense fusion gene was prepared by inserting a 644 bp fragment of mouse IRBP cDNA in the reverse orientation into the coding region of the CAT gene in a previously used human IRBP promoter-CAT fusion gene, which is expressed predominantly in the retina of transgenic mice at a time during photoreceptor differentiation. Among ten transgenic founders which had non-rd/rd genetic background, six showed greater than 50% photoreceptor loss at eight months of age. Four transgenic lines were established by outbreeding with Balb/C mice to dilute the rd mutation. At various ages, the retinal morphology of the transgenic mice was compared with non-transgenic controls. In two families, significant loss of photoreceptor nuclei was found. In another family, variable thickness of the outer nuclear layer and nuclear displacement were found. These changes may reflect the requirement of IRBP for the differentiation and organization of photoreceptor cells during development. Preliminary studies on antisense expression using RT-PCR method have shown transgene expression during embryonic development (E16 to E 19).

S 102 INHIBITION OF CLINICAL HIV-1 ISOLATES WITH A RETROVIRUS ENCODING AN HIV-REGULATED DIPHTHERIA TOXIN A (DTA) CHAIN GENE, Tyler J. Curiel*, Deborah Nicot*, Yang Wang+, Beatrice Hahn^, Sajal Ghosh^ and Gail Harrison+, University of Colorado Health Sciences Center, Divisions of *Infectious Disease and +Medical Oncology, Denver, CO, 80262 and ^Univ. of Alabama, Birmingham, AL.

We have previously demonstrated that H9 cells, ordinarily permissive for HIV infection, are rendered resistant to infection with HIV LAI strain when transduced with a retrovirus encoding either wild type (*wt*) or attenuated (*tox 176*) diphtheria toxin genes under control of HIV *tat* and *rev* (Harrison, et al., *Human Gene Therapy*, 1992;3:461). We now report that these same transduced H9 clones also protect against challenge with a variety of clinical HIV isolates expressing differing phenotypes and tissue tropisms, including A018a (18a), and the molecular clones YU-2 and SG3.1. Transduced H9 clones R9-D1 and R9-B4 (encoding *wt*), 3A5-A11 and 5B10-C8 (encoding *tox 176*) and control clone H9-X2 (encoding parental non-DTA) were infected by incubation with 500 to 5000 $TCID_{50}$ of LAI or 18a, or by electroporation with 2 μg of plasmid DNA corresponding to the molecular HIV clones. Supernatants were assayed for HIV p24 using a commercial kit (Coulter). R9-D1 inhibited LAI replication up to 31 d in culture (the last time point tested; p24 <1 pg/ml), compared to control H9X2 (p24 1425 pg/ml by d 11). Electroporated molecular HIV clones readily infected H9 and H9X2 (p 24 > 850 pg/ml by day 4), whereas infection was markedly inhibited in R9-D1 (p24 <1 pg/ml by d 18). All 4 transduced H9 clones were protected from 18a infection if virus was washed out after 2 h (p24 < 1 pg/ml on d 31), but were not if virus was not washed out (p24 = control by d 7). Work with other transduced H9 clones, and with other HIV isolates will also be presented.

S 103 REGULATION OF TUMOR NECROSIS FACTOR-α GENE EXPRESSION FOLLOWING TRANSFECTION: A GENETICALLY ENGINEERED MODEL FOR THE TREATMENT OF TOXIC SHOCK, Erickson K.L.[1] Kisich K.O.[1] Malone R.W.[2,4] Feldstein P.A.[3] and Powell J.S.[4] Departments of Cell Biology and Human Anatomy[1], Medical Pathology[2], Plant Pathology[3], and Medicine[4], University of California, Davis 95616.

Cytokine inflammatory mediators may play an important role in the pathogenesis of endotoxic shock. To develop specific therapeutic regimes in treatment, we have investigated the use of hammerhead ribozymes to inhibit tumor necrosis factor (TNF-α) expression in the induction of murine shock following endotoxin (lipopolysaccharide, LPS) challenge. Several TNF-α ribozymes varying in target site and recognition domain length were synthesized. Initial studies characterized the interaction of these ribozymes with cellular RNA and DNA *in vitro* to demonstrate the effects of hybridization domain length on specificity and catalytic turnover rate. We now report the successful transfection of these molecules into primary peritoneal macrophages both *in vitro* and *in vivo*. Linear ribozymes with 24 base target recognition domains directed against bases 266 or 453 of murine TNF-α mRNA, and ribozymes in autocircularizing RNA vectors having either 24 or 10 base target recognition domains directed against base 453 of murine TNF-α were prepared by *in vitro* transcription. Delivery vehicles of lipofectin and lipopolyamine containing cationic liposomes were formulated and tested for macrophage toxicity after transfection with and without added ribozyme RNA. Ribozyme or control RNA were complexed at a 1:10 mass ratio with the least toxic liposomes immediately prior to delivery. Complexes were then cultured with murine peritoneal macrophages *in vitro*, or delivered to peritoneal macrophages *in vivo* by a single intraperitoneal injection. Both groups of macrophages were stimulated *in vitro* with 1μg/ml LPS 4 hours post transfection. Macrophage TNF-α protein production was inhibited 60% after *in vitro* transfection or 85% after *in vivo* transfection of anti TNF-α ribozymes relative to cells transfected with control RNA; this effect appeared to correlate with the accumulation of TNF-α cleavage products. Thus, we have successfully transfected ribozyme/cationic lipid complexes both *in vitro* and *in vivo* which block the transcription and production of the inflammatory mediator TNF-α (Supported by CA 47050).

S 104 VITREAL INJECTION OF AN ANTISENSE OLIGONUCLEOTIDE FOR KINESIN INHIBITS RETINAL KINESIN SYNTHESIS AND RAPID ANTEROGRADE AXONAL TRANSPORT IN VIVO.
Richard E. Fine, Peter J. Morin, Kenneth S. Kosik* and Anil Amaratunga, Boston University Sch.of Med.Boston, MA 02118 and *Harvard Med. Sch., Boston, MA 02115.
Kinesin, a microtubule stimulated ATPase, was first isolated from squid axoplasm by virtue of its ability to move small vesicles along microtubules in a minus to plus direction at a speed approaching that of anterograde rapid axonal transport. Recently, it was demonstrated that an antisense oligonucleotide to 24 nucleotides including the start codon for the kinesin heavy chain can inhibit kinesin synthesis in cultured hippocampal neurons and concomitantly prevent two presynaptic membrane proteins from leaving the cell body (JCB 117:595,1992). We have injected a 24 residue phosphorothioate antisense oligonucleotide directed at the same sequence into the anaesthetized rabbit vitreous which directly overlies the retinal ganglion cells whose axons form the optic nerve, at a final concentration of approximately 50 uM. Kinesin synthesis is measured by the injection of ^{35}S-methionine cysteine into the vitreous 16 h later, followed after 3 additional hours by sacrifice and specific immunoprecipitation. The antisense nucleotide inhibits kinesin synthesis by 82±7% (n=4) while a sense oligonucleotide had no effect. The synthesis of other proteins was unaffected by kinesin antisense injection. The rapid axonal transport of the major membrane proteins into the optic nerve, as detected by radioautography after SDS-PAGE, was inhibited by 70±10% (n=4). These results provide direct evidence for the role of kinesin in anterograde rapid axonal transport in vivo and indicate the utility of antisense oligonucleotides to explore neuronal dynamics in a living animal. This work was supported by NIH grants R01EY08535-03 and R01AG05894-20.

S 105 **EXPRESSION OF THE LEISHMANIA GP46 PROTEIN IN RETROVIRAL VECTORS,** Suzanne Forry-Schaudies, Lori Clarke, Vina Patil, Michael Christy, Diana Moffat, Linda Weaver, Michael Kadan, Gerard McGarrity, Phillip Frost*, and Yawen Chiang. Genetic Therapy, Inc., Gaithersburg, MD 20878; and *Sandoz Research Institute, East Hanover, NJ 07936
The *Leishmania amazonensis* surface glycoprotein GP46 is highly antigenic and confers protective immunity from further parasite challenge in mice. We have made four retroviral constructs that include the coding region for GP46, and the neomycin resistance gene (Na). Two constructs contain the *Leishmania* antigen coding region and 1.3 kb 3'-untranslated region (La1); two constructs include only the coding region (La2). Each construct DNA was transfected into the ecotropic packaging cell line PE501 and PE501 supernatants were used to transinfect the amphotropic packaging cell line PA317. Individual producer cell clones were isolated from the G418-selected PA317 cell population. PE501 mixed, transfected cell populations and PA317 clones were tested by FACS for expression of the cell surface *Leishmania* antigen protein. PE501 mixed populations were 70-90% FACS-positive for all four constructs. However, PA317 clones were never FACS-positive, indicating that the *Leishmania* antigen is either not expressed, or is not located on the cell surface in PA317 cells. Southern and Northern analyses of the FACS-negative PA317 clones showed the presence of integrated retroviral DNA and abundant retroviral RNA. Analysis of retroviral RNA in PA317 producer cell supernatants indicated production of viral particles that contain both La and Na coding regions. The viral supernatant from clone PA317/G1La1SvNa.24 was used to transduce NIH 3T3 cells, a murine renal carcinoma cell line (RENCA), a poorly immunogenic murine colon tumor cell line (CT26), and a nonimmunogenic murine tumor cell line (CT26 clone 7). All of these transduced cell lines were FACS-positive, indicating that the PA317/G1La1SvNa.24 supernatant could successfully transduce and confer *Leishmania* antigen expression, although the PA317/G1La1SvNa.24 cells themselves are FACS-negative. We are currently testing whether the transduced tumor cell lines that express the *Leishmania* antigen will function as tumor vaccines in mice.

S 106 RETROVIRUS-MEDIATED TRANSFER OF WILD-TYPE *p53* GENE INHIBITS TGF-*α*-DEPENDENT GROWTH OF HUMAN LUNG CANCER SPHEROIDS. Fujiwara T, Grimm EA, Cai DW, Mukhopadhyay T, Roth JA. Dept of Thoracic Surgery, UT MDACC, Houston, TX 77030
Multicellular tumor spheroids (MTS) are a three-dimensional culture model in which the tissue approaches the degree of structural and functional differentiation of primary and *metastatic tumors*. We constructed LNSX retroviral vectors expressing wild-type *p53* (wt-*p53*) or mutant *p53* (mut *p53*) (codon 273) driven by a *β*-actin promoter. The effects of retrovirus-mediated transduction of wt-*p53* were studied on MTS of human non-small cell lung cancer cell line H322a, which has a homozygous mutated *p53* gene (codon 248). Transduction with wt-*p53* retroviral stocks was performed twice over 2 days in the presence of polybrene (10 M ug/ml). H322a spheroids grew exponentially in response to TGF-*α* (10 ng/ml). This growth was significantly inhibited by wt-*p53* transfection, whereas exposure to vector alone or the mut of the *p53* vector had no effect. Three cycles of serial transduction over 4 days by the wt-*p53* vector were more effective than a single transduction in suppressing spheroid growth. Histologic analysis showed dead cells with vacuole formation in an outer layer of spheroids 7 days after the infection began. These results suggest that wt-*p53* transduction induces not only growth arrest but also cellular death in TGF-*α*-stimulated tumor cells, and that MTS is a useful model for studying the effects of *p53* on three-dimensional tumor cell growth kinetics.

S 107 **TRANSCRIPTIONAL DEREGULATION OF *c-erbB-2* INVOLVES A NOVEL TRANSCRIPTION FACTOR, OB2-1: A NEW TARGET FOR TRANSCRIPTIONAL BLOCKADE.** **Donal P. Hollywood and Helen C. Hurst. Gene Transcripiton Laboratory, ICRF Molecular Oncology Group, Hammersmith Hospital, London, England.**
Overexpression of the *c-erbB-2* tyrosine kinase receptor is observed in 20-30% of human breast carcinoma, and has been correlated with both reduced patient survival and a lesser response to cytotoxic chemotherapy and endocrine therapies. Overexpression has been demonstrated in both the amplified and non-amplified *c-erbB-2* states, indicating that additional mechanisms beyond gene duplication must be involved in *c-erbB-2* mRNA accumulation.We have addressed this question by examining *c-erbB-2* expression levels, *c-erbB-2* mRNA stability, and the rate of *c-erbB-2* transcription in a panel of human breast cell lines. An increase in *c-erbB-2* transcripion in overexpressing cells has been confirmed. Further examination of the *c-erbB-2* promoter using promoter deletion mutants, DNaseI footprinting, and electromobility shift assays has identified a novel positive-acting transcription factor (OB2-1), that is involved in *c-erbB-2* upregulation. Interestingly, OB2-1 is more abundant in cell lines that overexpress from either an amplified or single copy *c-erbB-2* gene, suggesting that transcriptional deregulation of *c-erbB-2* is a frequent event in human breast carcinoma. Several mechanisms that might interrupt OB2-1 binding and permit the downregulation of *c-erbB-2* transcription are now being pursued, including the abrogation of OB2-1 binding by triple helix formation on the *c-erbB-2* promoter.

S 108 CELL TRANSFECTION WITH TGFβ TYPE II RECEPTOR (TβR-II) ALTERS THE PATTERN OF GROWTH AND TGFβ-INDUCIBLE GENE EXPRESSION, Mitsuhiro Inagaki, Brian I. Carr, Herbert Y. Lin, Aristidis Moustakas, and Harvey F. Lodish, Department of Surgery, University of Pittsburgh, PA 15213 and Whitehead Institute for Biological Research, Cambridge, MA 02142.

Hep 3B-TS human hepatoma cells express TβR-II and are growth inhibited by TGFβ protein. A clonal cell line that was adapted to growth in TGFβ, Hep 3B-TR, has lost the TβR-II and growth inhibition by TGFβ. After transfection of Hep 3B-TR with a vector containing TβR-II, two clones, 2 and 24 recovered expression of TβR-II protein and mRNA as well as growth inhibition by TGFβ1. Hep 3B-TS responded to TGFβ protein with an induction of TGFβ1, c-Jun and c-Fos mRNA and repression of c-myc mRNA. However, clones 2 and 24 had only minimal changes in the expression of these genes upon addition of TGFβ protein despite being growth-inhibited by it. These results show that growth inhibition by TGFβ can occur in the absence of changes in the expression of these genes.

S 109 CLONAL SUPPRESSION OF HUMAN LEUKEMIA CELL GROWTH BY ANTISENSE OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES Taira Maekawa, Akira Murakami, Shinya Kimura, Johji Inazawa, Kouichi Hirakawa, Tatsuo Abe and Keisuke Makino, Dept of Hygiene and Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602; Dept of Polymer Science and Engineering, Kyoto Institute of Technology, Kyoto 606, Japan

Patients with chronic myeloid leukemia (CML) have a specific chromosome abnormality, the Philadelphia chromsome generated by t(9;22)(q34; q11). The hibrid *bcr-abl* gene results in the formation of a p210 which contributes to the pathogenesis of CML. Decreased expression of c-*myc* has been demonstrated during the differentiation of HL60. Antisense and sense oligomers to the breakpoint region (b2a2 and b3a2 translocations) and to c-*myc* and c-*myb* were synthesized using phosphorothioate chemistry and purified by C18 polymer column. The former were capable of decreasing the growth of colony-forming cells in 5 of 7 cases in chronic phase and in 2 of 3 in myeloid blastic phase. The former more than 20μM in culture elicited a non-specific suppression on the growth of normal hemopoietic progenitors, whereas no suppression was observed in K562 cells even in the presence more than 40μM.

Addition of anti-c-*myc* and anti-c-*myb* oligomers resulted in the decrease of proliferation and colony forming capacity of HL60 cells in a dose-dependent manner. Clonal suppression of colony growth of antisense-treated HL60 cells in agar preceded the decrease of cell number in liquid culture. In HL60 cells treated with anti-c-*myc* oligomer, cells in G2/M phase were decreased and the fraction of S phase cells tended to have lower DNA values.

These results suggest that antisense oligodeoxynucleotides should provide powerful tools in the treatment of human leukemias.

S 110 p53 AS TRANSACTIVATOR IN HUMAN CYTOKERATIN 8 GENE EXPRESSION AND ITS EFFECT ON CELLULAR PHYSIOLOGY. Mukhopadhyay T, Roth JA, Dept Thoracic Surgery, UT MDACC, Houston, TX 77030

Role of p53 in the cellular physiology and malignant transformation is largely unknown. Antisense RNA approach is an intriguing way to evaluate the function of p53 and the genes whose expressions are controlled by p53. We introduced a recombinant plasmid DNA construct in human lung cancer cell lines with either wild-type or mutant p53 such that the β-actin promoter constitutively synthesized antisense p53 RNA. Northern and western blot analyses indicated endogenous p53 RNA and protein were expressed in significantly lower amounts in the transfectants than in the control cells. The transfectants showed rapid proliferation and grew large tumors in nu/nu mice, indicating that p53 acts as a tumor suppressor in these cell lines. Reduction of mutant p53 expression by the antisense construct also enhanced cell growth and tumorigenesis in nu/nu mice, suggesting that at least some mutations in the p53 gene retain the tumor-suppressor function. Antisense p53 reduced expression of other proteins. Purification and sequencing showed one of these was cytokeratin 8. We also found that p53 controls the expression of the cytokeratin 8 gene. Cytokeratin 8 is a marker protein specific for epithelial differentiation and an important component of the intermediate filament network. A 400-bp 5′ flanking promoter of the human cytokeratin 8 gene was isolated from a human non-small cell lung cancer cell line. Promoter activity was measured by CAT assay. Results showed that a reduced level of the p53 protein significantly inhibited expression of the cytokeratin 8 gene. A p53 DNA binding site has been identified close to the ATG start site. These results indicated that p53 transactivated the cytokeratin 8 gene in these cell lines and has a pleiotropic effect on cellular physiology.

S 111 MECHANISMS OF ANTISENSE MEDIATED MRNA STABILITY CONTROL. Wolfgang Nellen, Martin Hildebrandt*, Rebecca Oberhauser, and Beatrice Mahal
Max-Planck-Institut f. Biochemistry, D-8033 Martinsried, Germany.
*present address: Institut Pasteur, Paris, France.

Antisense transformation in plants and in *Dictyostelium* mostly results in destabilization and subsequent loss of the corresponding endogenous mRNA. Recent experiments suggest that this artificial system to control gene expression mimicks an endogenous regulatory system in *Dictyostelium*[1]. We have proposed a model to explain the mechanisms of antisense mediated mRNA stability control based on the regulation of antisense transcription and the involvement of three enzymatic activities in RNA-RNA interaction[2].

1. We have demonstrated differential expression of an endogenous antisense transcript in *Dictyostelium* which agrees with the differential destabilization of the corresponding mRNA. Both RNAs are transcribed frome the same locus.
2. We have characterized and partially purified a dsRNA degrading enzyme from *Dictyostelium* cytoplasm which is likely to digest sense - antisense hybrids in vivo. We also show that this activity can be modulated by phosphorylation and suggest the involvement of a recently proposed dsRNA-activated protein kinase[3].
3. RNA helicases may unwind sense - antisense hybrids and thus make them unavailable for degradation by dsRNase. Using PCR and antibodies directed against synthetic peptides, we have identified, partially cloned and purified four developmentally regulated nuclear proteins which are related to known or suggested RNA helicases. At least one of these proteins can unwind dsRNA in vitro and may reverse hybrid formation in vivo.
4. In vivo, RNA hybrid formation may be facilitated by hybrid-promoting proteins like the hnRNP protein A1. We are currently trying to identify such proteins in *Dictyostelium*.

