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Abstracts from Neurospora 1998

Abstract

Abstracts from the 1998 Neurospora Conference

Abstracts for Talks

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Session 1 - Cell Biology

The exocytic machinery in tip growth of *Neurospora crassa.*

Gagan Gupta and I. Brent Heath, York University Biology Department, North York, Ontario, Canada M3J 1P3

Tip growth of hyphae requires continuous and directed exocytosis of vesicles bearing new membrane and wall material. In other words, a gradient of vesicle fusions (highest at the hyphal apex) must be maintained so as to generate the hyphal shape. One model predicts that this gradient is due to the activity of a hypothetical vesicle supply center (VSC) which, due to its apical location, can concentrate hyphal tips with vesicles that subsequently undergo exocytosis. However, selective targeting and exocytosis of vesicles can also be achieved by a different, more ubiquitous mechanism. SNAREs are highly conserved integral membrane proteins that are believed to confer specificity to vesicle docking and fusion reactions on the basis of protein-protein recognition. They have been well characterized in many eukaryotes from yeast to plants and mammals, and it is therefore likely that they are also present in filamentous fungi. We present immunological, biochemical and genetic evidence for the presence of SNAREs in *Neurospora crassa* and its implications for tip growth models.

Apolar growth of *Neurospora crassa* leads to increased secretion of extracellular proteins.

In Hyung Lee[1], Rodney G. Walline[2], and Michael Plamann[1], [1]School of Biologcial Sciences, University of Missouri-Kansas City, Kansas City, MO 64110-2499, [2]Department of Human Anatomy, Texas A&M University,College Station, TX 77843-1114

Protein secretion in filamentous fungi has been shown to be restricted to actively growing hyphal tips. To determine whether an increase in the amount of growing surface area of a fungus can lead to an increase in the amount of protein secretion, we examined secretion in a temperature-sensitive *Neurospora crassa mcb* mutant that shows a loss of growth polarity when incubated at restrictive-temperature. Incubation of the *mcb* mutant at restrictive-temperature results in a three- to five-fold increase in the level of extracellular protein and a five- to 20-fold increase in carboxymethyl cellulase activity relative to a wild-type strain. A mutation in the *cr-1* gene has been shown previously to suppress the apolar growth phenotype of the *mcb* mutant, and we find that the level of extracellular protein produced by a *mcb*; *cr-1* double mutant was reduced to that of the wild-type control. Immunolocalization of a secreted endoglucanase revealed that proteins are secreted mainly at hyphal tips in hyphae exhibiting polar growth and over the entire surface area of bulbous regions of hyphae that are produced following a shift of the *mcb* mutant to restrictive-temperature. These results support the hypothesis that secretion of extracellular protein by a filamentous fungus can be significantly increased by mutations that alter growth polarity.

Type 2A protein phosphatase is involved in growth and reproduction in *Neurospora crassa.* Einat Yatzkan and Oded Yarden, The Department of Plant Pathology and Microbiology, Faculty of Agricultural, Food and Environmental Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

Protein phosphatases (PP) are an integral part of the reversible phosphorylation regulatory machinery, and are key elements in maintaining the balance of many cellular activities. The core structure of PP2A (which belongs to the ser/thr PPP gene superfamily) consists of a 35kDa catalytic subunit (PP2Ac) tightly complexed with a 65 kDa regulatory subunit (A-subunit). The core dimer complexes with a third, variable component (B-subunit) which controls enzyme activity and specificity. In the presence of the PP inhibitors Cantharidin and Calyculin A *N. crassa* hyphal growth and integrity were abnormal. The observed effects included higher branching frequencies and cell swelling and lysis in the vicinity of hyphal tips. We have analyzed the structure and function of two components of the PP2A holoenzyme in *Neurospora crassa*. The *pph-1* (encoding PP2Ac) and *rgb-1* (encoding the B-subunit) genes were isolated, sequenced and mapped. Both show high degrees of similarity to PP2A components isolated from other organisms. Failure to obtain viable progeny in which *pph-1* had been inactivated via the Repeat-Induced Point mutations (RIP) process and evidence that nuclei harboring a disrupted *pph-1* gene could only be maintained in a heterokaryon, indicated that a functional *pph-1* gene is essential for fungal growth. Reduced *pph-1* expression impaired hyphal elongation rates. Inactivation of *rgb-1* by RIP produced slow growing, female sterile, progeny which produce abundant amounts of arthroconidia yet hardly any macro or micro-conidia.

