METAL AND OXYGEN REGULATION OF GENE EXPRESSION

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Metal and Oxygen Regulation of Gene Expression

Prokaryotic Metal Metabolism/Homeostasis-I

C4-001 REGULATION OF THE BACILLUS SUBTILIS OXIDATIVE STRESS RESPONSE BY METAL IONS, John D. Helmann, Section of Microbiology, Wing Hall, Cornell Univ., Ithaca, NY. 14853-8101.

The *Bacillus subtilis mrgA* gene encodes a DNA-binding protein (Dps homolog) induced when cells enter stationary phase. MrgA, like the *Escherichia coli* Dps protein, protects cells against the lethal effects of hydrogen peroxide treatment. Transcription of *mrgA* is induced by hydrogen peroxide and is repressed by manganese and iron. To identify the important DNA and protein components of this regulatory system we have selected strains derepressed for transcription of an *mrgA-cat-lacZ* operon fusion in the presence of manganous ion. The resulting cis-acting mutants define an operator just upstream of the *mrgA* promoter. A highly similar sequence is located upstream of the promoter for the catalase gene, *katA*, and two copies overlap the promoter for the major heme biosynthesis operon of *B. subtilis*, *hemAXCDBL* (see Figure). The presence of similar sequences in the promoter regions of related genes from other gram positive organisms suggests that this is a broadly distributed regulatory system.

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TGATCTAAATTATAATTATTATAATTAGTATTGATTTTATTTAGTATATGATATAATTAA  (MRGA)
ATAACTATTTTATAATAATTATAAAATAATATTGACTTTTTACTTAGAGATGATTATTATGTT  (KATA1)
GAAACTATGTTATAATTATTATAAATAATGAGTT*                            (HEMA-1)
    *CTATGTTAGAATGATTATAAATTAAGATTGGGTGTTGGGGGTGTAATTAGAGCGATGC  (HEMA-2)
    CTAt-TTATAAT-ATTATAAattA       CONSENSUS
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Like *mrgA*, transcription of *katA* is also repressed by manganese which thereby potentiates the killing action of hydrogen peroxide. Two classes of trans-acting mutants which derepress *mrgA* transcription in the presence of manganous ion were identified. One group of mutations simultaneously derepresses catalase, heme biosynthesis, and the putative subunits of alkylhydroperoxide reductase and confers high level resistance to hydrogen peroxide. Phenotypically similar mutations are obtained by selection for hydrogen peroxide resistance. A second class leads to a loss of catalase activity. These data suggest that the inducible oxidative stress response of *B. subtilis* is regulated by a repressor, possibly a metalloregulatory protein, which senses hydrogen peroxide. Although hydrogen peroxide induces a similar set of genes in *B. subtilis* and *E. coli*, the regulatory mechanisms are quite different. Unlike the OxyR regulon, the *B. subtilis* peroxide regulon is under negative control and appears to involve a metalloregulatory protein. A model for the coordinate regulation of the oxidative stress response by hydrogen peroxide and metal ions will be presented.

C4-002 CHARACTERIZATION OF THE DIPHTHERIA TOXIN REPRESSOR AS AN IRON-DEPENDENT GLOBAL REGULATORY PROTEIN OF CORYNEBACTERIUM DIPHTHERIAE, Randall K. Holmes[1], Michael P. Schmitt[1], Zhaoxi Wang[1], Suping Zhang[1], Edda M. Twiddy[1], Jenny Liu[1], Xiayang Qiu[2], Christophe L. M. Verlinde[2], and Wim G. J. Hol[2], [1]Uniformed Services University of the Health Sciences, Bethesda, MD 20814, and [2]Howard Hughes Medical Institute and School of Medicine, University of Washington, Seattle, WA 98195.

Growth of C. diphtheriae C7(β) under high-iron conditions represses synthesis of diphtheria toxin, corynebacterial siderophore, and components of a siderophore-dependent iron uptake system. Coordinate repression of the corresponding genes is mediated by the diphtheria toxin repressor (DtxR), which is activated by Fe^{2+} in vivo and by Cd^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}, but not by Cu^{2+}, in vitro. The dtxR gene from C. diphtheriae was cloned, sequenced, and shown to encode a 226 amino acid polypeptide (25,316 Da). DtxR was overexpressed in E. coli and purified to homogeneity. Gel filtration chromatography, crosslinking experiments, and tests for protease-susceptibility established that DtxR is dimeric and undergoes a conformational change in the presence of divalent cation co-repressors. Gel retardation assays, DNaseI footprinting, hydroxyl radical footprinting, and methylation protection experiments demonstrated that DtxR binds to the diphtheria toxin (tox) operator symmetrically with respect to the pseudo-dyad axis of the operator. Analysis of random dtxR mutations that inactivated repressor activity and caused single amino acid substitutions in DtxR identified a predicted helix-turn-helix motif between residues 28 and 52 as the putative DNA-binding domain and a sequence that includes His-98, Cys-102, and His-106 as part of the metal-binding domain. An assay for binding of $^{63}Ni^{2+}$ by purified DtxR was developed, and the DtxR variant containing Tyr106 instead of His106 was shown to have decreased Ni^{2+}-binding activity. DtxR was crystallized in complex with various divalent cation co-repressors, and determination of their structures by X-ray diffraction is in progress. A set of DtxR-regulated promoter/operators from C. diphtheriae that was isolated in E. coli by expression cloning is being characterized. Comparison of the iron-regulated promoter/operators designated IRP-1 and IRP-2 with the tox promoter identified a palindromic 19 bp core with the consensus sequence ttAGGtTAGcctaACCTaa (capitals indicate the invariant residues). The gene downstream from IRP-1 encodes a lipoprotein that is homologous with FhuD, a ferrichrome receptor of B. subtilis. The corynebacterial lipoprotein regulated by IRP-1 is postulated to function as a ferric siderophore-binding protein for iron uptake in C. diphtheriae. A sequence upstream from an iron-regulated gene in Streptomyces pilosus is reported to be homologous with DtxR-regulated operators. DtxR has many similarities to, but differs in specificity from, the ferric uptake regulator (Fur) proteins of gram-negative bacteria. DtxR from C. diphtheriae is the prototype for a new family of iron-dependent global regulatory proteins in gram positive bacteria.

Prokaryotic Metal Metabolism/Homeostasis-II

C4-003 NICKEL METABOLISM IN *ALCALIGENES EUTROPHUS*, Bärbel Friedrich[1], Thomas Eitinger[2], Jens Dernedde[2], Christian Massanz[2], Sven Thiemermann[2], Edward Schwartz[1], [1]Humboldt University, 10115 Berlin, [2] Free University 14195 Berlin, Germany.

Nickel plays an important role in the nitrogen and energy metabolism of the chemoautotrophic proteobacterium *Alcaligenes eutrophus*. The bacterium forms three nickel enzymes: urease, membrane-bound hydrogenase (MBH) and cytoplasmic NAD-reducing hydrogenase (SH). We are studying two hydrogenases of *A.eutrophus* as a model for the biosynthesis and regulation of Ni-containing enzymes. The synthesis of MBH and SH is a complex and highly conserved pathway involving a series of ancillary proteins related to nickel metabolism. The genes coding for these functions are arranged in multiple operons and are expressed in response to various environmental signals such as external hydrogen, quality of the carbon and energy source, oxygen and the availibility of nickel (for review see 1). Nickel uptake in *A. eutrophus* is mediated by a high affinity transport protein encoded by the gene *hoxN*. Topological studies of HoxN predict an integral membrane protein with seven transmembrane helices (2). HoxN was functionally expressed in *E. coli* and significantly stimulated urease activity in a recombinant strain grown under Ni limitation. Once Ni is inside the cell it is further processed by a set of proteins (HypC,D,E (3)). Inframe deletions in *hypC,D,E* genes of *A. eutrophus* caused a pleiotropic phenotype. The resulting mutants contain catalytically inactive, Ni-free SH and MBH whereas urease is unaffected, indicating a specific function of HypC,D,E in Ni incorporation into hydrogenase. The role of the adjacent ORFs *hypA* and *hypB* in nickel metabolism remains to be defined. Recently an additional copy of each gene was mapped in the *hox* cluster of *A. eutrophus*. The structure of HypB predicts a GTP binding protein with three stretches of His-rich domains which share similarity with the urease accessory protein UreE. A function of HypB in Ni storage is discussed. The final target of Ni in hydrogenase biosynthesis are the catalytic subunits of MBH and SH. Site-directed mutagenesis of the *hoxH* gene which encodes the Ni-containing subunit of the heterotetrameric SH showed that out of 19 highly conserved amino acids two N-terminal and two C-terminal Cys residues are likely candidates for Ni-liganding residues. Analysis of maturation deficient mutants suggests that folding of the HoxH polypeptide and oligomerization to the SH holoenzyme requires cleavage of 24 amino acid residues from the C-terminal end of HoxH. This specific proteolysis is Ni dependent. To ensure coordination of the biosynthetic steps the overall process needs to be precisely regulated.

Two major transcriptional factors have been identified in the control circuit of the *hox* regulon: An alternative sigma factor (s^{54}) of RNA polymerase and the response activator HoxA. HoxA and at least one sensory protein (4) are the key components in a signal transduction pathway controlling hydrogenase expression. The role of Ni as an environmental stimulus in this pathway is currently under investigation.

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C4-004 EVOLUTIONARY RELATIONSHIPS OF METAL METABOLISM AND RESISTANCE IN BACTERIA, Simon Silver, Department of Microbiology and Immunology, University of Illinois, Chicago, IL 60612-7344

Bacterial plasmids contain highly specific systems for resistances to toxic heavy metal ions including Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, Te^{3+} and Zn^{2+}. Of these, Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} are also required micronutrients. Genetically regulated systems assure the adequate uptake of nutrients and avoid the toxic effects of excess inorganic ions. Separate transport systems (chromosomally encoded) transport essential micronutrients and different systems (often plasmid encoded) frequently efflux excess levels of the same ions . This Symposium features presentations on Fe^{3+}, Mn^{2+}, Mo_4^{2-}, Cu^{2+}, and Ni^{2+} uptake and gene regulation. I will briefly summarize the range of transport systems used for inorganic ions and of different patterns of gene regulation. These systems include simple chemiosmotic uptake transporters (for example for Mn^{2+}), single polypeptide P-type ATPases (Mg^{2+} and Cu^{2+} uptake; Cd^{2+} and Cu^{2+} efflux), newly-recognized three-polypeptide chemiosmotic efflux antiporters (for Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}), multi-polypeptide ABC Traffic ATPases (for example the Pst system for PO_4^{3-} uptake), and the arsenic efflux transporter (the first example of a family of systems that can function as one polypeptide chemiosmotic porters, or with an additional subunit, as ATP-coupled transporters. Gene regulation is equally varied, and includes single repressor protein systems (for gram positive cadmium and arsenic resistance), two polypeptide systems with major regulators and secondary minor regulatory proteins (MerD for Hg^{2+} and ArsD for oxyanions). The PcoR/PcoS system for Cu^{2+} regulation, as will be described by Cooksey and by Brown, is a two-component protein kinase sensor/responder system; the PhoR/PhoB for phosphate is another. The MerR protein, as studied by A.O. Summers, T.V. O'Halloran and others is a unique single protein positive activator. For the Czc (Cd^{2+}, Zn^{2+} and Co^{2+}) resistance system and its relatives, three regulatory genes are sometimes required, and in some cases these include representatives of a newly-recognized family of transcriptional σ factors regulating efflux or extracellular functions. In summary, the processes and gene regulation concerning inorganic cations and oxyanions are meticulously controlled the the levels of transcription and in function.

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Oxygen/Redox Regulation in Prokaryotes-I

C4-005 SIGNALING AND GENE CONTROL IN THE *soxRS* OXIDATIVE STRESS RESPONSE OF *E. COLI*. Bruce Demple, Elena Hidalgo, Ziyi Li, Beatriz Gonzalez-Flecha and Tatsuo Nunoshiba. Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115, USA

E. coli responds to the stress exerted by superoxide-generating agents, such as paraquat, and to nitric oxide, as generated by activated immune cells, by switching on a multicomponent defense system controlled by the *soxRS* locus. This *soxRS* regulon employs inducible activities that neutralize free radicals, generate NADPH, repair oxidative DNA damages and alter cellular permeability to various agents. Activation of the *soxRS* system occurs in two stages: pre-existing SoxR protein responds to an intracellular signal produced by the activating agents and stimulates transcription of the *soxS* gene. The increased expression of SoxS in turn activates the multiple promoters that comprise the *soxRS* regulon. SoxR protein is an unusual transcription factor whose stimulatory activity depends on the presence of binuclear iron-sulfur (Fe_2S_2) centers in the dimeric protein. A key question is how the activation of the SoxR metal centers occurs: possibilities include redox reaction(s) of the Fe_2S_2 clusters, and alteration of the protein's metal content. These possibilities are being explored by a combination of physical, biochemical and genetic studies, which will be discussed.

C4-006 CONTROL OF ANAEROBIC GENE EXPRESSION IN Escherichia coli: ROLES OF IRON, HEME AND MOLYBDATE. Robert P. Gunsalus, Robin Chiang, Peggy Cotter, and Silvia Daire. Department of Microbiology and Molecular Genetics, U.C.L.A., Los Angeles.

Escherichia coli, like many enteric bacteria, can utilize a variety of alternative electron acceptors for cellular respiration and energy generation. The respiratory substrates include oxygen, nitrate, nitrite, TMAO, DMSO and fumarate. Depending on the availability of one or more of these alternative electron acceptors, the cell preferentially synthesizes the terminal oxidoreductase needed for using the energetically more favorable substrate. The aerobic enzymes in E. coli include the cytochrome o oxidase (cyoABCDE), and cytochrome d oxidase (cydAB), while the anaerobic respiratory pathway enzymes include nitrate reductase (narGHJI), nitrite reductase (nir), DMSO/TMAO reductase (dmsABC), and fumarate reductase (frdABCD). We are investigating how the cell regulates expression of the respiratory pathway genes in response to the availability of i) the various respiratory substrates, and ii) the essential cofactors needed for assembly of the enzymes which include heme, non-heme iron and molybdenum. We have previously shown that the respiratory substrates are preferentially utilized in the order of oxygen > nitrate > TMAO = DMSO = fumarate (1). The aerobic/anaerobic control is provided by the ArcAB and Fnr regulatory proteins. Fnr transcriptionally regulates each of the respiratory operons while ArcAB control extends to only the cytochrome o and d oxidase genes. Expression of the anaerobic respiratory genes are regulated by the NarXLQ two-component regulatory system in response to nitrate. Cofactor availability is also an important determinant in control of respiratory gene expression. Limitation of cellular heme synthesis due to a hemA mutation results in aberrant expression of each of the respiratory pathway genes. This is seen regardless of whether the particular gene product contains a heme moiety. Interestingly, this control somehow also requires the action of the ArcAB and NarXLQ regulatory proteins although the mechanism is not well understood. Cellular iron limitation also causes reduced expression but only of the anaerobic respiratory genes, narGHJI, dmsABC, and frdABCD. These operons encode enzymes that contain multiple non-heme iron centers. The iron control appears to occur by a mechanism that is independent of Fnr, ArcAB or NarXLQ. Finally, the cell appears to regulate gene expression of the narGHJI, dmsABC, and frdABCD operons in response to molybdenum limitation. modC mutations that prevent normal molybdate uptake into the cell cause reduced transcription of the narGHJI and dmsABC genes that encode molybdenum-containing enzymes. This control was shown to be mediated by the NarXLQ regulatory proteins which in addition mediates nitrate-dependent expression of these genes.

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C4-007 THE OXYR REGULON, Gisela Storz, Shoshy Altuvia, Michel B. Toledano and Ines Kullik, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, Bethesda, MD 20892

Treatment of bacterial cells with low doses of hydrogen peroxide results in the induction of distinct groups of proteins that protect against killing by higher doses of hydrogen peroxide. The expression of at least nine hydrogen peroxide-inducible proteins including catalase, an alkyl hydroperoxide reductase, glutathione reductase, and Dps[1], a non-specific DNA binding protein, is controlled by the transcriptional regulator OxyR. *In vitro* transcription experiments with purified components showed that the oxidized but not the reduced form of the OxyR protein activates transcription, suggesting that oxidation of OxyR brings about a conformational change that leads to RNA polymerase activation. Recent studies on the DNA binding properties of OxyR have also shown that oxidized and reduced OxyR have different binding specificities[2]. Hydrogen peroxide treatment also causes the induction of a 109-nucleotide, non-coding RNA. This small RNA, denoted OxyS, acts as a regulator to activate and repress the expression of as many as 1% of the *E. coli* genes, including *uhpT* (a hexose phosphate transporter) and *fhlA* (an anaerobic transcription factor). The mechanism of this regulation by an RNA is currently being investigated. In summary, the genetic response to hydrogen peroxide in bacteria involves at least two distinct regulatory mechanisms: OxyR, a protein activator that is turned on by oxidation, and OxyS, a 109 nucleotide RNA that acts as both an activator and a repressor of gene expression.

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[2]Toledano, M. B. Kullik, I. Trinh, F. Baird, P. T. Schneider, T. D. and Storz, G. (1994) *Cell* **78**, 897-909.

C4-008 CORRELATION BETWEEN IRON METABOLISM, SUPEROXIDE DISMUTASE EXPRESSION AND OXIDATIVE STRESS IN *E. COLI*., Danièle Touati, Micheline Jacques, Sophie Despied, Brigitte Tardat and Laurence Bouchard, Institut Jacques Monod, CNRS-Université Paris VII, 2 place Jussieu, 75251 Paris cedex 05, France.

Iron and oxygen are essential for most living organisms, but their association is a source of problems. In oxygen-containing environment, iron is readily oxidized to insoluble ferric forms which render it scarcely available. In addition iron in conjunction with oxygen is a generator of highly deleterious oxygen species, such as hydroxyl radical. To cope with metabolic needs and avoid toxicity the processes of iron assimilation and storage are tightly regulated. In *E. coli* the Fur protein which acts with ferrous iron as cofactor as a transcriptional repressor, regulates all genes involved in iron assimilation. It also regulates expression of genes with general functions and the superoxide dismutase, thus coupling iron homeostasis with general metabolism and defense against oxygen toxicity.

A permanent deregulation of iron assimilation in a Δ*fur* mutant produces an oxidative stress consecutive to an intracellular iron overload. Δ*fur* mutants are oxygen sensitive in absence of recombiational DNA repair(*recA or recB*. mutants), show an increased spontaneous mutagenesis oxygen dependent, are sensitive to hydrogen peroxide. Iron chelator (ferrozine), surexpression of iron sequestering molecules(ferritin H-like protein), and block of ferric iron transport reduce iron intracellular iron concentration under the toxicity threshold.

Superoxide dismutase (SOD) deficiency results in an oxidative stress producing DNA damage: increased spontaneous mutagenesis, sensitivity to hydrogen peroxide, oxygen sensitivity of *sodAsodB recA* mutants. Block of ferric iron transport , but neither overexpression of iron storage protein nor iron chelation suppress oxygen sensitivity, indicating that increased superoxide-mediated reduction of ferric iron and not iron overload is responsible for toxicity.

Ferrous iron directly imported by *feo*- dependent transport system contributes in a minor way to iron toxicity in Δ*fur* mutants, but plays an important role for signaling iron scarcity. Elements participating to and rational for the coupling between iron metabolism and defense against oxygen toxicity will be further discussed.

Oxygen/Redox Regulation in Prokaryotes-II

C4-009 A HEME-BASED SENSOR OF OXYGEN, Marie A. Gilles-Gonzalez[1], Gonzalo Gonzalez[1], and Max F. Perutz[2], [1]The Ohio State University, Columbus, [2]Laboratory for Molecular Biology, Cambridge, UK.

Oxygen is a major biological regulator. In *Rhizobia*, the reduction in oxygen that results from the exchange of a vegetative lifestyle for a symbiotic one triggers nitrogen fixation, a process requiring the expression of more than 20 new gene products. We present the first characterization of a biological switch based on oxygen. The *Rhizobium meliloti* and *Bradyrhizobium japonicum* FixL's are heme proteins and they are also kinases. Oxy-FixL is an inactive kinase. At low oxygen pressure and high ATP/ADP ratio, deoxy-FixL transfers a phosphoryl group to transcription factor FixJ, enabling it to activate a cascade of *nif* and *fix* gene expression. Our studies show that the switch affecting FixL's kinase activity is a small motion of the heme iron during its transition from low to high spin. We propose that such heme-based sensors may have evolved to respond physiologically to a variety of heme ligands in addition to oxygen, including carbon monoxide and nitric oxide.

C4-010 REGULATION OF *sodA* EXPRESSION IN *Escherichia coli* BY FNR, FUR AND ARC: ROLES OF IRON, MANGANESE AND OXYGEN.
Hosni M. Hassan, North Carolina State University, Microbiology Department, Raleigh, N.C. 27695-7615.

In *Escherichia coli* the expression of the *sodA* gene, encoding the manganese-containing superoxide dismutase (MnSOD), is under rigorous control at both the transcriptional and post-transcriptional levels. The enzyme is induced in response to a variety of environmental stimuli including exposure to oxygen, redox cycling compounds, iron deprivation, and chemical oxidants. Studies using regulatory mutants revealed the roles of the *fur* and *arcA* gene products in the regulation of *sodA*. The role of FNR was deduced from the fact that the *arcA fur* double mutant was still inducible by 2,2'-dipyridyl; thus, the triple mutant *arcA fur fnr* was fully derepressed anaerobically with respect to *sodA* expression. We have also identified the role of IHF (Integration-Host Factor) in the regulation of *sodA*. Studies using UV-and spontaneous mutants have helped in identifying the cis-acting regulatory elements of *sodA*. We have also shown that the transcription of *sodA* is influenced by the degree of DNA-supercoiling. The positive regulatory role of SoxRS has been identified by other workers (B. Demple and his group and B. Weis and his group). The expression of *sodA* is regulated by three iron-dependent regulatory proteins: FUR, FNR and SoxRS. The roles of iron and manganese on the transcriptional and post-transcriptional regulation of *sodA* was also discovered. A conceptual model will be presented to accommodate these findings and explain the coordination of MnSOD biosynthesis with iron-uptake and the cell's ability to switch between anaerobic and aerobic life-style without suffering from oxygen toxicity.

C4-011 INDUCTION OF THE BACTERIAL *mer* OPERON BY EXPOSURE TO METALLIC MERCURY VAPOR, Hg(0): THE ROLE OF CATALASE/HYDROPEROXIDASE IN *Escherichia coli.* Fan Lee and Anne O. Summers, Department of Microbiology, The University of Georgia, Athens, GA 30602-2605.

The bacterial mercury resistance (*mer*) locus encodes several proteins including the enzyme, mercuric reductase (MerA), which detoxifies the reactive heavy metal ion, Hg(II), by reducing it to less reactive, mercury metal vapor, Hg(0). Synthesis of MerA and the other products of the *mer* operon is under transcriptional control by the regulatory protein, MerR, which represses synthesis of the structural gene mRNA in the absence of Hg(II) and activates it when Hg(II) is present. Once the MerA protein is synthesized it reduces Hg(II) to Hg(0), thus removing the inducer, and repression of the operon is restored. Surprisingly, when the structural gene promoter, P_{TPCAD}, is fused either to the *lacZ* or *luxAB* genes and the *merA* gene is deleted (*mer-lac* or *mer-lux, ΔmerA*) expression of the reporter genes can be induced by exposing the cells to metallic Hg(0) vapor. This paradoxical induction by Hg(0) vapor, ordinarily the product of the *mer* detoxification process, is dependent on the wildtype *merR* gene product. At least one gene encoded by the host cell chromosome, the multifunctional catalase/hydroperoxidase, HPI (product of the *katG* gene), is also required for efficient induction of the *mer* operon by Hg(0) vapor. *E.coli* strains mutant in *katG* induce a *mer-lux* fusion 3- to 5-fold more slowly than the parental strain in response to Hg(0), although they are not impaired in normal induction by $HgCl_2$. Moreover, a distinct *katG* regulatory mutant which over-expresses HPI is also enhanced in its ability to induce a *mer-lux* fusion in response to Hg(0). The basis for the role of KatG in Hg(0) induction of the *mer* operon is suggested by earlier observations of Hg(0) oxidation to Hg(II) by human and rat catalases. Although mechanistic details have not been defined, these eukaryotic heme-containing hydroperoxidases are thought to oxidize Hg(0) via a ferryl radical intermediate. Current studies focus on a kinetic characterization of Hg(0) oxidation by *E. coli* catalase and other hydroperoxidases and on their roles in induction of the *mer* locus by metallic mercury vapor.

Oxygen/Redox Regulation in Eukaryotes-I

C4-012 NF-κB: A TRANSCRIPTION FACTOR CONTROLLED BY INTRACELLULAR HYDROGEN PEROXIDE LEVELS, Patrick A. Baeuerle[1], Kerstin Schmidt[1], Paul Amstad[2], Peter Cerutti[2], and Britta-Mareen Traenckner[1], [1]Institute of Biochemistry, Albert-Luwigs-University, D-79104 Freiburg, Germany, [2]Swiss Cancer Research Institute, CH-1066 Épalinges/Lausanne, Switzerland.

Inducible transcription is frequently governed by transcriptional activator proteins that bind de novo to cis-regulatory elements. One important activator is NF-κB because it activates a plethora of genes in response to primarily pathogenic conditions. These include viral and bacterial infections, chemical and physical stresses and inflammatory cytokines. Among the target genes of NF-κB are cytokines, chemokines, hematopoetic growth factors, cell adhesion molecules and death genes. With this panel of activators and target genes, NF-κB is a central genetic switch in the early pathogen response. The transcription factor provides an attractive drug target to prevent toxic/septic shock, graft vs. host reaction, chronic inflammations, neurodegenerative diseases and malignant lymphoproliferation.