1 M. Hildebrandt & W. Nellen, Cell, 69, 197-204, (1992).
2Nellen et al., Biochem. Soc. Transact., 20, 750-754 (1992)
3T. Winckler pers. communication

S 112 *IN VITRO* INVESTIGATION OF KERATINOCYTE-SPECIFIC FACTOR IX GENE EXPRESSION CONSTRUCTS AND THEIR POSSIBLE THERAPEUTIC VALUE.

Caspar J. M. Robinson, Rosemary J. Akhurst.
Duncan Guthrie Institute of Medical Genetics, University of Glasgow, Glasgow, G3 8SJ, UK.

Haemophilia B is an X-linked genetic defect leading to a Factor IX protein deficiency. This condition is a potential candidate for somatic cell 'gene therapy' and because the missing blood clotting factor is a serum protein, many diferent tissues could act as potential sources of the protein. We have set out to investigate a possible therapeutic approach for this disease using keratinocytes as a tissue for delivery.

Three Factor IX gene expression constructs have been generated which are driven by a keratin gene promoter (K5). Stably transfected murine keratinocyte cell lines have been created using these constructs to investigate the efficiency of keratinocytes to transcribe, translate, post-translationally modify and secrete the Factor IX protein.

High levels of Factor IX mRNA have been seen in clones transfected with each of the three constructs, but only low levels of Factor IX protein have been detected intracellularly and in the conditioned media. Further investigations are continuing to examine and explain this observation.

S 113 ANTISENSE OLIGONUCLEOTIDE TREATMENT OF PROSTATE CANCER, Marvin Rubenstein, Sergey Muchnik, George Dunea and Patrick Guinan, Chicago, IL, 60612.

Antisense oligonucleotides (oligos) are artificial DNA sequences complimentary to known genes. When hybridized to mRNA, they may regulate expression of growth factors and their receptors, such as transforming growth factor-alpha (TGF-a) and the receptor for epithelial growth factor (rEGF) respectively. The PC-3 tumor is a hormone insensitive, prostatic tumor derived from a bone metastasis. Although hormone insensitive it produces and responds to autocrine growth factors. PC-3 cells were grown both in vitro and in vivo, utilizing nude mice, and treated with antisense oligonucleotides (oligos) complimentary to mRNA for TGF-a, rEGF or their combination.

In vivo tumors were injected (intratumoral) on two consecutive days with the combination of both antisense oligos. Three days later the animals were exanguinated and the tumors removed for histology and enzymatic assay for prostate specific acid phosphatase. Analysis of the tumors showed hemorrhagic necrosis with infiltration of immune cells. A sectioned PC-3 tumor had obviously reduced tartrate inhibitable, prostate specific acid phosphatase. In addition the serum from an oligo treated mouse contained no measurable prostate specific acid phosphatase, whereas non-prostate specific acid phosphatase was not affected.

In vitro, PC-3 cells were inhibited in growth by either antisense oligo alone, or most completely by their combination, which also prevented rEGF expression when its cells were immunohistochemically stained utilizing a monoclonal anti-rEGF. Treatment with the single antisense oligo directed towards rEGF alone also resulted in diminished rEGF expression. Growth inhibition, in vitro, was also measured utilizing the hormone sensitive LNCaP prostate tumor, but this line was statistically less sensitive to this treatment, possibly due to its dependence on androgen stimulated growth, rather than interactions at the rEGF.

We conclude that antisense oligonucleotide treatment of tumors which are responsive to autocrine factors may provide a useful therapy for metastatic, and often recurrent, hormone insensitive tumors.

S 114 ANTISENSE OLIGONUCLEOTIDES STABILIZED BY POLYALKYLCYANOACRYLATE NANOPARTICLES SPECIFICALLY INHIBIT ACTIVATED Ha-ras MEDIATED PROLIFERATION AND TUMORIGENICITY.

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We have shown before that the expression of mutated Ha-ras can be suppressed by an modified antisense nonamer targeted to a region of Ha-ras mRNA including the point mutation G → T at the 12th codon. In the present study we used three different 12 base sequences : an antisense oligonucleotide directed against and centered at the point mutation in codon 12 of the Ha-Ras mRNA, an antisense oligonucleotide targeted to the equivalent sequence of the normal Ha-Ras mRNA, and the 5'/3' inverted sequence of AS-Val. We tested the effect of these oligonucleotides on the proliferation of T24 cells that express only point mutated Ha-ras and of two clones of the human mammary cell line that differ only with respect to the expression of point mutated Ha-ras. The AS-Val oligonucleotide exhibited a cytostatic effect on cells that express point mutated Ha-ras (but not on the parent cell line), whereas As-Gly, directed against the same region in the mRNA of the protooncogene, did not exhibit any cytostatic effect. We have recently demonstrated that polyalkylcyanoacrylate (PIHCA) nanoparticules (NP) can function as carriers for antisense oligonucleotides. We made use of this approach adsorbing AS-Val and all control oligonucleotides to PIHCA NP in the presence of hydrophobic cations (CTAB). When cells that express point mutated Ha-ras were treated with these complexed oligonucleotides inhibition of proliferation occurred at 100-fold lower concentrations than with free oligonucleotide. Local subcutaneous injection of AS-Val adsorbed to NP in the site where transformed cells had been inoculated resulted in a considerable inhibition of tumor development.

S 115 **USE OF CHIMERIC REPORTER CONSTRUCTS TO TEST THE EFFICIENCY OF ANTISENSE INHIBITION OF CK-B.**

Erik Sistermans, Wilma Peters, Margo van Reen and Bé Wieringa; Department of Cell Biology and Histology, University of Nijmegen, PO Box 9101, 6500 HB, The Netherlands.

Creatine Kinase isoenzymes (CKs) catalyze the transfer of energetic phosphate between PCr and ATP and play an important role in the cellular energy metabolism of higher eukaryotes. In mammals four types of CK subunits, CK-B (Brain) and CK-M (Muscle) in cytosol, Ub-CKmit and Sc-CKmit in mitochondria, are found. These subunits are encoded by four separate genes whose expression is cell type-specific and developmentally controlled.

We have sought to use antisense RNA techniques in transgenic mice to gain more insight in the *in vivo* role of the most abundantly expressed CK-member, the cytosolic CK-B subunit. To set up the system and test the efficacy of our antisense vectors a rapid and generally applicable screening method in cell culture was developed. CK-B expression was uncoupled from its normal regulation and influences of cell type, cellular growth and differentiation. To this end, a 4 kb CK-B fragment containing the entire coding region and 750 bp 3' flanking sequences, but lacking the promotor sequences, was placed behind the PGK (Phosphoglycerate Kinase) promotor. The bacterial β-Gal reporter gene lacking its translational start codon was inserted in frame just before the CK-B stop codon, leaving the greater part of the mRNA structure intact. After transfection to a mouse N2A neuroblastoma cell line one blue colony expressing CK-B/β-Gal fusion protein (N2A-CKBlue) was selected as a substrate for all further assays. The CK-B/β-Gal fusion protein in these cells is deposited in the rough endoplasmatic reticulum (RER), a cellular location that does not show any overlap with the sites at which CK-B normally is found.

From computer analysis we predict that the CK-B mRNA is highly folded. In order to determine the region of the CK-B mRNA that was most accessible for antisense RNA binding vectors expressing different regions of the gene as antisense RNA were developed. Constructs were made with the constitutive SV40 early promoter, the inducible hs70 promoter, or based on tRNA and U1-snRNP RNA genes expressing antisense RNA predominantly in cytoplasm or nucleus respectively. Upon stable transfection of N2A-CKBlue cells either individual colonies or pooled clones were lysed and the efficiency of the antisense RNA inhibition determined by measuring the β-Gal activity using a simple spectophotometric assay.

S 116 DEVELOPMENT OF A "MODEL" ANTISENSE CONSTRUCT FOR DOWN REGULATION OF GENE EXPRESSION. Sokol, Deborah L., Murray, James D., Mackinley, Tony G., and Passey, Robert L, Department of Animal Science, University of CA, Davis, CA 95616 and School of Biochemistry, University of New South Wales, Kensington, Australia

Transgenic mice have been produced to compare the efficiency of antisense/catalytic RNA to antisense only for the down regulation of target gene expression in vivo. A model gene, targeted for mammary gland specific expression, composed of the bovine alpha-s1 casein promoter fused to chloramphenicol acetyl transferase (CAT) has been stably integrated into the genome of C57Bl/6 X CBA mice. CAT protein levels have been quantitated at 6, 12, and 18 days; protein expression is highest at 12 days with an average of 95.8 pg CAT activity/ul. Therefore, CAT expression levels at 12 days will act as the reporter for down regulated protein expression from antisense message.

One full length antisense CAT and one full length antisense/catalytic CAT construct have been stably integrated into the genome of mice. The full length antisense construct including four catalytic cleavage sites (Chopcat) has been shown to express mRNA in 3/4 founder lines. The lines containing the full length antisense construct are currently being analyzed for expression of mRNA. Mice expressing message from one of the antisense constructs are mated to mice containing the CAT construct to produce female progeny that are double hemizygous for determination of the levels of regulated CAT protein in mammary gland tissue. Currently, eight double hemizygote pups have been born containing the CAT and the antisense/catalytic constructs.

S 117 ANTISENSE OLIGODEOXYNUCLEOTIDES TO cAMP DEPENDENT PROTEIN KINASE (PKA) CATALYTIC SUBUNIT GENE ENHANCE EFFECTOR FUNCTIONS OF CYTOTOXIC T-LYMPHOCYTES (CTL). Hirotaka Sugiyama[1,2], Michail V. Sitkovsky[1], Satoshi Kimura[2] and Kaoru Shimada[2], [1]LI, NIAID, NIH, Bethesda, MD 20892 and [2]Dept. Applied Immunol. and Infect Dis, Institute of Med. Sci., Univ. of Tokyo, Tokyo 108 Japan.

Antisense oligodeoxynucleotides (oligomers) complementary to mRNA of catalytic (Cα) subunit of PKA were designed to inhibit the expression of Cα-subunit gene in mouse CTL, since Northern blotting with cDNA probes specific to Cα- and Cβ-subunit mRNAs showed predominant expression of Cα-subunit mRNA in the CTL. Synthesized oligomers were radiolabeled and their fate after incubation with the CTL was monitored to establish conditions for the most efficient protocol of CTL treatment. The effect of the anti-Cα antisense oligomers on the Cα gene expression in the CTL is demonstrated by using sensitive and specific substrate peptide phosphorylation assay in the presence and absence of specific peptide inhibitor of PKA's catalytic subunits. The pretreatment of the CTL with the Cα antisense oligomers, but not with sense nor nonsense oligomers, results in the inhibition of PKA activity of the CTL and in the enhancement of TCR-triggered exocytosis of granules and of Ag-specific cytotoxicity. In comparison with O-oligomers, S-oligomers revealed more pronounced and long lasting enhancing effects on the CTL's effector functions. The conclusion is reached that the antisense oligomers to PKA's Cα-subunit gene are useful tools for the enhancement of TCR-triggered effector functions in CTL.

S 118 EVIDENCE THAT THE RETINOBLASTOMA GENE PRODUCT IS A BIFUNCTIONAL REGULATOR OF CELL CYCLE PROGRESSION, Jane B. Trepel[1], Mahn Joon Ha[1], Seong Jin Kim[2], Charles E. Myers[1], Leonard M. Neckers[1], and Won Ki Kang[1], [1] Clinical Pharmacology Branch, Clinical Oncology Program, and [2] Laboratory of Chemoprevention, National Cancer Institute, NIH, Bethesda, MD 20892

The retinoblastoma protein (Rb) is generally considered the prototypical tumor suppressor gene product. The function of this class of proteins is presumed to be restricted to negative growth regulation. It is apparently paradoxical that the level of Rb protein increases approximately 8-fold when peripheral blood lymphocytes (PBL) transit from G_0 to S phase following mitogen activation (Furukawa *et al.* PNAS 87, 2770, 1990). The studies reported here examine the regulation of Rb as a function of cell cycle progression and use antisense technology to address the potential role of Rb as a positive growth regulator. Two systems have been utilized for these studies, PBL and hormone-refractory prostate cancer, to compare Rb regulation in normal cells and in a common adult carcinoma. We found that resting PBL, a G_0 synchronized population, had a very low level of Rb protein, that this protein was almost entirely underphosphorylated, and almost exclusively nonnuclear. Following mitogen activation total Rb levels increased dramatically, most of the Rb was in the phosphorylated form and nuclear. The increase in Rb protein occured prior to entry into S phase. In the human prostate carcinoma cell line PC-3, cells in logarithmic growth had high levels of Rb protein, and that protein was almost exclusively nuclear and in the phosphorylated form. PC-3 cells synchronized in G_1 by treatment with lovastatin showed a large decrease in Rb protein, with an especially prominant loss of the phosphorylated form. Addition of mevalonic acid restored the Rb content to pretreatment levels and the cells entered S phase. Thus the same pattern of Rb regulation is seen in tumor cells and in normal PBL, and that pattern is most consistent with a role for the Rb protein in entry into the cell cycle. To test this hypothesis directly we treated PBL and PC-3 cells with phosphorothioate-modified anti-Rb oligonucleotide antisense directed against the first 15 bases from the translation start site. In both systems there was a concentration-dependent inhibition of [^{3}H]-thymidine incorporation from 3 μM to 70 μM oligo, without loss of cell viability. A scrambled oligonucleotide control had little effect on thymidine incorporation. Comparable effects were seen in MTT assays of cell proliferation. Western blot analysis of PBL showed that the inhibition of thymidine incorporation in antisense-treated cells was paralleled by a loss of Rb protein. These data suggest a dual negative and positive growth regulatory role for the Rb protein.

S 119 USE OF EPISOMAL VECTORS FOR ANTISENSE RNA-MEDIATED INHIBITION OF ICAM-1 IN HUMAN BONE MARROW STROMAL CELLS, Matthew C. Weber and Mark L. Tykocinski, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

Bone marrow stromal cells regulate hematopoietic stem cell and leukemic cell proliferation and differentiation through a complex network of cytokines and cell surface molecular interactions. We employed stable gene tranfection to determine the role of the receptor:counter-receptor pair ICAM-1:LFA-1, in mediating stromal cell:hematopoietic cell intercellular adhesion. The KM-102 human bone marrow stromal cell line was found to express high levels of ICAM-1 on its surface, as determined by immunofluorescence staining, and this stromal line bound a variety of LFA-1-expressing human leukemic cell lines. Stable transfection of the KM-102 line with Epstein-Barr virus-based episomal vectors expressing either full-length sense or antisense ICAM-1 mRNA effectively enhanced or eliminated ICAM-1 surface expression, respectively. Leukemic cell binding to these transfectants was not significantly altered as compared to control transfectants. However, phorbol ester stimulation of the highest LFA-1 expressing line, JY, resulted in a 2.5-fold increase in binding to the sense KM-102 transfectant, but not to the ICAM-1-negative antisense KM-102 transfectant. This increase in binding for the sense ICAM-1 transfectant could be blocked by anti-ICAM-1 antibodies. These findings suggest that ICAM-1 binding to a phorbol ester-dependent conformational epitope on LFA-1 may contribute to stromal cell:hematopoietic/leukemic cell adhesion. Moreover, our data further confirm the utility of episomal vectors that we have previously developed for effective antisense RNA-mediated gene inhibition.

S 120 EFFECTS OF ANTISENSE p120 RNA on Hep G2 CELLS. Zhang W-W, Valdez B, Henning D, Wu Jim, Busch R, Busch H, Dept of Pharmacology, Baylor College of Medicine, Dept of Thoracic Surgery, UT MDACC, Houston, TX 77030

Human proliferation-associated nucleolar antigen p120 is expressed at high levels in Hep G2 cells. To assess the effect of antisense p120 RNA on tumor cell proliferation, the p120 cDNA in both orientations was introduced into Hep G2 cells by liposome-mediated DNA transfection and retroviral infection. The relative levels of the exogenous p120 RNA versus the endogenous p120 mRNA were quantitated by RNase protection assays. In the Hep G2 cells transfected with the EBV-based vectors using Rous sarcoma virus (RSV) promoter, which expressed low levels of the antisense RNA (3 times lower than the control), reductions of the endogenous p120 mRNA and p120 protein were not detectable. The cells infected with the retroviral vectors using cytomegalovirus (CMV) promoter expressed the antisense RNA at a higher level (8-10 times higher than the control), which resulted in 45% p120 mRNA reduction, 25% p120 protein decrease, and 35% cell growth inhibition. Secondary infection of the retroviral infected clones with higher multiplicity further increased the antisense RNA level 1.5 fold, which resulted in 62% p120 mRNA reduction, 45% p120 protein decrease, and 50% cell growth inhibition. CAT assays showed that the p120 transcription by the clones with high level expression of the antisense p120 RNA was decreased to 56%. The overexpression of the p120 antisense RNA caused morphology changes. The cells became round and were loosely attached. The initial dividing time was delayed for 3 days in the double infected antisense clones. These data indicate that inhibition of p120 gene expression by antisense RNA affected the Hep G2 cell proliferation.

Biological Expression and Genetically Targeted Molecules II

S 200 ADENOVIRUS VA_1 RNA AS A POTENTIAL SHUTTLE MOLECULE TO VEHICLE ANTISENSE RNA AGAINST HUMAN IMMUNODEFICIENCY VIRUS, Doglio A., Cagnon L. and Lefebvre J.C.,.Laboratoire de Virologie, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 2, France.

Antisense RNA may provide the basis for antiviral therapies of high selectivity. The adenovirus type 2 VA $gene_1$ is transcribed by RNA Polymerase III and this feature makes it an attractive vehicle for engineered antisense RNA against pathogenic virus like the Human Immunodeficiency Virus (HIV). The VA_1 gene was modified by the insertion of short DNA fragments designed to be complementary to the HIV tat or rev mRNA and cloned inside a plasmid harboring the antibiotic geneticin resistance gene. After transfection, geneticin resistant T-lymphoid cells were selected for constitutive intracellular expression of VA_1-antisense RNA. In these cell lines, HIV replication was strongly inhibited as measured by percentage of HIV-1 infected cells or viral P24 core-protein detectable in culture supernatant. Due to original features (easy genetic handling, transcriptional efficiency and remarkable RNA secondary structure) the VA gene 1 makes very compact shutttle molecules ideal for the expression of antisense sequences. We argue that if this model gives good results in our cell system it would be possible to extrapolate and to propose the use of some modified virus vectors, as retrovirus or adenovirus, for applications in the field of HIV therapy.

S 201 **EVALUATION OF PROMOTER STRENGTH IN RETROVIRAL VECTORS,** David L. Ennist, Michael J. Newell, Hitoshi Kotani, Perry B. Newton III, Gerard J. McGarrity, W. French Anderson*, Yawen L. Chiang, Department of Immunology, Genetic Therapy, Inc., 19 Firstfield Rd., Gaithersburg, MD 20878 and *Department of Biochemistry, University of Southern California, Los Angeles, CA, 90033

Adenosine deaminase (ADA) activity in ADA deficient human T and B cell lines was evaluated following electroporation and transduction with various retroviral constructs. Transient expression following electroporation of GM2471 B lymphoblastoid cells was used as a quick screen to evaluate a number of promoters, including the LTR, IL2, IL2 receptor, Mx, human and chicken β-actin and SV40 promoters. Of these, the most active was the SV40 promoter, followed by the β-actin promoters and the Mx and LTR promoters. The IL2 and IL2 receptor promoters were essentially background. Inducibility of the Mx promoter by type I interferons was further examined. The SV40 driven construct was used to optimize transduction protocols for these cells. By quantitative PCR and protein expression, this improved transduction procedure results in three to five fold greater transduction efficiencies than mere incubation of target cells with viral supernatants. The relative strengths of the various promoters were then evaluated using the improved transduction procedure.