Search for V-ATPase mutants results in interesting phenotypes. Emma Jean Bowman, Ryan Kendle, Forest O'Neill, and Barry J. Bowman, University of California, Santa Cruz, CA

The vacuolar ATPase generates an electrochemical gradient across the membranes of acidic compartments within the cell. We have taken two approaches to try to obtain mutants in this enzyme: (1) selection of strains resistant to concanamycin A, a specific inhibitor of V-ATPases and (2) the sheltered RIP method of Bob Metzenberg and co-workers to inactivate the catalytic subunit of the enzyme. Many concanamycin A resistant strains proved to be mutated in *pma-1*, which encodes the plasma membrane H+-ATPase. Others may be altered in the V-ATPase as suggested by a small, but reproducible increase in resistance to concanamycin in vitro and by their sensitivity to high pH or high histidine in the medium. Putative *vma-1RIP* strains appear to grow only as small, tight colonies under all conditions tested. Genetic or chemical inactivation of the vacuolar ATPase has a striking effect on hyphal branching patterns.

Characterization of three genes which encode amino acid and calcium transport proteins, and a comparison of the efficiency of RIPing these genes. Emilio Margolles-Clark, Ian Hunt, Stephen Abrue, and Barry J. Bowman, University of California, Santa Cruz, CA 95064

Three genes have been identified which encode transport proteins with 12 membrane-spanning helices. The *gap-1* gene appears to encode a general amino acid permease. We are pursuing experiments to determine if this is the same as the *pmg* gene previously identified by analysis of mutants; *aap-2* encodes a protein that may be homologous to GABA transporters; and *cax-1* encodes a calcium transporter in the vacuolar membrane. We have attempted to inactivate each

of these genes by RIPing. In each case a copy of the gene was introduced into the *his-3* locus, but the efficiency of RIPing was very different. The *cax-1* gene was RIPed at a relatively high rate (30%). No RIPed progeny have yet been identified in *gap-1* (40 analyzed) or in *aap-2* (20 analyzed).

Heterokaryon Incompatibility Revisited. J.F. Wilson, University of North Carolina at Greensboro

A gene with activity similar to that of the *I/i* genes described by Pittenger in Oak Ridge strains has been found in Rockefeller-Lindegren strains. We called this gene and its alternate allele, *Hi/hi*, for Heterokaryon Instability. The major difference in the two sets of genes is the *Hi* allele is dominant, even in extreme ratios, in the RL background. Results of crosses with appropriate strains indicate that *Hi/hi* and *I/i* are identical or closely linked, so operationally they can be considered the same. The OR-SL wild types are *i*; RL wild types, *I*; and the Wilson-Garnjobst testers, an *I/i* mixture. The *I,i* genotypes of FGSC *CDE* and *Cde* testers, and some other strains are now known. Contrary to expectations, some OR and SL mutants are *het-I*. Another source of variation in heterokaryon stability is the almost accidental nature of hyphal fusions between RL and OR-SL strains. The normal intra-strain stimulus-response mechanism is missing in inter-strain fusions. In combination, the two factors result in wildly different growth curves in heterokaryons. In addition, we have found two hyphal fusion mutants which form heterokaryons late or not at all. We now have *het* mutations involving fusions, the killing reaction, and stability. Perhaps it is time to consider a classification of *het* mutants based on the sequence of events that occur in the formation of a stable heterokaryon, instead of lumping all together under the term "incompatibility".

Loss of small, plasmid-like, mtDNA derivatives during sexual reproduction. Helmut Bertrand, Katherine A. Nummy and Georg Hausner, Michigan State University, East Lansing, MI