We have extensively analyzed the processes underlying the activation of NF-κB following pathogenic stimulation of cells. In unstimulated cells, the transcription factor is complexed to an inhibitory subunit, IκB. In the course of activation IκB is rapidly phosphorylated. This covalent modification does not release IκB but renders the protein susceptible to degradation by the multicatalytic 26S proteasome in the cytosol. The rapid and efficient decay of IκB ultimately releases active NF-κB which can migrate into the nucleus and initiate mRNA synthesis upon binding to regulatory sites in genes. While the regulation of many genes by NF-κB can be explained by the presence of high affinity binding sites for the factor in promoters and enhancers, it is very difficult to understand how very diverse conditions all cause IκB to be degraded in the cytosol. The strong inhibitory effect of many structurally unrelated antioxidative compounds on this reaction suggested to us that reactive oxygen intermediates (ROIs) serve as common intracellular messengers of NF-κB activation. This hypothesis gained support from numerous reports demonstrating that many NF-κB inducers lead to an increased production of H_2O_2 and superoxide within cells. Direct evidence for NF-κB being an oxidative stress-responsive transcription factor came from the finding that μ-molar concentrations of H_2O_2 strongly induced DNA binding and transcriptional activity of NF-κB in T cell lines, HeLa cells and endothelial cells.

Very recent data from our lab showed that overexpression of catalase interferes with NF-κB activation by TNF and okadaic acid. Cytosolic Cu/Zn superoxide-dismutase, an enzyme generating more H_2O_2 from superoxide in the cytosol, had the opposite effect. This enzyme caused a superinduction of NF-κB. This lends strong molecular genetic support to the involvement of H_2O_2, but not directly superoxide, as common intracellular second messenger of NF-κB activation. How H_2O_2 triggers the phosphorylation and subsequent depletion of IκB is not known. In the simplest scenario, H_2O_2 modulates the activity of a kinase and/or phosphatate by addition of glutathione to crucial cysteine residues, a reaction commonly observed in the cytosol upon H_2O_2-induced oxidative stress.

C4-013 TRANSCRIPTION REGULATION OF GLUTATHIONE S-TRANSFERASE GENE EXPRESSION BY OXIDATIVE STRESS, Truyen Nguyen, Thomas Rushmore* and Cecil B. Pickett, Schering-Plough Research Institute, Kenilworth, New Jersey, Merck Research Laboratories, West Point, Pennsylvania

Transcriptional activation of the rat liver glutathione S-transferase (GST) Ya subunit gene by xenobiotics is mediated by at least 2 *cis*-acting regulatory elements, the XRE and ARE, found in the 5'-flanking region of this gene.(1) The XRE contains a core sequence 5'-TNGCGTG-3' that is also found in multiple copies in the cyp1A1 gene and mediates an inducible response to planar aromatic compounds such as TCDD, benzo(a)pyrene, ß-naphthaflavone, and 3-methylcholanthrene. The ARE is a unique *cis*- acting element distinct from the XRE and is responsive to metabolizable planar aromatic compounds. In addition, the ARE also mediates gene activation in response to phenolic antioxidants (eg. *tert*-butylhydroquinone) and to oxidants such as H_2O_2 and menadione, suggesting a possible mechanism of activation in response to oxidative stress. Using point mutation and transfection experiments, the ARE core sequence required for xenobiotic inducibility in HepG2 cells has been identified as 5'-GTGACAAAGC-3'.(2) This core sequence has also been demonstrated to be directly involved in the interaction with a *trans*-acting factor found in the nuclear extracts prepared from HepG2 cells by *in vitro* binding assays. Based on the similarity in sequence between the ARE and consensus AP-1 binding site (5'-TGACTCA-3') as well as the recent demonstration of redox regulation of AP-1 activity, we investigated whether members of the AP-1 family of transcription factors can act through the ARE.

Our transfection data show that the phorbol ester, TPA, a known inducer of AP-1 activity, modestly activates transcription through the ARE as compared to t-BHQ. In gel retardation assays, however, the dimerized Jun/Fos protein (i.e. AP-1) binds readily to the AP-1 binding site but not to the ARE. When the two A nucleotides underlined in the ARE core sequence (5'-GTGACAAAGC-3') are changed to -TC-, this ARE sequence (ARE-TRE) becomes a high-affinity AP-1 binding site and is responsive to both TPA and tBHQ.(3) Mutation of the -GpC- dinculeotide at the 3'-end of this sequence abolishes its xenobiotic inducibility but has no effect on its TPA-responsiveness or as a high-affinity AP-1 binding site. These data suggest that transcriptional activation of the GST-Ya subunit gene through the ARE in response to oxidative stress is mediated via a unique signal transduction pathway and not directly by known members of the AP-1 family.

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Oxygen/Redox Regulation in Eukaryotes-II

C4-014 OXIDATIVE STRESS IN HUMAN CELLS: ROLE OF THE HAP1 PROTEIN. Ian D. Hickson, Gil Barzilay, Lisa J. Walker and Dominic Rothwell. Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, U.K.

The HAP1 protein (also called APE/Ref-1) is a human nuclear enzyme involved in both repair of oxidative DNA damage and redox regulation of transcription factor function. HAP1 is the major enzyme in human cells that repairs apurinic/apyrimidinic (AP) sites in DNA and also possesses the ability to alter the redox state of a cysteine residue in the DNA binding domain of several proto-oncogene products (e.g. c-Jun, c-Myb). We have shown that the DNA repair and redox functions of the HAP1 protein are directed by different domains of the protein and that the repair function alone requires divalent cations. The N-terminal domain contains the redox active site centred at cysteine-65, while the DNA repair active site residues are in a large C-terminal domain. The respective active sites for the two functions have been mapped to specific amino acid residues and the role of metal ions in the repair reaction of HAP1 has been clarified. The three-dimensional structure of the DNA repair domain has been predicted from the crystal structure of the closely related *E. coli* homolog of HAP1 (exonuclease III). The proposed mechanism by which HAP1 catalyzes phosphodiester bond cleavage will be presented. Depletion of HAP1 via antisense RNA expression sensitizes cells to several different forms of oxidative stress. Consistent with a protective role for HAP1 in human cells, expression of the HAP1 mRNA and protein is increased under oxidative stress conditions.

C4-015 METAL REGULATION OF HEAT SHOCK FACTOR ACTIVITY, Richard I. Morimoto, Michael Kline, Anu Mathew, Sameer Mathur, Sanjeev Satyal, Yanhong Shi, and Lea Sistonen. Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, Il. 60208.

The transcriptional activation of heat shock (HS) genes has provided a useful paradigm to understand how cells detect and respond to a range of environmental and physiological stresses including heat shock, oxidative stress, and transition heavy metals. The elevated synthesis of the stress-induced heat shock proteins (hsp) and molecular chaperones affords a protective mechanism to the cell by regulating the transport, translocation, and folding of proteins. At the transcriptional level, the response to stress is mediated by a family of heat shock factors (HSF) which are ubiquitously expressed and negatively regulated. HSF1, the predominant stress responsive factor is activated in response to heat shock, heavy metals (Cu, Zn, Cd), oxidative stress. Activation of HSF1 involves multiple steps including oligomerization, acquisition of DNA binding activity, translocation, and phosphorylation. Acquisition of DNA binding and oligomerization are linked and uncoupled from stress-inducible serine phosphorylation and transcriptional activation of HSF1. During attenuation of the heat shock response, HSF1 associates with specific heat shock proteins which provides a possible mechanism for autoregulation and recovery from stress. Another member of the HSF gene family, HSF2 is developmentally regulated, for example, during early mouse development and spermatogenesis and does not respond to physiological stress. The activation of HSF2 has been studied in some detail in human K562 erythroleukemia cells following treatment with hemin, which results in the conversion of HSF2 non-DNA binding dimers to the active DNA-binding trimeric state. The effects of hemin on HSF2 and consequently on hsp70 and hsp90 gene transcription are delayed, yet the activated state of the factor can be maintained for a period of 48-72hrs after which attenuation occurs and only the dimeric state is detected.

Zinc and Copper in Gene Expression

C4-016 STRUCTURE AND FUNCTION OF ZINC-CONTAINING TRANSCRIPTION FACTORS, Joseph E. Coleman, Kevin H. Gardner, Stephen F. Anderson, Matthew Junker and Karla, K. Rodgers, Dept. of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510.

Transcription factor IIIA, required for activating the transcription of the 5S RNA gene in Xenopus oocytes (Hanas, et al., J. Biol. Chem. 258, 14120, 1983) was the first of over 1000 proteins functioning in gene expression now recognized to be zinc proteins. The presence of zinc is required for most of these proteins to recognize specific DNA sequences. TFIIIA contains 9 sequential segments of amino acid sequence, -C-X_{4-5}-C-X_{12-13}-H-X_{3-4}-H-, each coordinating a Zn ion in a S_2N_2 tetrahedral complex, the motif called a "zinc finger" (Miller et al., EMBO J. 4, 1609, 1985). Recent hybridizations of human chromosomal DNA libraries with oligonucleotides coding the H-X_3-H-TGEKP-YE/K of the Kruppel-type zinc fingers have revealed the presence of 300-700 different zinc finger proteins of the C_2H_2 ligand type. This has led to suggestions that as much as 1% of human genomic DNA may code for zinc finger proteins. With continuing investigation, 8 or 10 zinc binding motifs, different from the zinc finger structure found in TFIIIA, have been found in eukaryotic transcription factors and structurally characterized. Seven of these motifs are listed below.

ZINC-BINDING MOTIF	NUMBER OF SITES	LIGANDS TO ZINC
1. CLASSICAL ZINC FINGER PROTEINS	1-37	CCHH
2. HORMONE RECEPTORS	2	CCCC and CCCC
3. ZINC CLUSTER TRANSCRIPTION FACTORS	2	Zn_2CYS_6 Binuclear Cluster
4. ZINC RING FINGER	2	CCCH and CCCC
5. LIM MOTIF	2	CCCC and CCCH
6. ZINC RIBBON STRUCTURE IN TFIIS	1	CCCC
7. TRANSCRIPTION FACTOR GATA-1	1	CCCC

The zinc-binding subdomains of all 7 classes have been cloned and overproduced and either a crystal structure or a solution structure (by NMR methods) has been determined. DNA binding studies show that zinc binding and DNA binding domains of the transcription factors overlap and that zinc induces the protein fold required to specifically recognize DNA. The structures of 7 different zinc-binding motifs found in eukaryotic transcription factors will be briefly outlined. Detailed discussion of the structure and its relation to function will be presented for the zinc cluster transcriptional activators, GAL4, LAC9 and HAP1, the transcriptional repressor, UME6 (a repressor of early meiotic genes) and the zinc ring finger protein, RAG1, required for VDJ recombination of antibody genes.

C4-017 COPPER REGULATION IN *PSEUDOMONAS*, Donald A. Cooksey, Department of Plant Pathology, University of California, Riverside, CA 92521-0122.

Copper-responsive transcriptional regulation has been investigated in plant pathogenic bacteria that are periodically exposed to high levels of copper salts used for plant disease control. Resistance to copper is determined by an operon (*copABCD*) that is usually plasmid borne in *Pseudomonas syringae* and in the related plant pathogen *Xanthomonas campestris*. The *cop* operon is also related, although distantly, to the *pcoABCD* operon of *Escherichia coli*. Although the structural genes are related, these systems differ considerably at both a functional and regulatory level. In *P. syringae*, a two-component regulatory system has been described, consisting of a putative copper sensor, CopS, and a transcriptional activator, CopR. Mutagenesis of *copS* is being performed to examine membrane topology and important domains for copper sensing and signal transduction. Mutations resulting in single amino acid changes in two domains of CopS caused overexpression of the *cop* promoter *in trans*. These mutations were near the probable histidine kinase domain and in another region near the carboxyl terminus that is conserved in several sensor proteins of two-component systems. CopR was purified and was shown to bind to a conserved domain (*cop* box) in both plasmid and chromosomal copper-inducible promoters of *P. syringae*. The *cop* box spans the -35 region with respect to the site of transcriptional initiation for the *copABCD* operon in *P. syringae*, and it contains an inverted repeat that may indicate the binding of CopR as a dimer. CopR did not bind to the promoter region of the *cop* operon from *X. campestris*, however, and a *cop* box was not identified in that sequence. This promoter from *Xanthomonas* did not function in *Pseudomonas*, and a much larger upstream sequence was required for copper-inducible activity than is known from the *cop* promoters from *P. syringae*. Included in this upstream region from *Xanthomonas* was an open reading frame, designated *copL*, that would encode a protein of 122 amino acids. A constitutive transcriptional start site was mapped 5' to *copL*, and a protein identified in crude extracts of *X. campestris* binds to this 5' region. Several mutagenesis experiments suggested that *copL* is needed for full copper-inducible expression of the *cop* operon in *X. campestris*, but mutations in *copL* could not be complemented *in trans*. Several large hairpin structures were predicted from analysis of folding of *copL* mRNA, suggesting that this region may function in attenuation of *cop* gene expression in *Xanthomonas*. The process of translation of *copL* might release transcriptional attenuation, which would be consistent with its *cis*, rather than *trans*, activity.

C4-018 STRUCTURAL STUDIES OF THE ADR1 DNA BINDING DOMAIN, Mia Schmiedeskamp, Bradley Bernstein, Peter Bowers, Angeline Kantola, Ross Hoffman, and Rachel E. Klevit, University of Washington, Seattle, WA 98195.

ADR1 is a zinc-containing yeast transcriptional activator that regulates alcohol dehydrogenase II expression. While the native ADR1 protein has 1323 residues, its DNA binding activity arises solely from a relatively short sequence at the amino terminus. The minimal DNA binding domain defined by deletion mutagenesis consists of about seventy-seven residues and includes a twenty-residue basic region and two Cys_2His_2 zinc finger motifs. The N-terminal basic region is required for high affinity DNA binding, while the zinc fingers are responsible for sequence specificity. The structures of the individual zinc finger motifs have been studied extensively using synthetic peptides and homonuclear ^1H NMR experiments. In addition a polypeptide encompassing the entire minimal DNA binding domain, called ADR1z, has been overexpressed in *E. coli*, allowing preparation of protein that is uniformly or specifically labeled with the NMR-active isotopes, ^{15}N and ^{13}C. NMR studies of ADR1z in both the presence and absence of its cognate DNA site will be presented.

C4-019 YEAST COPPER METALLOREGULATORY TRANSCRIPTION FACTORS, Dennis J. Thiele, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan, 48109-0606.

In all organisms studied to data, the redox-active metal copper is an essential component for catalytic function of a wide variety of enzymatic reactions including cytochrome oxidase, Cu, Zn superoxide dismutase, tyrosinase and lysyl oxidase. This same redox activity, however, renders copper an extremely potent cytotoxin due to its ability to engage in chemical reactions which generate destructive free radicals such as the hydroxyl radical. The essential yet toxic nature of copper dictates that all cells must possess mechanisms to both sense and appropriately respond to small fluctuations in copper levels to allow sufficient copper accumulation for biochemical reactions but to prevent the accumulation to toxic levels. The metallothionein (MT) proteins are a class of low molecular weight, cysteine-rich metal binding proteins which efficiently bind and sequester toxic metals and therefore constitute a critical component of the copper detoxification machinery in most organisms studied to date. Our laboratory has isolated the genes encoding copper-sensing metalloregulatory transcription factors ACE1 and AMT1 from the yeasts *Saccharomyces cerevisiae* and *Candida glabrata* respectively. These proteins are copper-activated sequence-specific monomeric DNA binding proteins which possess potent transcriptional activation function when bound to target gene promoters. Although the *ACE1* gene is constitutively expressed, the *AMT1* gene in *C. glabrata* is rapidly positively transcriptionally autoregulated and strains which are specifically defective in *AMT1* autoactivation fail to resist exposure to normally non-toxic levels of copper. The molecular interactions of AMT1 with *AMT1* promoter DNA, and *cis*-acting elements outside of the AMT1 binding site, play critical roles in the rapid autoactivation of the AMT1 metalloregulatory transcription factor gene. These studies demonstrate that rapid responses to toxic metals in yeast pivot on the sensory function of copper metalloregulatory transcription factors.

Prokaryotic Responses to Toxic Metals

C4-020 REGULATION OF CADMIUM RESISTANCE, Anita. L. Linet. Wayne State University School of Medicine, Detroit, MI 48201.

The *cadA* cadmium resistance determinant from *Staphylococcus aureus* plasmid pI258 contains two genes, *cadC* and *cadA*. Resistance encoded by this system is induced by a number of divalent cations, including Zn^{2+}, Bi^{2+}, Pb^{2+} and Co^{2+} in addition to Cd^{2+} (1). Recent data indicates that CadC protein is the transcriptional regulator of this system. Gel shift assays of the *cadA* operator/promoter DNA exhibit a specific association with CadC protein and release of CadC from the DNA was accomplished with the addition of Bi^{2+}, Pb^{2+} or Cd^{2+}. The CadC protein protects a region from nucleotides -7 to +7 of the operator/promoter as shown by DNA footprinting assays. Run-off transcription assays using the operator/promoter and the first 69 nucleotides of cadC gave the predicted mRNA product. Addition of the CadC protein to the assays inhibited *in vitro* transcription.

The second gene, *cadA*, encodes a protein that is a member of the P-ATPase class of ion-translocating enzymes. The N-terminal region of the predicted amino acid sequence contains a region of sequence similarity to other metal-binding proteins including the prokaryotic mercuric reductases and the putative human Menkes and Wilson disease proteins believed to be involved in copper metabolism. Within this conserved motif are two conserved cysteine residues in the pattern Cys-X-X-Cys. Site-directed mutagenesis was used to change each of the conserved cysteine residues to glycine and to serine. Mutants were characterized by cadmium transport into everted membrane vesicles made from *E. coli* cells containing the mutant proteins and by uptake into whole cells. Transport activity in everted vesicles of the mutants was decreased to 10 to 30% of wild-type activity. However, the reduction of cadmium uptake into whole cells that is seen with the wild-type protein was also present with the mutant proteins, suggesting that sufficient efflux activity remained in the mutant proteins to maintain the lower intracellular levels of cadmium seen with the wild-type protein. These results support the hypothesis that the cysteines are involved in protein function but that they are not required for efflux activity. We suggest that the N-terminal cysteine residues are not the substrate binding site for the cadmium ion that is transported. Instead, we propose that these residues may bind cadmium irreversibly as an activator of catalysis.

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C4-021 **REGULATION OF THE ARSENICAL RESISTANCE OPERON.** Barry P. Rosen, Weiping Shi and Jianhua Wu, Department of Biochemistry, Wayne State University, School of Medicine, Detroit, MI 48201.

Resistance to toxic oxyanions of arsenic and antimony in bacteria is conferred by conjugative resistance plasmids which carries *ars* operons (see [1] for a general review). The *ars* operon of resistance factor R773 encodes an oxyanion-translocating ATPase when expressed in *Escherichia coli*. Expression of the operon is inducible by arsenite and antimonite. The operon has five genes, including two regulatory (*arsR* and *arsD*) and three structural genes (*arsA*, *arsB* and *arsC*). The 13 kDa ArsR protein forms an arsenite-inducible dimeric repressor that controls the basal level of expression of the operon. It is a trans-acting regulatory protein that binds as a dimer to the operator region of the *ars* promoter. The 13 kDa ArsD protein apparently serves as a low affinity substrate-independent repressor that controls the upper level of operon expression. The ArsD protein is postulated to prevent overproduction of the ArsB protein, an inner membrane protein which is toxic when produced in high amounts. The concerted action of these two proteins forms a regulatory circuit that provides homeostatic control of the levels of the *ars* gene products, where the ArsR repressor sets the floor for expression, and the ArsD repressor sets the ceiling.

The ArsR protein is the first identified member of a newly recognized family of metal-responsive regulatory proteins, including cadmium, zinc, arsenic and antimony. To identify the inducer binding domain of the ArsR repressor, a positive selection involving resistance to expression of a gene for a toxic product was devised for mutant ArsR proteins that still formed an active repressor but no longer responded to inducer. Three mutants were isolated involving two cysteinyl residues in the ArsR protein. Mutants C32Y, C32F, and C34Y each had a noninducible phenotype. Each mutant protein was still capable of binding at the operator site on the DNA, but the response to inducer in vitro was greatly reduced. Thus this cysteine pair may be the inducer binding site. This cysteine pair is highly conserved in members of the ArsR family and is likely a portion of the metal responsive site in each.

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Eukaryotic Metal Metabolism/Homeostasis-I

C4-022 THE COPPER BINDING ATPASE DEFECTIVE IN WILSON DISEASE: FUNCTIONAL INFORMATION FROM MUTATIONS, Diane W. Cox, Gordon R. Thomas, John R. Forbes, and Jing Shi Wu, Research Institute, The Hospital for Sick Children and Departments of Molecular and Medical Genetics, and of Paediatrics, University of Toronto, Toronto, Canada.

Copper is an essential trace metal, which, like other heavy metals, can be toxic when tissues become overloaded. Effective means have evolved for maintaining a balance between copper absorption and excretion. Two disorders of copper transport are shedding light on the normal transport of copper: Menkes disease, an X-linked disease in which copper is not transferred out of intestinal cells, and Wilson disease.

Wilson disease (hepatolenticular degeneration) is an autosomal recessive disorder of impaired copper transport, in which copper accumulates, particularly in liver, brain, kidney and cornea. We have recently cloned the complete gene (*ATP7B*), predicted to be a copper binding ATPase (Bull et al, Nature Genet. 5:327, 1993) with 6 potential copper binding sites. Evidence from other laboratories suggests that efflux from the liver is ATP dependent, in support of the predicted protein model. The gene shows high homology with the Menkes disease gene (*ATP7A*). We have determined the structure of the Wilson disease (*ATP7B*) gene, which includes 22 exons in a region of about 80 kb. The most 3' exon differs between kidney and liver transcripts. Identification of all intron-exon boundaries has made possible the identification of mutations, using amplication of exons and single strand conformation polymorphism (SSCP) analysis. The severity of specific mutations is dependent upon the degree of conservation in the residue changed, the type of mutation, and the occurrence of the mutation in relation to alternately spliced exons. The calcium transporter from sarcoplasmic reticulum has been extensively studied and is helpful in interpreting the effect of mutations. While most patients are compound heterozygotes, with two different mutations, for 9 mutations we have been able to study the affected mutations in homozygotes. This is providing insight into the most important regions of the *ATP7B* gene. A mutation in Iceland due to a 7 bp deletion occurs in an alternately spliced region, which seems to modify disease severity. Alternate splicing occurs in the same positions in the Menkes and Wilson disease genes, suggesting that the alternate products may have a functional role.

We have cloned the homologous gene (*Atp7b*) in the rat, and have found a high degree of similarity, with the exception of absence of the fourth copper binding site. We have identified a deletion of several kb in the 3' end of the *Atp7b* gene of the mutant LEC rat, which is an excellent model for Wilson disease. The *ATP7A* and *ATP7B* genes show high similarity in their important functional regions with bacterial P-type ATPases, suggesting the importance of this type of transport system for all organisms. Furthermore, the recent identification of a homologous gene in yeast as a copper transporter, essential for iron transport, provides direct evidence for the interrelationship between copper and iron metabolism.

C4-023 COORDINATE, COPPER-RESPONSIVE EXPRESSION OF CYT C6, COPROPORPHYRINOGEN OXIDASE AND A COPPER UPTAKE SYSTEM, Sabeeha Merchant, Kent Hill, Jeanette Quinn and Hong Hua Li, Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024-1569.

The biosynthesis of plastocyanin (an abundant, copper-containing electron transfer protein in chloroplasts) and cyt c6 (a heme-containing substitute for plastocyanin) is regulated in a reciprocal fashion by a metal-sensing system: the presence of copper promotes plastocyanin synthesis but represses cyt c6 synthesis, whereas the absence of copper prevents plastocyanin accumulation but induces cyt c6 transcription, thus assuring the availability of one or the other of these structurally distinct, but functionally equivalent, proteins for photosynthetic electron transfer. In the case of plastocyanin, the last step of the biosynthetic pathway - copper insertion - is the critical one; when copper-deficiency precludes holoprotein formation, the apoprotein is rapidly degraded in vivo. In the case of cyt c6, regulation by copper occurs at the first step of its biosynthesis - initiation of transcription. The system is remarkable for its high sensitivity, selectivity for copper and its response range: in a copper-deficient cell, where cyt c6 function is essential for the continued operation of the electron transfer pathway, a greater than thousand-fold differential in the abundance of cyt c6-encoding messages is observed compared to a fully copper-supplemented cell where plastocyanin is functional. The transcriptional response to copper is effected via at least two redundant copper responsive elements (CuREs) that lie between nucleotides -127 and -56 relative to the start site of transcription and function as activators of transcription in copper-deficient cells. In addition to transcriptional activation of pre-apocyt c6 synthesis, other adaptations to copper-deficiency will be described. These occur coordinately with induction of cyt c6 accumulation and include the induction of an apoplastocyanin degrading activity and a high affinity copper uptake system (to increase the concentration of intracellular copper in response to nutritional deficiency), and induction of coproporphyrinogen oxidase (to increase flux through the tetrapyrrole biosynthetic pathway in response to the increased demand for heme).

C4-024 A GENETIC APPROACH TO THE STUDY OF THE REGULATION OF FERRITIN SYNTHESIS BY THE IRON REGULATORY PROTEIN, William E. Walden, Dept. Microbiology/Immunology, University of Illinois at Chicago, Chicago, IL 60612.