S 202 SEARCHING FOR GENES WITH A RECOMBINATION BASED ASSAY (RBA). A.J. Hanzlik, M.M. Osemlak-Hanzlik, M.A. Hauser, M. Van Keuren, G.-H. Xiao and D.M. Kurnit. Depts. of Pediatrics and Human Genetics and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109.

We developed a novel and efficient RBA for the isolation of genes. To demonstrate that the RBA works, we inserted the $(CGG)_{10}AGG(CGG)_9AGG(CGG)_9$ sequence from a fragile X gene into our specialized plasmid pMAD3. Clones were retrieved from multiple cDNA libraries, including from an 11 week human fetus and placenta, from a variety of 20 week human fetal tissues, including brain, spinal cord, eye, liver, kidney and skeletal muscle and from human adult jejunum. Recombination and counterselection retrieved the correct sequences as confirmed in each case by hybridization. This demonstrates that the fragile X sequence is transcribed in each of these tissues. Sequences in addition to the fragile X carrying this trinucleotide repeat are also transcribed as shown by this technology.

To extend this technology to phasmids, we utilize the strain, DM21 (pBR322), which prevents replication from the ColE1 origin in phasmids via incompatibility. We can then counterselect against *supF* using this strain, resulting in the elimination of the R6K plasmid and its insert and yielding the desired ColE1 plasmid and its cDNA insert. This technique includes advantages of ease of performance and the isolation of the desired counterselected cDNA sequence as molecules cloned in the high copy-number pBluescript plasmid.

This procedure can be performed in a stand alone fashion or in tandem with other methodologies (such as hybrid selection, exon trapping, computer analyses, zoo blotting and expression in somatic cell hybrids) to identify sequences that are transcribed in different tissues. In addition to this critical time and tissue profile, the technique also yields large cDNAs starting from a small (>25 bp) probe, as would be generated from the above methodologies. Rapid DNA sequencing then enables one to determine if the gene belongs to a previously described family. Thus, the recombination-based assay will enable us to append a "genic" initiative onto the larger "genomic" initiative.

S 203 **GENE THERAPY FOR AIDS: RETROVIRAL VECTORS ENCODING ANTISENSE RNA, RIBOZYMES AND TRANS-DOMINANT INHIBITORY MUTANT HIV-1 GENES.** Donald B. Kohn, Ingrid Bahner, Chen Zhou, Qian-Lin Hao, Gary Larson, and John Rossi. Childrens Hospital Los Angeles and City of Hope Medical Center.

We have examined a variety of gene therapy approaches targeted specifically against the critical regulatory genes of HIV-1, *tat* and *rev*. We have made retroviral vectors to encode antisense RNA, hammerhead ribozymes, RRE decoy RNA and *trans*-dominant inhibitory mutant (TDIM) *tat* and *rev* genes. The vectors were packaged as amphotropic virion and used to stably transduce CEM human T lymphocytes. The transduced CEM cells were challenged with HIV-1 and the effects on viral replication were determined by measuring the levels of HIV-1 *gag* p24 produced. Antisense RNA against the highly structured RNA motifs in either the TAR or RRE elements produces minimal inhibition of HIV-1 replication. Hammerhead ribozymes were designed which cleave sequences within the *tat* and *rev* coding sequences. Northern blot analyses and nuclease protection assays of T cells with numerous different vector constructs consistently demonstrate that the highest levels of ribozyme RNA are produced by simple vectors, in which the ribozymes are part of a single MoMuLV-directed transcript, without an internal promoter. Moderate inhibition of HIV-1 replication is seen in CEM cells expressing the anti-*tat* or anti-*rev* ribozymes to high levels. A vector encoding a TDIM *tat* gene with a premature stop codon at amino acid 54 possesses moderate inhibitory activity in transient transfection assays, but fails to significantly inhibit HIV-1 replication. The M10 *rev* mutant, with a two amino acid substitution, consistently shows strong *trans*-dominant inhibitory activity of HIV-1 replication. The highest inhibition is seen when M10 is expressed under the transcriptional control of a human CMV promoter; slightly less inhibition occurs when expression of M10 is controlled by the MoMuLV LTR. Minimal inhibition of HIV-1 replication is seen when the HIV-1 LTR controls the M10 gene. We are currently analyzing the effects of these inhibitory genes in human hematopoietic cells in long-term bone marrow culture, using clinical HIV-1 isolates. These studies may detect detrimental effects of these genes on normal hematopoiesis and may further demonstrate the potential utility of these vectors for gene therapy of AIDS.

S 204 IMPROVED METHODS OF RETROVIRAL VECTOR TRANSDUCTION AND PRODUCTION FOR GENE THERAPY, Hitoshi Kotani, Perry B. Newton III, Yawen L. Chiang, Edward Otto, Amy Roop, R. Michael Blaese, W. French Anderson and Gerard J. McGarrity. Genetic Therapy, Inc., Gaithersburg, Maryland 20878; National Institutes of Health, Bethesda, Maryland 20892; and University of Southern California, Los Angeles, California 90033.

There is a continuing need to improve and optimize vector transduction and production procedures to maximize clinical application of gene transfer technologies. Storage and shipment requirements are also essential concerns for large scale procedures and products. Significant titer enhancement of retroviral vectors from producer cells was demonstrated by employing roller bottle techniques and other cultivation procedures. These methods increased viral titers by 10 to 100 times (10^5 CFU/ml → 10^7 CFU/ml) in different producer cell lines. Viral titers were further enhanced 10 to 20 times (10^8 CFU/ml) using a refined concentration technique. A developed freeze-drying technique of viral vectors has demonstrated >60% preservationof viral infectivity. This technique has made practical an efficient and economical procedure for storage and shipment of retroviral vectors. A unique transduction methodology has achieved a transduction efficiency significantly greater than by conventional techniques. All tested target cells, including anchorage dependent (3T3 and tumor cells), hematopoietic and leukemia cells, demonstrated an increased transduction efficiency from 10 to 30 times.

S 205 CARTRIDGE METHOD FOR THE PURIFICATION OF OLIGONUCLEOSIDE METHYLPHOSPHONATES(ONMP) [1]Shwu-Bin Lin*, [1]Kuo-I Lin, [2]Gin-Wen Chang and [2]Lo-Chun Au [1]Graduate Institute of Medical Technology, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC. [2]Department of Medical Research, Veterans General Hospital-Taipei, Taiwan, ROC.

ONMPs as a class of oligodeoxyribonucleotide analog have nonionic methylphosphonate backbone, are resistant toward nuclease hydrolysis and have been shown to be an effective antisense agent to supress gene expression in cell culture. Although systhesis of ONMPs by automatic synthesizer is possible, purification of ONMPs is tedious and limits large scale production of the oligomer. ONMPs and amino-derivatized ONMPs with a 5′ phosphodiester linkage were synthesized in a dimethoxytrityl (DMT)-on mode and purified by reverse phase cartridges, OPC™, OPEC™, Poly-Pak™ and SEP-PAK®, using protocols modified from the procedure for the purification of oligodeoxynucleotides. Using a OPEC™ or a Poly-Pak™ cartridge, a total O.D. of 26-50 A_{260} (The yield is varied with the sequence) could be obtained in a 1 umol scale synthesis. The purity of the oligomer was analyzed by UV-spectroscopy, DEAE-cellulose chromatography, reverse phase HPLC, capillary electrophoresis and polyacrylamide gel electrophoresis of ^{32}P end-labelled oligomers. Product containing 90% of full length oligomer was usually obtained.

We have developed easy procedures for the purification of amino-modified and unmodified ONMPs. The procedure could be useful for the production of the oligomer in large quantity for *in vivo* experiments.

S 206 DESIGN OF QUASI-RANDOM RIBOZYME EXPRESSION VECTORS, Dennis G. Macejak and Kenneth Draper, Cell Biology, Ribozyme Pharmaceuticals, Inc., Boulder, CO 80301

Ribozyme expression vectors offer a unique approach to ablative gene therapy. In principle, ribozyme expression vector design is relatively more rigorous than the design of an mRNA expression vector. For example, the ribozyme must be present in the same cellular compartment as the target RNA in question. In addition, the ribozyme must be able to hybridize to the appropriate site on the target RNA in vivo. This site may be masked by RNA secondary structure in the target molecule itself or by RNA binding proteins within the cell. We have designed a strategy for the generation of a randomized library of ribozymes against a given target RNA molecule. The subsequent use of biological selection and amplification of the desired clones will generate ribozyme constructs which have potentially advantageous genetic or therapeutic activities. Moreover, use of this method allows one to clone target-specific ribozymes from any DNA fragment, even when the primary sequence or direction and dimensions of transcription units are uncertain. The DNA encoding the target in question is cleaved randomly into 20-40 nucleotide fragments and these fragments are then inserted in both orientations into a cloning vector. The basic requirement of the cloning vector in this scheme is that the restriction endonuclease site located immediately 5' of the cloned target DNA sequences be recognized by a restriction enzyme which cleaves the DNA at a distance 3' of the recognition sequence (and thus within the target DNA sequence). DNA encoding ribozyme core sequences are then inserted within the target sequence. The distance between the recognition and cleavage sites (along with the size of DNA fragments selected for cloning) will dictate the length of the ribozyme binding arms in the quasi-random constructs. Cloning sites were selected so that the ribozyme expression cassettes could be excised from the original plasmid vectors and inserted into eucaryotic expression vectors. The use of this approach for the development of quasi-random ribozymes against herpes simplex virus type 1 thymidine kinase will be presented.

S 207 DEVELOPMENT OF HERPES SIMPLEX VECTORS FOR VIRUS-MEDIATED GENE SUPPRESSION IN THE CNS, Christopher A. Meaney and Joseph C. Glorioso, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

Herpes simplex virus (HSV) derived vectors provide a method for the expression of manipulated genetic material within post-mitotic neuronal cells and may prove useful both in treatment of CNS disorders and for basic studies of nervous system function. Because of the additional need to eliminate function of mutant proteins in some genetic therapies we have begun to examine HSV based expression of genes transcribed by RNA polymerase III which are expressed at the high levels necessary for antisense RNAs and ribozymes to be effective (Jennings and Molloy 1987, Cotten and Birnstiel 1989). The VA RNAs of adenovirus are transcribed by RNA polymerase III and prevent shutoff of host protein synthesis by interferon induced dsRNA activated inhibitor kinase (DAI). The VA_I gene was recombined into the U_S3 locus of an attenuated virus and into the tk locus of a replication-defective backbone. Northern analysis of RNAs collected during the major stages of lytic gene expression from helper cells infected with the defective recombinant, $ICP4^-,TK^-$:: VA_I, revealed VA_I transcripts at all stages with particularly high levels seen after viral replication. Expression of VA_I was seen despite blockade of viral immediate-early protein production either by pre-treatment of cells with protein synthesis inhibitors or by non-productive infection of Vero cells. The level of VA_I transcript was dependent on the multiplicity of viral infection. We have also constructed an attenuated vector, U_S3^-::VAFox, in which the first exon and intron of the mouse *c-fos* gene are expressed by VA_I in antisense orientation. Because the cellular immediate early gene *fos* may participate in many neural processes, including HSV latency, we are interested in examining the effect of suppressing *fos* function in cell culture and in animals. Thus far *fos* specific transcripts have been detected only during late times of infection in culture. Experiments are now underway to examine the effects of VA_I expression on viral and cell protein synthesis, the expression of VA_I *in vivo* during viral latency, and the effects of antisense *fos* on cellular *fos* expression.

S 208 **PROGRESS TOWARD GENE THERAPY FOR AIDS: CHOICES FOR CLINICAL APPLICATIONS.** Richard A. Morgan*, Peter Bressler#, Jack Ragheb*, Richard Schneiderman&, Markus Dettenhofer@, Richard Markham@ and David Schwartz@ *Molecular Hematology Branch, National Heart, Lung, and Blood Institute; and #Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Bethesda, MD 20892, &Genetic Therapy Inc., Gaithersburg MD 20878, @Department of Immunology and Infectious Diseases, The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205.

Several approaches that use retroviral vectors to genetically modify lymphocytes such that they manifest anti-HIV activity were developed. Cells were modified such that they either directly possess anti-viral activities, or that they indirectly stimulate anti-viral activity. Retroviral vectors were constructed that express one or more of the following gene products: sCD4 (or a sCD4 derivative), transdominant HIV rev mutants, a tat and rev regulated human α2-interferon, and two HIV inducible suicide genes (diphtheria toxin and cytosine deaminase). Analysis of these AIDS gene therapy systems has demonstrated protection from HIV-1 infection in primary culture human lymphocytes from normal volunteers. Given these results, it is within reason to consider that the appropriate genetic engineering of lymphocytes could be of therapeutic benefit to individuals infected with HIV-1. In pre-clinical studies, we have engineered the lmphocytes from HIV-1 infected individuals using conditions that limit the spread of endogenous HIV-1 in culture. Further in vivo testing of engineered lymphocytes is in progress using the Hu-PBL-SCID mouse system. In addition to the lymphocyte as a cell target for anti-HIV gene engineering, we have begun to introduce genes into stem-cell-enriched (CD34+) human bone marrow and peripheral blood cells. The successful engineering of the hematopoietic stem cell with anti-HIV genes has both practical and scientific advantages to the engineering of lymphocytes alone and may lead to a more permanent and effective treatment for AIDS. The successful inhibition of HIV (in vitro) by several different strategies necessitates that the effectiveness and safety of the various approaches be further evaluated before clinical trials can be initiated.

S 209 PHENOTYPIC COMPLEMENTATION OF A FANCONI ANEMIA (GROUP A) FIBROBLAST CELL LINE AND RECOVERY OF THE cDNA BY PCR. Robb E. Moses, Petra M. Jakobs, Adam T. Hammond, Tina L. Neeley, Markus Grompe and Hiroshi Saito. Oregon Health Sciences University, Department of Molecular and Medical Genetics, Portland, OR. 97201

Fanconi anemia (FA) is an autosomal recessive disorder exhibiting aplastic anemia and multiple developmental anomalies. Cells from FA patients in many cases show defective DNA repair ability including cellular hypersensitivity to DNA crosslinking agents such as mitomycin C (MMC) and diepoxybutane (DEB). There have been at least four genetic complementation groups assigned by cell fusion experiments, and the cDNA for one of the FA groups was cloned recently by cDNA library complementation (Strathdee, et. al. Nature *356*:763 [1992]).

We have been attempting to isolate the cDNA for FA (group A) by complementation with cDNA. A cDNA library was constructed from DNA repair-proficient fibroblasts (GM639) after incubation with MMC. The cDNA was unidirectionally inserted into the expression vector pCMV4. The library covered 8×10^5 independent clones, with an average insert size of 1.5kb. The vector pCMV4 was generated from plasmid pRC/CMV (Invitrogen) by inserting a 12bp ClaI linker into the SfiI site within the SV40 replicative origin, thus inactivating the SV40 origin. The vector has a cytomegalovirus promoter for cDNA expression. It also contains the neomycin resistance gene to allow a positive selection by G418. A FA (group A) fibroblast cell line (GM6914), immortalized by SV40 is extremely sensitive to MMC and DEB, and was the recipient cell line for the cDNA library transfection. The cells were transfected, selected by G418 for one week and then exposed to MMC and DEB. Among 1.6×10^6 G418 resistant colonies, five were isolated as clones resistant to MMC(67nM) and DEB(1μM). Candidate genes for the FA group A were recovered from the three clones by PCR with primers flanking the insert. Restriction analysis showed that two of the three genes are identical.

This work was supported by Medical Research Foundation of Oregon and Fanconi Anemia Research Fund.

S 210 THE CD24 CELL SURFACE ANTIGEN AS A DOMINANT SELECTABLE MARKER IN RETROVIRAL MEDIATED GENE TRANSFER, Robert Pawliuk, Robert Kay, Peter Lansdorp, Margaret Hough and Keith Humphries, Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 1L3

As an alternative to drug resistance based selection systems (ie. G418) we have been investigating the use of cell surface antigens as selectable markers to be used in combination with fluorescence activated cell sorting (FACS). The use of such markers would enable the quantitative measure of expression and simultaneous phenotypic analysis and selection of retrovirally infected target cells. To explore this possibility we have employed the human cell surface antigen CD24. A JZen based retroviral vector harboring the extremely small coding region of the CD24 cDNA (approx. 240bp) under the regulatory control of the MPSV long terminal repeat enhancer and promoter elements has been constructed and used to infect NIH-3T3 fibroblast and BAF-3 pre-B cell lines. FACS analysis of infected cells showed that high levels of the CD24 antigen are expressed within 24 hours following the infection procedure. Further, FACS has been utilized to demonstrate that significant levels of CD24 are expressed on primary 5-fluorouracil pre-treated murine bone marrow cells within 48 hours following infection. Irradiated recipient mice reconstituted with bone marrow infected with the CD24 retrovirus show no major abnormalities in hemopoiesis and the CD24 antigen can be detected on the surface of cells of spleen and bone marrow for a minimum of 45 days post-transplantation. The use of the CD24 cell surface antigen as a dominant marker in retroviral vectors in combination with FACS should facilitate the rapid, efficient, and non-toxic selection of retrovirally infected cells in vitro.

S 211 SPECIFIC MODIFICATION OF MAMMALIAN GENES AND CHROMOSOMES IN SOMATIC CELLS VIA TARGETED AND SPONTANEOUS MUTAGENESIS, Andrew C.G. Porter, Philip A.C. Bates and Jane E. Itzhaki, Biochemistry Department, Oxford University, Oxford OX1 3QU, UK.

Genes artificially introduced at random sites in mammalian chromosomes are often poorly or inappropriately expressed and may interfere with the expression of genes at neighbouring sites. Such position effects could compromise many gene therapy strategies but could be avoided if the gene of interest is introduced either by homologous recombination with its endogenous counterpart or as part of an autonomous mini-chromosome. We have therefore been interested in the use of homologous recombination in somatic cells for both the targeted modification of specific genes and the construction of truncated chromosomes. We have developed two different enrichment procedures for the isolation of human fibrosarcoma (HT1080) cells in which both alleles of the interferon-inducible 6-16 gene of chromosome 1p35 have been modified. One of these procedures was adapted, by inclusion of human telomeric DNA into the targeting construct, so as to break chromosome 1 at the 6-16 gene locus. In separate experiments we transfected a somatic cell hybrid (853) carrying the human Y chromosome with plasmids designed for homologous integration at the Y190 or alphoid loci close to the Y centromere. Transfectants were isolated on the basis of alterations to the structure of the target locus. These were not the alterations predicted for homologous integration: most involved an apparently spontaneous deletion of some or all of the Y chromosome telomeric to the target locus. Thus, with the appropriate screening or selection procedure, either targeted or spontaneous mutations can be used to generate a defined chromosomal truncation.