Circular, plasmid-like elements (PLEs) consisting of tandem repeats of a segment of the mitochondrial chromosome (mtDNA) appear in high copy-numbers in the mitochondria of some cytochrome-deficient and/or UV-sensitive mutants of *Neurospora crassa*. Results obtained from 2-D gel electrophoresis and electron microscopy indicate that PLEs replicate by a recombination-dependent rolling circle mechanism. All PLEs are stably maintained in vegetatively propagated cultures, and, like true mitochondrial plasmids and suppressive mutant forms of mtDNA, they are transmitted horizontally and invasively through hyphal anastomoses. However, some small PLEs are inherited by only a small fraction of the ascospore progeny (about 1/20) from the female parent in crosses. Tetrad analyses show that such PLEs appear either in all or none of the products of any given meiotic event, whereas true mitochondrial plasmids and mutant mtDNAs are transmitted from the female parent to all the progeny in all the tetrads. This erratic pattern of maternal inheritance can not be attributed to heteroplasmy in the female parent, for there are no PLE-free segregants among single conidial isolates from these strains. Thus, the results suggest that some high-copy-number, replicatively-competent mtDNA derivatives lack an attribute required for consistent transmission of the mitochondrial chromosome and true plasmids from the maternal parent to the ascospore progeny.

Session 2 - Genomics

Control and Function of DNA Methylation in *Neurospora crassa*. Eric U. Selker, Institute of Molecular Biology, University of Oregon, Eugene, OR 97405

DNA methylation is not essential in Neurospora and only 2% of the genome is methylated. Nevertheless, a high fraction of sequences introduced by transformation, or mutated by repeat-induced point mutation (RIP) are methylated. DNA methylation is associated with gene silencing in many systems but the nature of the association is not well understood. Methylation of cytosines in sites recognized by transcription factors can interfere with factor binding, potentially blocking initiation directly. In addition, proteins that bind methylated sequences independent of sequence, may interfere with initiation and conceivably also interfere with elongation. We found that DNA methylation associated with products of RIP can prevent transcription elongation without affecting initiation. We have detected, and are currently purifying, Neurospora proteins that specifically recognize methylated sequences and may mediate this effect. Our work on the control of methylation revealed that de novo and maintenance methylation are operationally separable in Neurospora; preliminary results suggest they are mechanistically separable as well. I will summarize our latest understanding of the control and function of methylation in Neurospora including the relationship between RIP and DNA methylation.

Molecular analysis of regulated recombination hotspots in Neurospora P. Jane Yeadon, Frederick J. Bowring and David E. A. Catcheside, The Flinders University of South Australia

Local meiotic recombination in Neurospora is regulated by at least two types of genes, the *rec* loci and the recombinators with which they interact. Three polymorphic *rec* loci are known and in each case the dominant *rec* allele reduces recombination in specific regions of the genome. *cog,* a recombinator regulated by the unlinked gene *rec-2*, was recognised by the existence of two alleles with differing effects on recombination in the *histidine-3* region. The dominant allele of Lindegren Y8743 origin, cog^{La}, promotes recombination at a similar level to that seen at *Saccharomyces cerevisiae* (yeast) hotspots, significantly higher than the allele from Emerson, cog^{Ea}. The recombinator at the 5′ end of *am*, located in a recombination-cold region, is regulated by *rec-3* and seems functionally monomorphic.

We have cloned and sequenced both *cog* alleles within 10 kb that includes *his-3* and the next gene distal to *cog*, *lpl*. The *rec-2* locus has been located within a contig generated by a chromosome walk between *am* and *sp*. Sequence heterology, up to 6% close to *cog* and present at lower level in the *am* coding sequence, has been used in molecular analysis of chromatids that have experienced meiotic recombination at both *am* and *his-3* loci. Conversion at *his*-3 appears similar to that at yeast hotspots and seems highly associated with crossing over, though conversion tracts are more frequently discontinuous than in yeast. In contrast, conversion at *am* shows little association with crossing over. We are now analysing tetrads to extend the analysis.

Novel uses for Neurospora heterokaryons. W. Dorsey Stuart, Neugenesis Corporation, Honolulu, Hawaii

During the past year Neugenesis Corporation has continued to work with heterokaryons in Neurospora to produce heterodimeric molecules. The molecule we use as a model for this work is humanized IgG. We have previously shown that a Neurospora heterokaryon constructed so that one nucleus contains an expression cassette for an IgG kappa light chain and a second nucleus contains and expression cassette for an IgG gamma heavy chain will produce and secrete assembled and intact IgG molecules of 150,000 kD molecular weight. We have now shown that combinatorial panels of IgG molecules can be assembled using subunits from different antibody genes. We are also working on ways to increase the amount of IgG molecules produced by heterokaryons. We are utilizing classical mutagenesis, molecular genetic knockouts and fusion proteins as tools in this effort.