Synthesis of ferritin is regulated in animal cells in coordination with iron availability. This regulation is mediated by a sequence specific RNA binding protein, the iron regulatory protein (IRP). The IRP also mediates post-transcriptional regulation of transferrin receptor (TfR) synthesis in response to iron. The IRP binds a conserved 28 nucleotide sequence/structure (the iron responsive element; IRE) which is present in a single copy within the 5' UTR of ferritin mRNAs and in 5 copies in the 3' UTR of the TfR mRNA. In binding the IRE, the IRP mediates translational regulation of ferritin mRNA while it regulates the stability of the TfR mRNA, resulting in a reciprocal and coordinated regulation of iron storage and transport. In addition to its function as an RNA binding protein, the IRP is also an aconitase. This latter activity requires the assembly of an iron-sulfur cluster in the IRP which also inactivates the IRP for IRE binding. Thus, a mechanism for regulating gene expression through the IRP is via assembly and disassembly of an iron-sulfur cluster. We are investigating structure/function relationships in the IRP and the relationship of the multiple activities of the IRP to gene regulation and iron metabolism using yeast molecular genetic approaches. Mammalian IRP functions both as a translational repressor and as an aconitase in *Saccharomyces cerevisiae*. Translation of mRNAs containing IREs is specifically repressed by co-expression of the IRP in yeast cells. Repression is dependent on the specific IRP/IRE interaction, and it is IRP dose dependent. We are using this system to examine a variety of IRP mutations for their effects on IRE binding. The IRP also partially complements aconitase mutations in yeast. IRP mutations which eliminate its aconitase activity without affecting its RNA binding activity also eliminate its ability to complement aconitase minus strains. Several mutants have been isolated which enhance the ability of the IRP to complement aconitase minus yeast. A number of these mutants show an increased proportion of the IRP that is in the form of aconitase, suggesting that the mutations are in genes that affect either iron metabolism or the assembly of iron-sulfur clusters. Further analysis of these mutants is underway. The level of IRP aconitase activity is also modulated by iron status in yeast. Cells grown under iron limited conditions display lower IRP aconitase activity than cells grown under normal growth conditions. These results demonstrate that, similar to what has been shown in animal cells, iron-sulfur cluster assembly in IRP expressed in yeast is also regulated in coordination with iron availability.

Eukaryotic Metal Metabolism/Homeostasis-II

C4-025 MOLECULAR RESPONSE TO NICKEL: INHERITED FFFECTS ON TRANSCRIPTION, Max Costa, Department of Environmental Medicine and Kaplan Comprehensive Cancer Center, New York University Medical Center, 550 First Avenue, New York, NY 10016.

Certain particulate nickel compounds, i.e., crystalline Ni_3S_2, are well-established human carcinogens and induce tumors in animals at virtually any site of administration. Carcinogenic nickel compounds generate oxygen radicals in cells; however, these do not have mutagenic consequences probably because of the selective interaction of nickel(II) with genetically inactive heterochromatin. The oxidation potential of Ni^{2t} --> Ni^{3t}, or Ni^{4+} is lowered following binding to heterochromatin proteins and oxygen radicals are localized there. The avid binding of nickel to core histones and to H_1 as well as the general high affinity that Ni^{2+} has for the proteins compared to DNA drives the selective binding of Ni^{+2} to heterochromatin. We have previously reported that nickel-induced transformation of male Chinese hamster embryo cells was associated with inactivation of a senescence gene on the highly heterochromatic X chromosome by DNA hypermethylation (*Science* **251**:796, 1991). It was also found that the thrombospondin gene was inactivated in nickel-transformed cells by loss of transcription factors that positively regulate its promoter (*Mol. Cell Biol.* **14**:1851, 1994). Nickel-induced DNA methylation was implicated in the loss of expression of these transcription factors. Bacterial *gpt* transgenes inserted into V79 cells have yielded one particular cell line that was very responsive to nickel-induced 6TG resistance. However, the incidence of this resistance induced by nickel was too high for it to have been a mutagenic event (10^3). Subsequent studies revealed that the *gpt* gene was inactivated by nickel-induced increased chromatin condensation and hypermethylation of the flanking and coding regions of this gene. This gene was located near a dense heterochromatic region of chromosome 1. Additionally, Ni-induced 6TG resistance cell types were readily reverted to wild type with the drug azacytidine which prevents DNA methylation. Nickel induced 6TG resistant cells exhibited enhanced chromatin condensation around the *gpt* gene as evidenced by DNaseI sensitivity studies. Carcinogenic nickel compounds exhibited low activity in inducing 6TG resistance in other transgenic cell lines where the *gpt* gene was inserted in euchromatin. Chromatin fractionation studies demonstrated that the *gpt* gene was found in a biochemically distinct heterochromatin fraction in G12 cells and much less of the gene was associated with the heterochromatin in non-responsive transgenic cell lines. However, when cells became resistant to 6TG following nickel exposure, the gene was found to a much greater extent in the heterochromatin fraction. Recent studies suggest that the Rb gene may be inactivated in nickel transformed cells by DNA methylation. A model emerges from these studies with regard to how nickel might be producing the inactivation of genes that maintain a "normal" cell. Nickel has previously been shown to increase chromatin condensation by substituting for Mg^{2+} and excess Mg^{2+} inhibits both nickel induced damage to heterochromatin and nickel carcinogenesis. Ni^{2+} binds to heterochromatin causing the extension of heterochromatin into neighboring euchromatin resulting in genes that are active in euchromatin to become incorporated into heterochromatin. This can be visualized as a spool with thread where the inactive heterochromatin DNA is on the spool while the active euchromatic DNA is in the thread coming off the spool. Nickel causes more thread to be rolled up on the spool and thus initially genetically active DNA is inactivated by an increased condensed state. The newly condensed DNA is now methylated as a result of its presence in heterochromatin (on the spool), and since DNA methylation patterns are faithfully copied after DNA replication, this DNA will now be inherited in an inactive state in all daughter cells.

C4-026 ARSENITE AND ANTIMONIALS RESISTANCE IN *LEISHMANIA*. Marc Ouellette, Barbara Papadopoulou, Katherine Grondin and Anass Haimeur. Service d'Infectiologie, Centre Hospitalier de l'Université Laval, Ste-Foy, (Québec), Canada, G1V 4G2.

The protozoan parasite *Leishmania* is distributed worldwide with 400 000 new cases each year. The treatment of choice for all forms of leishmaniasis depends on pentavalent antimonials. Clinical resistance to these metals has been described in all endemic areas. In order to understand the mechanism(s) of resistance to antimonials, we have selected *Leishmania* cell lines in vitro for resistance to antimonials or to the related oxyanion arsenite in a step by step manner. Consistent with this selection procedure, characterization of the arsenite and antimonials resistant mutants has indicated the presence of several different resistance mechanisms, including the amplification of at least three different loci. The first amplified locus characterized encodes the P-glycoprotein related gene *pgpA*. Transfection of *pgpA* indicated that it is implicated in oxyanion resistance. However, levels of oxyanion resistance differed depending from which species the gene was isolated or in which species it was transfected suggesting that the *pgpA* gene itself and/or species-specific factors might also be involved in resistance. In *Leishmania tarentolae*, resistance mediated by pgpA does not seem to affect the steady-state accumulation of arsenite. A 50 kb linear amplicon unrelated to *ltpgpA* amplification was observed in all arsenite resistant mutants. Mutants grown in absence of arsenite lost the amplicon and part of their resistance to lead to partial revertants. Transfection of the linear amplicon in wild-type cells did not lead to an increase in oxyanion resistance, but transfection in a partial revertant restored the resistance levels of the parent mutant. When *pgpA* is co-transfected with the linear amplicon in the partial revertant resistance levels observed are higher than expected than if resistance was due to the simple addition of the contributions of pgpA and the linear amplicon in resistance. Therefore, several independent mutations are present in oxyanion resistant *Leishmania* mutants and some of these act cooperatively to confer high level of resistance. A third unrelated amplified locus derived from one of the largest *Leishmania* chromosome was observed in several mutants selected for pentavalent antimony resistance. In a mutant grown in absence of the drug, the copy number of the circular amplicon as well as the level of resistance decreased. The gene part of this amplicon has been isolated by transfection and is currently being characterized. Finally, in all oxyanion resistant mutants studied, a rapid efflux of arsenite was observed that was independent of any of the three loci amplified currently characterized. Transfection experiments are in progress to isolate the gene coding for the efflux system, to look at the individual contribution of each isolated resistance gene and to reconstruct the resistance levels of the parent mutants. These studies will in turn be useful to look whether any of the gene characterized is involved in antimony resistance in clinical isolates.

Metal and Redox Regulation in Prokaryotes

C4-100 GENETIC AND BIOCHEMICAL ANALYSIS OF SENSORY TRANSDUCTION COMPONENTS CONTROLLING ANAEROBIC INDUCTION OF PHOTOSYNTHESIS GENE EXPRESSION IN *R. CAPSULATUS*. C. E. Bauer, C. S. Mosley, J. L. Kouadio, K. Inoue, Indiana University, Department of Biology, Bloomington, Indiana 45750.

Several studies have established that expression of the *puf, puc* and *puh* operons that code for apoproteins of light harvesting-I and reaction center complexes occurs only under anaerobic conditions (1). Recent genetic analyses has revealed that anaerobic induction of these operons involves two *trans* acting factors, RegB and RegA (2,3). Sequence analysis indicates that RegB is a member of the histidine sensor kinase family of eubacterial sensory transduction proteins and that RegA is of the response regulator class. *In vitro* biochemical analysis has confirmed that RegB has autophosphorylation activity as well as the capability of phospho-transfer to RegA (4). Interestingly, *in vitro* autophosphorylation of a truncated cytosolic portion of RegB is still redox dependent. We are currently using molecular genetic and biochemical techniques to investigate the mechanism whereby the truncated version of RegB can still respond to redox poise.

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C4-101 RNA POLYMERASE MUTATIONS AFFECT EXPRESSION OF THE *mer* OPERON. Laurie F. Caslake and Anne O. Summers, Department of Microbiology, The University of Georgia, Athens, GA 30602-2605.

The mercury resistance (*mer*) operon is transcribed from overlapping divergent promoters: P_R for the regulatory gene, *merR*, and P_{TPCAD} for the structural genes, *merTPCAD*. The dyadic binding site for MerR lies within the 19-bp spacer of the sigma-70-dependent structural gene promoter. MerR represses transcription of *merTPCAD* without Hg(II) and activates transcription with Hg(II). Even in the absence of Hg(II), MerR causes RNA polymerase (RNAP) to bind stably to the promoter. The current model of *mer* activation is that Hg(II) provokes MerR to distort the spacer DNA, thereby allowing the prebound RNAP to contact the -10 sequence and initiate transcription.

Mutations in regions 2.1 or 4.2 of sigma-70 (Siegele et al., 1989) effect 4-8X higher expression of P_{TPCAD} in the absence of MerR than the wildtype (wt) sigma-70. With MerR but no Hg(II) (the repressed condition), the sigma mutants have from 2-5X higher expression, indicating an interference with repression. However, activated expression of P_{TPCAD} [MerR +Hg(II)] was not enhanced by these sigma-70 mutants. Substitutions in the -35 hexamer of P_{TPCAD} abolish the high expression of the derepressed promoter, consistent with a requirement by these mutants for canonical contacts in the -35 region. These sigma-70 mutants also effect higher activity (up to 4.5X) than wt sigma-70 at P_R which has a short interhexamer spacing of 15-bp. Mutations in the extreme C-terminus of sigma-70 (R596) decreased MerR-Hg(II)-induced activation of P_{TPCAD} by as much as 55% in contrast to their effects with other activator proteins.

Mutations in the C-terminus of the alpha subunit have differential effects on *mer* expression depending on the amino acid substitution. A proline to leucine substitution (PL323) caused 2-fold higher Hg(II)-induced activation than the wildtype RpoA, whereas a serine substitution at the same position (PS323) lowered activation by 40%. In the repressed condition, the *rpoA* PL323 mutant exhibits 50% lower expression than the wt, indicating tighter repression; this mutant has no effect on the derepressed (-MerR) activity. The MerR-dependent *in vivo* effects of the P323 mutants contrasts with the apparent lack of effect of C-terminal deletions in *rpoA* on MerR-mediated expression *in vitro* (Ishihama, 1993) suggesting a specific interaction between these two proteins.

C4-102 THE BACILLUS SUBTILIS MRGA PROTEIN IS A DPS/PEXB HOMOLOG AND PROTECTS AGAINST OXIDATIVE STRESS, Lei Chen and John D. Helmann, Section of Microbiology, Wing Hall, Cornell Univ., Ithaca, NY. 14853-8101

Upon the cessation of exponential growth, *Bacillus subtilis* cells enter a transition phase which leads to either spore formation or a non-sporulating, stationary phase. During this transition period cells secrete degradative enzymes, become competent for DNA transformation, are actively motile, and acquire resistance to oxidative killing. The *mrgA* gene was originally identified as a gene fusion induced in stationary phase. This induction could be prevented by addition of metal ions in the order manganese > iron = cobalt > copper. Sequence analysis indicates that *mrgA* encodes a member of the Dps /PexB family of general stress proteins. Like the *Escherichia coli* Dps protein, MrgA forms highly stable, multimeric protein-DNA complexes which accumulate in stationary phase cells and protect against oxidative killing. The *mrgA* gene is part of the inducible oxidative stress response of *B. subtilis*: *mrgA* is induced by hydrogen peroxide and a strain lacking MrgA displays increased sensitivity to oxidative killing. Conversely, hydrogen peroxide resistant mutants which constitutively overproduce catalase and alkyl hydroperoxide reductase also overproduce MrgA. The stationary phase expression of *mrgA-lacZ* transcription is elevated several-fold in these mutants, but in some cases this expression can still be repressed by manganese. These results indicate a complex interplay between expression of the *B. subtilis* oxidative stress response and metal ions. Models to explain the coordinate regulation of *B. subtilis* genes by hydrogen peroxide and metal ions will be presented.

C4-103 COPPER RESISTANCE DETERMINANTS IN *A. EUTROPHUS*. Corbisier P., Wouters I., van der Lelie D., and Mergeay M. Environmental Technology, Flemish Institute for Technological Research (VITO), Boeretang 200, B-2400 Mol, Belgium.

Copper resistances have been detected on three megaplasmids of *Alcaligenes eutrophus*. The plasmids were self-transferred into a plasmid-free *A. eutrophus* strain to analyse their copper resistances determined as the MIC values on minimal medium. This plasmid-free strain showed a MIC of 0.85 mM for copper.

① The highest MIC (1.40 mM) was obtained with pMOL30 (czc^+, 238 kb) from strain CH34, whereas a hypersensitivity (0.43 mM) was found for its deletion derivative pMOL53 (ΔpMOL30, czc^+, 121 kb). pMOL53 hypersensitivity may be due to a partial deletion of the copper resistance genes while keeping intact some functions involved in the uptake of copper. The *cup* locus of pMOL30 was cloned via complementation by an *A. eutrophus* CH34 genomic bank transferred into an *A. eutrophus* plasmid free strain.

② pMOL85 (czc^+, 250 kb) and pMOL90 (260 kb) of the Copper resistant *A. eutrophus* DS185 were also analysed. A small resistance was found on pMOL85 (MIC=1.1 mM). Two inducible outer membrane proteins Cup1 (23-kDa) and Cup2 (21.6-kDa) were detected in copper-stressed cultures of *A. eutrophus* containing pMOL85. Those proteins are very similar to excreted proteins observed in *V. alginolyticus* and *P. aeruginosa* copper-stressed cultures.

③ No Cu-resistance was found on pMOL90 (MIC=0.9mM). However, a DS185 mutant containing only pMOL90::Tn*4431* (obtained by random-lux fusion) emitted light in the presence of Cu^{2+} ions. It suggests that pMOL90, which coexists with pMOL85 in *A. eutrophus* DS185, carries an incomplete copper resistance that is still inducible by this metal. This hypothesis gets support from the fact that a diploïd (pMOL53 + pMOL90::Tn*4431*) restore full resistance to copper. Eight hundred bp of the copper inducible element on pMOL90::Tn*4431* have been sequenced and were shown to encode the internal part of an ORF. The encoded theoretical protein showed similarity (49 %) with a group of proteins formed by 3-isopropylmalate dehydratases and bacterial aconitases that contain an iron-sulfur cluster identical to the mammalian iron-responsive element binding proteins (IRE-BP).

C4-104 OSMOTIC SHOCK-SENSITIVE PROTEINS OF THE *ESCHERICHIA COLI* ENTEROBACTIN SYNTHETASE COMPLEX, Charles F. Earhart and Feras Hantash, Department of Microbiology, The University of Texas at Austin, 78712-1095

Escherichia coli synthesizes enterobactin (Ent), a small iron-specific chelating molecule used for iron uptake, in iron-poor environments; the holorepressor for genes specifically required for ferriEnt assimilation consists of Fe(II) and the Fur protein. The latter stages of Ent biosynthesis are carried out by the products of the *entD, E, F* and *B/G* genes, which are hypothesized to form a membrane-associated complex (Ent synthetase) that catalyzes the reaction 3 L-Ser+3 Dihydroxybenzoic Acid + 6ATP → Ent + 6AMP + PPi. We now show that osmotic-shock and freeze-thaw procedures but not spheroplasting release more than 70% of EntE and Ent B/G and variable amounts of EntF. Ent synthetase proteins were identified by Western blotting. Absence or inactivation of one of the Ent synthetase proteins had no effect on the release of the others and the specific activity of extracts from shocked cells was decreased by 33% compared to extracts prepared from normal cells. In cell fractionation experiments, 50% of EntB/G could be detected associated with cytoplasmic membrane whereas EntE and EntF were both found exclusively in the soluble fraction. The results indicate that some Ent synthetase components are localized to osmotic shock-sensitive regions of the cell; this compartmentalization may be necessary for proper Ent secretion. If a complex exists, it must be transitory or composed of loosely-associated proteins.

C4-105 IRON TRANSPORT IN MYCOBACTERIA: IDENTIFICATION OF GENES INVOLVED IN THE EXOCHELIN BIOSYNTHETIC PATHWAY Ellen H. Fiss and William R. Jacobs. Jr. Dept of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y.

In response to iron deprivation, mycobacteria produce soluble (exochelins) as well as cell-wall associated (mycobactins) siderophores. A *Mycobacterium smegmatis* mutant mc^2848, defective in the production of the soluble siderophore, was isolated using agar medium containing chrome azural S for the sensitive detection of siderophores. Cosmids from mycobacterial genomic libraries were identified by complementation. A cosmid was subcloned to a 4.3 kb fragment which was required for siderophore activity. Sequencing of the DNA revealed 4 open reading frames. FxuA shares homology with the *E. coli* iron permease FepG (48% identity in 304 amino acids). FxuB shares homology with the iron permease FepC from *E. coli* (56% identity in 222 amino acids). FxbA did not demonstrate homology with any siderophore biosynthetic genes. However, it did demonstrate homology with a number of enzymes containing formyl transferase activity such as the *E. coli fmt* gene (36% identity in 234 amino acid). Deletion analysis identified fxbA as the gene required to restore activity in the exochelin minus mutant.

Beta galactosidase fusions of FxbA demonstrated regulation by iron, identifying the the iron regulated mycobacterial promoter region. The ability of mc^2848 to synthesize mycobactin in the absence of exochelin confirmed that exochelin is not a precursor of mycobactin and supports the hypothesis that the two siderophores have independent biosynthetic pathways. The iron uptake system in mycobacteria appears to utilize a pathway which is conserved among many different species, both Gram negative and positive.

C4-106 CHARACTERIZATION OF A FERRIC UPTAKE REGULATOR (Fur) HOMOLOG IN PORPHYROMONAS GINGIVALIS. Ren-Yo Forng and Caroline A. Genco, Department of Microbiology and Immunology, Morehouse School of Medicine, Atlanta, GA 30340

In Escherichia coli and Salmonella typhimurium, transcriptional regulation of iron-regulated genes occurs by the action of ferric uptake regulator protein (Fur). Fur acts as a classic negative regulator inhibiting the transcription of both genes involved in iron transport and virulence determinants. The influence of iron/hemin on the expression of serveral putative virulence factors produced by the periodontal pathogen, Porphyromonas gingivalis, has also recently been documented. However, the mechanisms involved in the iron regulation of specific virulence genes have not been defined. Using antisera specific to Pseudomonas aeruginosa Fur protein, an immunoreactive antigen was detected in whole cell lysates of P.gingivalis indicating the potential existence of a Fur homolog. We provide evidence here that P.gingivalis produces a Fur-like protein which may function in the transcriptional regulation of iron-regulated genes. Degenerative primers designed from conserved amino acid regions among Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, and Yersinia pestis Fur proteins were used to amplify a P.gingivalis fur homolog by the polymerase chain reaction. In this abstract, we report on the identification and cloning of the putative P.gingivalis fur homolog. Currently, we are sequencing this putative fur homolog and examining the ability of the P.gingivalis fur to complement E.coli fur mutants.

C4-107 TRIGGERING OF OXIDATIVE STRESS RESPONSES DURING AEROBIC GROWTH. Beatriz Gonzalez-Flecha and Bruce Demple. Department of Molecullar and Cellular Toxicology. Harvard School of Public Health. Boston, MA, 02115.

Oxidative stress, defined as an increase in the intracellular concentration of oxygen free radicals, can mediate either adaptative responses or oxidative damage to cellular components depending on the magnitude of the increase. We have measured the intracellular concentration of hydrogen peroxide (H_2O_2) in intact *E. coli* during aerobic growth to evaluate the putative occurrence of oxidative stress during exponential growth. The rate of H_2O_2 production in *E. coli* AB1157 markedly increased upon entering exponential growth. This increase linearly correlated with the number of respiratory chain units per cell and then, to the respiratory activity. Catalase-hydroperoxidase I activity was regulated both during aerobic growth and in strains defective in respiratory chain components in order to compensate for changes in the H_2O_2 production rates. Catalase activity increase was *katG*- and *oxyR*-dependent and it followed the same pattern than *oxyR* expression. These results suggest that catalase induction during exponential growth is not only due to an increase in H_2O_2 production. The regulation of *oxyR* expression will be discussed.

C4-108 THERMODYNAMIC STABILITIES OF CO(II)-COMPLEXES FORMED BY WILD-TYPE AND METAL-LIGAND SUBSTITUTION MUTANTS OF T4 GENE 32 PROTEIN, Juqian Guo, Huawei Qiu and David P. Giedroc, Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128

Phage T4 gene 32 protein (gp32) is a zinc metalloprotein which binds cooperatively and preferentially to single-stranded nucleic acids and functions as a replication- and recombination accessory protein. We have shown that the Zn(II) coordination by gp32 employs a metal ligand donor set unrelated to any known zinc-finger motif thus far described and is derived from the His64-X_{12}-Cys77-X_9-Cys87-X_2-Cys90 sequence in the ssDNA-binding core domain of the molecule. In studies directed toward understanding the origin of the stability of the metal complex, we have employed an anaerobic optical spectroscopic, competitive metal binding assay to determine the association constants for the binding of Co(II) to wild-type, H64C and C87S gp32 at 25 °C, pH 7.50 and 0.1 M KCl. The Co(II) complexes of wild-type and H64C gp32s have thermodynamic stabilities of -12.2 (±0.1) and -12.0 (±0.1) kcal mol^{-1}, respectively. Although substitution of His64 with a liganding Cys creates a tetrathiolate Co(II) coordination complex of comparable stability to wild-type gp32, it's susceptibility to oxidation by O_2 is far greater, and the core domain is much more susceptible to limited proteolysis by trypsin than the wild-type HCCC complex. In contrast, replacement of Cys87 with a nonliganding Ser destabilizes the complex considerably, giving a loading free energy of -7.2 (±0.2) kcal mol^{-1}. As the fourth ligand in C87S gp32 is presumably a solvent molecule, it's accessibility to exchange is being probed by the addition of exogenous ligands in a first step toward the creation of novel metalloenzyme catalysts.

C4-109 THE IRON-SULFUR CLUSTERS PRESENT IN SoxR REGULATE ITS ACTIVITY AS A TRANSCRIPTIONAL FACTOR, Elena Hidalgo and Bruce Demple, Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115

In the presence of superoxide stress and nitric oxide, *Escherichia coli* activates a defense regulon, *soxRS*. The SoxR protein, constitutively expressed in the cell, is both the sensor of the stress and the activator of the second regulatory gene, *soxS*. We are studying the mechanism by which SoxR senses superoxide and nitric oxide and becomes an activator of *soxS* transcription. SoxR contains two iron-sulfur clusters, required for its activity. An apo-form of SoxR (lacking iron) can be reactivated by reconstitution of its iron-sulfur clusters. The clusters are also redox-sensitive, and we demonstrated before that the oxidized iron-containing SoxR is transcriptionally active. Therefore, the mechanism of activation of SoxR *in vivo* can be due to either a reconstitution of the iron-sulfur clusters or to an oxidation of pre-existing and reduced Fe-SoxR. We will present experiments that will try to demonstrate one of these two hypothesis.

C4-110 OXYGEN REGULATION OF DENITRIFICATION GENES IN *Paracoccus denitrificans*

Andrew Hinsley[1], Matthew Duchars[2] and Stephen Spiro[1]
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In facultative anaerobes which couple anaerobic energy generation to the reduction of nitrate to dinitrogen, the expression of alternative respiratory chains is regulated by the availability of electron acceptors. The molecular genetics of denitrification are best characterised in *Pseudomonas stutzeri* and *Paracoccus denitrificans*, where there is evidence for the regulation of expression of structural genes by oxygen, nitrate, nitrite and nitrous oxide. The precise nature, and mechanism, of regulation is poorly understood, although there is some evidence implying a role for an analogue of the *Escherichia coli* transcriptional regulator FNR, which activates gene expression in response to anaerobic growth. We have isolated the *fnr* homologue of *P. denitrificans* by selecting clones from a genomic library which activate the transcription of an FNR-dependent promoter in *E. coli*. We shall present evidence to suggest that the FNR-like protein of *P. denitrificans* detects anoxia by monitoring the activity of the aerobic electron transport chain. Regulatory mutants with pleiotropic defects in anaerobic respiratory pathways have been isolated and the corresponding genes cloned by complementation. Results of these studies, and their implications for the mechanism of the response to oxygen limitation will be presented.