S 212 **RETROVIRAL MEDIATED GENE TRANSFER AS A POLYPHARMACEUTICAL ANTI-HIV AGENT,** Jack A. Ragheb, Richard A. Morgan, Peter Bressler*, and W. French Anderson. Molecular Hematology Branch, National Heart, Lung, and Blood Institute; and *Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Bethesda, MD 20892

Using retroviral mediated gene transfer, we have shown that several anti-HIV agents can diminish the spread of HIV in a genetically engineered population of peripheral blood lymphocytes (PBL) from normal donors. Among the agents we have tested are: soluble CD4, human alph interferon, HIV Rev transdominants, and diphtheria toxin.

Given the rapidity with which HIV evolves, it is likely that any therapeutic approach that relies on a single anti-viral agent will eventually be unsuccessful. In order to reduce the likelihood that HIV will circumvent any of our singular gene therapy agents, they have been packaged together into triple gene retroviral vectors. This has been accomplished by incorporating the internal ribosome entry sites (IRES) found in picornoviruses into our murine based retroviral constructs. Placement of these IRES sequences 5′ to the initiation codon of a downstream gene in the vector results in the creation of a functional polycistronic mRNA by allowing internal translational initiation of the downstream gene. By conferring upon a single engineered cell multiple independent mechanisms of defense against the virus, these polycistronic vectors reduce the chance of escape by HIV.

In order to increase the functional capacity of our retroviral vectors and broaden our gene therapy armamentarium against HIV, we have begun to constuct polygene vectors that encode anti-HIV pol III transcipts in a double copy configuration, in addition to a polycistronic mRNA.

S 213 Retroviral vectors for secretable TNF and hypersecretable TNF: Construction, characterization of expression in TIL and tumor cells, and large scale GMP production of clinical lots. R. Ralston, M. Kriegler[1], C. Perez[1], K. DeFay, D. Lowe and B. Maiorella. Chiron Corporation, Emeryville, CA 94608.

Intralesional administration of tumor necrosis factor (TNF) has been shown to promote tumor regression in human subjects (Bartsch et al., 1988; Kahn, et al., 1989). Expression of TNF by transduced tumor infiltrating lymphocytes (TIL) could mediate or evoke a significant anti-tumor response. Retroviral vectors for expression of secretable TNF were constructed in the LXSN backbone (Miller & Rosman, 1989), using cDNA clones for either full length TNF or a hypersecretable 17 kD form containing the γIFN signal sequence (Perez *et al.*, 1990). (Master cell banks were prepared from single cell clones of PA317 cells (Miller & Buttimore, 1986) containing a single provirus (LTNFSN or LTγsigSN). Production cells consistently generated titers of $>10^6$ cfu/ml. Clinical lots of 9 L each were prepared under GMP, including release testing for absence of adventitious viruses (assays for MLV, BVDV, PPV, LCMV, HIV-1, HTLV-1, MAP and CPE), adherence to post-thaw specifications for recombinant virus titer ($>10^6$), and for TNF expression on NIH 3T3 cells. Clinical lots of TNF retrovirus were provided to NCI for transduction of human TIL and tumor cells from study subjects (S.A. Rosenberg, M.D., principal investigator). Transduction efficiency in TIL ranged from 4-16% without selection and up to 86% after 5 days of selection with G418 ([2]P. Hwu, M.D., personal communication). Expression of TNF by LTγsigSN-transduced TILs was approximately 5-fold higher than for LTNFSN-transduced TILs. Establishment of transduced carcinoma cells required extended culturing (>60 d) but the resulting lines expressed TNF very efficiently. Clinical evaluation of the transduced TIL and tumor cells is in progress.

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S 214 VECTORS AND PROMOTERS FOR EXPRESSION OF ANTISENSE RNA IN CARCINOMA CELLS, Edward J. Shillitoe, J-Numa Lapeyre, Frank Marini and Cherrilee Steele, University of Texas Health Science Center and M.D. Anderson Cancer Center, Houston TX 77225

The transformed phenotype of squamous carcinoma cells that contain DNA of human papilloma-viruses (HPV) can be modified by antisense RNA. We have begun to screen virus vectors and promoters to find the best system for delivery of antisense molecules to this cell type.

Carcinoma cells that express genes of HPV-18 have been transfected with shuttle vector plasmids that express antisense RNA under control of the MMTV promoter. Eight cell lines have been derived that show various levels of inhibition when expression is induced. Dot blot and northern blot hybridizations will now be used to determine the copy number and level of expression that is necessary for inhibition. For selection of viral vectors we are comparing derivatives of the Moloney murine leukemia virus, adeno-associated virus and herpes simplex virus type-1. Up to 40% of cells could be infected with a retrovirus. However more efficient infectivity was found with herpes simplex, which infected 79% of cells as shown by staining with antibody to ICP-4. For accurate comparisons of promoters it will be necessary to express the same gene product from each. A FLAG peptide-encoding sequence was cloned into the shuttle vector and the peptide was readily detected in transfected cells by western blots. The coding sequences will now be cloned into each viral vector. These experiments will allow selection of the optimal promoter and viral vector for gene transfer to carcinoma cells.

S 215 DEVELOPMENT OF RECOMBINANT ADENOVIRUS VECTORS FOR CLINICAL USE IN GENE THERAPY FOR GENETIC AND ACQUIRED DISORDERS, Bruce C. Trapnell, Nanette Mittereder, Soonpin Yei and Yung-Nien Chang, and Paul Tolstoshev, Genetic Therapy, Inc., Gaithersburg, Maryland, 20878.

Development of gene therapy approaches for genetic and acquired disorders in which tissues to be genetically reprogrammed cannot be removed, corrected *in vitro* and re-implanted will require *in vivo* delivery of the therapeutic genetic information. This presents special problems related to the efficacy and safety of the delivery vehicle. First, such a vector designed for *in vivo* delivery must be able to transfer genes to quiescent cells as somatic cells in many tissues proliferate only slowly or not at all. Second, the vector must genetically modify enough cells within the target tissue to achieve a therapeutic benefit. Third, the overall treatment must be safe enough to achieve an acceptable therapeutic index. Recombinant retroviral vectors currently employed in *ex vivo* gene therapy strategies require division of the target cell for successful gene transfer and expression and are difficult to produce in the high concentrations likely to be necessary for efficient *in vivo* gene transfer. Adenovirus-based vectors represent a powerful alternative as an *in vivo* delivery vehicle in that they readily infect non-dividing as well as dividing cells, can be produced in very high titers and can be rendered replication deficient. We have developed vectors derived from human serotype 5 adenovirus, a linear, 36 kb, double-stranded DNA virus naturally tropic for the human respiratory epithelium, but which posseses a broad host cell range. The general structure of these first generation adenoviral (Avl) vectors consists of deletions within early regions 1 (ΔE1) and 3 (ΔE3) of the viral genome. The ΔE1 deletion renders the recombinant vector replication deficient and ΔE3 creates space for insertion of an expression cassette containing a therapeutic minigene. Avl vectors have been developed for treatment of a variety of disorders which include the fatal pulmonary component of cystic fibrosis, and other pulmonary diseases, cancer, and cardiovascular applications. We are currently evaluating the molecular biology and pharmacokinetics of these vectors, both *in vitro* and *in vivo*, as well as issues regarding the efficacy and safety of their use in humans.

S 216 **ADENOVIRUS VECTORS FOR GENE THERAPY OF CANCER, Ken N. Wills, Patricia Menzel, CANJI Inc., 3030 Science Park Rd., San Diego, CA 92121**

The Retinoblastoma (Rb) and p53 genes are classical examples of the "tumor suppressor" class of genes, whose normal function appears to be involved in the control of cellular proliferation. It has been demonstrated that replacement of a normal Rb or p53 gene into several different tumorigenic cell lines lacking these functional gene(s) has resulted in significant suppression of their tumorigenicity. While the majority of these studies have used retroviral vectors to deliver the normal genes, an adenoviral-based delivery system has some distinct advantages when considering gene therapy for cancer. Results comparing adenoviral versus retroviral delivery of tumor suppressor genes and their effects will be discussed.

S 217 TEST FOR THE TUMORIGENICITY OF RETROVIRAL VECTORS IN MICE. EFFECTS OF ANIMAL AGE AND PRIOR CHEMICAL CARCINOGEN TREATMENT. Wen K. Yang, Tzu-Hao Wang, Den-Mei Yang, Donald C. Henley and Lan-Yang Ch'ang, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831 and Clinical Oncology Research Laboratory, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, ROC.

Possibility of insertion mutagenesis by retroviral vectors used in gene therapy has been of concern particularly for tumorigenic effects. We have constructed a *neo*-carrying retroviral vector of endogenous ecotropic virus origin and produced high-titer amphotropic virion preparations in packaging cells. S^+L^- assay showed less than 0.1% of replication-competent virus contaminating the vector preparations. Single or multiple intraperitoneal injections of 10^4 to 10^7 neo-resistant units of these preparations were given to groups of young adult FVB/N, C3H, C57BL/6 and B6C3F1 strain mice; all showed no increase in tumor incidence over the control up to 2 years of observation. However, if the same vector preparations were injected from 1 to 5 days of age, thymic lymphoma was induced in 30-50% of the animal within 6 months. In mice injected with 5-azacytidine at 3, 5 and 7 days of age, 70-80% developed thymic lymphomas in 4 to 7 months; if the 5-azacytidine treatment was followed by the vector injections at 9, 11 and 13 days of age (by itself not tumorigenic), the thymoma incidence was raised to 100% with an accelerated rate. In mice injected with diethylstilbestrol daily from 1 to 5 days of age and with retroviral vector at 7, 9 and 11 days of age, T lymphomas were induced in 4 to 7 months; no such lymphoma was obtained in control mice receiving the same diethylstilbestrol or the same viral vector treatment alone. DNA gel blot analysis revealed that 1/5 of lymphomas induced in FVB/N mice by combined azacytidine/viral vector treatment contained single *neo*-carrying integrated proviruses while the remaining showed the ecotropic type LTR sequences that are normally absent in the FVB/N mouse genome, implicating *in vivo* proliferation and proviral integration of the contaminating replication-competent virus during leukemogenesis. [Research supported by US-DOE/OHER under contract DE-AC05-840R21400 with Martin Marietta Energy Systems,Inc. and US-NIEHS contract YO1-ES-40118 and also by DOH of ROC].

Delivery and Fate of Genetically Targeted Molecules

S 300 EVALUATION OF BIODEGRADABLE POLYMERS FOR THE SUSTAINED DELIVERY OF ANTISENSE OLIGODEOXYNUCLEOTIDES, S. Akhtar and K.J. Lewis, Drug Development and Research Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK.

Recent studies suggest that the in vivo efficacy of antisense oligonucleotides, especially for target proteins that have a slow turnover, is likely to require repeated parenteral administration of nucleic acids. This is partly due to their poor biological stability and partly because of their rapid clearance from the body. Thus, sustained-release devices which provide a "reservoir" of oligonucleotide that can be delivered in a controlled manner to the target tissue over a period of days whilst protecting it from degradation will be useful from a therapeutic viewpoint.

Although osmotic or infusion pumps can be used to provide long-term treatment of antisense oligonucleotides in animals, these devices suffer the disadvantage that they have to be removed from the body once release has been completed. An alternative approach is the use of biodegradable and biocompatible polymer matrix devices which slowly degrade to non-toxic metabolites within the body. In such a system the oligonucleotide is entrapped within the polymer and is released slowly with reproducible kinetics. The rate of release may be controlled by several factors including the rate of degradation of the polymer device.

In this study we have monitored the in vitro release profiles of c-myc and HIV-tat antisense oligonucleotides from a series of biodegradable polyesters of both natural and synthetic origin as matrices for the sustained delivery of antisense agents. Depending on the nature of the polymer and/or the fabrication technique used, sustained delivery of oligonucleotides could be obtained for a period several weeks. Unmodified DNA and phosphorothioate oligonucleotides entrapped within the polymer matrix (films or microspheres) were resistant to degradation by serum nucleases. In vitro release profiles suggested that entrapped oligonucleotide was released biphasically from the polymer. Release was more rapid from microspheres than from thin films. Both types of devices may have uses in the controlled delivery of antisense oligonucleotides particularly to targets with a slow turnover.

S 301 **A NEW METHOD FOR LIPOSOME-MEDIATED GENE TRANSFER,** Alain R. Thierry, Mira Jung, and Anatoly Dritschilo, Department of Radiation Medicine, Lombardi Cancer Research Center, Georgetown University, Washington, DC 20007

We report a new preparation for liposomally encapsulated DNA for use in gene transfer. The preparation is efficient and results in a non-toxic delivery system, potentially suitable for clinical applications. Plasmid DNA between 5 to 18 kb in size can be efficiently encapsulated. Using ^{32}P-labelled plasmid DNA prepared by nick translation, entrapment rate was found to be 70-90% of the initial input dose. Cellular uptake of liposomal DNA varies with the cell line used and may reach concentrations up to 1pg/cell. Autoradiography using ^{35}S-labelled plasmid DNA shows the high cellular internalization of liposomal DNA. Liposomes of various phospholipid compositions have been tested and have shown different abilities to accumulate in cells. *In vitro* stable transfec-tion efficiency was determined in squamous carcinoma (SCC) 35 cells transfected with pSV40 *neo* plasmid. Transfection efficiency using this liposomal DNA preparation was 7,4, and 3 times greater than DEAE-dextran, calcium phosphate, and lipofectin reagent (Gibco, BRL) methods, respectively. Transient transfection efficiency was ascertained following transfection of SCC 35 cells with the pRSV *cat* plasmid and identification by immuno-fluorescence of cells producing CAT protein. Our preliminary results show a 14 and a 2-fold increase of transfection efficiency using liposome-encapsulated DNA compared to DEAE-dextran and calcium phosphate methods, respectively. High transfection rates using this new liposomal method have been shown in various cell lines such as NIH/3T3, cos-7 and lymphoma MOLT-3 cells. Additional studies show successful delivery and expression of marker genes such as β-gal and luciferase genes, in peripheral blood lymphocytes and hepatocytes. Liposomally encapsulated DNA prepared by this method may be promising for use in gene therapy.

S 302 ENHANCED BIOLOGICAL ACTIVITY OF ANTISENSE OLIGONUCLEOTIDES WITH NOVEL CATIONIC LIPOSOME FORMULATIONS, C. Frank Bennett, Jiin Felgner, C. N. Sridhar, Carl J. Wheeler, and Philip L. Felgner, ISIS Pharmaceuticals 2280 Faraday Ave., Carlsbad, CA 92008, and VICAL Inc., 9373 Towne Center Drive, San Diego, CA 92121

Cationic liposome formulations such as DOTMA:DOPE (50:50) increased the biological potency of ICAM-1 phosphorothioate antisense oligonucleotides by greater than 1000-fold in cell culture. Cationic liposomes enhanced cellular association of ^{35}S-labelled phosphorothioate oligonucleotides by 10 to 20-fold and changed the subcellular distribution of the fluorescein labelled antisense oligonucleotides from vesicular cytoplasmic compartments in the absence of liposomes to localization in the nucleus in the presence of cationic liposomes. A novel series of dialkyloxypropyl quaternary ammonium compounds, containing a hydroxyalkyl moiety on the quaternary amine were synthesized and tested for enhanced delivery of ICAM-1 antisense oligonucleotides. The rank order potency for substituents on the quaternary amine were hydroxypropyl > hydroxylethyl = methyl > hydroxybutyl > hydroxypentyl. Different length saturated and unsaturated alkyl groups were examined for their effects on the activity of cationic liposomes. Oleic acid ($C_{18:1}$) and myristic acid ($C_{14:0}$) containing cationic lipids were significantly more active than palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) containing cationic lipids. The effects of different co-lipids on the biological activity of cationic lipids were also examined. With the exception of dimyristyl containing cationic lipids, phosphatidylethanolamine was required to support activity of the lipids. Neither phosphatidylcholine nor cholesterol would substitute for phosphatidylethanolamine. These studies suggest optimal activity was dependent on both a cationic group which interact with the oligonucleotide and a fusogenic lipid which aids in the delivery of the oligonucleotide to the cytoplasm of the cell.

S 303 **TARGETING OF ANTISENSE OLIGODEOXYNUCLEOTIDES SPECIFIC FOR HIV TO KUPFFER CELLS BY LACTOSYLATED LOW DENSITY LIPOPROTEIN**

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The therapeutic application of antisense oligodeoxynucleotides (ODN) is hampered by their poor capacity to reach in vivo the target cells in sufficient quantities. This problem may possibly be overcome by association of ODN to carriers that are specifically internalized by the target cells. We showed earlier that ODN derivatized with cholesterol (cholODN) associate readily with plasma lipoproteins. In this study, cholesterol was coupled to the 3' end of a ^{32}P-labeled 19-mer ODN specific for human immunodeficiency virus (HIV). The cholODN was incorporated into lactosylated low density lipoprotein (LacLDL), a modified lipoprotein that is rapidly and specifically internalized by galactose-specific receptors on Kupffer cells (liver macrophages). The [^{32}P]cholODN-LacLDL complexes were injected into rats (0.20 pmol cholODN/g body wt). The complexes were rapidly cleared from the circulation: only 19.0 ± 1.4 % of the dose was left in plasma at 10 min after injection. The tissue distribution of cholODN was studied at 10 min after injection, and it was found that the association to the liver (0.80 ± 0.03 pmol cholODN/g fresh wt.) was at least 2-fold higher than that of any other tissue. Liver cells were isolated at 10 min after injection. Kupffer cells showed by far the highest uptake: 9.23 pmol cholODN/g fresh wt. Parenchymal and endothelial cells contained only 0.08 and 1.22 pmol cholODN/g fresh wt, respectively. Preinjection of rats with N-acetylgalactosamine reduced uptake by Kupffer cells by > 70%, which indicates that cholODN-LacLDL is taken up by galactose-specific receptors. Furthermore, cholODN incorporated in unmodified LDL, a carrier that is not rapidly taken up, showed low uptake by Kupffer cells (0.95 pmol cholODN/g fresh wt at 10 min after injection).

We conclude that by derivatization with cholesterol, and subsequent incorporation into LacLDL, ODN can be specifically targeted to Kupffer cells. The ODN become highly concentrated in this cell type, which may be therapeutically beneficial as macrophages have been reported to play a role in infection with HIV.

S 304 OLIGONUCLEOTIDE HYBRID FORMATION AND DISRUPTION IN SOLUTION AND LIVING CELLS IS REVEALED BY FLUORESCENCE ENERGY TRANSFER, Daniel J. Chin[1], Sophie Sixou[2], Francis Szoka, Jr[2]. [1]Agouron Institute, La Jolla, CA 92037 and [2]School of Pharmacy, UCSF, San Francisco, CA 94143

Antisense oligonucleotides (oligos) have been the focus of great interest but their ability to form hybrids within cells is still unclear. Herein, we employ fluorescence resonance energy transfer to study oligo hybrid formation and disruption *in vitro* by spectrofluorimetry and *in situ* within living cells by microinjection and confocal scanning laser microscopy. The translation initiation region of the human immunodeficiency virus *rev* region was synthesized as an unlabeled 28-mer phosphodiester or labeled with a 3' rhodamine moiety (+PD-R). The complementary 5'-fluorescein labeled phosphorothioate (-PR-F) oligo was specifically quenched by (+PD-R) under various conditions; including physiological salts at 37° C ($T_{1/2}$ ~ 3 min). The stability of the hybrid complex was characterized by nuclease digestion, competititon by unlabeled oligo or nonspecific DNA and thermal denaturation using fluorescence energy transfer. Injection of pre-formed hybrids or serial injections revealed that hybrids within living cells were transient ($T_{1/2}$ ~ 20 min). *In vitro*, the extent of fluorescein quenching (74% at a 1:1 molar ratio of +PD-R:-PT-F) and acceptor transfer (76% increase in rhodamine emission at a 1:1 ratio) were more efficient than the energy transfer resulting from serial injections in living cells (~50% fluorescein quenching and ~25% increase in rhodamine emission at a 2:1 +PD-R:-PT-F ratio). This may result from the non-uniform, speckled distribution of oligo complexes in the nucleus and self-quenching. The potential for sequence-specific nuclear distribution patterns is being investigated.