Chromosome rearrangements that will not follow the rules. Edward G. Barry, University of North Carolina, Chapel Hill

Reciprocal translocations, insertional translocations, quasiterminal translocations, pericentric inversions, and an intrachromosomal transposition have been analyzed and mapped to linkage groups. Most rearrangements are identified also by the type of ascospore patterns observed in crosses of rearrangement by normal sequence. The patterns are ascertained from collections of eight ascospores shot from perithecia. Ratios of octads with black (B) viable to white (W) aborted spores reveal the type of rearrangement in many or most rearrangements studied (D. D. Perkins, 1997, Adv. Genet. 36:239-398). Only paracentric inversions have not been detected by spore patterns. Two chromosome rearrangements, which are not inversions, have been very difficult to work out: T(IIR;VIL)R2459 and T(II→ (X);IV;V)AR179 (and also a derivative of AR179 with aberration only in linkage group IV). All three frequently produce asci containing seven or fewer spores. Sister spores, which should be identical, may not match, yielding ratios of, for example, 3B : 5W or 5B : 2W or even 3B : OW. When counting only groups of eight spores, this significant feature of these aberrations is overlooked. Furthermore, isolation of ordered octads from R2459 crosses to normal wild type sequence reveal unexpected allele ratios: 8:0 or 5:3 for one marker while adjacent linked heterozygous markers are 4:4. Other spores may show a mixed genotype as if they were partial diploids receiving both copies of the heterozygous alleles yet there is no corresponding deficiency, recognized as an aborted spore, in any other member of the octad.

The Neurospora Genome Project at the University of New Mexico: update. Donald O. Natvig[1], Mary Anne Nelson[1], Patricia L. Dolan[1], Matthew E. Crawford[1], Edward L. Braun[1,2] and Seogchan Kang[3], [1] Department of Biology, University of New Mexico, Albuquerque, NM 87131, [2]National Center for Genome Resources, Santa Fe, NM 87505 and [3]Department of Plant Pathology, Pennsylvania State University, University Park, PA 16802

We have been sequencing clones from four *N. crassa* cDNA libraries: germinating conidial, advanced-growth mycelial, perithecial and unfertilized sexual tissues. Our strategy has employed both random clone selection and subtraction of clones representing highly-expressed genes. To date, we have identified more than 1600 different genes. We have also developed a system for classifying encoded proteins predicted from the results of data base searches. Recent

improvements at our World Wide Web site provide rapid review of proteins for which corresponding *N. crassa* cDNAs have been sequenced, along with links to homologous genes in GenBank and relevant clones from our libraries. As a pilot project in genomic sequencing, we have nearly completed sequencing a cosmid clone that carries the *N. crassa* genes for *snz-1* and *sno*-1 and at least eight additional genes. The results of this project support previous estimates of gene density in *N. crassa*, with a predicted total of over 10,000 genes.

Session 3 - Development

A G protein α subunit, *gna-3*, regulates cAMP metabolism and aerial hyphae formation in *Neurospora crassa*. Ann M. Kays, Patricia S. Rowley, Rudeina Baasiri, and Katherine A. Borkovich, University of Texas Medical School - Houston.

In eukaryotic systems, heterotrimeric αβγ guanine nucleotide proteins are used to convey extracellular signals. Signals such as light, pheromones, and neurotransmitters activate G protein coupled signal transduction pathways. Cellular responses are generated by the conversion of the initial signal to production of intracellular secondary messengers. Our lab previously identified and characterized two G protein α subunits, *gna-1* and *gna-2* . A third novel G protein α subunit, *gna-3*, has been cloned and found to be 70% and 67% similar to *gna-1* and *gna-2* , respectively, in *Neurospora crassa* . A deletion of *gna-3* was marked by resistance to the bacterial hygromycin B phosphotransferase *(gna-3::hph)*. Western blot analysis indicated that GNA-1 and GNA-2 were present at wild-type levels in *Δgna-3* strains. On solid medium, Δ *gna-3* strains exhibited denser conidiation and stunted aerial hyphae growth, but were not significantly affected in their apical extension rate. The level of cAMP in Δ *gna-3* mutants was only 20% of wild-type on solid medium. The reduced cAMP levels suggest a role for GNA-3 in the metabolism of cAMP in Neurospora crassa