C4-111 REGULATION OF MOLYBDENUM TRANSPORT AND METABOLISM IN *AZOTOBACTER VIINELANDII*, Nigel J. Mouncey, Lesley A. Mitchenall and Richard N. Pau, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK.

Molybdenum is required for the cofactors of about 20 redox-active enzymes. All the cofactors consist of a molybdopterin, with the exception of that of Mo nitrogenase, which has the composition 1Mo:7Fe:9S:1 homocitrate. In *Azotobacter vinelandii*, high-affinity Mo uptake is carried out by a transporter of the ATP-binding cassette (ABC) family, encoded by three genes (*modABC*) in the *mod* operon. Initial molecular selectivity for Mo depends on the specificity of the periplasmic molybdate-binding protein, the product of *modA*, which binds molybdate and tungstate, but not sulphate or vanadate. Strains lacking the high-affinity transporter do not show nitrate reductase activity when grown in medium containing less than 10μM Mo. A separate low-affinity transport system, responsible for Mo uptake at higher concentrations, is expressed when the high-affinity transporter is lacking or repressed. The first gene in the *mod* operon, *modP*, encodes a 29 kDa protein whose C-terminal half consists of a tandem repeat which is highly homologous to the sequence of a 6.5 kDa molybdopterin-containing protein called Mop isolated from *Clostridium pasteurianum*. *MoqA*, the gene immediately upstream and divergently transcribed from the *modPABC* operon, encodes a protein that consists solely of a tandem repeat of Mop, and is thus similar to the C-terminal half of *modP*. These homologies suggest that a molybdopterin may be involved in the sensing and/or early metabolism of Mo. Mutational analysis shows that Mo repression of both *modABC* and *moqA* transcription is mediated by the product of *modP*, but not by the product of *moqA*. Mo repression of alternative nitrogenase expression is not regulated by the product of *modP*, suggesting that there are two pathways for gene regulation by Mo. Nitrate reductase expression is not regulated by Mo.

Although the products of *modP* and *moqA* individually do not affect high-affinity Mo uptake, together they have different effects on the biosynthesis of the cofactors of nitrate reductase and Mo-nitrogenase. A strain containing mutations in both *modP* and *moqA* exhibits nitrate reductase activity at 100-fold lower Mo-concentration than the wild-type strain, indicating that the products of *modP* and *moqA* both limit the availability of Mo for nitrate reductase Mo-cofactor synthesis. The *modP/moqA* double mutant fails to grow diazotrophically in both the presence and absence of Mo.

C4-112 OXYGEN REGULATION OF TRANSCRIPTION AND SIGNAL TRANSDUCTION OF THE TYROSINE HYDROXYLASE (TH) GENE IN PC12 CELLS. M.L. Norris, R.M. Raymond, and D.E. Millhorn, *Department of Molecular and Cellular Physiology, The University of Cincinnati, Cincinnati, Oh. 45267.*

Reduced oxygen tension (hypoxia) is a fundamental physiological stimulus in the regulation of expression of a number of genes involved in environmental adaptation. Gene expression for tyrosine hydroxylase (TH), the rate limiting enzyme for catecholamine synthesis, is increased in O_2 sensitive carotid body type I cells, which mediate the cardiopulmonary responses to hypoxia. Current studies have been undertaken in PC12 cells, which are functionally and morphologically similar to carotid body type I cells, to elucidate the molecular mechanisms involved in the O2 regulation of tyrosine hydroxylase gene expression. We found that induced gene expression of TH is due to regulatory changes in both transcripton and stability (*Czyzyk-Krzeska et al.*, **JBC** 269: 760-764). CAT assays were employed to determine the 5' flanking region(s) responsible for oxygen regulation of transcription of the TH gene. TH-CAT promoter analysis of cells exposed to 21% O_2 (normoxia) or 5% O_2 (hypoxia) revealed a region from -272 to -90, relative to transcription start site, which mediates the increased transcription rate during hypoxia. Several *cis* regulatory elements (*eg* AP2, AP1, SP1) are located within this flanking region, as well as a newly characterized hypoxia inducible element (HIE) (*Semenza et al.*, **MCB** 12: 5447-5454). An increase in protein binding activity to the AP1 element was measured when PC12 extracts from cells exposed to 6-12 hours of hypoxia were utilized in DNA-protein binding studies. Numerous transcription factors (*eg* Fos, Jun) mediate gene transcription rate through DNA-protein interactions involving this *cis* regulatory element. Techniques such as UV crosslinking, immunoblotting, supershifts, and shift-westerns have been employed to further elucidate the factors involved in this regulatory complex. Initial evidence also suggests the possible involvement of a heme protein in this complex signalling pathway. Experiments have focused on the possible role of soluble guanylate cyclase, a heme protein, in mediating this transcriptional response to hypoxia. Our preliminary results show that pharmacological blockade of the cyclic guanosine monophosphate (cGMP) pathway prevents increased transcription of the TH gene during hypoxia, suggesting that cGMP is indeed involved in this complex system of second messengers and transcriptional modulators. These data taken together should help to elucidate the various mechanisms involved in the physiological response to oxidative stress.

C4-113 ROLE OF A BACTERIAL SUPEROXIDE STRESS RESPONSE *soxRS* IN COUTERACTING ATTACK BY MAMMALIAN MACROPHAGE, Tatsuo Nunoshiba[†] and Bruce Demple, Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115

Activation of *E. coli* superoxide stress regulon genes such as *sodA, zwf, soi, fumC* and *nfo* is mediated by two-stage *soxRS* system. In this system, the redox-sensitive SoxR protein transcriptionally activates the *soxS* gene, whose product then stimulates the transcription of regulon genes. The *soxRS* system also responded to nitric oxide (NO•) in a manner independent of oxygen. NO• is produced through an enzymatic oxidation of L-arginine in various mammalian cells for intercellular signaling and in copious quantities to cause cytotoxicity. In the latter, NO• is a deliberate cytotoxic radical of activated macrophages. Phagocytosis by mice-derived murine peritoneal macrophages also caused activation of the *soxS* transcription that was completely inhibited by NO• synthase inhibitor, *N*-methyl-L-arginine. The activation evidently provided bacterial resistance to the macrophages, with kinetics that parallel the NO• production from the macrophages. The required functions include DNA repair endonuclease IV, *micF* antisense RNA for major outer membrane porin OmpF and glucose-6-phosphate dehydrogenase. These results demonstrated that SoxR senses the cellular exposure to NO•, and that the SoxRS response may contribute to defense against counteracting attack by activated macrophages.

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C4-114 MOLECULAR CLONING, CHARACTERIZATION AND INTERACTION WITH THE DIPHTHERIA *tox* OPERATOR OF THE *Corynebacterium diphtheriae dtx*R HOMOLOG FROM *Brevibacterium lactofermentum*, José A. Oguiza[1], Xu Tao[2], Ana T. Marcos[1], Marcos Malumbres[1], Juan F. Martín[1], and John R. Murphy[2], [1] Area of Microbiology, Faculty of Biology, University of León, 24071 León, Spain and [2]Evans Department of Clinical Research and Department of Medicine, Boston University Medical Center Hospital, Boston, MA 02118

The diphtheria *tox* repressor, DtxR, is a transition metal ion-activated regulatory element that has been shown previously to control the expression of diphtheria toxin as well as several genes involved in siderophore synthesis in *Corynebacterium diphtheriae*. Since DtxR appears to be analogous to Fur (ferric uptake regulator) in *Escherichia coli* and Fur-like proteins in other Gram negative organisms, we reasoned that the structural gene encoding DtxR-like regulatory elements may have been conserved in Gram positive organisms. In the present report, we have cloned the *dtx*R homolog from *Brevibacterium lactofermentum*; this gene encodes a 25,416 molecular weight protein in which the amino acid sequence is 70.8% identical to that of the DtxR from *C. diphtheriae*. DtxR from *B. lactofermentum* is immunoreactive with polyclonal antisera raised against *C. diphtheriae* DtxR and is also a transition metal ion-activated repressor that is capable of regulating in an iron-dependent fashion the expression of β-galactosidase from a diphtheria *tox* promoter/operator transcriptional fusion in recombinant *E. coli*. Gel mobility shift assays have shown that the purified *B. lactofermentum* DtxR protein specifically binds to the *tox* regulatory region. Also we demonstrate by DNase I footprint analysis, that the *B. lactofermentum* DtxR footprints for the *tox* regulatory region in the presence of a divalent heavy metal ion are similar to those obtained previously for *C. diphtheriae* DtxR. These results suggest that there may be a family of transition metal ion-activated DtxR related regulatory elements in Gram positive organisms that are involved in iron-dependent control of gene expression.

C4-115 A NOVEL PARAQUAT-INDUCIBLE (pqi) GENE REGULATED BY soxRS IN E.coli, Jung-Hye Roe, Young-Sang Koh, Department of Microbiology and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, KOREA

We isolated promoters inducible by paraquat, a superoxide radical generating agent, from *Escherichia coli* using promoter probing plasmid, pJAC4. One of the promoter clones was named *pqi5* where *pqi* denotes paraquat-inducible gene. Mapping analysis using the *E. coli* phage library made by Kohara revealed that this gene is located at about 21.8 min on the chromosome where no known gene was reported to be inducible by paraquat or other superoxide generating agents. We constructed an operon fusion of *E. coli lacZ* gene to *pqi5* promoter to monitor transcriptional level of the gene in a single copy state. Transcription from *pqi5* promoter was induced about 4 to 8-fold by 100-780 μM paraquat. Other superoxide generators such as menadione and plumbagin, or ethanol also induced the expression of β-galactosidase in this fusion strain. On the other hand, no significant induction was observed by treatment with hydrogen peroxide and heat shock. Induction of β-galactosidase was significantly reduced by introducing mutations Δ*sox-8*::cat or *soxS3*::Tn*10* into the fusion strain, indicating that this gene is member of *soxRS* regulon. DNA fragment containing *pqi5* promoter was cloned from the Kohara phage E2E5. DNA sequence analysis revealed the presence of one open reading frame (ORF), which encodes a predicted protein of 37.9 kDa which might be an integral membrane protein. Transcription start sites from *pqi5* promoter were determined by primer extension and S1 nuclease protection analysis. S1 analysis of 5' and 3' ends of *pqi5* transcripts indicated that the mRNA levels increased 20-fold by paraquat treatment and the *pqi5* transcripts could cover the entire ORF.

C4-116 X-RAY STRUCTURE OF THE DIPHTHERIA *TOX* REPRESSOR FROM *CORYNEBACTERIUM DIPHTHERIAE*, Nikolaus Schiering, Xu Tao[2], John R. Murphy[2], Gregory A. Petsko & Dagmar Ringe, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02154, U.S.A. [2] Section of Biomolecular Medicine Boston University Medical Center Hospital Boston, MA 02118, U.S.A.

The crystal structure determination of the diphtheria tox repressor is currently underway in our laboratories. We are confident that by the time of the meeting there will have been enough progress to present an interesting poster.

The final abstract will be provided at the latest time convenient for you.

C4-117 **IDENTIFICATION OF THE INDUCER BINDING SITE FOR As (+3) AND Sb (+3) ON THE ArsR REPRESSOR,** Weiping Shi, Marina Yu. Ksenzenko and Barry P. Rosen, Dept. Biochem., Wayne State Univ. Sch. Med., Detroit, MI 48201. The arsenical resistance (*ars*) operon of the plasmid R773 encodes an arsenite-translocating ATPase and confers major resistance to the *metalloid oxyanions antimonite and arsenite in Escherichia coli.* Transcription of the *ars* operon is negatively controlled by the dimeric ArsR repressor and induced by arsenite or antimonite. The ArsR protein is a member of a newly identified family of metalloregulatory proteins termed the ArsR family. Members of this family also include Cd^{2+} and Zn^{2+} regulatory proteins. In these proteins the sequence $ELC_{32}VC_{34}DL$ is highly conserved. We previously reported the isolation of three *arsR* mutants by hydroxylamine mutagenesis: C32Y, C32F and C34Y and proposed that this cysteine pair is an important component of the metal recognition site of this family of metalloregulatory proteins (J. Biol. Chem. **269**, 19826-19829(1994)). In this study site directed mutagenesis was used to alter C_{32} and C_{34} to G, alone and in combination, and C_{37} was altered to S. *In trans* each mutant *arsR* gene repressed expression of a reporter gene controlled by the *ars* promoter. Addition of inducer did not relieve repression of the C32G, C34G and C32/34G proteins but could relieve that of the wild type and C37S proteins. Similar results were obtained from *in vitro* DNA gel retardation assays. Wild type and mutant ArsR proteins were examined by affinity chromatography using columns with 4-aminophenylarsine oxide (PAO) bound to agarose. The wild type ArsR protein bound tightly, requiring 0.6 M 2-mercaptoethnol to elute. The altered proteins C32G, C32Y, C32F and C34G bound less tightly, and the C32/34G protein did not bind. However, the binding ability of C37S and C34Y were close to wild type ArsR protein. These results suggest that binding to the PAO column reflects ability to respond to inducer and that C_{32}, C_{34} and at least one residue within the DNA binding region form the inducer binding site. *Supported by USPHS grant AI19793.*

C4-118 REDOX-DEPENDENT SHIFT OF OxyR-DNA CONTACTS ALONG AN EXTENDED DNA-BINDING SITE: A MECHANISM FOR DIFFERENTIAL PROMOTER SELECTION, Michel B. Toledano[1,2], Ines Kullik[1], and Gisela Storz[1], [1]Cell Biology and Metabolism Branch National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892 and [2]Department of Pharmacology and Toxicology, Rutgers College of Pharmacy/UMDNJ, EOHSI Building, 681 Frelinghuysen Road, Piscataway, NJ 08855-1179.

The redox-sensitive OxyR protein activates the transcription of antioxidant defense genes in response to oxidative stress. OxyR also acts to repress its own expression under both oxidizing and reducing conditions. Previous studies showed that OxyR binding sites are unusually long with limited sequence similarity. To explore how OxyR carries out dual regulatory functions and to investigate the nature of sequence-specific DNA recognition by OxyR, we selected binding sites from pools of random oligonucleotides and identified the OxyR-DNA contacts by hydroxyl radical footprinting and missing nucleoside interference assays. We found that the oxidized form of OxyR recognizes a consensus motif comprised of four ATAGnt elements spaced at 10 bp intervals. The oxidized protein contacts these elements by binding in four adjacent major grooves on one face of the DNA helix. In contrast, reduced OxyR only recognizes a subset of the sites bound by oxidized OxyR and contacts two pairs of adjacent major grooves separated by one helical turn. The two modes of binding are essential for OxyR to function as both an activator and a repressor in vivo. We propose that specific DNA recognition by an OxyR tetramer is achieved with four contacts of intermediate affinity dispersed across an extended binding site. The intermediate affinities allow OxyR to reposition its DNA contacts and thereby target alternate sets of promoters as the redox state of the cell is altered.

Oxygen/Redox Regulation in Eukaryotes

C4-200 THE INVOLVEMENT OF STRESS-RELATED TRANSCRIPTION FACTORS IN THE IRON-DEPENDENT REGULATION OF THE *FRE2* FERRIC REDUCTASE GENE OF *SACCHAROMYCES CEREVISIAE*,
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FRE2 gene encodes for one of the two membrane-associated ferric reductases in *S. cerevisiae*. It is required, together with *FRE1*, for the conversion of the insoluble environmental iron to the soluble ferrous form transported intracellularly. The activity of both genes is induced by iron depletion but with distinct kinetics. This induction is mainly due to transcriptional regulation. Deletion analysis of the *FRE2* promoter fused to a heterologous reference gene defined distinct regions responsible for the iron-dependent transcriptional regulation of the gene. The dependence of the iron-regulated *FRE2* promoter on transcription factors known to be involved in stress tolerance was also examined. This analysis has demonstrated that the Yap1 transcription factor is necessary for *FRE2* gene transcription. The involvement of the other members of the same leucine-zipper family,Yap2 and Gcn4, is under investigation.

C4-201 THE *FRE1* AND *FRE2 SACCHAROMYCES CEREVISIAE* GENES ESSENTIAL FOR IRON UPTAKE PARTICIPATE ALSO IN COPPER METABOLISM,
Georgatsou E.[1], Mavrogiannis L.[2], Alexandraki D.[1,2]
Foundation for Research and Technology-HELLAS, Institute of Molecular Biology and Biotechnology[1] and University of Crete, Department of Biology[2], P.O. Box 1527, Heraklion 711 10 Crete, GREECE

Iron and copper are trace elements whose biological importance is well established. Their common properties have been frequently underlined as regards to their participation in similar fundamental biochemical reactions as well as their role in oxygen dependent toxicity. However, the cellular mechanisms by which living organisms exploit these similarities in order to fulfil their needs and simultaneously protect themselves are only beginning to be unravelled.
In *Saccharomyces cerevisiae,* Fre1 and Fre2 are ferric reductases, responsible for the total plasma membrane associated activity of the cell. The reaction they catalyse is a prerequisite for iron uptake by the organism. Both corresponding genes are negatively regulated by the extracellular iron concentration, at the transcriptional level. The two products have however distinct temporal regulation of their activities during growth. We have shown that they are also regulated differentially under copper deprivation: although both enzymes can reduce copper, only the *FRE1* gene is induced in its absence. This induction also takes place at the transcriptional level. Moreover, Fre1 and Fre2 are the only copper reducing activities detected at the plasma membrane of *Saccharomyces cerevisiae.* We are currently investigating the factors involved in the differential regulation of the two genes in relation to the interconnection of iron and copper metabolism.

C4-202 IRON REGULATES CYTOPLASMIC LEVELS OF A NOVEL IRON-REGULATORY PROTEIN WITHOUT ACONITASE ACTIVITY, Bing Guo, Yang Yu, Fritz Brown, John Phillips and Elizabeth Leibold, Program in Human Molecular Biology & Genetics, and Department of Medicine, University of Utah, Salt Lake City, UT 84112
Iron-regulatory proteins (IRPs) are cytosolic proteins that bind to a conserved RNA stem-loop termed the iron-responsive element (IRE). IREs are located in the 5'- and 3'- noncoding regions of mRNAs including ferritin and the transferrin receptor (TfR) mRNAs, respectively. IRPs were formerly termed as the iron-responsive element binding protein (IRE-BP), iron-regulatory factor (IRF) and the ferritin repressor protein (FRP). Binding of the IRP to the 5' IRE represses translation, whereas binding to the 3' IRE stabilizes the mRNA. IRP1 has a molecular weight of 98,000 and has sequence homology with the iron-sulfur protein aconitase. The iron-sulfur cluster is important for iron-dependent regulation: IRP1 containing iron has low affinity for the IRE and contains aconitase activity, whereas IRP1 lacking the iron has high affinity for the IRE and lacks aconitase activity. A second IRP termed IRP2 has been identified in rat tissues and cultured cells by RNA-band shift analysis. IRP2 was purified from rat liver and rat hepatoma cells by IRE-affinity chromatography, and the molecular weight was estimated by SDS-polyacrylamide gels to be 104,000. Although IRP1 and IRP2 contain similar characteristics in that they bind the ferritin and the TfR IREs with similar affinities, function as translational repressors of IRE-containing RNAs in vitro, and have decreased RNA binding activity in the extracts of iron-treated cells, they differ in three important aspects: first, although the amino acid sequences of IRP1 and IRP2 share about 88% identity, IRP2 contains an unique insertion of 73 amino acids near its amino terminal end; second, both IRP1 and IRP2 activity are negatively regulated by iron, however, IRP1 activity decreases without a significant change in the total IRP1 levels, whereas IRP2 levels decrease to undetectable levels during iron treatment, and is due to the increased turnover of the protein; and third, in contrast to IRP1, IRP2 does not contain aconitase activity. Finally, both IRP1 and IRP2 are ubiquitously expressed in all rat tissues examined, however, their levels vary among the different tissues. These data indicate that IRP1 and IRP2 are distinct proteins that have similar specificity for IRE binding, and function similar in translation, but are regulated by iron by different mechanisms.

C4-203 COPPER HOMEOSTASIS IN C6 RAT GLIOMA CELLS: EVIDENCE THAT COPPER EFFLUX, NOT INFLUX, REQUIRES A COPPER-TRANSPORTING ATPase, Edward D. Harris, Yongchang Qian, and Evelyn Tiffany-Castiglioni, Departments of Biochemistry and Biophysics and Veterinary Anatomy and Public Health, Texas A&M University, College Station TX 77843-2128
Copper is a cofactor for some 30 enzymes and its defective metabolism is the basis for two inherited human disorders, Menkes' disease and Wilson's disease. The disease gene candidates have been identified as encoding P-type ATPases with a cysteine-rich N-terminal region. A link between membrane ATPase and copper homeostasis has not been reported. In our work, we have used sulfhydryl (-SH) and carboxyl-binding agents to inhibit P-type ATPases and characterize the copper transport mechanism. Rates of ^{67}Cu influx and efflux were temperature-dependent and showed saturation kinetics. The apparent K_m for uptake at 37°C was 0.51 ± 0.1 μM (n=7); V_{max} was 3.29 ± 0.6 pmol Cu/mg protein (n=7). The apparent K_m for efflux was 0.1± 0.02 μM (n=4); V_{max} was 1.01 ± 0.22 pmol Cu/mg protein (n=4). The -SH agent *p*-chloromercuriobenzoate (PCMB) at 0.2 mM totally blocked efflux while having virtually no effect on the influx of ^{67}Cu; Km 0.47 ± 0.04 μM (n=5), V_{max} 3.41 ± 1.06 pmol Cu/mg protein (n=5). PCMB restored the linear rate of efflux beyond 20 min confirming that accumulation of ^{67}Cu was caused by an efflux block. A second -SH agent iodoacetamide at 1.0 mM also prevented efflux as did 0.5 N,N'-dicyclohexylcarbodiimide, a P-type ATPase inhibitor. PCMB-treated cells retained twice the amount of ^{67}Cu as untreated cells. Subcellular fractionation, however, revealed that the ratio of ^{67}Cu/total ^{67}Cu in PCMB-treated cells was 7% as compared with 30% for the untreated cells. The data suggest that copper influx and efflux operate through two separate copper carriers or one carrier in two distinct membrane environments. Efflux depends on -SH groups accessible to PCMB. The data are consistent with the involvement of a P-type ATPase in copper efflux. Rapid export of copper by a high affinity efflux system may be a primary factor maintaining copper homeostasis in C6 glioma cells.

C4-204 IDENTIFICATION OF CADMIUM-INDUCED MUTATIONS IN THE MuSVts110 DNA OF 6M2 CELLS, Caroline A. Heckman and Edwin C. Murphy, Jr., Department of Tumor Biology, U. T. M.D. Anderson Cancer Center, Houston, TX 77030

Epidemiological and experimental data have proven cadmium to be a potent carcinogen in both humans and animals. However, cadmium is weakly or non-mutagenic in prokaryotic assays. To assess the damage to mammalian genes by carcinogens such as cadmium, we developed a mutagenesis assay based on MuSVts110-infected normal rat kidney cells (6m2 cells). MuSVts110 is a mutant murine sarcoma virus conditionally defective for cell transformation. At high growth temperatures, MuSVts110 is transformation-incompetent due to a frameshifting deletion between the viral *gag* and v-*mos* genes. At low growth temperatures, however, MuSVts110 RNA can be spliced, allowing the translation of the v-*mos* gene product. Carcinogen-induced mutations to MuSVts110 DNA can be detected by "reversion" of 6m2 cells to the transformed state at high growth temperatures. When cadmium was used as a potential mutagen, 6m2 revertants were induced in which the MuSVts110 gene product at 39 °C was either an 85 kd or a 100 kd *gag-mos* fusion protein rather than the wildtype 58 kd truncated *gag* gene product. These variations in v-*mos* expression indicated possible alterations to MuSVts110 sequences. Analysis of the MuSVts110 DNA from two cadmium-induced revertants has revealed a variety of mutations. One revertant, expressing p85*gag-mos* at 39 °C, contained a G to T and an A to T transversion, plus an A to G transition. The transversions, which are 84 and 100 bases downstream from the MuSVts110 5' splice site, and the transition, which is 48 bases downstream from the 3' splice site, may create alternative splicing signals or enhance present signals to generate the oversplicing seen in this revertant. The second revertant was found to contain a two base deletion in the MuSVts110 intron that permits a shift from the *gag* gene reading frame to the v-*mos* gene reading frame and thereby allows the translation of p100*gag-mos*. The variation among these cadmium-induced mutations suggests that cadmium may act to increase the rate of spontaneous mutations or decrease the fidelity of the DNA repair system.

C4-205 STUDIES ON THE ROLE OF METALLOTHIONEIN IN ZINC AND COPPER HOMEOSTASIS BY MANIPULATION OF THE MOUSE GENOME

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The proposed functions of metallothionein (MT) include heavy metal detoxification, protection against reactive oxygen species and metal homeostasis. To investigate these proposed functions, we have generated strains of mice that either lack the ubiquitously expressed isoforms, MT-I & MT-II, or have amplified levels of MT-I. Mice that lack MT-I and MT-II are normal under standard vivarium conditions but they are particularly sensitive to the toxic heavy metal cadmium. To continue this study we are testing the sensitivity of MT-null mice to zinc and copper. To address the question of MT's role in zinc homeostasis, we are subjecting animals that have varying levels of MT to alterations in dietary zinc intake. The administration of 25 mM zinc sulfate in the drinking water results in a modest increase in hepatic zinc in control animals that is matched in MT-null mice. There is, however, no pathology associated with this treatment. We are also subjecting mice to either a moderate (5 ppm) or severe (1 ppm) restriction in dietary zinc intake and then examining the changes in bodily distribution of zinc as well as any associated histopathological changes. We are using a genetic approach to test the function of MT in copper homeostasis that entails breeding MT-null mice with the Mottled-Brindled strain of mice. Mottled-Brindled mice are a model of Menkes disease in man that involves a defect in copper transport. Comparison of the results of these studies should provide insight into the role(s) of metallothionein in mammalian zinc and copper homeostasis.