S 305 GENE TRANSFER INTO SKELETAL MUSCLE *IN VIVO* OPTIMISATION AND EXPERIMENTAL USES.

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Physiotherapy Program, University of Ottawa, Canada. * Dept. de Biologie Moléculaire, Institut Pasteur, Paris, France.

Somatic gene transfer into skeletal muscle has therapeutic and experimental potentials. However, a drawback to both applications at present is high variability of expression. We have approached the problem at two levels. First we tested whether efficiency of transfer and stability of expression could be improved in mammalian systems. Various methods of introducing DNA into normal and regenerating skeletal muscle were tried. Pre-injection of sucrose solution ameliorates distribution and expression of genes, which is less variable if DNA is injected in a larger rather than a smaller volume. The choice of promoter is also important[1]. Second, in Xenopus, we developed a procedure for double DNA injection into muscle which allows simultaneous *in vivo* expression of a gene under physiological study and of a luciferase gene driven by a constitutive promoter. Gene expression : 1) is strong and reproducible 2) is highly correlated to the amount of injected DNA and 3) increases continually over time. This study demonstrates for the first time that somatic injection of DNA into skeletal muscle may be used to analyse the physiological regulation of muscle gene transcription *in vivo*.

1.Davis H.L.,Whalen R.G.&Demeneix B.A.; Human Gene Therapy, (in press)

S 306 ENHANCED GENE DELIVERY AND MECHANISM STUDIES WITH A NOVEL SERIES OF CATIONIC LIPID FORMULATIONS, Philip L. Felgner, C.N. Sridhar, Carl J. Wheeler, and Jiin Felgner, VICAL Inc., 9373 Towne Centre Drive, San Diego, CA 92121

The application of cationic liposome reagents has simplified DNA and mRNA transfection and a growing body of research is demonstrating their utility as in vivo gene delivery vectors. However, chemical structure activity data leading to a better mechanistic understanding of their biological activity is still limited. Results are presented here showing the dependency for neutral phospholipids in cationic lipid DNA transfection reagents. For these studies a novel series of 2,3-dialkyloxypropyl quaternary ammonium compounds containing a hydroxyalkyl moiety on the quaternary amine were synthesized and tested for in vitro transfection efficacy using a β-galactosidase plasmid. The rank order of potency for substituents on the quaternary amine were hydroxyethyl > hydroxypropyl > hydroxybutyl > hydroxypentyl ~ methyl. Vesicles formulated with 50 mole% dioleoylphosphatidyl ethanolamine (DOPE) are 5-10 fold more active than formulations with 50 mole% dioleoylphosphatidylcholine or than formulations without any neutral lipid, and the level of DOPE required for optimal activity is 50 mole%. Phosphatidylethanolamine (PE) analogs were studied for their transfection enhancing activity. PE analogs with increasing acyl chain saturation were progressively less active than unsaturated analogs; analogs with increasing numbers of methyl or methylene groups added to the primary amine of PE were also progressively less active. Lyso-PE neither enhanced nor inhibited the activity of the reagents. Cells transfected in the presence of 10% serum expressed higher levels of β-galactosidase activity than cells transfected under serum free conditions. Some of the formulations were enhanced by the addition of chloroquine, while others were unaffected or decreased. These results have implications regarding the design of new cationic and neutral lipid molecules for use in the development of improved cationic lipid gene delivery vectors.

S 307 CYTOPLASMIC GENE EXPRESSION BY CO-DELIVERY OF T7 RNA POLYMERASE AND T7 PROMOTER SEQUENCE BY CATIONIC LIPOSOME, Xiang Gao and Leaf Huang, Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA15261

T7 RNA polymerase produced intracellularly by recombinant vaccinia virus has been used to overproduce foreign genes driven by T7 promoter. We reported here that purified T7 RNA polymerase can be delivered directly by cationic liposomes along with a CAT construct driven by T7 promoter. The condition of transfection was optimized in terms of amount of enzyme used as well as DNA and liposome dose. CAT enzyme was transiently expressed in the transfected cells in a T7 RNA polymerase specific and dose dependent manner. The kinetics of expression was somewhat faster than that of pSV2CAT, a CAT construct driven by SV40 early promoter. Several cell lines were successfully transfected by this method *in vitro*. Under optimal conditions the toxicity to treated cells was minimal. Delivery of T7 RNA polymerase into eukaryotic cells allows cytoplasmic expression of a foreign gene and the expression is independent from host gene regulatory factors. This alternative approach should be useful in molecular biology and gene therapy research. Supported by NIH Grants AI 29893, HL 50256 and CA 59327.

S 308 PHARMACOKINETICS AND DISPOSITION OF OLIGONUCLEOTIDES INFUSED INTO THE RAT CENTRAL NERVOUS SYSTEM. Daniel A. Geselowitz, Christine Chavany, Brigid Fahmy, Luke Whitesell, and Len Neckers. Clinical Pharmacology Branch, NCI, NIH, Bethesda, MD 20892.

The cerebrospinal fluid (CSF) bathing the rat central nervous system provides a unique site for administration of oligonucleotides due to its small volume and clearance mechanisms which are quite different from those of the bloodstream. Its potential relevance as a site of administration for treatment of human brain tumors and metastases to the spinal cord led us to explore the fate of oligonucleotides administered directly into the CSF space. We report studies on the distribution and stability of phosphodiester and phosphorothioate oligonucleotides infused intraventricularly into rat brains. Our findings demonstrate that phosphodiester oligonucleotides are short-lived in the CSF when injected in this way, despite considerable stability in CSF*in vitro*. We attribute this to degradation during active exchange with brain tissue. Autopsy of the brains after infusion with fluorescently labelled oligonucleotides reveals substantial penetration of the oligonucleotide into brain tissue. Additionally, studies using gadolinium-tagged oligonucleotides to allow magnetic resonance imaging of intrathecal infusion will be presented.

S 309 THE IN VITRO SELECTION OF ATP SPECIFIC DNA APTAMERS

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The study of structural requirements for specific ligand binding activity of single stranded oligonucleotides will lead to an enhanced ability to design therapeutic oligonucleotides. ATP is an interesting ligand because of the broad role it plays as an enzymatic cofactor and as a precursor to RNA. Starting from a random pool of oligonucleotides we have used repeated cycles of amplification and in vitro selection by affinity chromatography on an ATP-agarose column to isolate single stranded DNA molecules which bind ATP specifically. The starting pool had a complexity of approximately $5X10^{14}$ molecules and contained a random region of 72 nucleotides flanked by defined regions. After seven rounds of selection the ATP binding DNA molecules were cloned and further characterized. No significant homology was found among the clones which indicates more then one solution to the ATP binding function. As expected from previous studies[1] there is no similarity between the sequences of the ATP binding domains of RNA and DNA molecules selected using the same experimental conditions. We are in the process of investigating ligand specificity using analogs of ATP. and determining the minimal sequence requirements of the aptamer through deletion analysis, and site specific mutagenesis of specific clones shown to bind ATP individually. We plan to determine ligand-aptamer disassociation constants using Isocratic Elution from an ATP agarose column.

1. Ellington, A.D. and J. Szostak (1992) Nature 355, 850-852.

S 310 RECOMBINANT ADENOVIRAL VECTORS FOR HEPATIC GENE THERAPY, Mark A. Kay[2,3], Quitang Li[2], Milton Finegold[4], Leslie D. Stratford-Perricaudet[5] and Savio L.C. Woo[1,2,3]. (1) Howard Hughes Medical Institute, Departments of (2) Cell Biology, (3) Molecular Genetics and (4) Pathology. Baylor College of Medicine, Houston, TX 77030 (5) Institut Gustave Roussy, Centre National de la Recherche Scientifique UA 1301,94805 Villejuif, France.

We have used the recombinant RSV.Bgal adenoviral vector that encodes the beta-galactosidase gene under the transcriptional control of the RSV-LTR to investigate the feasibility of the vector for gene transfer to mouse hepatoyctes in vivo. Different quantities of the purified RSV.Bgal vector was infused into the portal veins of mice. Various times later, the animals were sacrificed for hepatocyte isolation followed by x-gal staining and/or histologic liver preparation for pathologic analysis. The frequency of gene-transfer was determined by counting the proportion of hepatocytes that stained blue. The frequency of gene transduction was proportional to the number of viral particles injected. When 1×10^{10} viral particles were infused, 95% to 100% of the hepatocytes stained blue. The transduction protocol is relatively safe in that there is no detectable helper virus production in transduced animals and that very few extra-hepatic cells are transduced by this method. There is also no evidence of significant liver pathology unless substantially greater quantities of virus are used. However, the transduced hepatocytes do not appear to persist in vivo as the percentage of hepatocytes expressing b-gal declined over time. Four months after the infusion only 0.5% to 10% of the hepatocyes contain detectable b-gal activity in vivo. Thus, current recombinant adenoviral vectors may have clinical applications in gene therapy for acute hepatic disorders and repeated adenovirus administration may be necessary for chronic disorders.

S 311 MODIFICATIONS TO MOLECULAR CONJUGATE VECTORS INCREASE THE POTENTIAL FOR CELL SPECIFIC GENE TRANSFER, Sharon I. Michael, Chien-hui Huang, Maria U. Rφmer, Santosh Agarwal, Ernst Wagner, Ping-chuan Hu, and David T. Curiel, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 and Research Institute of Molecular Pathology, A-1030 Vienna, Austria

High efficiency gene transfer may be accomplished via the receptor-mediated endocytosis pathway employing molecular conjugate vectors facilitated by adenovirus. These ligand-adenovirus-polylysine-DNA complexes are internalized by virtue of the ligand domain and accomplish cell vesicle escape on the basis of adenovirus mediated endosomolysis. One of the potential advantages of this system is its ability to target specific cell types based on the specificity of the ligand for its receptor. Despite this, however, the polylysine moiety may bind nonspecifically to certain cell types. To address this, we blocked nonspecific electrostatic binding using an irrelevant polynucleotide, yeast tRNA. For this study, we examined free adenovirus facilitation of pL-DNA complexes. It was shown that nonspecific pL-DNA uptake in HeLa cells could be significantly reduced by the tRNA (pL-DNA=1,514 L.U.; pL-DNA+Ad=1,630,779 L.U.; pL-DNA+tRNA+Ad=6,882 L.U.). To show that this treatment did not also block specific uptake by virtue of the ligand, we repeated the experiment with human transferrin-polylysine-DNA complexes, (hTfpL-DNA=539 L.U.; hTfpL-DNA+Ad=37,656 L.U.; hTfpL-DNA+tRNA+Ad=23,052 L.U.). Thus, tRNA can block nonspecific polylysine binding without blocking specific ligand uptake. An additional factor potentially undermining specificity is the adenovirus moiety. Adenovirus, which like molecular conjugates, also enters cells via the receptor-mediated endocytosis pathway, can act as a competing ligand when introduced into the conjugate design. To overcome this, we have treated adenovirus with an anti-fiber antibody, which renders the virus binding-incompetent. It was shown that antibody pretreated adenovirus could not efficiently facilitate gene transfer of hTfpL-DNA complexes since the free adenovirus could not internalize (hTfpL-DNA=630 L.U.; hTfpL-DNA+Ad=232,238 L.U.; hTfpL-DNA+Ad+αfiberAb#7=26,555 L.U.; hTfpL-DNA+Ad+PY203 (irrelevant antibody)=155,199 L.U.). However, when antibody pretreated virions were linked to hTfpL-DNA complexes, they could significantly augment gene transfer efficiency (hTfpL-DNA=630 L.U.; hTfpL/AdpL-DNA=5,514,994 L.U.; hTfpL/AdpL-DNA+αfiberAb#7=4,971,577 L.U.; hTfpL/AdpL-DNA+PY203=7,241,683 L.U.). This result indicates that, in the linked configuration, binding incompetent virions were internalized by an alternate pathway but retained their ability to disrupt endosomes and thus facilitate molecular conjugate mediated gene transfer. We have shown that nonspecific polylysine binding to cell surface components can be blocked using an irrelevant polynucleotide such as yeast tRNA. Adenoviral binding can also be ablated employing a neutralizing anti-fiber antibody. These results represent a step towards constructing molecular conjugate vectors which are cell type specific.

S 312 CATIONIC LIPID IS NOT REQUIRED FOR UPTAKE AND INHIBITORY ACTIVITY OF ICAM-1 ANTISENSE OLIGONUCLEOTIDE IN KERATINOCYTES. F.O. Nestle, R.S. Mitra, C.F. Bennett, B.J. Nickoloff. Dept of Path, Univ of Mich, Ann Arbor, MI, and Dept of Cell Biology, ISIS Pharmaceuticals, Carlsbad, CA.

Aberrant keratinocyte (KC) ICAM-1 expression has been pathophysiologically implicated in retention of lymphocytes within the epidermis in a wide variety of T-cell mediated dermatoses. In this study we asked whether an 20-mer ICAM-1 phosphorothioate antisense oligonucleotide (IPAO) directed at specific sequences of the 3' region of ICAM-1 mRNA (ISIS - 2302) would have biological activity in-vitro as a prelude to in-vivo topical clinical trials. Multipassaged human KCs grown in serum free KGM were treated with gamma interferon (IFN-γ, 50-500 U/ml) to induce HLA-DR and ICAM-1. KCs were pre-treated with IPAO in presence/absence of cationic lipid (lipofectin 5 μg/ml) and analyzed by Northern blot hybridization, ELISA, and FACS. Fluorescein-labeled IPAO (5 μM) incubated with KCs without lipofectin revealed only granular cytoplasmic reactivity after 30 min in >50% of the cells but prominent accumulation in both nuclei and nucleoli after 120 min. Adding lipofectin enhanced nuclear uptake of IPAO in 60-70% of the KCs after 30 min of addition of FITC-IPAO. This direct cellular uptake in the absence of lipofectin by KCs has not been observed using cultured fibroblasts or endothelial cells. Functional studies revealed a dose-response effect in which IPAO (0.5-5 μM) maximally inhibited ICAM-1 expression by 25-40% in the absence of lipofectin (compared to IFN-γ treated KCs not exposed to IPAO), which was further enhanced by addition of lipofectin. All 3 assays (Northern blot hybridization, ELISA, and FACS analysis) yielded similar relative degrees of ICAM-1 inhibition. This inhibition was ICAM-1 specific as no changes were observed in the ability of IFN-γ to induce HLA-DR, or in the constitutive expression of the p60 TNF-α receptor, or glyceraldehyde 3-phosphate dehydrogenase.

These results indicate that KCs have the capacity to directly and rapidly take up IPAO inside their nucleus, with the consequent reduction in their ability to produce ICAM-1 mRNA/protein after exposure to IFN-γ. These results suggest that epicutaneous application of this low molecular weight oligonucleotide may significantly and selectively reduce KC ICAM-1 expression, thereby interrupting the intraepidermal trafficking pattern of lymphocytes. Such inhibition may have an anti-inflammatory and immunosuppressive effect that will benefit dermatology patients.

S 313 USE OF RIBOZYMES TO DEVELOP AND MODIFY MODELS OF HUMAN DISEASE, Matthew G.F. Sharp and John J. Mullins, AFRC Centre for Genome Research, Edinburgh University, West Mains Road, Edinburgh, Scotland EH9 3JQ.

Antisense RNA and catalytic RNA (ribozymes) are being used increasingly as tools to reduce the level of expression of target genes. Ribozymes have been used most successfully *in vivo* in high concentrations relative to the target mRNA. One possible explanation for this is that only a small proportion of the ribozyme RNA is coming into contact with the target mRNA, due to differences in subcellular localisation. We have attempted to address this possibility during the construction of ribozyme genes targetting a murine gene for the aspartyl protease *renin* (a key regulator of blood pressure control and homeostasis). In an attempt to channel the ribozyme RNA into the same cellular compartment as the target mRNA molecules, hammerhead ribozyme domains were inserted into a gene encoding the small nuclear RNA species U1. These genes encode the RNA component of a snRNP involved in pre-mRNA splicing. It was hoped that the use of this kind of ribozyme-U1 fusion RNA would lead to the nuclear localisation and accumulation of ribozyme molecules. If the ribozyme-U1 transcript is capable of associating with other splicing components, then this may provide a vehicle by which the catalytic RNA can be brought into contact with every spliced pre-mRNA transcribed within a cell. Ribozyme-U1 genes were introduced into cultured hepatoma cells constitutively expressing renin mRNA, and were shown to have nuclear localisation of the ribozyme-U1 RNA. The efficiency of ribozyme cleavage was assessed by northern blotting and ribonuclease protection. These ribozyme-U1 genes, and similarly constructed antisense genes are being introduced into transgenic animals in order to show the utility of these tools in gene inhibition strategies in real physiological situations.

S 314 ATTEMPT TO CHANGE THE SUBCELLULAR DISTRIBUTION OF OLIGODEOXYNUCLEOTIDES CONTAINING PHOSPHOROTHIOATE LINKAGES. Yoko Shoji, Kazuhiro Doken, Jingoro Shimada, and Yutaka Mizushima. Division of Clinical Pharmacology, Institute of Medical Science, St. Marianna University School of Medicine, Sugao, Miyamae-Ku, Kawasaki 216 JAPAN

Antisense DNA seem to be promising therapeutic reagents in the next century. From the pharmaceutical view of point, there are several obstacles to be overcome. First of all, the amount of oligodeoxynucleotides(ODNs) taken up by cells are not still sufficient enough. Major mechanism of cellular internalization of ODNs is via endocytosis, which ends up lysosome. To increase the antisense effects there are two possible ways, 1)increase the membrane permeability, and 2)avoid to distribute to lysosomal compartments. In this study, we observed subcellular distribution pattern of ODNs containing phosphorothioate linkages(S-ODNs) by using Confocal Laser Scan Microscope(CLSM). And we tried to change the subcellular distribution pattern of S-ODNs under the several conditions. CLSM observation well documented that internalization of S-ODNs has occurred via cell surface binding site. Gradually S-ODNs were internalized and were distributed in cytoplasm punctately . Within 1 hour little portion of S-ODNs were recognized in nucleus. Small fluorescent vesicles were still recognized even 24 hours later. In the next experiments, we tried to change the subcellular distribution of S-ODNs under the several conditions. Distribution of S-ODNs in lysosomal compartments was altered when cells were treated with several drugs including chloroquine. S-ODNs were distributed in nucleus when cells were treated with cationic lipid (lipofectin). Since lipofectin or chloroquine have been reported to enhance the antisense effects, change the distribution pattern of S-ODNs would be the way to be concerned to get sufficient antisense effects.

S 315 in vivo GENE TRANSFER INTO RABBIT THYROID BY DIRECT DNA INJECTION: A NOVEL STRATEGY FOR GENE THERAPY. Michael L. Sikes, Bert W. O'Malley, Jr., Fred D. Ledley. Departments of Cell Biology, Pediatrics, and Otorhinolaryngology, Baylor College of Medicine, Houston TX.