C4-206 DETECTION OF TWO DIFFERENT NUCLEAR PROTEINS WHICH INTERACT WITH THE METAL REGULATORY ELEMENTS OF THE GENE ENCODING MOUSE METALLOTHIONEIN-I. Simon Labbé, Raffaella Faraonio, Marie-Josée April and Carl Séguin, Centre de recherche en cancérologie de l'Université Laval, l'Hôtel-Dieu de Québec, Québec, Canada, G1R 2J6.

Metallothioneins (MTs) are small cysteine-rich metal binding proteins which are thought to function in heavy metal detoxification and metabolism. In higher eukaryotes, the genes encoding MTs are inducible at the transcriptional level by a wide variety of chemical agents and by a number of stresses. Metals are the most general and potent of these inducers. Metal activation of MT gene transcription is dependent on the presence of *cis*-acting elements, termed MREs, adjacent to MT genes, and involves *trans*-acting factor(s) interacting with the MREs, present in six non-identical copies (MREa through MREf) in the 5' flanking region of the mouse MT-I gene. MEP-1 is a nuclear protein that binds to the different MRE elements of the mouse MT-I gene promoter. Although we have shown that purified MEP-1 is sufficient to generate a footprint over the MRE elements of the mouse MT-I promoter, it is yet unclear how many classes of nuclear proteins interact with the MRE elements. In this study, we have fractionated a mouse L-cell nuclear extract using an improved purification strategy involving a combination of low pressure heparin-Sepharose chromatography with salt gradient elution, and two steps of affinity chromatography using mutant and wild-type MRE-DNAs as the affinity substrat. Using this procedure, we detected, in addition to MEP-1, another MRE-binding protein interacting with the MREc element. The two proteins can be distinguished on the basis on their elution profiles, and their respective affinities towards different MRE competitor oligonucleotides, as assayed by DNaseI footprinting. We also detected a nuclear protein which specifically interacts with the transcription initiation site. These results show that at least two different nuclear proteins interact with the MRE elements of the the mouse MT-I gene. The role of these two proteins in the regulation of MT gene transcription, and the relationship between them or with the other MRE-binding proteins reported in the literature, remain to be determined. Supported by the MRC and the NIH.

C4-207 IDENTIFICATION OF GENES INVOLVED IN ZINC HOMEOSTASIS IN BHK CELLS

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BHK cells that do not express metallothionein were transformed with a zinc-responsive reporter gene, MRE-βGeo. This reporter gene is tightly regulated by zinc and therefore provides a sensitive indicator of "free" zinc. These cells were subsequently mutagenized and a zinc-sensitive derivative was isolated that has a high basal expression of the reporter gene. The relationship of total cellular zinc to induction of βGeo in these and the parental cells was examined. The mutant cells have elevated zinc content under normal conditions which accounts for the high basal expression of βGeo and zinc content rises to toxic levels with relatively small changes in extracellular zinc. There is only a 2-fold difference in zinc content between conditions of starvation and toxicity in both mutant and normal cells. The mutant cells are sensitive to zinc toxicity due to a recessive defect in zinc efflux. Zinc-resistant transformants were selected after transfection of a cDNA expression library and the plasmids have been rescued and characterized. One class of revertants encodes a zinc transporter that stimulates zinc efflux from the cell. Another class encodes a similar protein that appears to transport zinc into a cellular organelle. Zinc resistance can also conferred by various metallothionein genes. The roles of these gene products in regulating the ability of BHK cells to adapt to changes in extracellular zinc are being pursued.

C4-208 FUNCTIONAL ANALYSIS OF THE IRON-REGULATORY PROTEIN 2 IN *SACCHAROMYCES CEREVISIAE*, John D. Phillips, Bing Guo, Elizabeth A. Leibold, Program of Human Molecular Biology and Genetics, University of Utah, Salt Lake City, UT 84112

Iron-Regulatory Proteins (IRP) are cytosolic RNA-binding proteins that bind to stem-loop structures known as iron-responsive elements (IRE). These IREs are located in the 5'- or 3'- untranslated regions of mammalian mRNAs generally involved with the metabolism, transport or storage of iron. Binding of IRPs to 5' IREs represses translation of the mRNA, while binding to 3' IREs stabilizes the mRNA. Two IRPs have been identified in mammalian cells, and are termed IRP1 and IRP2. Both IRPs have been cloned and sequenced from rat liver. IRP1 and IRP2 share about 61% amino acid identity, and both have about 28% identity with the iron-sulfur protein mitochondrial aconitase. Importantly, both IRP1 and IRP2 contain the three cysteines that are ligands for the iron-sulfur cluster in mitochondrial aconitase and IRP1. The RNA-binding activities of IRP1 and IRP2 are decreased in extracts from iron-treated cells. The decrease in IRP1 activity is due to conformational changes within the protein itself without significant changes in IRP1 levels. In contrast, the decrease in IRP2 activity is due to an increase in the turnover of the protein. To study the regulation of IRPs, we expressed IRP1 and IRP2 in yeast. *S. cerevisiae* lacks the homologous IRPs, and can be used to study the biological functions of IRP1 and IRP2. The yeast expressed rat IRP1 and IRP2 have an identical mobility on SDS-polyacrylamide gels to their rat liver counterparts. RNA-bandshift analysis indicates that both IRP1 and IRP2 bind the IRE with high affinity. To determine if the yeast expressed IRP1 and IRP2 are capable of repressing translation, a LacZ reporter gene was constructed that contained an IRE in its 5' UTR. When expressed in yeast, both IRPs bound the IRE and repressed translation of the IRE-LacZ mRNA. IRP1 repressed translation approximately 10-fold, while IRP2 repressed translation 5-fold. Iron administration to yeast expressing IRP1 or IRP2 does not modulate their RNA-binding activities suggesting that yeast lacks the distinct IRP1 and IRP2 iron regulatory systems present in mammalian cells. Finally, when IRP1 and IRP2 is expressed in an *aco1* yeast strain, only IRP1 contains aconitase activity after reconstitution with iron. These data indicate that both IRP1 and IRP2 function as translational repressors of IRE-containing RNAs, and that yeast contains the necessary translational machinery for this regulation. The reconstituted IRP/IRE interaction in yeast provides a system to study structure-function aspects of IRP regulation.

C4-209 **Insect metal-responsive genes as biomonitors of heavy metal pollution.** **Alfredo Rayms-Keller**, Laura Klimowski, Ken Olson, and Barry J. Beaty. Department of Microbiology. **Colorado State University**. Fort Collins, CO 80523

Metal-responsive genes isolated from aquatic insects, such as mosquito larvae may prove to be useful tools for biomonitoring heavy metal pollution.

The toxicological impact of heavy metals on *Ae. aegypti* has been demonstrated by viability and morphological studies. Metal-, dose-, and time-dependant effects in larval mortality rates has been determined. Exposure of *Ae. aegypti* larvae to heavy metals results in the obliteration of the peritrophic matrix (PM). The PM is an extracellular matrix that lines the gut of most arthropods. This is the first demonstration of a heavy metal effect on PM status.

Metal-responsive genes from an Ae. aegypti cDNA library have been isolated by a differential screen with radioactively labelled cDNA probes made from unexposed and metal exposed larvae polyA+RNA. Four independent metal responsive cDNA clones have been recovered. **Northern blot analysis indicates that these metal responsive cDNAs code for an abundant RNA transcripts that are induced by heavy metals in the larval midgut**. Further characterization of these metal responsive cDNAs, their corresponding genomic DNAs, and their regulatory elements is under way.

C4-210 M96,A ZINC-DEPENDENT MRE BINDING PROTEIN, MAY BE INVOLVED IN THE ACTIVATION OF MT GENES BY HEAVY METAL IONS INDUCTION.
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A cDNA clone encoding for a MRE binding protein (M96) has been previously isolated by the use of an *in vivo* screening of expression lybraries in *Saccharomices Cerevisiae*.M96 has the potentiality to form three zinc finger domains omologous to the ones revealed the trithorax genes family.As shown by Northern blot analysis M96 appears to be costitutively expressed which is consistent with the observation that metal induction of MT genes does not require *de novo* protein synthesis.M96 has the ability to recognize MREs *in vitro* when mobility shift assays were performed in the presence of a bacterially expressed glutathione S-transferase-M96 fusion protein (GST-M96).Methylation interference analysis revealed that GTS-M96 specifically interacts with tandem MRE repeats (MREd/c) present on the mouse MT-Ia promoter region.Mutations in the sites of interaction with M96 abolished the ability of MREd/c region to confer metal inducibility to an eterologous promoter as revealed by transfection experiments.Expression of a M96 antisense cDNA in transfected human 293 cells inhibited both basal and metal induced activation of a reporter gene driven by hMTIIa promoter .Analysis of the effect of metal ions on the M96-MREs interactions revealed that M96 binding activity is positively modulated by zinc ions.Taken together these data suggest that M96 protein,if involved in the regulation of MT genes,is present prior the induction and,upon metal exposure,becomes activated in a manner analogous to the yeast MT regulator CUP2/ACE1.

C4-211 MOLECULAR MECHANISMS OF ARSENITE RESISTANCE IN MAMMALIAN CELLS, Toby G. Rossman and Zaolin Wang, The Nelson Institute of Environmental Medicine, NYU Medical Center, New York, NY 10016

Chinese hamster cells have an arsenite- and antimonite-inducible tolerance mechansim to arsenite, which differs from the heat shock response. A number of stable arsenite-sensitive and arsenite-resistant sublines of Chinese hamster V79 cells were isolated. The arsenite-sensitive line, As/S5, shows a faster accumulation of labeled arsenite and a slower efflux rate compared with wild-type cells, suggesting the possibility that this line may have a defective efflux pump. The arsenite-resistant line has a slower accumulation and a faster efflux rate, suggesting possible up-regulation of an efflux pump. The arsenite efflux rate can be increased by prior exposure to arsenite. Another arsenite-resistant subline, As/R28A, is cross-resistant to arsenate and antimonite but is very sensitive to cadmium, copper and mercury. Fusion of As/R28A with wild type V79 cells results in hybrids which are arsenite-resistant but have an intermediate resistance to cadmium. Northern blots show that As/R28A (unlike wild-type V79) has undetectable basal expression of metallothionein, but is able to induce metallothionein in response to zinc, cadmium, and arsenite. Induction of metallothionein by zinc or cadmium is protective against arsenite. However, the arsenite-tolerance induced by arsenite-pretreatment cannot be accounted for only by metallothionein. By expression cloning of cDNA from arsenite-induced As/R28A cells (transfecting into arsenite-sensitive cells and selecting for arsenite-resistance), we have identified two other genes which confer some arsenite resistance. One shows almost complete homolgy with the rat *fau* gene, a putative tumor suppressor. The other is a novel cDNA.

C4-212 HUMAN ZINC-REGULATORY FACTOR (ZRF) ACTIVATES HUMAN AND TROUT METALLOTHIONEIN PROMOTERS *IN VITRO*, Susan L.-A Samson[1], Fuminori Otsuka[2], Kaoru Suzuki[3], Shinji Koizumi[3], and Lashitew Gedamu[1], [1]Dept. of Biology, Univ. of Calgary, Calgary, CANADA, T2N 1N4;. [2]Dept. of Environmental Toxicology, Teikyo University, JAPAN, [3]Dept. of Experimental Toxicology, Nat. Institute. of Industrial Health, JAPAN.

Metal induction of metallothionein (MT) gene transcription is dependent on multiple copies of Metal Responsive Elements (MREs) present in the promoter. ZRF is a 116 KDa HeLa cell protein which was affinity purified using the human MT-IIA gene MREa sequence and binds other MT-IIA MREs[2,3]. However, its role in transcription regulation of MT genes was unknown. We have employed a rat liver nuclear extract *in vitro* transcription system to test the functional interactions of ZRF with MT promoters. ZRF is able to activate both rainbow trout MT-B and human MT-IG promoters substantially in the presence of zinc, confirming the functional conservation of MRE binding factors. Further, this activation is correlated with binding to native MRE sequences from both promoters *in vitro*. Mutations which inactivate MRE activity also diminish ZRF binding. Since MREs have been shown to act synergistically, we are currently studying the functional and physical interactions of ZRF with single and multiple MREs of MT promoters. Also, we are investigating if the metal requirement for ZRF activity is a function of the DNA binding domain or a separate metal responsive domain.

(Supported by the Medical Research Council of Canada).

C4-213 ZN-DEFICIENT FIBROBLASTS: MOLECULAR & CELLULAR ROLES OF ZINC. David A. Suhy[*] and Thomas V. O'Halloran [*#]. Department of Biochemistry, Molecular Biology, and Cell Biology[*], and Department of Chemistry[#], Northwestern University, Evanston, Il. 60208.

A structural class of transcription factors, as well as over 300 metabolic enzymes have been shown to contain zinc, yet little is known about the cell biology of this metal.[1] We have generated a zinc-deficient mammalian cell line to probe uptake, distribution, and homeostasis of this metal as well as to determine if fluxes in intracellular concentration can alter patterns of gene expression. Media in which zinc concentrations are reduced from the normal micromolar levels to less than 30 nM still allow proliferation. Cells grown up in this media exhibit a drastic morphology change within five or six passages that is "curable" upon the addition of zinc chloride into the media - yet remain unresponsive to the substitution of copper, cadmium, or cobalt. As $[Zn]_{media}$ is varied from 3 x 10^{-2} to 50 uM, an 18-fold increase in intracellular zinc is observed. In contrast, a non-adapted control fibroblast cell line exhibits only a 3-fold increase in intracellular zinc over the same range of extracellular zinc. Zinc-specific fluorescent dyes reveal similar changes of intracellular zinc concentration as a function of $[Zn]_{media}$. Finally, changes in subcellular distribution of this metal with increasing concentrations of zinc in the external media are observed. These fibroblasts provide models in assessing the biological roles of zinc.

[1] Vallee, B. L. , and K. H. Falchuk. (1993) Physiological Reviews 73(1), 79-118.

C4-214 VANADATE METABOLISM IN *S. cerevisiae*, Gail R. Willsky and David Pawlowski, Department of Biochemistry, SUNY at Buffalo, Buffalo NY 14214

The addition of 5 mM vanadate (VO_4^{3-}, V) to the growth medium stops the growth of *S.cerevisiae*. After removal of vanadate growth resumes after a two hour lag as previously demonstrated using growth curves. Individual cell viability was determined after the exposure of yeast to 5mM vanadate for 24 hours and removal of vanadate from the medium. A 40 to 50% decrease in cell viability was observed. These results imply that growth inhibitory amounts of vanadate can kill a significant fraction of the cells, while growth of the remaining cells will continue after the removal of vanadate. Using magnetic resonance spectroscopy (ESR and ^{51}V-NMR) we have monitored the processing of intracellular vanadium in cells exposed to 5 mM vanadate in the growth medium for 24 hours. Intracellular accumulation of dimeric or tetrameric and decameric vanadate along with vanadyl (VO^{2+}, IV) can be seen. Cell which grew in the presence of 1 mM vanadate did not accumulate any cellular vanadate, but did have cell associated vanadyl.

To monitor the affects of intracellular oxovanadium compounds on cellular metabolism, intracellular glutathione levels and calcium levels were measured in cells exposed to 1 mM and 5 mM vanadate in the growth medium. The amount of glutathione in the cells increased proportionally to the amount of vanadate in the growth medium. Cells grown in 5 m M glutathione had twice as much glutathione (97 ng/cell) as those cells grown in the absence of vanadate. Calcium imaging fluorescence microscopy of cells containing Fura-2AM demonstrated that free intracellular calcium decreased as the concentration of vanadate in the growth medium increased.

Using a complementation assay for vanadate sensitivity and a yeast genomic library, DNA which complements a class 1 vanadate-resistance mutation was isolated. This mutation has been mapped to yeast chromosome V. Analysis of the gene which complements the class 1 vanadate-resistance mutation will help explain how the cell metabolizes excess vanadium.

C4-215 AFT1: ACTIVATOR OF FERROUS TRANSPORT REGULATES IRON UPTAKE TRANSCRIPTIONALLY IN S. CEREVISIAE. Yuko Yamaguchi-Iwai, Daniel S. Yuan, Andrew Dancis, Richard D. Klausner.
Cell Biology and Metabolism Branch, NICHD, NIH, Bethesda, MD 20892.

Iron is an essential yet toxic element for virtually all organisms.The uptake and metabolism of iron is tightly regulated. Using a genetic screen to identify genes involved in iron metabolism in S. cerevisiae, we have identified an activator of ferrous transport, AFT1, that mediates the control of iron uptake. This control is exerted at the level of transcription of several genes that have been shown to be regulated by environmental iron and required for the high affinity uptake of environmental iron including the plasma membrane ferric reductases, FRE1 and FRE2, and the copper dependent membrane oxdase,FET3. AFT1 encodes a 78kD protein that is characterized by a highly basic amino termenal region and a glutamine rich carboxy terminal region, reminiscent of transcriptional activators.In addition, the AFT1p contains two histidine rich domains. A dominant mutant, termed AFT1-1^{up}, demonstrates constitutively high levels of uptake due to high and unrepressable levels of FRE1, FRE2 and FET3 transcription. *AFT1-1up strains demonstrate greatly enhanced susceptibility to* the toxic effects of environmental iron. Interruption of the AFT1 gene results in a loss of expression of FRE1, FRE2 and FET3 and the resultant absence of high affinity iron uptake. These interruption strains are highly susceptible to iron deprivation. Thus, AFT1 appears to encode a novel iron dependent transcriptional regulator that plays a central role in the controlled expression of genes involved in iron uptake.

C4-216 Characterization of the *CUP14* Gene Involved in Copper Tolerance in *S. cerevisiae*. Wei Yu and Dennis R. Winge, Department of Biochemistry, University of Utah Health Sciences Center, 50 N Medical Drive, Salt Lake City, UT 84132 Telephone: (801)581-6713

All cells require metal ions for cellular physiology, yet an excess concentration of most metal ions causes toxicity. Cells have evolved homeostatic mechanisms to regulate the intracellular concentration of metal ions. Laboratory yeast strains can tolerate a wide range of copper levels and the mechanisms by which yeast control the intracellular copper level are being resolved.

Wild-type *Saccharomyces cerevisiae* strain X-2180 can usually grow in medium containing upwards of 2 mM Cu(II). In an early genetic screen, a series of yeast mutants were isolated by Dr. S. Fogel at the University of California, Berkeley with the phenotype of copper sensitivity (Cu^S). These Cu^S mutants were unable to grow in medium containing Cu(II) salts with a range from 0.1 mM to 1.5 mM. Genetic analysis identified 12 complementation groups implying that multiple genes are necessary for Cu tolerance in yeast (Fogel and Welch PNAS 79: 5342, 1982). These were designated as *cup* (**C**opper **U**tilization **P**rotein) mutants. Of these *cup* mutants, *CUP1* was identified as a yeast metallothionein gene and *CUP2* as a transcription activator for *CUP1* gene expression. *CUP5* encodes one of the subunits of the vacuolar ATPase. Since *cup3* and *cup14* exhibited a marked phenotype of Cu sensitivity, we set out to identify the genes responsible for these two interesting mutants. Original mutants were without any auxotrophic markers and were HO^+. Strains of ho^- were generated that had auxotrophic markers. We cloned the *CUP14* gene by complementing the mutant with a high copy yeast genomic bank. The identified *CUP14* gene encodes a predicted ORF of 447 a.a. with no significant homology to the current database. The validity of this ORF was proved by the lack of function after a frameshift was introduced. Overexpression of this gene in a wild-type *S. cerevisiae* strain conferred resistance to copper salts. Disruption of this gene in yeast also resulted in slightly enhanced tolerance to Cu(II). More interestingly, analysis of the original and the null mutant showed normal level of intracellular glutathione, normal *CUP1* expression, and normal initial uptake of Cu salts. However, the long term accumulation of cellular Cu was consistently higher in the mutant than in the wild type cell. The mechanisms by which this gene controls yeast copper tolerance are being further investigated.

C4-217 GENETIC ELEMENTS REQUIRED FOR THE RAPID AUTO ACTIVATION OF THE *AMT1* METALLOREGULATORY TRANSCRIPTION FACTOR GENE IN THE YEAST *Candida glabrata*, Zhiwu Zhu and Dennis J. Thiele, Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI 48109-0606.

Copper is an essential yet toxic metal ion in biological systems. The dual nature of copper demands that cells constitute a rapid sensory and responsive mechanism to differentiate copper concentrations and therefore maintain a delicate balance between essential and toxic levels. Many organisms protect themselves from copper toxicity by synthesizing metallothioneins (MTs), low molecular weight, cysteine-rich metal-binding proteins. In the opportunistic yeast *Candida glabrata*, AMT1, the copper sensing metalloregulatory transcription factor, activates a family of metallothionein genes (*MT-I, MT-IIa and MT-IIb*) to modulate the bioavailability of copper and therefore prevent adverse effects of high level of copper ions. Unlike its counterpart in the yeast *S. cerevisiae, ACE1, AMT1* is transcriptionally regulated by its own product, extremely rapidly in response to copper. This metal-responsive nature of the *AMT1* gene is achieved in part, through an AMT1 binding site within *AMT1* promoter. Our recent experiments have elucidated other distinct *AMT1* promoter elements which are absolutely essential for the rapid auto activation by AMT1 and which strongly contribute to the magnitude of gene transcription by copper-activated AMT1. Therefore, these elements and the AMT1 binding site within the *AMT1* promoter are critical constituents of important Metalloregulatory Transcription Factor Gene in defense against copper toxicity.

Metal/Oxygen Regulation in Eukaryotes

C4-300 IMPAIRMENT OF PROTEIN SYNTHESIS IS CLOSELY ASSOCIATED TO CHANGES IN ENVIRONMENTAL OXYGEN LEVELS RATHER THAN TO INTRACELLULAR ATP CONTENT, Pedro Buc Calderon and Véronique H. Lefebvre, Unité de Biochimie Toxicologique et Cancérologique, Département des Sciences Pharmaceutiques, Université Catholique de Louvain, BCTC 7369, B-1200 Brussels, Belgium

Acute responses of cell to hypoxia involve mutliple and complex events (1). Besides such effects, many energy-dependent metabolic pathways are inhibited when cells are submitted to hypoxia (2). Indeed, we observed that freshly isolated rat hepatocytes incubated under hypoxic conditions (N_2/CO_2, 95%:5%) loose rapidly their ability to synthesize proteins. Such an early decrease of protein synthesis (as measured by the incorporation of radiolabelled leucine into proteins) is unlikely the result of ATP depletion, inhibition of amino acid uptake, enhanced protein degradation or decreased RNA synthesis (3). On the basis of the experimental data obtained, it appears that the presence or the absence of O_2 may turn on/off the control of major metabolic process. In the absence of O_2, cells are under metabolic arrest and kept their ATP molecules for more critical and essential functions (i.e. ionic homeostasis). Considering the rapid inhibition of protein synthesis, when cells are deprived of O_2, we will discuss that liver cells might contain an "oxygen sensor" indicating to cells whether to stop or to reinitiate their biosynthetic activities.

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(2) Seglen P.O., Biochim. Biophys. Acta 338, 367, 1974
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C4-301 CHARACTERIZATION OF EUKARYOTIC DNA TOPOISOMERASE I CATALYSIS. Kent Christiansen, Birgitta R. Knudsen, and Ole Westergaard, Department of Molecular Biology, University of Aarhus, C. F. Møllers Alle', 8000 Århus C, Denmark.

Eukaryotic type I DNA topoisomerases comprise a class of monomeric enzymes, which catalyzes changes in the topological state of negatively or positively supercoiled DNA by coordinated cleavage and religation of one strand of the DNA. The existence of a covalent intermediate in topoisomerase I catalysis allows uncoupling of the cleavage and ligation half-reactions on partially single-stranded DNA substrates containing a highly preferred interaction site. Utilizing this DNA substrate system we have found that the cleavage reaction requires bipartite interaction with two distinct DNA duplex regions located around the cleavage site (region A) and on the side holding the 5'-OH end generated by cleavage (region B). Postcleavage complexes containing the enzyme covalently attached at an internal or a terminal position are capable to ligate DNA strands matching the noncleaved strand or duplex DNA fragments carrying blunt-ends, respectively (1). In addition, topoisomerase I-DNA intermediates are able to catalyze pH dependent hydrolysis and alcoholysis of the 3'-phosphotyrosyl bond by accepting water or alcohol compounds as substitutes for 5'-OH DNA ends (2). Topoisomerase I-mediated DNA cleavage, DNA ligation, and non-DNA utilization during hydrolysis and alcoholysis are inhibited to the same degree by modification of the enzyme with the sulfhydryl-specific agent N-ethylmaleimide. Since these reactions require different extent of DNA interaction or none, it suggests a role for cysteine residue(s) in catalysis rather than in DNA binding activity. When these features are considered together with an approximately 2 fold stimulation of topoisomerase I activity by reducing agents (dithiothreitol), it indicates a possible redox regulation of eukaryotic topoisomerase I catalysis.

1. Christiansen, K. and Westergaard, O. (1994) *J. Biol. Chem.* **269**, 721-729
2. Christiansen, K., Knudsen, B.R., and Westergaard, O. (1994). *J. Biol. Chem.* **269**, 11367-11373

C4-302 A HOMOLOGUE OF HUMAN HYPOXIA-INDUCIBLE FACTOR-1 IS PRESENT IN SACCHAROMYCES CEREVISIAE. Peter T. Curtin, Stephen L. Hatch II and Ashima Madan, Dept. of Medicine, Tufts/New England Medical Center, Boston, MA 02111 and Dept. of Pediatrics, UCSF, San Francisco, CA 94143

The increased expression of the human erythropoietin (Epo) gene in response to hypoxia is mediated in part by the interaction of a 3' enhancer element with a hypoxia-inducible nuclear factor, designated HIF-1. HIF-1 binds to an 8 base pair sequence that is necessary for enhancer activity. HIF-1 induction can be blocked by inhibitors of transcription, translation or phosphorylation. In addition, HIF-1 binding activity can be abolished by treatment of nuclear extracts with phosphatase. A wider role for this regulatory system was suggested by the finding that the hypoxia-inducible enhancer is functional in a number of non Epo-producing cells and that hypoxic nuclear extracts from these cells contain HIF-1. Thus, this regulatory system appears to play a more general role in the control of mammalian cellular response to hypoxia. In order to examine the conservation of this regulatory system among eukaryotes, normoxic and hypoxic nuclear extracts from mammalian and avian cells and from *Saccharomyces cerevisiae* were assayed for HIF-1. Cells incubated for four hours in 21% or 1% O_2 were harvested and nuclear protein extracts were prepared. Mobility shift assays were performed by incubating each extract with a radiolabeled Epo enhancer probe. HIF-1 binding activity was present in hypoxic extracts from a number of mammalian cell lines including Hep 3B and Hep G2 (human hepatoma), U937 (human monocyte), cos7 (monkey renal fibroblast) and MEL (mouse erythroleukemia cells). It was also present in hypoxic extracts from primary cultures of neonatal rat cardiac myocytes and chick embryo fibroblasts. Hypoxic extracts from *Saccharomyces cerevisiae* also contained a factor that bound specifically to the enhancer fragment. The electrophoretic mobility of the inducible band in yeast samples was greater than that observed in mammalian and avian samples. Competition experiments demonstrated the same specificity of binding for the yeast factor and mammalian HIF-1. Pre-incubation with cycloheximide blocked the induction of both the yeast factor and HIF-1. Phosphatase treatment of hypoxic mammalian or yeast extracts resulted in a loss of inducible binding activity. Together these findings indicate that a yeast homologue of HIF-1 exists and that the mechanism of its induction is similar to that of mammalian HIF-1. They also suggest that a molecular mechanism underlying transcriptional response to hypoxia has been conserved from yeast to man.

C4-303 REGULATION OF TYROSINE HYDROXYLASE mRNA STABILITY BY OXYGEN AND REDOX STATE IN RAT PHEOCHROMOCYTOMA (PC12) CELLS. M.F. Czyzyk-Krzeska, D.E. Millhorn. Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267-0576.

Moderate reduction in O_2 tension in the environment (10% O_2) causes 3 fold increase in half-life of mRNA for tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamines synthesis, in pheochromocytoma (PC12) cells [*M.F. Czyzyk-Krzeska et al., JBC, 269, 760-4, 1994*]. The increase in TH mRNA stability during hypoxia is accompanied by enhanced binding of cytoplasmic proteins to a pyrimidine rich sequence between bases 1552-1578 in the 3' untranslated region of TH mRNA [*M.F. Czyzyk-Krzeska et al., JBC, 269, 9940-5, 1994*] that occurs within first hour of exposure. Using oligonucleotides antisense to different fragments of this 28 base sequence followed by RNase H digestion and gel retardation assays we showed that two different RNA-protein complexes were formed; both of which were enhanced by hypoxia and had similar molecular weight. A short sequence (bases 1564-1578) was sufficient for binding of one of the protein factors, while the whole length of 1552-1578 was required for the binding of another.

The signal transduction pathway mediating increased TH mRNA stability in response to hypoxia is unknown. We have hypothesized that hypoxia affects redox potential of the RNA binding proteins by increasing number of free thiol groups that participate in the binding. Oxidation of the protein extracts with diamide abolished the binding. Subsequent reduction of oxidized proteins with β-mercaptoethanol restored it. Higher doses of diamide were required to reduce binding of proteins to TH mRNA when proteins were extracted from cells exposed to hypoxia. This indicates that a larger number of SH groups participates in the binding of proteins to TH mRNA during hypoxia. We have further studied if the change in the redox potential caused by reducing agents (i.e. antioxidants) mimics effects of hypoxia on TH mRNA stability. PC12 were treated with either N-acetyl-L-cysteine (NALC), reduced glutathione or N-(2-mercaptopropionyl)-glycine. All three drugs are known to increase intracellular concentration of thiol groups. These treatments increased TH mRNA stability, similar to that observed during hypoxia. In addition, treatment of PC12 cells with NALC enhanced binding of a protein complex to the same sequence on TH mRNA identified previously to bind the hypoxia inducible factor.

C4-304 REDOX REGULATION OF AP-1 INDUCTION AND GLUTATHIONE S-TRANSFERASE GENE EXPRESSION

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Exposure of mammalian cells to a variety of chemical agents induces the expression of drug-metabolizing enzymes, glutathione S-transferases (GSTs), glucuronosyl transferases and NAD(P)H:quinone reductase. We have recently shown that Fos and Jun transcription factors are involved in the regulation of this expression. We demonstrated that a regulatory element composed of two adjacent AP-1-like binding sites and activated by Fos/Jun heterodimeric (AP-1) complex is responsible for the induction of GST Ya and quinone reductase gene expression by chemical agents.
We show now that the induction of the drug-metabolizing enzymes is mediated by the activation of c-*fos* and c-*jun* proto-oncogene expression and an increase in AP-1 binding activity. The present findings indicate that treatment of cell cultures with a variety of chemical agents such as planar aromatic (β-naphthoflavone, 3-methylcholanthrene) and electrophilic (*tert*-butylhydroquinone, dimethylfumarate, *trans*-4-phenyl-3-butene-2-one) compounds, tumor promoters (TPA, phenobarbital, dioxin), H_2O_2, arsenite, arsenate and heavy metals (Pb, Cd, Zn), leads to a rapid induction of AP-1 binding activity. We present evidence that the chemical induction of AP-1 activity is prevented by thiol compounds and is enhanced by a thiol oxidant or intracellular glutathione (GSH) depletion. These findings suggest that diverse chemicals that induce the AP-1 complex leading to the AP-1 mediated transcriptional activation of GST Ya gene expression may act through a common mechanism involving the production of reactive oxygen species and depletion of reduced glutathione levels. By monitoring quinone-generated oxygen radicals we show a correlation between the production of·OH radicals, GSH level, induction of AP-1 binding activity and GST Ya gene expression.

C4-305 ANTIOXIDANT INHIBITION OF NF-κB BLOCKS ENDOTHELIAL CELL ACTIVATION, Christiane Ferran, Maria Millan,Vilmos Csizmadia, Christine Brostjan , Fritz H. Bach and Hans Winkler. Sandoz Center for Immunobiology Department of Surgery, New England Deaconess Hospital, Harvard Medical School, Boston , MA, 02215.

Endothelial cells (EC) respond to activating stimuli by rapidly upregulating a characteristic set of genes. Induction of these genes represents the molecular basis of a newly-acquired EC phenotype that promotes leukocyte adhesion, coagulation and thrombosis and involves production of inflammatory cytokines. Transcriptional activation of these genes is protein-synthesis independent suggesting that preexistant transcription factors are responsible for their expression. Given the presence of NF-κB binding sites in the promoter regions of all of the upregulated genes tested (with the exception of junB), we hypothesized that NF-κB plays a key role in EC activation. Based on data showing that reactive oxygen intermediates act as second messengers in activation of NF-κB, we have used the antioxidant pyrrolidine dithiocarbamate (PDTC) to test the effectiveness of NF-κB inhibition on TNF-induced EC activation in vitro.

Confluent PAEC (porcine aortic EC) monolayer cultures were activated with TNFα (100U/ml), with or without pretreatment for 1.5 hours with PDTC (100μM). RNA was extracted 1,2,4,6 and 24 h later. Northern blot analysis showed that PDTC significantly reduced (80 to 90%) the TNF-mediated induction of E-selectin, VCAM-1, ICAM-1, IL-8, PAI-1 (plasminogen activator inhibitor), TF (tissue factor) and IκBα. As already mentioned, all these anti-oxidant sensitive genes have NF-κB binding sites in their 5' flanking regions. Importantly, neither the TNF-inducible, NF-κB-indepedent protooncongene junB nor the constitutively expressed thioredoxin or GAPDH mRNA levels were affected by PDTC treatment. Nuclear extracts from the same cell treatment groups were examined using electrophoretic mobility shift assay to evaluate NF-κB binding.Results showed that PDTC totally abrogated NF-κB binding activity in extracts from TNF-activated PAEC. PDTC pretreatment also induced an increase of the binding of the E-selectin specific transcription factor, NF-ELAM1. The significance of this increased binding, and whether it is related to inhibition of E-selectin is yet unclear.

Many inhibitors of EC activation are selective and inhibit a few of the genes up-regulated by TNF; different inhibitors inhibit different subsets of genes. In contrast, NF-κB appears to function as a central regulatory switch that when inhibited leads to a block in induction of those genes tested that are involved in the proinflammatory and prothrombotic phenotype of activated EC. NF-κB may thus represent a potential therapeutic target in diseases where EC activation is a central pathogenetic component.

C4-306 REGULATION OF ENDOTHELIAL CELL GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE EXPRESSION BY HYPOXIA. Krista K. Graven and Harrison W. Farber, The Pulmonary Center, Boston University School of Medicine, Boston, MA 02118.

Exposure of endothelial cells (EC) to hypoxia results in the increased expression of a distinct set of proteins with molecular masses of 56, 47, 39, 36 and 34 kDa. The 36 kDa protein has been identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Northern blot analysis demonstrates that the time course and extent of GAPDH mRNA accumulation during hypoxia is similar to that of the protein. Nuclear run-off assays suggest that GAPDH mRNA is upregulated primarily through an increase in the rate of transcription.. We have now performed RNA stability studies using actinomycin-D which confirm that GAPDH upregulation by hypoxia is primarily due to an increase in the rate of transcription with a small contribution from an increase in RNA stability. Studies using cycloheximide show that protein synthesis is required for GAPDH mRNA upregulation. Transient transfection assays using a GAPDH-CAT construct that contains the GAPDH promoter region (-487 to +20 nucleotides in relation to the transcription initiation site) into bovine EC demonstrate a hypoxic regulatory element in this region. These studies show a 3- to 4-fold increase in CAT activity following exposure of transfected EC to 0% oxygen for 18 hours. Interestingly, the GAPDH gene contains a 6 base pair hypoxia-inducible factor 1 (HIF 1) site (-990 to -985), however , it is not included in the constructs used. Whether the HIF 1 site is active in regulating GAPDH gene expression in response to hypoxia or whether GAPDH is regulated by a unique hypoxic regulatory element, as suggested by our preliminary data, is under further study.

C4-307 REDOX REGULATION OF PEBP2/AML1/CBF/SEF1 PROTEINS, Magnus Holm, Xiao-Qi Xie and Thomas Grundström, Department of Cell and Molecular Biology, Umeå University, Umeå, Sweden.

SL3-3 Enhancer Factor 1 (SEF1) sites are found in regulatory regions of many lymphoma and sarcoma retroviruses as well as in the regulatory regions of many cellular genes with high expression in T-cells. Proteins binding to SEF1 sites are more abundant in T-cells than in other cell types tested. Recently cDNAs denoted PEBP2αA and PEBP2αB (AML1) encoding proteins able to bind to a consensus SEF1 site were cloned. cDNAs encoding a protein denoted PEBP2β or CBFβ have also recently been cloned. This protein is by itself unable to bind to DNA but can bind to PEBP2α proteins and augment their DNA binding. Cloning studies revealed that at least 3 chromosomal translocations, t(3,21), t(8,21) and [inv(16)(p13q22)], frequently seen in human myeloid leukemias, disrupt genes encoding SEF1 binding proteins.

Analysis of SEF1 binding proteins purified from bovine thymus showed requirement for reducing conditions for strong DNA binding. Recent data have indicated a possible role of redox regulation in many normal (e.g. apoptosis) and pathological processes. We have cloned the published PEBP2α and β cDNAs using PCR, and expressed them *in vitro* and in *E. coli*. Both the PEBP2αA and B proteins show the corresponding redox dependency as the purified proteins. We have found that substitution of conserved cysteines in the PEBP2αA and B proteins render them insensitive to oxidation *in vitro*. Cysteines are important both for DNA binding and for interaction with PEBP2β proteins. In addition, the activity of the β proteins is affected by oxidising conditions. PEBP2β contains four cysteines and we have identified cysteines important for the redox regulation of β. We have also shown that transcriptional activation by transiently expressed PEBP2α proteins is regulated *in vivo* in response to modulation of the redox state of the transfected cell.

C4-308 OXIDANT SCAVENGING BY PARASITIC FILARIAL WORMS (NEMATODA). Eric R. James, David C. McLean Jr., Heather L. Callahan, Lakshmi Venkatakrishnaiah, Preeti Gupta Lal, and Gautami Guha. Department of Ophthalmology, Medical University of South Carolina, Charleston, SC 29425-2236.

In addition to oxygen derived by-products of cellular metabolism, parasitic helminths must cope with oxidants generated by host metabolism and also by host phagocytic cells. Filarial worms normally tolerate superoxide ($O_2^{\cdot-}$) but not H_2O_2, yet low concentrations of H_2O_2 can induce tolerance to a lethal concentration of H_2O_2 suggesting at least partial regulation of oxidant scavenging by oxidants. As a first step towards examining this process we have attempted to identify the major oxidant scavenging enzymes. We have shown previously that filariae contain high concentrations of Cu-Zn superoxide dismutase (SOD), but the H_2O_2-detoxifying enzymes glutathione peroxidase (GSH-px) and catalase (Cat) are essentially undectable. Cu-Zn SOD is also secreted *in vitro*. We have screened cDNA and genomic libraries by hybridization and by PCR using degenerate primers whose designs are based on known sequences for the genes for the antioxidant enzymes of other organisms. In addition to a cytosolic Cu-Zn SOD (CySOD) we have identified a novel extracellular form of Cu-Zn SOD (EcSOD) from both *Onchocerca* (causative agent of River Blindness) and *Dirofilaria* (causative agent of Dog Heartworm Disease) and a surface expressed Se-independent GSH-px from *Dirofilaria* which may function as a lipid hydroperoxidase (GSH-Lpx) (according to others who have identified a homologuous protein in *Brugia*). The GSH-Lpx is absent from *Onchocerca*. No Se-dependent GSH-px or Cat sequences have been located using both PCR and low stringency hybridization screening with GSH-px or Cat probes from other organisms. Both parasites also posess a glutathione S-transferase (GST) and others have reported a second GST from *Onchocerca*. We speculate currently that filariae secrete EcSOD to regulate the amount of H_2O_2 and other oxidants produced from the inflammatory cells' $O_2^{\cdot-}$ and utilize GSH-Lpx and/or GST to detoxify lipid hydroperoxides resulting from membrane interaction with H_2O_2 and/or the other oxidants; parallel intracellular protection from metabolically produced oxidants being produced by CySOD and GST. It is not yet known which enzyme(s) account for the induced tolerance to extrinsic H_2O_2. Support from NIH EY07542, WHO TDR, Clark Foundation, Research to Prevent Blindness Inc. and MUSC is acknowledged.

C4-309 MODULATION OF AP-1 ACTIVITY BY D-α-TOCOPHEROL AND HYDROGEN PEROXIDE IN VASCULAR SMOOTH MUSCLE CELLS. ROLE OF PROTEIN KINASE C, Dominique Marilley, Barbara Chatelain-Stäuble, Daniel Boscoboinik, Andrea Tasinato and Angelo Azzi, Institut für Biochemie und Molekularbiologie, Universität Bern, Bern, Switzerland.

Growth of vascular smooth muscle cells (VSMC) is an important event in the pathogenesis of atherosclerosis. VSMC are stimulated to grow by mitogens such as platelet-derived growth factor. We have shown that VSMC growth is inhibited by d-α-tocopherol. This inhibition is mediated by the decrease of protein kinase C (PKC) activity in a cell cycle-dependent manner. When the cells were treated with H_2O_2, DNA synthesis and PKC were activated. Since AP-1 is a mediator of growth, differentiation and cellular stress, its activity in VSMC was analysed. Treatment of quiescent rat VSMC with the PKC activator PMA increased AP-1 binding to its consensus DNA element. Simultaneous addition of H_2O_2 almost completely prevented it. When PKC was inhibited, H_2O_2 activated AP-1 binding. D-α-tocopherol increased AP-1 binding activity in the absence of PMA but inhibited it in its presence. D-β-tocopherol, an analogue of d-α-tocopherol, did not show any effect either on cellular growth, PKC activity or AP-1 binding, indicating that the effects of d-α-tocopherol are not due to its antioxidant properties. A compositional change of the AP-1 complex is observed after treatment with PMA or d-α-tocopherol, as shown by supershift analysis of AP-1 with antibodies raised against the members of the Fos and Jun families. The effectiveness of the AP-1 complexes obtained upon treatment with H_2O_2 and d-α-tocopherol to produce gene expression, tested by transfecting CAT-constructs containing several AP-1 binding elements, will be reported.

C4-310 PROSTAGLANDIN H SYNTHASE-1 DEFINES A NSAID-INSENSITIVE REACTIVE OXYGEN PATHWAY OF NF-κB ACTIVATION, Donald G. Munroe, Elizabeth Y. Wang, J. Philip MacIntyre, Susanna S. C. Tam, Daniel H. S. Lee, Gareth R. Taylor, Lubing Zhou, Richard K. Plante, Syed M. I. Kazmi, Patrick A. Baeuerle§ and Catherine Y. Lau, The R. W. Johnson Pharmaceutical Research Institute, Toronto, Ont, Canada M3C 1L9 and §Institute of Biochemistry, Albert-Ludwigs-University, Freiburg, Germany

Transcription factor NF-κB is involved in the induction of numerous genes during inflammatory reactions. In most tissues, NF-κB exists in a latent form, which can be induced by a variety of seemingly unrelated pathogenic stimuli. It has been proposed that reactive oxygen species (ROS) play a common role as intracellular messengers in NF-κB activation; however, the enzyme(s) that produce this ROS signal has not been identified. Here we show that the widely-expressed prostaglandin H synthase-1 (PGHS1) enzyme, targeted by many NSAIDs, contributes to NF-κB activation via a ROS-mediated pathway which is insensitive to NSAIDs. Overexpression of PGHS1 in COS7 cells resulted in a dramatic increase in peroxide-induced ROS production, and potentiated both basal and PMA-induced NF-κB activities. The NSAID-sensitive cyclooxygenase activity of PGHS1 was not required for ROS production or NF-κB potentiation, while the NSAID-insensitive PGHS1 peroxidase was indispensible. Furthermore, ROS production was efficiently suppressed by the antioxidant pyrrolidine dithiocarbamate, which blocks NF-κB activation by phorbol esters in a variety of cell types. Thus, PGHS1 can generate dual extracellular and intracellular signals, effected respectively through prostaglandin synthesis and ROS-mediated activation of NF-κB.

C4-311 PRODUCTION OF HYDROGEN PEROXIDE BY TGF β1 AND ITS INVOLVEMENT IN INDUCTION OF EGR1 IN MOUSE OSTEOBLASTIC CELLS, Kiyoshi Nose, Motoi Ohba, Motoko Shibanuma. Department of Microbiology, Showa University School of Pharmaceutical Sciences, Hatanodai 1-5-8, Shinagawa-ku, Tokyo 142, Japan.

Transforming growth factor (TGF) β1 is a multifunctional cytokine regulating cell proliferation, differentiation, and other biological processes. It controls the expression of numerous genes, including early response genes and cellular matrix genes. However, the signal-transducing mechanism underlying this regulation of gene expression is not fully understood. In this study, we investigated whether redox regulation plays a role in the TGF β1 signal transduction in the mouse osteoblastic cell line (MC3T3-E1). The overall intracellular oxidized state of the cells, when measured using 2',7'-dichlorofluorescin diacetate by laser-scanning confocal microscopy was increased transiently after the addition of TGFβ1. This increase was abolished by the addition of oxygen radical scavengers, such as catalase and N-acetyl-cysteine. In a variant cell line lacking the TGFβ1 receptor, the intracellular oxidized state was not modulated by treatment with TGFβ1. We then examined the expression of early growth response-1 (egr-1) gene, which is induced by TGFβ1 and H_2O_2. Radical scavengers inhibited the induction of egr-1 by TGFβ1, but not that by phorbol ester. A nuclear run on assay indicated that this inhibition was at the transcriptional level. From transient expression experiments using chloramphenicol acetyltransferase gene linked to serially deleted egr-1 gene 5'-upstream region, the CArG element in the 5'-flanking region of egr-1 gene was identified as an essential sequence in the transcriptional activation for both TGFβ1 and H_2O_2-stimulation. These results suggest that H_2O_2 acts as a mediator for the TGFβ1-induced transcription of egr-1 gene.

C4-312 **SIGNIFICANT LIPID PEROXIDATION IN PREGNANCY INDUCED HYPERTENSION AS INDICATED BY ELEVATED PLASMA MALONALDEHYDE LEVELS.**

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Malonaldehyde *(MDA)*, a common product of lipid peroxidation is formed from cyclic endoperoxides produced by autooxidation of fatty acids. Pregnancy induced hypertension *(PIH)* which occurs in the last trimester of pregnancy is characterised by an elevation in the arterial blood pressure, proteinuria and edema. the mean levels of MDA were found to be significantly increased in PIH when compared to control groups viz. normotensive pregnant controls *(t=2.17; df=398)*, normotensive nonpregnant controls *(t=11.75; df=298)* and non-pregnant hypertensive women without the history of PIH during their reproductive life. The levels were found to be elevated with severity of the condition and in primigravid women as compared to multigravid women. No variation was observed in cases with positive family history of PIH, while elevated levels were observed in cases with positive family history of essential hypertension.

The elevated levels of MDA suggest an increase in lipid peroxidation and oxygen free radical formation leading endothelial cell damage caused due to activated neutrophils. The free radicals so formed form cross links with DNA disturbing the template and affecting the protein metabolism. The rise in the antioxidants in the present PIH cases probably is of compensatory nature responding to a rise in peroxide load and the extent of severity of the condition.

C4-313 Modulation of NF-kB by reactive oxygen species and cAMP
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The transcription factor NF-kB may play an important role in glomerular injury in the kidney. We therefore examined the regulation of NF-kB in glomerular mesangial cells. Treatment of mesangial cells with TNF-α increased nuclear proteins that bound to NF-kB specific DNA oligonucleotide. IgG aggregates also increased nuclear NF-kB, showing for the first time Fc-γ receptor mediated activation of NF-kB. Treatment of a cytostolic preparation with the detergent deoxycholate also activated NF-kB. The binding characteristics were typical for NF-kB transcription factors as determined by competition experiments with wild type NF-kB binding DNA oligonucleotides and mutated oligonucleotides. Furthermore a monoclonal antibody against the p65 subunit of NF-kB prevented the binding of NF-kB to the kB-oligonucleotide. To evaluate the potential role of reactive oxygen intermediates in the activation of NF-kB we used PDTC as a scavenger and HMAP as an inhibitor of NADPH-dependent oxidase. Both PDTC and HMAP attenuated the increase in nuclear NF-kB in response to either TNF-α or IgG. Finally generation of superoxide anion by xanthine oxidase activated NF-kB, an effect also mitigated by PDTX. In contrast, exogenous H_2O_2 did not activate NF-kB. Preincubation of cells with 8 Br-cAMP, forskolin or PGE_2 attenuated the increase in nuclear NF-kB in response to TNF-α, IgG and superoxide anion. Our results provide support for a role of reacitve oxygen intermediates as mediators for activation of NF-kB in MC after stimulation with TNF-α or IgG aggregates. As an unexpected novel finding we report that cAMP can inhibit activation of NF-kB in MC. These observations may help to explain effects of TNF-α, IgG aggregates and cAMP on generation of cytokines by mesangial cells and the resulting glomerular pathophysiology.

C4-314 INDUCTION OF THE STAT PATHWAY BY OXIDATIVE STRESS, Amy R. Simon[1], Barry Fanburg[1] and Brent Cochran[2], [1]Department of Pulmonary and Critical Care and [2]Department of Physiology, Tufts University School of Medicine, Boston, MA 02111

Reactive oxygen species have important effects in normal physiologic processes as well as in the pathogenesis of disease. Oxidative stress has been shown to lead to the activation of latent transcription factors and many immediate early genes. Our lab has previously identified a growth factor inducible signaling pathway that is comprised of members of the STAT family of transcription factors. We therefore set out to examine whether the STAT family is induced by oxidative stress. We show that a superoxide stimulus, generated from xanthine/xanthine oxidase, induces STAT family DNA binding activities. This induction occurs rapidly, is seen in multiple cell lines and is suppressed by the antioxidants N-acetyl-L-cysteine and pyrrolidine dithiocarbamate. In addition, we have identified STAT 3 as the factor induced by superoxide exposure in bovine pulmonary artery endothelial cells. In contrast, hydrogen peroxide is a weak inducer of STAT binding activities. We are currently investigating the mechanisms by which oxidative stress induces the STAT pathway and what the consequences of this activation are on the transcriptional response of the cell.

C4-315 CATALASE GENE AMPLIFICATION IN H_2O_2- AND O_2-RESISTANT CHINESE HAMSTER FIBROBLASTS, Douglas R. Spitz*, Shannon J. Sullivan*, Chris Von Kapp-Herr[1], Wendy Golden[1], Randy A. Hock[1], R. Ariel Gomez[1] and Clayton R. Hunt*, *Radiation Oncology Center, Section of Cancer Biology, Washington University School of Medicine, St. Louis, MO 63108 and [1]University of Virginia, Charlottesville, VA 22908

Chinese hamster fibroblast cell lines which are stably resistant to oxidant-mediated toxicity have been isolated following prolonged exposure (>240 days) to progressively increasing concentrations of either H_2O_2 (50 to 800 μM) or O_2 (80 to 95%) (*J. Cell. Physiol.* 139: 592; *Am. J. Physiol.* 262:L748). These H_2O_2- and O_2-resistant cell lines demonstrate steady state increases in activity and immunoreactive protein of several antioxidant enzymes including catalase, superoxide dismutase, glutathione transferase, glutathione peroxidase, as well as increased total glutathione content (*Biochem. J.* 267:453; *Arch. Biochem. Biophys.* 279:249). To determine molecular mechanisms responsible for the stable increases in catalase activity and immunoreactive protein demonstrated by these resistant cells, steady state levels of RNA were determined using Northern blotting techniques as well as DNA copy number using Southern blotting techniques. Both H_2O_2- and O_2-resistant cell lines demonstrated 10-20 fold greater levels of RNA as well as a 10-20 fold increase in DNA copy number when probed with a catalase cDNA (pCAT10). *In situ* hybridization on chromosome spreads from H_2O_2-resistant cell lines demonstrated that the amplified catalase genes were chromosomally integrated. These results show that catalase gene amplification can occur in hamster fibroblasts following prolonged exposure to either H_2O_2 or 95% O_2. Furthermore, the chromosomal integration of the amplified genes appears to account for the stability of the increased expression of catalase protein in the absence of selective pressure. These results also suggest that amplification of genes coding for antioxidant proteins may represent an important mechanism whereby mammalian cell lines become resistant to chronic oxidative stress.