The thyroid represents a potentially useful target for somatic gene therapy. We have previously described ex vivo strategies for gene delivery to the thyroid using retroviral vectors. We now describe a strategy for somatic gene therapy involving direct administration of DNA into the thyroid. Previously, only muscle tissue has demonstrated significant levels of gene expression after injection of unmodified plasmid vectors. We injected plasmids encoding either CAT or nuclear-targeted ß-galactosidase under control of the CMV immediate early promotor into the rabbit thyroid gland under direct visualization. CAT activity was expressed at levels equivalent to those in muscle injected in control experiments. No expression was seen in other control tissues including kidney, liver, and salivary gland. Significant levels of expression were evident in animals sacrificed 3-5 days after injection. Histochemical analysis with X-gal staining demonstrated scattered cells expressing ß-gal along the track of injection but not elsewhere in the gland. Some X-gal-positive cells were positioned within the follicular wall and exhibited the histological appearance of thyroid follicular cells; others were present within disrupted tissue. PCR and southern analysis demonstrated that the loss of CAT activity paralleled the disappearance of plasmid sequences, but that plasmid was not completely eliminated up to 34 days after injection. The persisting plasmid exhibited a bacterial pattern of methylation suggesting it was not replicated or integrated. These studies introduce a new target for gene therapy by direct administration of DNA which might be useful for expressing gene products such as growth factors, clotting factors, cytokines, or neurotropic for 3-5 days in conjunction with surgery, anaesthesia, acute disease, or injuries. Further studies will be directed at developing regulated expression from the thyroid and evaluating strategies for prolonging the period of expression.

S 316 THE IMPORTANCE OF COLOCALIZATION OF RIBOZYME AND SUBSTRATE CONTAINING RNAS FOR TRANS CLEAVAGE IN VIVO, Bruce Sullenger and Thomas Cech, University of Colorado, Boulder, CO 80309 The hypothesis, that ribozyme mediated in vivo trans cleavage of a substrate RNA is limited by the rate which substrate and ribozyme RNAs are colocalized within a cell, has been tested by employing two retroviral vectors and taking advantage of the mechanism by which retroviruses sort their own RNAs in vivo. The transcripts generated from a Lac Z containing retroviral vector were targeted for cleavage within a clonal ecotropic packaging cell line. Such transcripts have two fates. Some serve as messenger RNAs being translated to produce ß-gal enzyme within these cells; while, others serve as genomic RNAs and are packaged into viral particles emerging from the packaging cells. This latter process is mediated by the retroviral encapsidation machinery which recognizes the viral packaging signal,Ψ. Two hammerhead ribozymes designed to cleave the Lac Z transcripts were cloned into and expressed from neo containing retroviral vectors. Ribozyme and mutant ribozyme containing vectors were transfected into an amphotropic packaging cell line, and the resulting virus was used to infect 10^4 Lac Z vector containing packaging cells at an MOI of 10 to avoid artifacts associated with clonal selection. Both anti-Lac Z ribozyme and Lac Z substrate RNAs contain Ψ. Thus ribozyme and Lac Z genomic RNAs should be colocalized by the viral encapsidation machinery; whereas, transcripts serving as mRNAs should not be colocalized. Lac Z viral titer is reduced by 90-95% from active anti-Lac Z ribozyme containing cells as compared to control and mutant containing cells; while, no reduction in ß-gal enzyme activity within the cells or in neo viral titer emerging from the cells is observed. These results suggest that in vivo trans cleavage is limited by the rate of colocalization of ribozyme and substrate containing RNAs within a cell.

S 317 DELIVERY OF STABLE RIBOZYMES TO VIRALLY - INFECTED CELLS, Garry B. Takle, Alain R. Thierry*, Prema Narayan, Paul Gottlieb, Shaji T. George and Allan R. Goldberg, Innovir Laboratories, Inc., New York, NY10021 and * Georgetown University Medical Center, Washington, DC20007.

The delivery of ribozymes and other therapeutics to target cells and tissues poses a considerable obstacle to their effective use. We have developed a spectrum of stable ribozymes by incorporating modified ribonucleotides during their synthesis, and have shown their efficacy in cleaving hepatitis B surface antigen (HBsAg) mRNA. These ribozymes have been administered to cell lines expressing HBsAg using a range of different targeted and non-targeted delivery systems, and data from these experiments will be described.

S 318 3' END MODIFIED OLIGODEOXYNUCLEOTIDES PHOSPHOROTHIOATE: PHARMACOKINETICS AND STABILITY IN MICE, Temsamani, J, Tang. J-Y, Padmapriya. A, Kubert, M. and Agrawal,S., Hybridon, Inc. One Innovation Drive, Biotechnology Research Park. Worcester, MA. 01605.

Antisense oligonucleotides have been shown to inhibit replication of the human immunodeficiency virus and several other viruses (1). A limiting factor in the use of phosphodiester oligonucleotide backbone is their rapid degradation by various nucleolytic activities in cells. Modifications of the phosphodiester backbone have been used to generate oligonucleotides with enhanced nuclease sensitivity. Phosphorothioate analogs have shown promise as agents capable of modulating gene expression . In earlier studies (2), we showed that after either intravenous or intraperitoneal administration of a single dose, oligonucleotide phosphorothioate (^{35}S-labeled at each internucleotide linkages) was biodistributed in most of the tissues. In most of the tissues the oligonucleotide was stable except in kidney and liver where 50% of oligodeoxynucleotide phosphorothioate was degraded after 48 hours.

To increase the in vivo persistence of oligonucleotide phosphorothioate, several 3' end modified oligonucleotides phosphorothioate have been synthesized and studied for their pharmacokinetics, biodistribution, excretion and metabolic stability in mice after intravenous administration (3). The overall biodistribution and excretion of the 3' end modified oligonucleotides were found to be independent of 3' end modification. However significant differences in stability of the oligonucleotide phosphorothioate extracted from tissues was observed between the unmodified and 3' end modified oligonucleotides phosphorothioate . For unmodified and 5' end modified oligonucleotides phosphorothioate, out of the total bioavailable concentration in liver and kidney, only 50% was found to be intact oligonucleotide after 24 hours. In contrast, for the 3'-end modified oligonucleotide phosphorothioates, almost all oligonucleotides were found to be intact.

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S 319 IN VITRO AND IN VIVO TARGETING OF GENE EXPRESSION TO MELANOMA CELLS, Richard G. Vile, Martin Goss and Ian Hart, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom.

Gene therapy protocols for cancer usually involve removal of tumour cells, culture *in vitro* to allow gene transfer, and subsequent reintroduction *in vivo*. Targeting therapeutic genes to tumour cells *in situ*, requires an accuracy of gene delivery that currently is not possible using existing techniques. To overcome these limitations we have used two promoters, which are preferentially active in melanocytic cells, to direct gene expression specifically to melanoma cells both *in vitro* and *in vivo*. Here, we describe experiments showing that as little as 769 bp of the 5' flanking regions of the Tyrosinase, and 1.4 kbp of the Tyrosinase Related Protein-1, genes are sufficient to direct expression of the ß-galactosidase gene to both human and murine melanoma cells and melanocytes, whilst not permitting expression in a range of other cell types *in vitro*. These promoters showed high levels of activity (between 20-50,000 arbitrary enzyme units per 10^7 transfected cells) in 12 out of 14 murine and human melanoma cell lines tested but showed only basal levels of activity, similar to that of a promoterless construct, in a range of 12 other cell types (<1,000 arbitrary enzyme units per 10^7 transfected cells). Cell type specificity is maintained when the construct is delivered to cells either by physical means or by inclusion of the cell type-specific expression cassette into a retroviral vector. Direct injection of DNA, encoding the ß-galactosidase gene expressed from either promoter, into established B16 melanomas or Colo 26 tumours in syngeneic mice resulted in extensive transduction of tumour cells in the B16 melanomas (~10% of tumour cells expressing 10 days after DNA injection), whereas no blue-staining cells were seen in the Colo 26 tumours . The reporter gene was expressed in melanoma cells, and in some normal melanocytes, but not in other surrounding normal tissue. Similar results have been confirmed using reverse transcriptase PCR when the reporter gene was replaced with the murine interleukin-2 gene. We propose that the combination of a tissue-specific promoter driving a therapeutic gene, with delivery of such a construct directly to sites of tumor growth *in vivo*, either by direct DNA injection or by retroviral infection, provides a means of achieving selective targeting of gene expression to a specific tumour type.

S 320 RECEPTOR-MEDIATED GENE DELIVERY AND ITS AUGMENTATION BY ENDOSOME-DISRUPTIVE VIRUSES OR PEPTIDES, Ernst Wagner, Matt Cotten, Kurt Zatloukal, Christian Plank, David T. Curiel#, Georg Stingl*, Max L. Birnstiel, Research Institute of Molecular Pathology, Vienna, A-1030, Austria; #Department of Medicine, University of North Carolina, Chapel Hill, NC 27599; * 2nd Clinic of Dermatology, University of Vienna School of Medicine, Vienna, A-1090, Austria

We have developed a novel gene transfer system which uses the receptor-mediated endocytosis route to import DNA into mammalian cells. DNA gene constructs complexed with polylysine-conjugated transferrin (which serves as a ligand for uptake via the transferrin receptor) and polylysine-conjugated, replication-defective adenovirus (which serves as an endosome-disruption agent and allows cytoplasmic entry of the DNA) have been delivered to and expressed at very high level in a large proportion of target cells (30% to 80% in primary fibroblasts, primary endothelial cells or primary human melanoma cells). Gene transfer *in vivo* has been demonstrated by local injection of the complexes into the liver of rats.

The use of replication-defective and chemically (psoralen /UV) inactivated adenovirus avoids most of the potential hazards associated with viral vectors. The delivered gene is carried on the exterior of the adenovirus, being therefore far less restricted in size or sequence of the DNA to be delivered. Transfer of 48 kb DNA molecules, and high level expression of the full length (8 kb) human factor VIII cDNA in primary fibroblasts, myoblasts and myotubes has been demonstrated.

Murine M3 melanoma cells transfected with cytokine gene constructs produce high levels of cytokines (24 000 units IL-2 / 10^6 cells/24 hrs; 1200 ng IFN-γ/10^6 cells/24 hrs). Transplantation of $1x10^5$ IL-2 transfected M3 cells into syngeneic DBA/2 mice showed loss of tumorigenicity (0/6 animals), whereas 6/6 animals treated with $1x10^5$ untransfected M3 cells developed tumors.

In order to generate a synthetic virus-free gene transfer system, we have taken synthetic peptides derived from the N-terminal sequence of influenza virus hemagglutinin HA-2 and linked them to a complex containing DNA, transferrin and polylysine. The peptides within the complex disrupt endosomal membranes triggered by the change to lower pH and substantially (up to 1000-fold) augment the gene transfer.

S 321 INHIBITION OF HIV-1 REPLICATION IN CULTURED CELLS WITH IMMUNO-LIPOSOME-ENCAPSULATED ANTISENSE OLIGONUCLEOTIDES OR PHOSPHORYLATED DIDEOXYURIDINE DERIVATIVES, Olivier Zelphati and Lee Leserman, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille CEDEX 9, France.

Both *REV*-specific and control phosphorothioate antisense oligonucleotides inhibit HIV-1 proliferation in acutely infected cells in vitro, while phosphodiester antisense agents are without effect, because of their rapid degradation in culture medium. Liposomes containing antisense phosphorothioate or phosphodiester oligonucleotides targeted to acutely infected CEM cells by specific antibodies inhibited HIV-1 p24 and RT with comparable efficiency. The antiviral effect was not seen for targeted empty liposomes or for oligonucleotide-containing liposomes bearing control antibodies.

2',3'-Dideoxyuridine 5'-triphosphate is the most powerful inhibitor, in cell free systems, of HIV RT among the 2',3'-dideoxynucleoside 5'-triphosphates containing a physiological base, but this agent is inactive in culture, since it is a poor substrate for cellular nucleoside kinases. Phosphorylated ddU is unable to cross cell membranes. Nevertheless, ddU nucleotides remain phosphorylated for several weeks after encapsulation in immunoliposomes, and exert potent antiviral activity when delivered into infected cells by cell-specific antibodies ($ED_{50} \leq 1$ μM on CEM). In contrast, no inhibition was observed with non-targeted liposomes containing phosphorylated ddU, or with empty liposomes, whether targeted or not. Our results reveal that it is possible to efficiently deliver antisense oligonucleotides and phosphorylated ddU into HIV-infected cells via immunoliposomes. Once inside the cell, these antiviral agents profoundly inhibit HIV replication. Liposome-mediated delivery can thus ameliorate problems of stability and intracellular transport.

Chemistry and Biochemistry of Genetically Targeted Molecules

S 400 SYNTHETIC OLIGONUCLEOTIDES: POTENT APTAMERIC INHIBITORS OF PROTEIN TYROSINE KINASES, Raymond C. Bergan, Yvette Connel, Brigid Fahmy, Edward Kyle, and Len Neckers, Clinical Pharmacology Branch, NCI, NIH, Bethesda, MD 20892

Protein tyrosine kinases (PTKs) are well recognized as important modulators of cellular function, and as a group comprise a significant fraction of the known oncogenic proteins. While investigating inhibition of certain tyrosine kinases via application of antisense technology, we observed a unique sequence-specific, but non-antisense, inhibitory process known as aptameric inhibition. Extensive evaluation of short synthetic oligonucleotides (ODNs) has revealed a group of sequence-related ODNs to be potent non-competitive inhibitors of PTKs. The activity of several serine/threonine kinases studied appears unaffected by these ODNs. Effective inhibitory concentrations are in the sub-micromolar range. While effective *in vitro* against several different PTKs, these ODNs can also reduce intracellular protein phosphotyrosine content and cell growth rate. Entirely phosphorothioate or phosphorothioate-capped ODNs are as effective as unmodified constructs. The sub-micromolar effectiveness of this class of ODNs, their specificity, and the importance of the targets involved make these molecules ideal candidates for investigative tools as well as pharmacotherapeutic development.

S 401 RIBONUCLEASE P CAN CLEAVE HEPATITIS B VIRUS RNA IN THE PRESENCE OF EXTERNAL GUIDE SEQUENCES, Jeffrey M. Bockman, Prema Narayan, Garry B. Takle, George T. Drivas, Shirin Siony, Shaji T. George, and Allan R. Goldberg, Innovir Laboratories, New York, NY 10021

Ribonuclease P (RNAse P) is an endogenous ribozyme system present in all cells which processes precursor tRNAs to yield the mature 5' termini. The RNA component of RNase P is the catalytic moiety. Heterologous RNAs can be targeted for cleavage by RNAse P by the use of an external guide sequence (EGS). Hybridization between a "human EGS," resembling three-quarters of a tRNA molecule, and an appropriate site in the target RNA results in the formation of a tRNA-like complex. This tRNA precursor-like structure is cleaved by RNAse P in the target RNA as if it were the 5' leader sequence. Four EGSs were designed against a target RNA corresponding to the first 837 nucleotides of hepatitis B virus (strain adw), which includes parts of the pre-S2 and S coding region of the surface antigen (HBsAg). All EGSs elicited varying degrees of cleavage of the HBV RNA by human RNAse P or the catalytic RNA "M1" of *E.coli* RNAse P. Since resistance to nucleases is an important aspect of a viable ribozyme therapeutic, these three-quartered EGSs were synthesized as deoxynucleotides, phosphorothioate deoxynucleotides, or phosphorothioate ribonucleotides. The phosphorothioate RNA EGSs were several orders of magnitude more resistant to nucleases than their entirely RNA counterparts in a HeLa cell S100 or tissue culture medium with 10% serum. Some of these modified EGSs were able to elicit cleavage of the target RNA by M1 RNA and human RNAse P. One of these all phosphorothioate EGSs was approximately 50% as efficient as its all RNA counterpart. Phosphorothioate RNA EGSs were much more efficient at eliciting cleavage than those composed of either DNA or modified DNA. Experiments involving liposome-mediated delivery to an HBsAg-expressing hepatoma cell line of chemically synthesized nuclease-resistant EGSs, and mammalian expression vectors encoding the anti-HBV EGSs, will be described.

S 402 RIBOZYMES SPECIFIC FOR THE L6 *BCR-ABL* FUSION mRNA. Leslie R. Coney[†], Fred T. Oakes[‡], Karin Moelling[*] and Catherine Pachuk[†]. [†]Department of Molecular and Cellular Biology, [‡]Department of Chemistry Research and Development, Apollon, Inc., Malvern, PA 19355 and [*]Max Planck Institut fur Molekulare Genetik, Inhestrasse 73, D-1000 Berlin 33, FRG

Chronic Myelogenous Leukemia (CML) is often associated with two *bcr-abl* mRNAs, both of which encode a protein kinase important in the establishment of the disease. The L6 mRNA can be expressed either alone, or in conjunction with the K28 mRNA; therefore, treatment of CML may require a therapeutic specific for the L6 mRNA.

We have designed ribozymes to specifically target the L6 mRNA. The first set of ribozymes was targeted to the GUA site located 19 nucleotides downstream of the L6 *bcr-abl* fusion. The distance of the cleavage site from the *bcr* sequences made it necessary to utilize regions of non-contiguous homology to target the ribozyme. This ribozyme cleaved up to 40% of the L6 substrate *in vitro* when equimolar amounts of ribozyme and substrate were used. The effect of ribozyme modifications on the efficiency of cleavage are being tested.

The second set of ribozymes was designed to cleave at the CUU motif located 7 nucleotides downstream of the L6 *bcr-abl* junction. The ribozyme having 100% homology with the *bcr-abl* junction region completely cleaved the L6 *bcr-abl* substrate when equimolar amounts of ribozyme and substrate were used. However, this ribozyme cleaved the K28 substrate with equal efficiency. To decrease complementarity of this ribozyme to *abl* sequences between the translocation junction and the cleavage site, 2, 3, or 4 mismatches to this region were introduced. These modifications decreased the rate of cleavage, but did not alter the specificity of cleavage.

Targeting with complementarity to non-contiguous regions of homology is of use for ribozymes that recognize any fusion mRNA resulting from a translocation. The L6 specific ribozymes generated using this approach might be useful in the treatment of patients who express the L6 mRNA alone or in conjunction with the K28 mRNA.

S 403 A 3' SECONDARY STRUCTURE MODIFICATION OF OLIGONUCLEOTIDES CONFERS RESISTANCE TO 3' EXONUCLEASE - POTENTIAL NOVEL ANTISENSE AGENTS, Judy M. Coulson, Ilyas M. Khan, Alan D.B. Malcolm & Leonard C. Archard, Biochemistry Department, Charing Cross & Westminster Medical School, St. Dunstans Rd, London, W6 8RF, ENGLAND.

We have investigated the use of antisense oligonucleotides as antiviral agents in two systems, targeting (1) the E6 and E7 transforming genes of the Human Papilloma virus HPV16 and (2) the viral DNA polymerase gene essential for the replication of vaccinia virus. We have previously described target sites for antisense oligonucleotides which are effective at inhibiting translation of RNA transcribed from these genes in a cell free system. It has been shown by others that unmodified oligonucleotides are rapidly degraded by 3' exonucleases in cell culture, and this was confirmed for our antisense sequences. Means of stabilising oligonucleotides against such degradation generally involve chemical modification of the phosphate backbone or of the bases. We describe an alternative approach based on the formation of secondary structure at the ends of an oligonucleotide. For example, short sequences have been identified which are capable of forming stable secondary structures [I. Hirao, Y. Nishimura et al (1989) Nuc. Acids Res. 17 (6) 2223-2231]. We have shown that the inclusion of such a sequence at the 3' end of an antisense oligonucleotide can reduce the degradation of phosphodiester oligonucleotides by exonucleases. Antisense sequences known to be effective in a cell free system were synthesised including an additional eight base sequence at the 3' end. We report here the electrophoretic and enzymatic characterisation of such antisense oligonucleotides and investigations of their inhibitory effects in rabbit reticulocyte lysate and cell culture systems.