C4-316 THE ROLE OF HYDROGEN PEROXIDE IN GROWTH FACTOR SIGNALLING. Maitrayee Sundaresan*, Zu-Xi Yu, Kaikobad Irani, and Toren Finkel. Cardiology Branch, NHLBI, National Institutes of Health, Bethesda, MD, 20892. *HHMI-NIH Research Scholar.

Reactive oxygen species (ROS) have been implicated in a variety of biological events including inflammation, apoptosis, and atherosclerosis. Vascular smooth muscle cell (VSMC) chemotaxis and proliferation are profoundly influenced by growth factors like platelet-derived growth factor (PDGF), which is present in large quantities in atherosclerotic plaques. We therefore sought to assess whether ROS and in particular hydrogen peroxide (H_2O_2), might modulate PDGF signal transduction as a potential mechanism linking ROS with atherosclerosis.

We demonstrated by spectrofluorometry using an H_2O_2-sensitive fluorophore, that H_2O_2 is produced by VSMCs in response to PDGF stimulation. In addition, exposure of VSMCs to H_2O_2 (10μM) in the presence of vanadate stimulates tyrosine phosphorylation of multiple proteins. Similarly, the same H_2O_2 concentration independent of vanadate exhibited a small mitogenic effect in VSMCs. To better understand H_2O_2's role in PDGF signaling, PDGF-stimulated H_2O_2 increase was inhibited in VSMCs by: 1) transfecting cells with a replication-deficient recombinant adenovirus (Ad5) harboring the cDNA encoding catalase (Ad5-Cat); 2) exposing VSMCs to purified catalase protein. VSMCs which had increased catalase activity had blunted H_2O_2 release following PDGF stimulation. In addition, increased catalase levels blocked PDGF-stimulated tyrosine phosphorylation, mitogenesis and chemotaxis. These data suggest that H_2O_2 is important in the PDGF signal transduction pathway.

C4-317 A REGULATORY NETWORK IS INVOLVED IN OXYGEN-DEPENDENT EXPRESSION OF GENES IN *S. Cerevisiae*. J.Verdière,[1]M.Gaisne,[1] J.M. Amillet[2] and R.Labbe[2], (1)Centre de Génétique Moléculaire du CNRS, 91190 Gif sur Yvette, FRANCE.(2)Institut J. Monod, Université Paris VII, 2 Place Jussieu, 75251,Paris, FRANCE.

The goal of our research is to characterize and determine the mechanisms by which *S.cerevisiae* is able to control the expression of a large number of genes according to the availability of Oxygen. Two sets of such genes are regulated by oxygen and/or heme. Heme is believed to mediate oxygen dependent regulation for two reasons:1-its absence mimic a deficiency in oxygen 2-its synthesis requires molecular oxygen at two steps.

CYP1 (*HAP1*)activates the expression of several genes whose transcription is heme and/or oxygen dependent. It exerts regulatory functions, even in the absence of heme usually considered to be its effector. It mediates both positive and negative effects depending on the target gene and on the red-ox state of the cell.In aerobic conditions, heme repression is mediated by *ROX1*. The transcription of *ROX1* is heme dependent and activated by *CYP1* (1).

We will present data which show that in anaerobic conditions the cyp1p is an efficient repressor of *ROX1*.A model in which the complex regulatory properties of *CYP1* are explained by a regulatory cascade involving *ROX1* will be presented.

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C4-318 PHORBOL ESTER DEPENDENT CONTROL OF AP-1 AND NF-*k*B TRANSCRIPTION FACTORS IN THE REGULATION OF HIV-1 LTR ACTIVITY BY THE ANTIOXIDANT PYRROLIDINE DITHIOCARBAMATE. Kenji Watanabe, Unna Venkatachalam and Steven C. Miller, Cell and Molecular Biology Laboratory, Life Sciences Division, SRI International, Menlo Park, CA 94025

Pyrrolidine dithiocarbamate (PDTC) is an antioxidant that has been shown by a number of laboratories to inhibit NF-*k*B dependent gene expression. In contrast, PDTC has recently been shown to stimulate AP-1 dependent gene expression. In light of recent evidence for combinatorial mechanisms of gene regulation through AP-1 and NF-*k*B interactions, we have used PDTC to evaluate the phorbol ester (TPA) dependent control of AP-1 and NF-*k*B acting on the HIV1-LTR. Human promonocytic cells were electroporated with a chloramphenicol acetyl transferase (CAT) reporter gene directed by the HIV1-LTR. Replicate cultures were treated with increasing doses of TPA to activate HIV1-LTR directed CAT protein. One set of cells were treated with PDTC for 1 h before the addition of TPA. Samples were harvested after 24 h and the level of HIV1-LTR-directed CAT protein induced by TPA in the control or PDTC treated cultures was measured in cell extracts with a CAT ELISA assay. Our results demonstrate that PDTC has stimulatory and inhibitory activity on the signal transduction pathways integrating at the level of the HIV1-LTR. Moreover, the dose-dependent effect of PDTC to stimulate gene expression was suppressed by TPA in a dose-dependent manner. Electrophoretic mobility shift assays (EMSAs) demonstrated that PDTC treatment inhibited NF-*k*B and induced AP-1 DNA binding activity that was also dose-dependent with TPA. Our results demonstrate that AP-1 activity is responsible for the stimulation of HIV1-LTR activity by PDTC and are consistent with other studies demonstrating the cross-coupling of NF-*k*B and AP-1 transcription factors.

C4-319 REDOX REGULATION OF THE ENDOTHELIN GENE: MECHANISM AND TISSUE SPECIFICITY. Keith A. Webster, Ilona Bodi, Daryl J. Discher, and Nanette H. Bishopric. Cell and Molecular Biology Department, Program in Molecular Cardiology, SRI International, Menlo Park, CA 94025.

Endothelin-1(ET-1) is a member of a family of vasoactive peptides that are involved in regulating regional vascular tone and blood flow in the heart, kidney, and brain. ET-1 is a potent vasoconstrictor that is induced by a number of stimuli including thrombin, transforming growth factor β, sheer stress, calcium ionophores, phorbol esters, and hypoxia. The expression and secretion of ET-1 are elevated during myocardial ischemia and ET-1 probably has a critical role in the regulation of vascular tone and possibly vascular and cardiac remodeling during both acute and chronic phases of cardiovascular disease. Our studies focus on the regulation of ET-1 expression by hypoxia. The level of ET-1 mRNA was increased by 9± 1.2 fold in human arterial endothelial cells (HAEC) that were subjected to hypoxia (0.2 % O_2, 5% CO_2, bal N_2) for 1 day (n=3). ET-1 mRNA was also induced 3 fold by treating HAEC cultures with the free radical generating agent pyrogallol (100 μM). Unstimulated levels of ET-1 mRNA could be detected by Northern blots or quantitative polymerase chain reaction in HeLa, Hep-G2, cardiac fibroblasts, and cardiac myocytes but not in mouse NIH 3T3 fibroblasts. The amount of ET-1 mRNA in cardiac myocytes was only a fraction (< 5%) of the corresponding levels in endothelial cells (ECs), (n=5). The induction by hypoxia was observed only in the ECs. Hypoxia had no effect on ET-1 transcript levels in Hep-G2 cells or either neonatal or adult heart cells (n=5). Hypoxia depressed ET-1 levels in HeLa cells (n=3). The induction of ET-1 in HAECs was reduced by increasing passage of the primary cells and was no longer apparent after 24 passages, although the cultures retained other EC characteristics. ET-1 was transiently induced by 2-4 fold, in all cells by treatment with the phorbol ester PMA, suggesting different pathways for induction by hypoxia and phorbol esters. The expression and induction of ET-1 was eliminated in all cells by treatment with the broad range protein kinase inhibitor staurosporine. Induction by hypoxia, but not basal expression was inhibited 30% by either the PK-C selective inhibitor GF 109203X or the tyrosine kinase selective inhibitors genistein and lavendustin, and these effects were additive when the inhibitors were used in combination. The results are consistent with an EC specific hypoxia response factor and multiple kinase steps in the regulation of ET-1 expression by hypoxia *in vitro*.

Eukaryotic Metal Metabolism/Homeostasis

C4-400 OXIDATIVE DAMAGE IN YEAST CAN BE SUPPRESSED THROUGH GENES CONTROLLING INTRACELLULAR TRANSPORT OF COPPER OR MANGANESE, Valeria C. Culotta, Su Ju Lin, Paula Lapinskas and Xiu Fen Liu, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md 21205.

Strains of *Saccharomyces cerevisiae* lacking both the copper/zinc cytosolic and manganese mitochondrial superoxide dismutases (SOD) are hyper-sensitive to oxygen toxicity and are auxotrophic for lysine and methionine when grown in air. All these defects of SOD mutants are suppressed through additional mutations in either the *BSD1* or *BSD2* gene. Notably, both *BSD* genes were found to function in the intracellular transport of transition metals. *BSD1* is equivalent to *PMR1*, a member of the P-type ATPase family that localizes to the Golgi and plays an important role in the cellular accumulation and distribution of manganese. *BSD2* was found to encode a novel 37.5 kd polypeptide that controls intracellular accumulation of copper. Inactivation of either *BSD1/PMR1* or *BSD2* serves to alter cellular pools of manganese and copper. These metal ions in turn may reverse oxidative damage through metal-catalyzed scavenging of reactive oxygen species. The aerobic defects of yeast lacking SOD can also be suppressed through over-expression of the *ATX1* gene. *ATX1* encodes a small molecular weight protein (8.2 kd) exhibiting significant homology with the MERp mercury transport proteins of bacteria. Over-expression of *ATX1* causes cells to accumulate elevated levels of copper, indicating a normal role for *ATX1* in copper ion transport and homeostasis. A deletion in the *ATX1* gene is associated with a strong sensitivity towards paraquat, a superoxide anion generating agent. These studies provide evidence for a dual function for *ATX1* in the homeostasis of both metals and oxygen free radicals. Collectively our studies on *ATX1* and the *BSD* genes have established novel biological links between metal ion transport and the detoxification of oxygen free radicals.

C4-401 CONTROL OF IRON REGULATORY PROTEIN ACTIVITIES BY NITRIC OXIDE, Jean-Claude Drapier and Cécile Bouton, U 365 INSERM, Institut Curie, Section de Biologie, 26, rue d'Ulm, 75005 Paris, France.

Nitric oxide (NO) is a gaseous radical synthesized by many cell types, and is involved in many vital functions, including the regulation of vascular tone, neurotransmission and the macrophage-mediated immune response. NO may exist in several redox forms and thus reacts with thiols, transition metals and oxygen. We previously showed that NO alters the activity of iron-dependent enzymes in mitochondria, particularly aconitase, an [Fe-S] enzyme. Further, we also showed that Iron Regulatory Protein (IRP), an RNA-binding protein which controls iron homeostasis through binding to iron-responsive elements (IRE) and which also functions as an aconitase in the cytosol, is sensitive to the effect of NO. Indeed, in response to NO synthesis, IRP commutes from its RNA-binding form to its aconitase form *(Drapier et al., 1993, 12, 3643)*. Mutual exclusion of both activities of IRP is thought to be due to an allosteric switch *(Klausner et al., Cell, 1993, 72, 19)*, and we investigated whether NO can mediate this transition by targeting the [Fe-S] cluster. Crude preparations of IRP from RAW 264.7 macrophages were treated in vitro with increasing amounts of exogenous NO-donors. Aconitase activity, assessed spectrophotometrically, was followed in parallel to IRE-binding activity determined by gel retardation using radiolabeled IRE probe. We showed that NO-donors induce stable change to IRP as increase of IRE-binding of IRP resisted to gel filtration. Furthermore, IRP was treated by SIN-1 or thionitrites in the presence of aconitase substrates, known to bind one of the iron atom of the [Fe-S] cluster of the mitochondrial form of aconitase. Data showed that citrate or *cis*-aconitate dose-dependently prevented NO to induce binding of IRP to IRE. In contrast, tricarballylate and *trans*-aconitate, two weak subtrates, were far less potent in this regard. These results suggest that NO and citrate compete for the same site of ligation. Altogether, our data bring evidence that NO targets the [Fe-S] cluster of IRP and support the view that the cluster is in the vicinity of the RNA-binding domain. Moreover they allow to propose that the balance between the relative amounts of citrate and NO within cell cytosol is crucial to select the function of IRP : RNA-binding protein or aconitase.

C4-402 IDENTIFICATION OF THE Zn(II) DOMAIN IN THE Cu-ACTIVATED TRANSCRIPTION FACTOR, AMT1.
Rohan A. Farrell, Joanne L. Thorvaldsen, and Dennis R. Winge, Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, UT 84132.

The AMT1 metalloregulatory *trans*-acting factor of *Candida glabrata* is functionally and structurally homologous to the ACE1 transactivator of *Saccharomyces cerevisiae*. AMT1 regulates the expression of 3 metallothionein genes in *C. glabrata* in a Cu(I)-dependent manner. Cu(I) binding to AMT1 stabilizes a conformation capable of binding to specific upstream activation sequences (UAS) in the MT gene promoters.

We have shown that both the AMT1 and ACE1 proteins bind four copper atoms as a tetracopper center and one zinc atom within the DNA-binding, N-terminal half of each molecule. Site specific mutagenesis of a synthetic gene, encoding the metal-binding domain of the *AMT1* gene, has been utilized to identify residues that may act as zinc- and/or copper-binding ligands. The results of these studies indicate that the N-terminal third of the metal-binding domain to be critical for zinc-binding. Mutations of cysteinyl and histidinyl residues within the N-terminal segment affects the Zn(II) but not the Cu(I) stoichiometries. The mutations that affect Zn(II) binding alter, but do not prevent, binding of AMT1 to DNA. In contrast, mutations of cysteinyl residues that appear to function in formation of the tetracopper center prevent binding of AMT1 to the UAS sequence.

A Co(II) complex of a synthetic peptide of residues 1-42 of AMT1 yeilds a similar d-d transition envelope as a Co(II) complex of the intact AMT1 which, further supports the notion that the N-terminal region of AMT1 and likely ACE1, is a Zn(II) binding module. ^{113}Cd NMR spectroscopy of the Cd:peptide complex revealed a signal at ~670 ppm, which suggests that 3 cysteinyl thiolates and 1 histindyl imidazole act as metal ligands. The implication is that AMT1 and ACE1 metal-binding domains infact consist of two structural sub-domains, the N-terminal region forming a Zn(II) module and the C-terminal region enfolding the tetracopper center. The sequence forming the Zn module is conserved in another transcription factor, MAC1. Studies are underway to further elucidate the role of Zn(II) in these proteins.

C4-403 THE INDUCTION OF IMMUNE GENES IN INSECTS H_2O_2 AUGMENTS, AND THIOL REAGENTS DIMINISH.
Ingrid Faye and Shao-Cong Sun*, Department of Microbiology, Stockholm University, S-106 91 Stockholm, Sweden, *Department of Microbiology and Immunology, College of Medicine, University Hospital, The Milton S. Hersey Medical Center, Hersey, Pennsylvania 17033

Insects have an effective humoral immune system, and its synthetic activity is centered largely in the fatbody (Faye and Hultmark, 1993). The means by which insect immune genes are regulated has been the focus of our attention. We have extended our studies of a transcription factor for the insect immune genes, the Cecropia immunoresponsive factor, CIF from the giant silkmoth *Hyalophora cecropia* (Sun and Faye, 1992). CIF shares many characteristics with the mammalian transcription factor NFκB (Baeuerle and Henkel, 1994). It binds to κB-like motifs, which confer high levels inducible immune gene expression in insects (Engström et al. 1993). We sought evidence that oxygen derived active species might modulate the activation of CIF and the expression of *Attacin A*, and *B* two of the immune protein genes. Aracidonic acid, TPA, phytohem-agglutinin, bacteria, and components of microbial cell envelopes stimulate expression of *Attacin A* and *B*, both *in vivo* and *in vitro* (in tissue cultures of fat body). H_2O_2 stimulated expression of these genes and the weak immune response to wounding was greatly augmented by administration of H_2O_2 . These responses to mitogens and to H_2O_2, stimulating the production of attacin RNA, were largely or entirely inhibited by dithiothreitol or N-acetylcysteine. Nonspecific responses were excluded by failure to immunostimulatory induce the expression of a constitutive retrotransposase-like protein. We found that both H_2O_2 and bacteria , when administered *in vitro*, could activate CIF in fatbody cells and that dithiotreitol and N-acetylcystein prevented this.

Taken together the simplest general explanation of these data is that the induction of the immune protein genes in insects is mediated through the activation of CIF, contingent upon thiol oxidation induced by oxidative stress.

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C4-404 REGULATION OF Mn PEROXIDASE GENE TRANSCRIPTION BY Mn ION, HEAT SHOCK, AND OXIDATIVE STRESS, Michael H. Gold, Bruce Godfrey, Lakshmi Akileswaran, Dan Li, Jessica Gettemy and Margaret Alic, Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, OR 97291-1000

The basidiomycete *Phanerochaete chrysosporium* degrades lignin and a wide variety of aromatic pollutants during secondary metabolic (idiophasic) growth. Manganese peroxidase (MnP) is an extracellular component of this organism's lignin degrading system. MnP oxidizes Mn^{II} to Mn^{III} and the latter, complexed with a dicarboxylic acid chelator such as oxalate, diffuses into the wood to oxidize the terminal phenolic substrate, lignin. We have sequenced cDNA and genomic clones encoding two MnP isozymes. These gene sequences have been used to study the transcriptional regulation of MnP. Northern and western blot analysis has shown that *mnp* gene transcription is regulated by Mn ion, heat shock and oxidative stress, and the promoter regions of both *mnp* genes contain putative metal response elements (MRE) and heat shock elements (HSE). We developed a *mnp* promoter-orotidylate decarboxylase (ODase) reporter gene system to delineate the *cis*-acting sequences involved in the transcriptional regulation of *mnp* genes. Using this system, we have shown that fusion of 750 bp of the *mnp* promoter immediately upstream of the translation start site of the *odase* gene is sufficient to regulate the reporter gene transcription by Mn. We have also created a homologous reporter system based on the deletion of several hundred bp from the coding region of each of the sequenced *mnp* genes. Transcripts of these truncated genes can be differentiated from endogenous transcripts on northern blots. Our results with these truncated transcripts suggest that both *mnp* genes are regulated by Mn. Using these promoter reporter constructs we are now creating *mnp* promoter mutants in order to define the role of the putative MRE in Mn regulation of *mnp* gene transcription.

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C4-405 GENES THAT REGULATE EXPRESSION OF YEAST Cu Zn SUPEROXIDE DISMUTASE IN RESPONSE TO OXIDATIVE STRESS, Edith B. Gralla, Vanessa Moy, Lee-Loung Liou, Lisa Ellerby, and Joan S. Valentine, Department of Chemistry and Biochemistry, UCLA, Los Angeles, CA 90024

The expression of CuZnSOD (product of the *SOD1* gene) in *Saccharomyces cerevisiae* is increased in conditions of oxidative stress, respiratory growth, or stationary phase. Several yeast transcriptional activators have been described that regulate other genes in response to such signals. We report that *HAP2,3,4*, a heme-responsive transcriptional activator that has been shown to increase the expression of a number of respiratory proteins following shift to a non-fermentable carbon source, is involved in the regulation of CuZnSOD in response to carbon source shift. A potential binding site was located in the promoter region, and confirmed by gel shift and other assays. We have constructed a fusion of the *SOD1* promoter to the *lacZ* gene, and used it to study regulation of the *SOD1* gene in response to a number of oxidative stresses. We have also studied the expression of the fusion gene in a *yap1*$^-$ strain, where the response to hydrogen peroxide is reduced. The variety of stressors to which expression of this gene responds indicates that regulation may be complex and interconnected.

C4-406 EFFECTS OF IRON EXCESS ON *NICOTIANA PLUMBAGINIFOLIA* : IMPLICATIONS TO OXIDATIVE STRESS, Karlheinz Kampfenkel, Marc Van Montagu and Dirk Inzé, Laboratorium voor Genetica, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

Iron is essential for many cellular processes of higher plants, most notably photosynthesis and respiration. However, very little is known at the molecular level about plant iron metabolism including transport, storage, and homeostasis. Iron excess is believed to generate oxidative stress. To contribute to the understanding of Fe metabolism, Fe excess was induced in *Nicotiana plumbaginifolia* grown in hydroponic culture upon root cutting. Toxicity symptoms became already visible after 6 hours leading to brown spots covering the leaf surface. Photosynthesis was heavily affected within 12 hours. The photosynthetic rate was decreased by 40%. Inhibition of photosynthesis was accompanied by photoinhibition, increased reduction of PSII, and higher thylakoid energization. Fe excess seemed to stimulate photorespiration since catalase activity doubled. To cope with cellular damage respiration rate increased and cytosolic glucose-6-phosphate dehydrogenase activity was more than doubled. Simultaneously, the content of free hexoses was strongly reduced. Indicative for a generation of oxidative stress was doubling of ascorbate peroxidase activity within 12 hours. Contents of the antioxidants ascorbate and glutathione were reduced both by 30%, resulting in equivalent increases of dehydroascorbate and oxidized glutathione. Taken together, already moderate changes in leaf Fe content have a dramatic effect on plant metabolism. This indicates that cellular Fe concentrations must be fine regulated to avoid cellular damage, most likely due to oxidative stress induced by Fe. Further studies are needed, to pinpoint how higher plants sense the intracellular iron status and whether plants repress the uptake system and/or induce detoxification mechanisms in response to a surplus of iron. Experiments are in progress to characterize the iron uptake system of higher plants via the functional complementation of yeast mutants perturbed in iron acquisition.

C4-407 MOLECULAR INTERACTIONS OF THE AMT1 COPPER METALLOREGULATORY TRANSCRIPTION FACTOR FROM THE YEAST *Candida glabrata*. Keith A. Koch, and Dennis J. Thiele, Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI 48109-0606

The redox activity of copper is utilized by a large number of enzymes as a cofactor for catalysis. This redox activity also lends itself to cytotoxicity, presumably through the generation of reactive oxygen species, when copper is present at elevated levels within the cell. When cells encounter dangerous levels of copper, a major cellular homeostatic response is the production of copper-chelating metallothioneins. In the yeast *Candida glabrata*, three distinct metallothionein (MT) genes are transcriptionally activated in response to copper via the metalloregulatory transcription factor *AMT1*. The AMT1 protein has a cysteine-rich amino terminus which coordinates Cu(I) ions, which ultimately induces a conformational change in AMT1 that allows for sequence specific interactions between AMT1 and the metal responsive elements (MRE's) within the promoters of its target genes. The carboxy terminus of AMT1 is highly acidic and is thought to harbor the putative transcriptional activation domain. Importantly, AMT1 is involved in copper-dependent activation of the *AMT1* gene. Furthermore, the transcriptional autoactivation of the *AMT1* gene has been shown to be essential for cell survival at high copper levels. Understanding the mechanism utilized by AMT1 to activate transcription from its own gene, requires that we understand the molecular interactions of AMT1 with the *AMT1* promoter MRE. We have purified full length Cu-AMT1 produced in *E. coli* to homogeneity, and have used Cu-AMT1 in gel mobility shift assays to ascertain the affinity of Cu-AMT1 for the *AMT1* promoter. We have also determined that the high affinity interactions of Cu-AMT1 with its own promoter occur through Cu-AMT1 binding to the *AMT1* promoter MRE as a monomer. Through the use of the missing nucleoside experiment, we have established the critical residues required for AMT1 binding to the *AMT1* promoter, and circular permutation analysis has revealed that Cu-AMT1 induces DNA distortion when bound to the *AMT1* promoter. The molecular details of AMT1-MRE interactions provide critical insight into the mechanism by which rapid transcriptional autoactivation of the *AMT1* promoter can occur.