S 404 CHARACTERIZING THE CLEAVAGE OF FULL-LENGTH βAPP-751 mRNA BY A HAMMERHEAD RIBOZYME, Robert B. Denman and David L. Miller, Department of Molecular Biology, NYS Institute for Basic Research, Staten Island, NY 10314

The sequences surrounding the first 5'GUC3' in the mRNA encoding the Alzheimer Amyloid precursor protein (βAPP) were used to construct a pair of trans-acting hammerhead ribozymes. Each ribozyme contained the conserved core bases of the hammerhead motif found in the positive strand of satellite RNA of tobacco ring spot virus [(+)sTRSV] and two 7 base stems complementary to the target, βAPP-751 mRNA. However, one of the ribozyme RNA cleaving strands was lengthened at its 3' end to include the early splicing and polyadenylation signal sequences of SV40 viral RNA. This RNA more closely mimics transcripts produced by RNA polymerase II from eucaryotic expression vectors *in vivo*. RNA, prepared by run-off transcription of cDNA oligonucleotide or plasmid constructs containing an T7 RNA polymerase promoter (Denman *et al*, 1992 BBRC 186, 1171-1177) was used to characterize several properties of the cleavage reaction. Magnesium-ion dependent, site-specific cleavage of a model 26 base βAPP substrate RNA and full-length βAPP-751 mRNA was observed in the presence of both ribozyme cleaving strands at the hammerhead consensus cleavage site. Neither ribozyme was active against non-message homologs of βAPP mRNA, nor was cleavage detected when point mutations were made in the conserved core sequences. However, the k_{cat}/K_m at 37°C in 10 mM Mg^{+2} of both ribozymes was reduced 25-fold when model and full-length substrates were compared. The use of short deoxyoligonucleotides (13-17 mers) that bound upstream of the ribozyme was found to enhance the rate of cleavage of the full-length, but not βAPP model substrate RNAs. The rate of enhancement depended on both the length of the deoxyoligonucleotide used and its site of binding on βAPP mRNA. For example, addition of 10 μM of a deoxyoligonucletide complementary to bases 112-128 of βAPP-751 mRNA to the ribozyme reaction resulted in a 15-fold enhancement of the cleavage rate. These data demonstrate the utility of ribozymes to cleave target RNAs in a catalytic, site-specific fashion *in vitro* and allow a direct comparison of the efficiency of different ribozyme constructs and different modulating activities.

S 405 Specific Cleavage of the Chimeric BCR/ABL RNA of Chronic Myelogenous Leukemia by Ribozymes Directed against the Fusion Junction. George T. Drivas, Shaji T. George, Andy Shih, Jeffrey M. Bockman and Allan R. Goldberg, Innovir Laboratories, Inc., New York, NY 10021.

In chronic myelogenous leukemia (CML), 95% of affected individuals carry the abnormal Ph' chromosome, t(9;22)(q34;q11). This reciprocal translocation results in the fusion of the *abl* gene on chromosome 9, with the *bcr* gene on chromosome 22. The resulting hybrid mRNA encodes a chimeric protein with deregulated tyrosine kinase activity and demonstrated transforming potential, that is capable of producing CML-like syndromes when expressed in mice. This evidence suggests that the BCR/ABL protein plays a causal role in the progression of this human leukemia. Ribozymes offer a distinct advantage over many current modalities proposed for the treatment of CML, in that they could be designed to produce specific cleavage and inactivation of the unique *bcr/abl* mRNA present in CML leukemic cells. To explore the utility of this approach, we made hammerhead ribozymes directed against the common K28-type CML breakpoint, and examined their ability to cleave test RNA substrates (500-800 nt.) representing the *bcr*, *abl*, and fusion messages. Cleavage reactions were done in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$ at 37°C, at a variety of ribozyme to substrate ratios, and analyzed by denaturing gel electrophoresis of the labelled RNA substrates. The ribozymes produced a significant amount of cleavage of the fusion *bcr/abl* substrate at an equimolar ratio, but no detectable cleavage products of either the *bcr* or *abl* substrates at enzyme:substrate ratios of up to 500:1. An additional ribozyme targeted to the *bcr* sequence 40 nt. upstream of the fusion junction effectively cleaved both the *bcr* and *bcr/abl* substrates under these conditions. These results indicate that the region surrounding the fusion junction is accessible to ribozymes in both the normal and chimeric substrates and that we have succeeded in developing ribozymes that are capable of eliciting specific cleavage of the fusion RNA. We are now examining the activity of these ribozymes against the full-length *bcr/abl* mRNA, with the goal of introducing these reagents into cells to produce specific inactivation of the unique chimeric mRNA of CML leukemic cells.

S 406 PEPTIDE NUCLEIC ACIDS (PNA) CONTAINING ALL FOUR NUCLEOBASES, Michael Egholm,[a] Peter E. Nielsen,[b] Rolf H. Berg,[c] and Ole Buchardt[a]
Research Center for Medical Biotechnology, [a]Chemical Laboratory II, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark and [b]Department of Biochemistry B, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark. [c]Polymer Group, Materials Department, Risø National Laboratory, DK-4000 Roskilde, Denmark

We have recently described the design, synthesis and properties of a new class of DNA analogues termed peptide nucleic acids (PNA). In PNA the backbone of DNA is replaced by an achiral peptide-like backbone which turns out to give good properties of the resulting oligomers. The enhanced binding strength to DNA and RNA compared to DNA and analogues is the most surprising property of PNA oligomers.

In the case of homo-pyrimidine PNA oligomers the neutral backbone strongly favors the formation of $(PNA)_2(DNA)$ triplexes upon binding to complementary oligodeoxynucleotides. These triplexes display an extraordinary stability and when homo-pyrimidine PNA oligomers bind to complementary targets in double stranded DNA it proceeds with displacement of the non-complementary strand.

We have now extended the synthetic scheme to include all four nucleobases, and oligomers containing all four nucleobases are now synthesized routinely. PNA oligomers with mixed purine-pyrimidine sequences form stable Watson-Crick duplexes with both DNA and RNA.

The initial attempts to employ the duplex and triplex formation between PNA and RNA or double stranded DNA for the inhibition of gene expression will be described.

S 407 **RIBOZYME MEDIATED CLEAVAGE OF HEPATITIS B VIRUS SURFACE ANTIGEN mRNA**
Shaji T. George, Andy Shih, Garry B. Takle, Shirin Siony and Allan R. Goldberg. Innovir Laboratories Inc., 510 East 73rd Street, New York, N.Y. 10021.

The hepatitis B virus surface antigens (HBsAg) which form the envelope of the viral particle are encoded by three overlapping genes, the pre-S1, pre-S2 and S genes. The major HBsAg protein is the 226 amino acid S gene product derived from a 2.1 Kb subgenomic RNA. Since HBsAg is essential for the production of infectious virus, the cleavage of this RNA by ribozymes could form the basis for a novel therapy. There are nine canonical "GUC" hammerhead ribozyme cleavage sites in the open reading frame of the S mRNA. We have synthesized hammerhead ribozymes against all nine sites and assessed the ability of these ribozymes to cleave both a 0.8 Kb fragment and the full length mRNA. Of the nine ribozymes only two could cleave the mRNA at 37°C in 10 mM $MgCl_2$ as assessed by the identification of radiolabeled cleavage products on a polyacrylamide gel. However, upon repeated heating and cooling of the enzyme and substrate (three rounds of heating to 65°C followed by snap cooling and incubation at 37°C), the expected cleavage products were seen in all cases. This demonstrates that all nine ribozymes are capable of cleaving the mRNA if higher order structures are disrupted by heat denaturaion. The two sites that were cleaved by the ribozymes without thermal denaturation were not in regions that were predicted to be unstructured by computer simulated folding programs based on least energy structure. The efficacy of these ribozymes in inhibiting the virus production is being tested in cell culture.

S 408 ANTISENSE AND ANTIGENE PROPERTIES OF PEPTIDE NUCLEIC ACIDS Jeffery C. Hanvey, Nancy J. Peffer, John E. Bisi, Stephen A. Thomson, Rodolfo Cadilla, John A. Josey, C. Fred Hassman, Michele A. Bonham, Karin G. Au, Stephen G. Carter, David A. Bruckenstein, Ann L. Boyd†, Stewart A. Noble and Lee E. Babiss, Departments of Cell Biology and Medicinal Chemistry, Glaxo Inc. Research Institute, Research Triangle Park, N.C. 27709 and †Department of Biology, Hood College, Frederick, MD

Peptide nucleic acids (PNAs) are novel polyamide oligomers with high affinity for both DNA and RNA, providing both antisense and antigene approaches for regulating gene expression. PNAs can strand invade duplex DNA, causing displacement of one strand and formation of a D-loop. PNA binding to the transcribed strand of a G-free transcription cassette caused 90-100% site-specific termination of pol II transcription elongation. In contrast, when PNA was bound on the non-transcribed strand, site-specific inhibition never exceeded 50%. PNA binding to RNA resulted in site-specific termination of both reverse transcription and *in vitro* translation, precisely at the position of the PNA•RNA heteroduplex. In contrast to oligodeoxynucleotides (ODNs), PNAs inhibited cDNA synthesis and ribosome elongation in an RNase H independent manner. Following nuclear microinjection of cells constitutively expressing SV40 large T antigen (T Ag) with either a 15-mer or 20-mer PNA targeted to the T Ag mRNA, suppression of T Ag expression was observed. The specificity of this effect was confirmed by demonstrating: 1) no reduction in β-galactosidase (β-gal) expression from a co-injected expression vector , and 2) no inhibition of T Ag expression following microinjection of a 10-mer PNA. Our results suggest that PNAs offer a promising means of regulating expression of specific cellular or viral genes at the DNA and/or RNA level.

S 409 TRIPLE-HELIX FORMATION AT SEQUENCES CONTAINING SHORT PURINE AND PYRIMIDINE BLOCKS: SEQUENCE DEPENDENCE OF ALTERNATE STRAND RECOGNITION, Brian H. Johnston, Sumedha D. Jayasena, and Amy Liu, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025

Triple helical DNA increasingly shows potential for applications in the control of gene expression (including therapeutics) and the development of sequence-specific DNA cleaving agents. A major limitation in this technology has been the requirement of homopurine sequences for triplex formation. We have developed a simple approach (1) that relaxes this requirement, by utilizing both Pu•PuPy and Py•PuPy base triplets to form a continuous DNA triple-helix at tandem oligopurine and oligopyrimidine tracts. The third strand, consisting of both purine and pyrimidine blocks, pairs with purines in the Watson-Crick duplex, switching strands at the junction between the oligopurine and oligopyrimidine blocks, but maintaining the required strand polarity without any special linkage.

Using chemical probing, affinity cleaving and DNase I footprinting, we showed (1, 2) that intra- and inter-molecular triplexes can be formed at both 5'-$(Pu)_n(Py)_n$-3' and 5'-$(Py)_n(Pu)_n$-3' target sequences. Although triplex formation at one 5'-$(Py)_n(Pu)_n$-3' sequence has recently been reported to require a 2-nucleotide spacer in the third strand between the purine and pyrimidine blocks (3), we found that triplex formation occurred without any linker for a different sequence (5'-CCCCCCCCTTAAGAAAAAAA-3'), but with lower yield than for 5'-$(Pu)_n(Py)_n$-3' sequences (2). For a third sequence (5'-C_8A_8-3'), triplex formation did not occur at all even when a spacer was available (4). A possible explanation, based on computer modeling studies, for the lower yield and the sequence-dependence at 5'-$(Py)_n(Pu)_n$-3' sequences is proposed in terms of the distance the third strand must traverse in switching its pairing interactions from one Watson-Crick strand to the other.

These results suggest that triplex formation via alternate strand recognition at sequences made up of blocks of purines and pyrimidines may be feasible for many sequences but prohibited for others.

1. S.D. Jayasena & B.H. Johnston, *Biochemistry* **31**, 320-327 (1992).
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4. S.D. Jayasena & B.H. Johnston, *Biochemistry*, in press.

S 410 PPT AND PST OF HIV-1 AS TARGETS FOR TRIPLEX OR ANTISENSE OLIGODEOXYNUCLEOTIDES, Karin Moelling*, Silke Volkmann*, Jens Dannull*, Andrey Surovoy**, *Max-Planck-Institut fuer Molekulare Genetik, Berlin, **Department of Chemistry, University of Tübingen, Germany.

Replication of retroviral RNA into double-stranded DNA by the viral reverse transcriptase (RT) and its associated RNase H activity involves initiation of plus-strand DNA synthesis at the polypurine tract, PPT. The PPT is highly conserved among HIV-1 retroviral isolates. It occurs twice, adjacent to the 3' LTR and in the coding region of the integrase. Recognition of the PPT by the RNase H is essential for virus replication since a mutant RNase H defective for recognition of the PPT fails to replicate. Antisense oligonucleotides, a 20mer and 40mer, complementary to the PPT induce complete block of DNA synthesis *in vitro* whereas another antisense oligonucleotide does not.
Previously polypurine sequences have been used by several groups for triplex-formation. During replication the HIV-PPT is present as RNA-DNA hybrid. Triplex-formation with a third DNA-strand as triplex-forming oligonucleotide TFO was tested here by resistance to RNase H cleavage *in vitro*. A pyrimidine in parallel orientation to the PPT RNA shows some protection. GT-containing pyrimidine-purine mixed TFO's (16mer, 25mer) led to up to 50 % protection independent of their orientation.
The Psi-region of HIV-1 is essential for packaging into virus particles by the nucleocapsid protein NCp7. We succeeded in demonstrating specific RNA-protein interaction with a synthetic NCp7 protein in filter binding assays. Antisense oligonucleotides against three different regions of the Psi region were analyzed *in vitro*. Only one of them interferes with the specific RNA-protein interaction. The effect of antisense oligonucleotides interfering with the PPT or Psi region during viral replication at the cellular level is under investigation.
Furthermore the effect of NC protein on protection of oligonucleotides or ribozymes against nucleases or as carrier for cellular uptake is being analyzed.

Wöhrl et al., JMB 220, 801 (1991).
Volkmann et al., JBC, in press.
Surovoy et al., JMB, in press.

S 411 NUCLEASE RESISTANT RIBOZYMES AGAINST HEPATITIS B SURFACE ANTIGEN mRNA.
Prema Narayan, Umberto Pace, Shaji T. George and Allan R. Goldberg, Innovir Laboratories Inc. New York, N.Y. 10021.

To develop a ribozyme based therapeutic against Hepatitis B virus infections. We have designed hammerhead ribozymes against a 800 nt fragment of the Hepatitis B surface antigen (HBsAg) mRNA. One of the main requirements for a successful ribozyme based therapeutic is that the ribozyme is resistant to nucleases present in the blood and in the cell. Therefore, we have introduced several nucleotide modifications at specific positions of the ribozyme. Using an enzymatic approach we have introduced phosphorothioate linkages in 60 % of the ribozyme molecule. This enzyme retains 80% of the catalytic activity of the all RNA ribozyme with phosphodiester linkages. Upon incubation of the phosphorothioate ribozyme with a HeLa cell S-100 fraction, 70% of the enzyme remained intact at the end of 30 min. In comparison, 99% of the all RNA ribozyme was degraded in 30 min. Increasing the phosphorothioate linkages decreased catalytic activity to 10% of the all RNA enzyme without further increasing nuclease resistance in the S-100 fraction. We have also chemically synthesized ribozymes with various types of modifications. These modifications were aimed at increasing nuclease resistance while still maintaining most of the catalytic activity. All of these synthetic ribozymes contain a minimum number of ribonucleotides comprising the catalytic core. The rest of the molecule contains either deoxyribonucleotides, 2'O-methylribonucleotides, phosphorothioate linkages or a combination thereof. Preliminary results indicate that complete susbstitution of the hybridizing arms with 2'O-methylribonucleotides or phosphorothioate linkages maintains most of the catalytic activity while increasing the half life of the ribozymes in the S-100 fraction. The efficacy of these stable ribozymes is being tested in cells expressing HBsAg.

S 412 CONSTRUCTION OF RIBOZYMES WHICH DISCRIMINATE BETWEEN CLOSELY RELATED mRNAs, Umberto Pace, Jeffrey M. Bockman, Barbara MacKay, and Allan R. Goldberg, Innovir Laboratories, New York, NY 10021
We have been investigating the use of ribozymes as therapeutics for acute promyelocytic leukemia (APL). APL is associated with a balanced t(15;17) chromosomal translocation which results in the fusion of genes encoding a putative transcription factor (PML) and the retinoic acid receptor α (RARα). It has been hypothesized that the PML-RAR fusion protein blocks myeloid differentiation. We have hypothesized that inactivation of the *PML-RAR* mRNA by ribozyme-mediated cleavage will induce terminal differentiation of the leukemic cells. We have therefore been developing hammerhead ribozymes which preferentially cleave the fusion mRNA over either parental transcript. Two hammerhead cleavage sites have been focused on: site 1, an AUU located 4 nucleotides 3' to the fusion junction; and site 2, a UUC located 26 nucleotides 3' to the junction. Using a 788 nucleotide fragment of the *PML-RAR* mRNA and a 930 nucleotide fragment of the *RARα* mRNA, we have determined the enzyme to substrate ratio which results in 50% cleavage of the substrate (C_{50}) under our standard reaction conditions (50 mM Tris-HCl, 30 mM $MgCl_2$, 37°C, 3 hours). One construct, IHRZ1.18, directed against site 1, cleaved the *PML-RAR* RNA with much greater efficiency than it cleaved the *RARα* RNA, having a C_{50} of 3 and 80, respectively. Variations in the length of the hybridizing sequences resulted in a construct, IHRZ1.23, with enhanced selectivity for the fusion RNA over the *RAR* RNA, but with a concommittant reduction in activity, having a C_{50} of 20 and 500, respectively. A standard hammerhead ribozyme directed to site 2, IHRZ1.28, did not demonstrate the selectivity that IHRZ1.18 displayed against site 1, having a C_{50} of 1.5 and 15, respectively. Interestingly, however, a modified hammerhead directed against site 2, IHRZ1.3, resulting in a non-canonical stem-loop structure in stem III of the ribozyme-substrate complex, displayed the highest degree of selectivity with only a moderate decrease in activity. This construct had a C_{50} for the *PML-RAR* RNA of 20 but one of greater than 900 for the *RARα* RNA.