C4-408 LUNG INJURY INDUCED BY METALS FOUND IN RESIDUAL OIL FLY ASH IS ASSOCIATED WITH UPREGULATION OF CYTOKINE GENE EXPRESSION, Urmila P. Kodavanti[1], Richard Jaskot[2], Daniel L. Costa[3] and Kevin L. Dreher[3], [1]CEMLB, University of North Carolina, Chapel Hill, [2]ManTech Environmental Inc., and [3]US Environmental Protection Agency, Research Triangle Park, NC 27711

The purpose was to investigate the roles of three predominant metals, Fe^{3+}, V^{2+}, and Ni^{2+}, of residual oil fly ash (ROFA), in inducing lung injury and their relationship with cellular adhesion molecule and cytokine gene expression. Male Sprague-Dawley rats (60 day old) were intratracheally instilled with either saline, $FeSO_4$ (0.54 μmol/rat), VSO_4 (1.7 μmol/rat), $NiSO_4$ (1.0 μmol/rat), a mixture of all three metals (MIX) or ROFA (2.5 mg/rat) in 0.3 ml saline. The quantity of metals instilled reflected the amount found in 2.5 mg fly ash. After 3, 24, or 96 h, the left lung was used for histopathology while the right caudal lobe was used for RNA extraction. Histopathological findings indicated that Ni^{2+}, MIX, and ROFA induced marked focal lung injury characterized by inflammatory cell infiltration, edema, alveolar cell hyperplasia, thickening of the terminal bronchiolar region, and bronchiolar mucous cell hypertrophy. V^{2+} and Fe^{3+} alone produced less severe effects. Reverse transcriptase-PCR for cytokine gene expression indicated that macrophage inflammatory protein-2 and interleukin-6 were induced by all metals, MIX, and ROFA as early as 3 h after instillation and declined to nearly control level by 24 h in all treated groups except Ni^{2+}. Interleukin-5 also appeared to be upregulated through 96 h in all treated groups, but especially with the MIX and ROFA. Expression of tumor necrosis factor-α mRNA appeared to be non-specifically induced by saline, all metals and ROFA at all times. Adhesion molecules, E-selectin, and VCAM-1 expression were slightly increased by all metals, the MIX and fly ash at 3 h and remained slightly elevated at 96 h in Ni^{2+} instilled animals. These studies suggest that lung damage induced by metals present in an emission source particle which contributes to existing air pollution, may be mediated through induction of cellular adhesion molecule and cytokine gene expression, and may ultimately determine the chronicity of the lung damage (This abstract does not necessarily reflect EPA policy, Supported in part by EPA through CEMLB, #CR817643).

C4-409 THE MAC1 PROTEIN, A HOMOLOG OF COPPER-DEPENDENT TRANSCRIPTION FACTORS, IS REQUIRED FOR TRANSCRIPTIONAL ACTIVATION OF STRESS RESPONSE GENES IN *S. CEREVISIAE*, Daniel Kosman, Jason Plotkin, Annette Romeo, Mihaela Serpe, and Richard Hassett, Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York, Buffalo, NY 14214

MAC1 (metal-dependent activator, S. Jentsch *et al.*, **EMBO J.** 12, 5051-5056) encodes a nuclear protein of 417 residues which includes consensus CCHC box and nuclear targeting domains as well as a duplicated $CysXCysX_4CysXCysX_2CysX_2His$ motif. The N-terminal CCHC box (zinc finger) domain is 53 and 48% identical to the potential Zn-binding regions in Ace1p and Amt1p, respectively, which are Cu-dependent yeast transcription factors. We have shown that Mac1p is required for expression of Fe- and Cu-handling functions in yeast, *eg*, metal reductase and transporter activities. Deletion of *MAC1* also confers H_2O_2, thermal, and Cd^{2+} sensitivity, and respiration deficiency. These phenotypes are not complemented by expression of Mac1p containing a "CSHS" box, indicating that this motif is physiologically functional. In contrast, overexpression of Yap1p, a *jun*-family yeast homolog, partially represses these phenotypes. Induction of *CTT1* (catalase T) by H_2O_2 is absent in the *mac1Δ*-containing strain. Northern and reporter gene analyses show that peroxide induction of *CTT1* is Hap1 and UAS1 mediated indicating that Mac1p functions upstream from Hap1 in this pathway. Peroxide also activates *via* a Map kinase pathway; the proximal *cis* element in this pathway is an STRE sequence, AGGGG, found in a number of stress-response genes. Mac1p is also required for signal transduction in this pathway as is Yap1p indicating that these two proteins functionally interact. The respiration deficiency in *mac1Δ* correlates to reduced or absent glucose derepression of *CTT1*, *CYC1*, and *COX6*. Epistatic tests indicate that Mac1p is not a proximal element and are consistent with a failure to detect Mac1p DNA-binding activity. Substitution of Gln for the terminal His in the first Cys-rich repeat alters the transcriptional regulation due to Mac1p suggesting a model in which the redox state of a bound metal at this site may provide the sensor for this signal. This metal could sense the intracellular redox potential as set either metabolically (carbon source), by metal level (iron or copper), or environmentally (peroxide addition). Supported by NIH RO1GM46787.

C4-410 OXIDATIVE STRESS AND IRON CHELATORS INCREASE GLUCOSE TRANSPORT IN MUSCLECELLS IN CULTURE BY ELEVATION IN GLUT1 TRANSCRIPTION. Nitzan Kozlovsky, Ruth Potashnik and Nava Bashan. Department of Clinical Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel.

Oxygen free radicals have been implicated as etiologic agents in a number of widely occurring clinically conditions such as arthritis, carcinogenesis, tumor promotion, radiation injury, ischemic damage to heart, brain, kidney and intestine. In addition there are evidences that oxygen free radicals are involved in the toxicity of iron, reported to be involved in the development of diabetes in certain cases. This led us to investigate the effect of various oxidants, and iron chelators on the regulation of glucose transport into muscle cells in culture.

Incubation of L6 myotubes for 24 hours in the presence of hydrogen peroxide (generated by incubation in the presence of 5 mM glucose and 50 mU/ml of glucose oxidase), and superoxide (generated by 50 μM xanthine and 20 mU/ml xanthine oxidase) resulted in 2.1 ±0.35 and 3.3±1.3 fold increase in 2-deoxyglucose uptake respectivly. Exposure of the cells to 10^{-7}M insulin for one hour activate glucose uptake in control cells by 1.8±0.2 fold, however insulin had no effect on 2-deoxyglucose uptake in oxidized cells. Addition of cycloheximide or actinomycin D to the medium of cells incubated in the presence of the oxidants prevented the increase in 2-deoxyglucose uptake indicating that *de novo* synthesis of glucose transport proteins was the major means by which this cells increased their ability to take up glucose. Western and Northern blot analysis revealed that expression of GLUT1 protein and mRNA were significantly elevated in cells incubated in the presence of the oxidants. The level of insulin sensitive glucose transporter (GLUT4) did not changed by exposure to hydrogen peroxide but was reduced by exposure to superoxide. Incubation of the cells in the presence of the iron chelators deferoxamide (0.5 mM) or bipyridyl (0.1 mM) resulted in elevation of both GLUT1 protein and mRNA leading to increase by 3-4 fold in 2-deoxyglucose uptake. No change in the amount of GLUT4 protein or in the insulin stimulation of 2-deoxyglucose uptake under those conditions was observed. In conclusion, the present results indicate that muscle cells in culture respond to both oxidative stress and reduction of iron by increase cellular glucose uptake through accelerated expression of GLUT1 which is known to be elevated in various cell types under stress conditions.

C4-411 HEAT SHOCK TRANSCRIPTION FACTOR ACTIVATES YEAST METALLOTHIONEIN GENE EXPRESSION IN RESPONSE TO THE PRO-OXIDANT MENADIONE. Xiaodong Liu, Nicholas Santoro and Dennis J. Thiele, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

Metallothioneins (MTs) constitute a class of low molecular weight, cysteine-rich metal binding stress proteins which are biosynthetically regulated at the level of gene transcription in response to metals, hormones, cytokines and other physiological and environmental stresses. Recent work in our laboratory, in collaboration with E. Gralla, L. Ellerby and J. Valentine at UCLA, has demonstrated that the *S. cerevisiae* MT, encoded by the *CUP1* gene, suppresses the growth defects of cells harboring a deletion of *SOD1*, the Cu-Zn superoxide dismutase gene. These observations suggest that the MT encoded by *CUP1* is a potent line of defense against oxidative stress. Furthermore, these studies also demonstrated that *CUP1* gene transcription is induced under conditions of enforced respiration, which are known to generate superoxide radicals. To investigate whether *CUP1* mRNA levels are elevated in response to oxidative stress, we exposed cells to the potent superoxide generator menadione. We demonstrated that menadione induces *CUP1* gene expression through the yeast Heat Shock Transcription Factor (HSF). *CUP1* transcription in response to Menadione occurs very rapidly and is sustained for over two hours. Consistent with menadione activation of *CUP1* transcription via a mechanism that directly or indirectly involves superoxide anion, *CUP1* transcription is hypersensitive to induction by very low concentrations of menadione in *sod1Δ* strains as compared to isogenic wild type strains. Furthermore, cells harboring deletions of both the *CUP1* and *SOD1* genes are more sensitive to menadione toxicity than cells with a *SOD1* deletion alone. Taken together, these results demonstrate that the yeast metallothionein protein encoded by the *CUP1* gene plays an important role in protecting cells from oxidative stress and that yeast Heat Shock Transcription Factor activates transcription of *CUP1* in response to oxidative stress.

C4-412 *Abstract Withdrawn*

C4-413 REGULATION OF ENDOTHELIAL CELL HYPOXIA ASSOCIATED PROTEINS: EFFECTS OF HYPOXIA AND HEAVY METALS. Robert J. McDonald, Krista K. Graven and Harrison W. Farber. Pulmonary Center, Boston University School of Medicine, Boston, MA 02118

Endothelial cells (EC) exposed to acute and chronic hypoxia upregulate a unique set of stress proteins, termed hypoxia associated proteins (HAPs). The 36kD has recently been identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To investigate whether induction of GAPDH is analogous to the induction of erythropoietin (EPO), bovine aortic and pulmonary arterial EC were exposed to hypoxia or varying concentrations of heavy metals. New protein production was evaluated by ^{35}S-methionine labelling and SDS-PAGE; mRNA levels were evaluated by Northern blot using a ^{32}P-labelled human GAPDH cDNA probe. Upregulation of both GAPDH protein and mRNA occurred with exposure to hypoxia or the heavy metals, cobalt ($CoCl_2$; max 150um), nickel (max 400um) and manganese (max 400um). Concomitant exposure to hypoxia and these metals did not induce protein or mRNA to a greater extent than hypoxia alone. Exposure to iron (100-600um) or zinc (100-300um) did not upregulate EC GAPDH. Exposure to 4,6 dioxoheptanoic acid, an inhibitor of aminolevulinate dehydratase did not upregulate GAPDH synthesis whereas exposure to deferrioxamine, an iron chelator, caused upregulation of GAPDH protein and mRNA. In contrast to EPO induction, neither agent inhibited GAPDH upregulation by either hypoxia or $CoCl_2$. Likewise, in contrast to EPO induction, other heme binding agents, such as NO (1mM sodium nitroprusside) or 10% CO failed to inhibit the upregulation of GAPDH protein and mRNA by hypoxia or $CoCl_2$. These findings suggest that the mechanism of the GAPDH upregulation by hypoxia and heavy metals is different than that of EPO.

C4-414 IRON REGULATORY PROTEIN (IRP) RESPONDS TO IRON LEVELS AND NITRIC OXIDE (NO): ROLE OF POST-TRANSLATIONAL CONVERSION OF IRON-SULFUR TO APO-PROTEIN, K. Pantopoulos and M. W. Hentze, EMBL, Gene Expression Programme, Heidelberg, Germany.

Expression of genes involved in iron uptake, utilization and storage is controlled post-transcriptionally by mRNA/protein interactions. When iron is scarce, an RNA-binding protein, referred to as "iron-regulatory protein" (IRP), binds with high affinity to iron-responsive elements (IREs), structural motifs within the untranslated regions (UTRs) of mRNAs. IRE/IRP complexes in the 5' UTR blocks the translation of ferritin and eALAS mRNAs, while IRP stabilizes the transferrin receptor (TfR) mRNA by binding to IREs in the 3' UTR. When iron is abundant, IRP assembles a 4Fe-4S cluster that prevents IRE-binding and converts IRP to a cytoplasmic aconitase. The dual activities of IRP are reversibly regulated at the post-translational level by a Fe-S cluster assembly-disassembly mechanism.

Nitric oxide (NO) represents a second signal which regulates the activities of IRP. In B6 fibroblasts stably transfected with a murine macrophage iNOS cDNA (B6.NOS), constitutive NO-biosynthesis activates IRE-binding, which in turn represses ferritin mRNA translation and stabilizes TfR mRNA. When B6.NOS cells are co-cultured with B6 fibroblasts stably transfected with an iron-regulated IRE-containing hGH indicator, NO release by B6.NOS cells regulates hGH biosynthesis in B6.IRE-hGH cells. Abrogation of NO biosynthesis in the transfected fibroblasts by the NOS substrate analog N^G-methyl-L-arginine reverses the induction of IRE-binding. The activation of IRE-binding by NO appears to result from a switch of IRP to the apo-form. However, iron starvation and NO release represent independent signals to IRP. Our findings demonstrate that the post-transcriptional control of iron metabolism is intimately connected with the NO pathways.

C4-415 **TRANSCRIPTIONAL REGULATION OF RAT HEME OXYGENASE GENE WITH CADMIUM**

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Heme oxygenase, a key enzyme of heme degradation, is a major stress-related protein, which is induced with heme, heavy metals, heat shock, organic compounds, UV radiation, hydrogen peroxide and hypoxia. In order to elucidate the transcriptional regulation, we isolated the 4.3 kbp 5' flanking region of rat heme oxygenase gene. To localize heme and cadmium responsive elements, various regions of 5' upstream sequence were fused to the chloramphenicol acetyltransferase (CAT) gene, and a transient expression assay was conducted in the rat glioma cells (C6) treated with or without 10 μM $CdCl_2$ or 10 μM hemin. These studies indicated that the sequence from -3.3 to -2.8 kbp was essential for the induction by cadmium. However, hemin failed to induce CAT activity. Then, we prepared hepatic nuclear extracts from rat treated with or without cadmium (14 mg $CdCl_2$/kg). Using gel retardation assay, a DNA-protein complex band was observed only when nuclear extracts prepared from Cd-treated rat were used. In this region, we found a MRE like core sequence which may play an important role for the induction of heme oxygenase with cadmium.

Our findings suggested that a transcription factor affected with cadmium binds to this region and regulates the transcription of heme oxygenase gene.

C4-416 ROLE OF HEAT SHOCK TRANSCRIPTION FACTOR IN METAL RESISTANCE IN YEAST. Andrew K. Sewell, Fumihiko Yokoya and Dennis R. Winge, Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah 84132.

Whereas normal, wild-type yeast are tolerant to only 15 μM cadmium salts, strain 301N, created by subculturing in incremental Cd(II) concentrations, is able to grow in media containing in excess of 500 μM Cd(II) (T. Murayama, Ehime University, Japan) Cadmium tolerance in 301N is dependent on the CUP1 metallothionein. We have shown that, in addition to the usual copper induction of *CUP1* expression through the ACE1 transcription factor, 301N exhibited the ability to induce *CUP1* in response to cadmium stress. We have determined that the 301N HSF gene (*hsf301*) is the key to both this induction and the metal-resistance, it presumably acts through the minimal heat shock element (HSE) in the *CUP1* promoter. *hsf301* gives a 1000 fold increase in the basal expression from the *CUP1* promoter compared to the wild type factor and gives a further stimulation when cells are exposed to Cd(II). Cells carrying the mutant *H5F* gene on a YCp plasmid exhibit significant tolerance to Cd(II). In addition to giving cadmium tolerance, this gene also imparts limited copper tolerance to cells with a disrupted *ACE1* allele and gives some cells limited copper and cadmium inducibility of *CUP1* in the absence of ACE1. No such effects are observed from similar plasmids carrying the wild type *HSF1* gene. This up regulation is not limited to the *CUP1* promoter, *hsf301* affords cells increased heat shock survival and is observed to up regulate synthetic HSE/*lacZ* reporter genes.
Sequencing of *hsf301* shows it to have two codon changing mutations in the DNA binding domain of the molecule (R206S and F256Y). Both the R206S and the previously reported V203A give cadmium-resistance but not the F256Y mutation singly. HSF301 but not the wild type protein is observed to give a specific DNA complex with the two GAA box proposed heat shock element in the *CUP1* promoter. Thus *hsf301* appears to have two phenotypes, one of increased binding to the *CUP1* promoter and one of reduced low temperature repression. The R206S mutation appears to allow binding to HSE(*CUP1*), work in progress is aimed at examining the reduced low temperature repression of HSF301 and the role of the F256Y mutation.
Overexpression of HSF1 in cells which lack the *CUP1* metallothionein gene affords cells some tolerance to cadmium and copper. Similar expression of HSF301 gives only cadmium tolerance. This suggests a role for the 'general stress response' in metal-resistance which differs for cadmium and copper ions. We propose that cells may have a three tier repertoire of metal resistance; a 'constitutive defense', the 'induced response' and the 'general stress response' with the possible involvement of *HSF1* at each level.

C4-417 METALLOTHIONEIN-1 (MT-1) GENE REGULATION BY HEME-HEMOPEXIN (HPX): ROLE FOR AN ANTI-OXIDANT RESPONSIVE ELEMENT (ARE). Ann Smith and Yafei Ren, MBB-Biological Sciences, University of Missouri, Kansas City, MO 64110. Heme-HPX coordinately increases MT-1 and heme oxygenase-1 gene transcription and helps maintain homeostasis when intracellular heme and iron increase and thus alleviates injury, infection and other stresses. Reactive oxygen species (ROI's) regulate MT expression since substances which induce ROI's (*e.g.* metals and cytokines) or which undergo redox cycling or decrease GSH (*e.g.* heme) increase MT mRNA and/or protein. Receptor-mediated heme transport *via* HPX activates PKC. Notably, heme may raise the normal levels of ROI's, and a putative ARE resides at -98 to -89 bp in the mouse MT-1 gene. Northern blot analyses and transient transfection studies with p3055B, containing 750 bp of the mouse MT-1 promoter linked to the *lacZ-neo* gene, showed that the increase in β-galactosidase activity by heme-HPX is equivalent to endogenous MT-1 mRNA induction. When the region from -150 to -40 bp was deleted (p3055A), the response to heme-HPX was lost, and placing these 110 bp in front of the basal promoter (p3055C) conferred inducibility on the reporter gene. Induction of MT-1 mRNA by heme-HPX was abrogated by extracellular superoxide dismutase and catalase. H_2O_2 induced MT-1 mRNA levels, and a superoxide generating system raised the β-galactosidase activity of p3055C. The thiol reagents, GSH and N-acetyl cysteine, inhibited MT-1 mRNA induction and transcriptional activation of p3055C by heme-HPX. The reporter gene activity of a construct containing two copies of the native putative MT-1 ARE ($pARE_2$-βGeo) was induced by heme-HPX or H_2O_2, but pMRE-βGeo containing five MRE's did not respond to HPX. Thus, the MT-ARE sequence functions in the regulation of the MT-1 gene by heme-hemopexin and acts as an "oxidant-responsive" element, termed anti-oxidant (ARE), since the gene products act as anti-oxidants. PDTC and DDC, which inhibit SOD by chelating Zn, augment the induction of p3055C by HPX, possibly by releasing MRE-binding proteins and implicating interactions between proteins binding to ARE and MRE elements. (USPHS grant, DK-37463).

C4-418 $V^{V/IV/III}$ METABOLISM IN ASCIDIAN HOMEOSTASIS, Mitchell J. Smith, Div. of Natural Products, CFSAN, FDA, Wash., DC 20204 (mks@fdacf.ssw.dhhs.gov) Despite nearly a century of scientific scrutiny the manifest presence of vanadium in diverse animals has eluded virtually all attempts to disclose a natural purpose, *e.g.*, it is considered an essential trace element for mammals and birds. To this end, studies world-wide are investigating just how and why ascidians assimilate up to decimolar concentrations of this metal against 2-5 million-fold concentration gradients and reduce it to an air-sensitive oxidation state within anomalous, yellow-green blood cells. These enigmatic filter-feeders have intrigued scientists ever since Aristotle's time, are often placed at the very origin of vertebrate evolution, and inhabit all oceans, at all depths. To elucidate the transient and mutually exclusive vanadium chemistries that comprise this convoluted redox cycle and distinguish facts from experimental incongruities is nontrivial. Moreover, a variety of technical and conceptual barriers continue to confound a thorough evaluation of the purpose(s) of vanadium for ascidians; virtually all proposals pertain to either recognition/defense processes or stem from an unorthodox formulation involving (microaerobic) homeostasis [Smith *et al.*, Metal Ions in Biological Systems *31*, 423, in press]. Even so, several novel findings and convergent, evolutionary/ecophysiological trends derived from the voluminous literature offer astonishing and compelling support for the latter formulation, all of which will be presented in detail. The notorious challenge now is to perform the definitive tests, whose design remain provocatively elusive.

C4-419 **REGULATED EXPRESSION OF NUCLEAR OR CYTOPLASMIC-TARGETED HUMAN METALLOTHIONEIN FUSION PROTEIN IN CHINESE HAMSTER OVARY CELLS.** **Elizabeth S. Woo and John S. Lazo. Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261.** Metallothioneins (MT) comprise a family of low molecular weight proteins, whose sole undisputed function is to bind and thereby protect cells from heavy metal toxicity. The examination of putative roles for MT in conferring resistance to electrophiles, including cisplatin, alkylating agents, and mutagens, as well as oxidizing agents are limited by the promiscuity of MT inducers. We have created a MT fusion gene comprising the minimal SV40 T antigen nuclear localization sequence (NLS), human MT IIA cDNA, and LacZ, the expression of which can be regulated by cellular exposure to 2.5mM IPTG. Following lipofection of this construct or a cytoplasmic counterpart lacking the NLS, nuclear or cytoplasmic localization of the fusion construct, respectively, was evidenced by X-gal staining. In the absence of IPTG inducer, minimal or undetectable staining was observed, indicative of repressed fusion gene expression. Both cell growth inhibition and clonal survival assays showed protection from cadmium cytotoxicity in the MT fusion gene transfectants, irrespective of MT subcellular location; thus, the fusion constructs retain MT functionality. These cells provide a useful model for examining the importance of MT subcellular location in its protective role against electrophilic agents without the ambiguities associated with traditional MT inducers.

C4-420 FUNCTIONAL REGULATION OF ZINC FINGER TRANSCRIPTION FACTORS BY REDOX STRESS *IN VIVO*. Xiaosu Wu, Daryl J. Discher, Nanette H. Bishopric, and Keith A. Webster. Cell and Molecular Biology Department, Program in Molecular Cardiology, SRI International, Menlo Park, CA 94025.
Redox regulation of nucleic acid binding proteins through the reversible oxidation of key cysteine sulfydryl groups has been demonstrated to occur *in vitro* for a range of factors including AP1, NF-κβ, v-rel, c-myb, E2, IRE-BP, AUBP, and USF. Neither the redox regulation of binding, nor transcriptional effects have been described *in vivo*. In our studies we have demonstrated such effects on zinc finger factors including the Sp1-Egr family. In HeLa cells these proteins not only displayed a higher sensitivity to redox regulation when compared to other redox responsive factors *in vitro*, but they were also responsive *in vivo* as demonstrated by both physical and functional assays. Oxidative stress was imposed by subjecting the cells to 50μM L-buthionine-S,R-sulfoximine to lower cellular glutathione, and 200-800 μM diamide to oxidize sulfydryl groups. At intervals during the treatments, cells were harvested and nuclear proteins were extracted under non-reducing conditions. The extracts were used to determine protein-DNA binding with specific probes for AP1, serum response factor (SRF), NF-κβ, and the zinc-finger factors, Sp1 and Egr, by using gel mobility shift. Specific binding was detected in the unstressed extracts for all probes (n=5). While oxidative stress *in vivo* did not effect the binding of AP1 or SRF proteins (n=5), Sp1, Egr, and NF-κβ binding was reduced under the mildest conditions and eliminated at the higher stress levels (n=4). This loss in binding was reversed by treating extracts with DTT. To analyze for a functional effect HeLa cells were transfected with two low affinity Sp1-dependent, and two Sp1-independent promoters linked to the CAT gene. Transfected cells were treated with the oxidizing conditions that reduced Sp1 binding by 80% as determined by the gel-shift assay, and the transcriptional activity of each construct was measured by quantitative polymerase-chain-reactions. The half-life of CAT mRNA, measured using Actinomycin D was 1.05 h under all conditions. Transcription from the Sp1 promoters was reduced >70 ± 10 % (n=4); transcription directed by the non-Sp1 promoters was not effected (n=4). Cell viability was not effected by the treatments. We conclude that acute severe oxidative stress can directly impact the binding and function of zinc finger, and other thiol-containing transcription factors and this may be a novel method of gene regulation by redox stress.

Late Abstract

GENOMIC FOOTPRINTING ANALYSES OF THE *B. japonicum fixRnifA* UPSTREAM PROMOTER REGION.
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The *B. japonicum* NifA protein is the central regulator for nitrogen fixation gene expression. This protein is directly regulated by oxygen, such that it can only activate *nif* and *fix* gene expression during microaerobiosis or in root-nodule bacteroid state. NifA activates transcription by binding to enhancer-like elements, located around 120bp from the transcription start and melting the closed promoter complex to the active open form. The *nifA* gene is encoded in the *fixRnifA* operon and is expressed from multiple and overlapping promoters (1). The *fixR*p1 promoter is of the -24/-12 class and is recognized by the RNA polymerase σ^{54}. This promoter is autoactivated by NifA in microaerobic conditions and in bacteroids. The *fixR*p2 promoter is of the *B. japonicum* -35/-10 class and is recognized by a form of the RNA polymerase likely to be $E\sigma^{?0}$, the most abundant form found in this bacteria. The -10 region of this promoter completely overlaps the -12 region of *fixR*p1. The *fixR*p2 promoter is also positively regulated by an unknown protein and is expressed in both aerobic and microaerobic conditions but it is not expressed in 30 days-old bacteroids (1, 2).

We showed by *in vivo* genomic DMS and $KMnO_4$ footprinting analyses that the *fixRnifA* promoter region is differentially protected and melted depending upon which RNA polymerase holoenzyme is bound. A guanine (G) residue located at position -75, in a region previously demonstrated to be critical for the aerobic expression of the *fixRnifA* operon (2), was specifically protected from methylation in the wild type promoter but not in a promoter that bears a A to C transversion at -68. On the other hand, two G residues, located at -100 and -119, were protected from methylation. These Gs form part of TGT motifs of UAS half-sites and are very likely binding sites for NifA.

These results add direct physical evidence of the great complexity of the organization, regulation and function of the *fixRnifA* promoter region.

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