S 413 SOLID-PHASE INTRODUCTION AND INTRA-CELLULAR PHOTOINDUCED REACTION OF A WATER- SOLUBLE meso- tetraCARBOXYPORPHINE-OLIGONUCLEOTIDE CONJUGATE, J.F. Ramalho Ortigão, A. Rück, K.C. Gupta, and H. Seliger, Sektion Polymere, Universität Ulm, D-7900 Ulm, Germany
Oligonucleotide conjugate with water soluble meso tetra(4-carboxyphenyl) porphine ($TPPC_4$) have been prepared by a support synthesis and novel solid-phase conjugation strategy. This procedure, which can be extended to other modifications as well, has the advantage that excess porphine and side products are removed simply by filtration.
Clevage specificity was determined by hybridizing 1pmol $TPPC_4$-conjugate 17mer M13 sequencing primer, after metallation with Fe^{+2}, to an equimolar amount of complementary synthetic 35mer sequence. On incubation with dithiothreitol, highly specific cleavage pattern could be obtained. This oligonucleotide $TPPC_4$ conjugate was able to cleave dsM13 DNA in a similar assay. Additionally, on incubation of RR 1022 rat epithelial cell culture with 0.6µM non-metalated oligonucleotide $TPPC_4$ conjugate, directed against rat α-actin mRNA, cytotoxic effect was detected after irradiation with laser light at 635nm. With $TTCP_4$ alone, this effect could not be observed with a concentration bellow 10µM. Furthermore fluorescenc microscopy studies shows that the conjugate after internalizations have clearly cytoplasmatic localization.

S 414 END-BLOCKING OF ANTISENSE AGENTS ENHANCES ANTIPROLIFERATIVE ACTIVITY AGAINST CHRONIC MYELOID LEUKEMIA CELLS, Peter T. Rowley, Margaret Thomas, Barbara Kosciolek, Yu-Waye Chu, and Douglas H. Turner, Departments of Medicine and Chemistry, University of Rochester, Rochester, NY 14642.

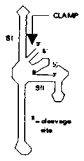
Antisense agents targeting the molecular sequence responsible for a given type of malignancy may have the cell specificity lacking in current anticancer drugs. Chronic myeloid leukemia cells have a translocation of the *abl* proto-oncogene on chromosome 9 to the *bcr* region on chromosome 22 resulting in a hybrid gene product. We have investigated the activity of antisense agents and their end-blocked derivatives in inhibiting the proliferation of K562 chronic myeloid leukemia cells. We have investigated the effect of antisense *bcr-abl* phosphodiester oligomers to three different target regions. One was the junction of *bcr* exon b3 and *abl* exon a2 in K562 cells. The other two target regions were selected from these two exons using the method of free energy minimization as regions most likely to be single-stranded, viz. nucleotides 16-37 and 104-123 of *abl* exon a2. Although none inhibited growth in suspension, the 104-123 oligomer reduced colony number in semisolid culture. This inhibition was sequence-specific since oligomers with the same nucleotide composition but in random sequence had no effect. A junction antisense oligomer with a 5'-dimethoxytrityl group, unlike the unprotected agent, had an antiproliferative effect on K562 cells in suspension. Although little effect was seen with oligomer addition on the first day only, daily antisense addition reduced growth and caused cell number to fall after day 4. Since the principal exonuclease appears to be of the 3' type, 3',5'-derivatives were also studied. 3'-amino, 5'-dimethoxytrityl antisense oligomer caused a plateauing of growth by day 3 and a subsequent fall in cell number, whereas the nonsense oligomer had no effect. We conclude that a *bcr-abl* antisense agent may be more effective if blocked at the 3' and 5' ends, presumably by reducing degradation by exonucleases.

S 415 PYRENE-MODIFIED OLIGODEOXYNUCLEOTIDES: ENHANCED DUPLEX BINDING AND STABILITY TOWARDS EXONUCLEASE DEGRADATION *IN VITRO*, Yoko Shibata, Jeffry S. Mann, Alan R. Wolfe, and Thomas Meehan, Departments of Pharmaceutical Chemistry and Pharmacy, University of California, San Francisco, San Francisco, CA 94143

Antisense oligodeoxynucleotides (ODNs) have been used as sequence specific regulatory agents for the studies of protein function. The use of ODNs for therapeutic purposes has also been suggested. However, the usefulness of ODNs for these purposes is hindered by their rapid degradation by nucleases, their relatively low binding affinity to target strands, and their limited cellular uptake. We have attempted to overcome these difficulties by conjugating the 5' end of an ODN with a neutral polycyclic aromatic hydrocarbon, pyrene. Our preliminary thermodynamic data showed that the pyrene-modified ODN attached through a tetramethylene linker increased the stability of the duplex with its complementary strand by 2.6 kcal/mol compared to an unmodified equivalent. Kinetic data showed that this 5' modification reduced the rate of digestion by an order of magnitude with Phosphodiesterase II, an exonuclease which attacks ODNs from the 5' end. In a cell-free translation system, pyrene modified ODNs inhibited Brome Mosaic Virus protein synthesis more effectively than their unmodified counterparts. We are currently investigating the stability of duplexes with different linker lengths and are modifying other positions in the ODNs, in order to further improve their properties. We are also testing pyrene-modified ODNs in RSV infected chicken cells as inhibitors of *src* gene expression.

S 416 A NOVEL CLAMP-LIKE STRUCTURE ENABLES RETARGETING OF A RIBOZYME DERIVED FROM THE ANTIGENOME OF HEPATITIS DELTA VIRUS AND TRANS-CLEAVAGE OF HETEROLOGOUS RNA, Andy Shih, Barbara MacKay, Allan R. Goldberg, and Shaji T. George, Innovir Laboratories, Inc., New York, NY 10021

We have recently developed an efficient trans-cleaving genomic ribozyme which contains structural motifs distinct from those in either the previously reported cis- or trans-acting systems (Shih et al, submitted). We have applied one of these novel structures, a clamp-like stem situated between stems I (SI) and II (SII), towards the retargeting of an antigenomic-derived ribozyme. Construct IDRZ104 had a 26 nucleotide substrate consisting of 5 non-HDV nucleotides at the 5' end, which are predicted to form a 5 base pair clamp with 5 non-HDV nucleotides at the 3' terminus of the enzyme domain, followed by 21 HDV nucleotides, 7 of which



are predicted to form stem II with the enzyme. This construct, at an enzyme to substrate ratio of 1:4, cleaved approximately 75% of the substrate at 37°C and 90% at 50°C after 90 minutes in the presence of 30 mM Mg^{2+}. Having demonstrated the functionality of the clamp for an antigenomic HDV ribozyme, this approach was used to retarget the ribozyme to non-HDV sequences. For the antigenomic ribozyme construct IDRZ155, the substrate consisted of 26 nucleotides of HBV (strain adw) sequence corresponding to nucleotides 358-383 within the coding region for the HBV surface antigen. The 5 residues at the 3' terminus of the enzyme were engineered to be complementary to the 5' terminal residues of the substrate, and the enzyme portion of stem II to be complementary to the 3' end of the substrate. Under identical conditions to those used above, IDRZ155 cleaved 55% of the substrate at 37°C and 70% at 50°C. This is the first reported retargeting and trans-cleavage by an HDV antigenomic ribozyme. An analogous construct, IDRZ158, which lacked the clamp cleaved only 16% and 18% of the substrate under identical conditions. IDRZ159, with an 8 base pair stem, cleaved 30% and 50%, respectively.

S 417 3' END MODIFIED OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES: SYNTHESIS, PROPERTIES AND ANTIVIRAL ACTIVITY, Tang, J.Y., Guo, Q., Roskey, A. and Agrawal, S., Hybridon, Inc., One Innovation Drive, Biotechnology Research Park, Worcester, MA 01605

Oligonucleotide phosphorothioates have been used to inhibit viral as well as cellular gene expression. In order to increase the in vivo persistance of oligonucleotide phosphorothioates, several 3'-end modified oligonucleotide phosphorothioates were synthesized by attaching different linkers or modifying the phosphorus backbone and studied for their nuclease sensitivity, affinity to the target nucleic acids and RNase H activity. The antiviral activity in tissue culture has also been studied. Our results showed that 3' end modified oligonucleotide phosphorothioates were more resistant to nucleolytic degradation. Some 3' end modified oligonucleotide phosphorothioates had increased cellular uptake and in vivo persistance. Most of the 3'-end modified oligonucleotide phosphorothioates showed increased antiviral activity.

S 418 SITE SPECIFIC TERMINATION OF TRANSCRIPTION IN EUKARYOTIC CELL EXTRACTS BY PEPTIDE NUCLEIC ACID (PNA) OLIGOMERS. T.A. Vickers, S.M. Freier, & D.J. Ecker. ISIS Pharmaceuticals, 2280 Faraday Ave., Carlsbad, CA 92008. R.H. Berg[1], M. Egholm[3], P.E. Nielsen[2], & O. Buchardt[3]. Polymer Group, Materials Dept., Riso National Laboratory, DK-4000 Roskilde, Denmark[1]. Dept. of Biochemistry B, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark[2]. The H.C. Orsted Institute, Universitetsparken 5, DK-2100 Copenhagen 0, Denmark[3].

Plasmid constructs have been designed in which viral sequences were inserted downstream of the CMV LTR. Mixed homopyrimidine PNA oligomers were hybridized to complementary homopurine regions of the viral sequences. Run-off transcripts produced from these templates in HeLa cell extracts were specifically aborted at the site of PNA binding. The effect was shown to be sequence specific and dependent upon hybridization of the PNA to the template strand. Thermal melts and gel shift assays were performed to determine stoichiometry and binding affinity of the PNA's. Stoichiometry measured against single stranded DNA complement was determined to be 2:1 (PNA:DNA). Hybridization of PNA to DNA duplex was found to be salt and pH dependent. Lowering either salt concentration or the pH resulted in an increase in binding. Although affinity to single stranded target was also increased by lowering pH, it was not effected by changes in salt concentration. It has recently been proposed that homopyrimidine PNA's invade duplex DNA binding the complementary DNA strand while displacing the non-complementary strand. A second PNA then interacts with the PNA/DNA duplex forming a triple stranded structure with increased stability. Our results are consistent with this model as decreasing salt concentrations favors strand invasion by decreasing the onrate constant and lowering pH favors third strand interactions due to the protonation of cytosines in the PNA.

S 419 C-MYC GENE REGULATION BY TRIPLE HELIX-FORMING OLIGONUCLEOTIDES: ANTISENSE APPROACH TO THE INTRA-MOLECULAR TRIPLE HELIX OF THE C-MYC PROMOTER, Kyonggeun Yoon and Meiquing Lu, Department of Molecular and Cellular Biology, Apollon Inc., 200 Great Valley Parkway, Malvern, PA 19355

Oligonucleotide-directed triplex formation within upstream regulatory sequences is envisioned as a potential tool for gene inhibition. Homopurine-homopyrimidine sequences are frequently found in upstream regulatory regions, in which they are suspected to play a function in gene expression. The nuclease-sensitive site containing C/T rich sequence was detected in cells, in which c-myc gene was actively transcribed. The structure of the C/T rich sequence was shown to be consisted of an intramolecular DNA triplex region with the pyrimidine-rich strand donating the third strand and leaving the complementary region of the purine-rich strand single stranded.[1] We compared the efficacy of various oligonucleotides (oligos) targeted to C/T rich region of the c-myc promoter in their abilities to inhibit c-myc transcription: 1) G-rich oligos designed to bind the duplex target in antiparallel manner (G:GC, A:AT or T:AT). 2) G-rich oligos with a scrambled sequence. 3) C-rich oligos which target duplex DNA in parallel or antiparallel manner (C:GC). We report the surprising finding that a C-rich oligo exhibits a remarkable inhibition of c-myc transcription and thymidine uptake in HL60 cells. In contrast, neither G-rich oligos targeted for duplex DNA nor G-scrambled sequence oligos were similarly inhibited. The inhibiting capactiy of the C-rich oligo in HL60 cells does not correlate with the affinity of triple helix-forming oligos binding to the duplex target. Only antiparallel G-rich oligos, not the C-rich oligo were shown to bind to the duplex target. However, the C-rich oligo may be able to bind to the putative single-stranded region of an intramolecular triplex in an antisense manner. This result suggests that a non-B DNA structure may exist in cells where c-myc is actively transcribed.

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[1]Kollwin, R., Torrey, T.A., Kinniburgh, A.J. (1992) Nucl. Acid Res. 20:111-116.

Late Abstracts

ANTISENSE RNA AGAINST THE TRANSACTIVATOR NS-1 PROTEIN OF THE PARVOVIRUS MVMp, INHIBITS CYTOTOXICITY AND NUCLEAR ACCUMULATION OF VIRAL TRANSCRIPTS, Jose M. Almendral[1,2], Juan F. Santaren[2] and Juan C. Ramirez[1,2], 1.Centro de Biología Molecular (UAM-CSIC) 28049 Madrid, and 2.CIEMAT 28040 Madrid, Spain.

The Minute Virus of Mice (MVMp), an autonomous Parvovirus that replicates cytolytically in the A9 mouse fibroblast cell line, was interfered by constitutive expression of an antisense RNA targeted against the major non-structural NS-1 protein. Permanently transfected A9 clones expressing NS-1 antisense, showed increased proliferative capacity upon virus infection, and likewise cultures infected at low multiplicity by MVMp reached confluence overcoming virus growth. Correspondingly, an inhibition in virus multiplication was demonstrated by a significant lower virus production and plaque forming ability in clones expressing antisense RNA.
At the molecular level, several fold reduction in viral DNA,RNA and proteins was quantitated by respective analysises of Southern, RNase protection and bidimensional gels. Remarkably, the accumulation of all three viral messengers (R1,R2,R3) was decreased both in the cytoplasm and in the nucleus, suggesting that antisense-mediated inhibition is primarily exerted at the level of viral transcription or nuclear post-transcriptional events. Thus, this system illustrates the possibility to create an antisense-mediated protective stage to highly cytotoxic viruses in permissive cells, by down-modulation the expression of a transactivator of virus genes.

SELF-SPECIFIC T LYMPHOCYTE LINES AS VEHICLES FOR GENE THERAPY : MYELIN SPECIFIC T CELLS CARRYING EXOGENOUS NERVE GROWTH FACTOR GENE

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At least in theory, self-reactive T lymphocytes should be useful vehicles to deliver exogenous gene products into specific target tissues. In order to test this therapeutic concept, we introduced the nerve growth factor (NGF) gene under control of a viral promoter into a myelin basic protein (MBP) specific rat T lymphocyte line, and examined the expression of NGF *in vitro* . As target cells, we used T cell line Z85, a long term CD4+ T cell line, which had been isolated from a MBP primed Lewis rat , and which recognizes the MBP epitope p68-88 in the context of MHC class II determinants. Z85 cells are strongly cytotoxic in vitro, and secret cytokines like IL-2, interferon-γ and TNF-α. They do not , however, release NGF wether in a resting or in an activated stage . The human NGF gene was stably introduced into Z85 cells by a retroviral vector, and expression verified by Northern blot analysis. The secretion of NGF in resting, transduced Z85 cells was barely above background. In striking contrast, T cell activation by presentation of MBP initiate a burst of NGF production lasting several days , with concentrations of more than 1 ng/ml in the culture medium. NGF secretion was quantified by ELISA , and verified by a chick DRG neuronal survival bioassay. The results are of interest by two reasons :
A. Exogenous genes can be introduced stably into self-specific T cell lines.
B. Transgene expression is remarkably enhanced by specific antigen recognition. It thus appears that self-specific T cells carrying exogenous gene products will be of use as powerful vehicles to import desirable therapeutically relevant factors into tissues as secluded as the CNS.
This work was supported by a grant from Volkswagen Foundation

THE ROLE OF p53 AND RB GENES IN GROWTH REGULATION OF NORMAL RAT HEPATOCYTES:INVESTIGATION BY ANTISENSE OLIGONUCLEOTIDES

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The role of Rb and p53 genes in growth regulation of normal rat hepatocytes was investigated by the antisense oligonucleotide technique. Phosphorothioated oligonucleotides were prepared to recognize the translation initiation codon(ATG) of p53 and Rb mRNA. Treatment of primary cultured newborn rat hepatocytes with the p53- or Rb-antisense alone had no effect on their growth after stimulation by insulin/EGF. However, co-treatment of the cells with the p53- and Rb-antisense significantly enhanced their growth. Our results suggested that the p53 and Rb genes cooperate to regulate the growth of normal rat hepatocytes.

HEMOPHILIA: AN IMPORTANT TARGET FOR GENE THERAPY,

Margaret V. Ragni, Michael T. Lotze, Department of Medicine and Surgery, University of Pittsburgh School of Medicine and Pittsburgh Cancer Institute, Pittsburgh, PA 15261

Occurring one in 10,000 male births, hemophilia is an X-linked disorder characterized by deficiency of coagulation factor VIII:C (Hemophilia A) or factor IX:C (Hemophilia B). The defect results in bleeding into joints, hemarthroses, and into muscles, hematomas, which when chronic and recurrent, leads to crippling degenerative arthritis with associated disability and morbidity. Perhaps the most disabling complication has been the viral infections transmitted through clotting factor concentrate treatment. The burden of disease is high, with over 45% infected with human immunodeficiency virus (HIV), and over 20 cases of AIDS per 100. Nearly 90% are infected with hepatitis B (HBV) and hepatitis C (HCV) viruses with chronic liver function abnormalities, and have biopsy-proven chronic liver disease in 20% or more. The introduction of newer, purer clotting factor concentrates has markedly reduced viral transmission, with no new HIV infection in this population since 1986, and with a marked reduction in HBV and HCV infection. However, treatment of hemophilia is costly, over $100,000 per year for severe patients, is associated with immunosuppression related to chronic foreign antigen exposure, and because treatment is "reactive" rather than prophylactic, continues to result in significant morbidity and mortality. The introduction of recombinant factor VIII treatment, while free of human viruses, has resulted in a two-fold higher cost, but as a "reactive" treatment will not eliminate morbidity and mortality. Gene therapy for hemophilia has the potential to reduce or eliminate hemorrhages, avoid transmission of viral pathogens, and significantly reduce life-time health care costs. Hemophiliacs are an ideal group to target for gene therapy, as they are a well-characterized group, have frequent medical followup, and welcome clinical trials of new therapies leading to "cure". We are developing a strategy for gene therapy for hemophilia A and B, using retroviral vectors for factors VIII and IX with colleagues at Genetic Therapy, Inc.

Comparison of various types of Antisense TGF-ß1 oligonucleotides to release early human hematopoietic progenitors from Go phase

(Jean Pierre Levesque[1], Pascal Batard[2], Hemchand Sookdeo[2], Eugene L. Brown[2], Angelo Cardoso[1], Ma Lin Li[1], Antoinette Hatzfeld[1] and Jacques Hatzfeld[1]. 1) CNRS UPR 272 rue Guy Mocquet 94802 Villejuif France, 2) Genetics Institute, Inc., Cambridge, Massachusetts 02140.

Human hematopoietic stem cells are primarily quiescent which prevents efficient gene transfer using retroviral vectors. We have shown that antisense Transforming Growth Factor ß1 ($TGFß_1$) or Retinoblastoma (Rb) oligonucleotides can release early hematopoietic progenitors from quiescence (Hatzfeld et. al., J. Exp. Med. 174, 925-929:1991). For these studies SBA^-CD34^+ $CD38^-$ cells were purified and developed, in culture, into large colonies containing granulocytes, monocytes, megakaryocytes and erythroid cells when appropriate cytokines were added. Addition of antisense TGF-ß1 oligonucleotides to these cultures stimulated a 100% increase in the number of CFU-GEMM. The new CFU-GEMM represent even earlier progenitors as the colonies are much larger and contain up to $1.5x10^5$ cells. In these experiments utilization of the antisense oligonucleotide actually enhanced stem cell activation which provides an excellent assay system to discriminate between the specific and toxic side effects of various modified antisense oligonucleotides. We will present data on the use of this assay to compare various types of oligonucleotides for their stability, penetrability and toxicity.