Altered cAMP levels, adenylyl cyclase and cAMP phosphodiesterase activities associated with mutation of G protein α subunits in Neurospora crassa. F. Douglas Ivey*, Qi Yang, and Katherine A. Borkovich, University of Texas Medical School, Houston, TX

Heterotrimeric GTP-binding proteins are key components of signal transduction in eukaryotic organisms. Previously, we reported that the filamentous fungus Neurospora crassa possesses two G protein α subunits, GNA-1 and GNA-2. Loss of gna-1 leads to multiple phenotypes, while Δ gna-2 strains do not exhibit visible defects. However, Δ gna-1; Δ gna-2 mutants are more affected in all Δ gna-1 phenotypes. This report presents results of a biochemical investigation of the roles of GNA-l and GNA-2 in cAMP metabolism. Comparison of Mn^{2+}ATP-dependent adenylyl cyclase activities indicated that the levels of enzyme were normal in Δ gna-2 mutants, but reduced 40% in Δ gna-1 and Δ gna-1; Δ gna-2 mutants. Assays of Mg^{2+}ATP-dependent adenylyl cyclase activity (± GppNHp) indicated that Δ gna-2 strains were normal, whereas Δ gna-1 and Δ gna-1; Δ gna-2 strains had only 10-15% the activity of the wild-type control. Mg^{2+}ATP-adenylyl cyclase activity in wild-type cell extracts could be inhibited using anti-GNA-l IgG, suggesting a direct interaction between GNA-1 and adenylyl cyclase in N. crassa. The

intracelluiar levels of cAMP in Δ gna-1 and Δ gna-1; Δ gna-2 mutants were reduced relative to wild-type under conditions that result in morphological abnormalities. cAMP phosphodiesterase activities in Δ gna-1 and/or Δ gna-2 strains were lower than in wild-type controls; the individual deletions were additive in decreasing phosphodiesterase activity in the Δ gna-1; Δ gna-2 double mutant. Thus, G protein α subunits differentially influence adenylyl cyclase and cAMP phosphodiesterase activity in N. crassa.

Nutritional regulation of conidiation. Daniel J. Ebbole, Texas A&M University, College Station, TX

Wild type Neurospora does not conidiate in media with adequate carbon and nitrogen sources. Repression of conidiation occurs with a variety of carbon sources. We are interested in understanding how nutrient sensing regulates development. The rco-3 mutant was isolated because of its elevated expression of a developmentally regulated gene, con-10. The mutant was found to be 2-deoxyglucose resistant and defective in glucose repression. Low affinity glucose transport was strongly inhibited in the mutant. The mutant also fails to repress macroconidiation in minimal medium regardless of carbon source suggesting that the defect is not glucose-specific. Thus, RCO3 appears to be a regulatory protein involved in glucose repression and carbon source repression of conidiation. The RCO3 protein has sequence similarity to sugar transporters. Our current model is that RCO3 acts as a membrane receptor of glucose and that ligand binding signals glucose availability.

N. crassa ras-3 defines a novel class of Ras protein. Peter Margolis and Charles Yanofsky, Stanford University, Stanford, CA.

Using degenerate PCR primers, we amplified from N. crassa DNA three apparent ras genes: previously characterized ras-1 and ras-2, and a third locus (ras-3) that maps to LG VII R. Putative RIP mutants of ras-3 are female sterile, aconidial, slow-growing and defective in carotenogenesis. True null mutations in ras-3 may be lethal.

The RAS3 protein, encoded as a single exon, belongs to the Ras class of small GTPases, as judged by overall homology and by the presence of Ras-specific sequence motifs. However, RAS3 differs from standard Ras proteins in several respects: 1) sequence divergence at many typically conserved residues; 2) absence of a typical C-terminal "-CAAX" farnesylation site; 3) unusually large size (34 Kd, vs. a typical 21-25 Kd). The increased size of the predicted RAS3 reflects the presence of 3 "insertions" compared to a standard Ras; notably, RAS2 also contains one such "extra" protein sequence. These new polypeptide segments lack obvious similarity to known structural motifs. Computer modeling suggests that the extra RAS3 sequences could form a novel domain on one face of the canonical Ras structure.

A subsequent PCR-based phylogenetic survey indicates that ras-3 homologs are present in many Euascomycetes, but are not found outside of this class of fungi. By contrast, distinct ras-1 and ras-2 homologs are observed in most of the filamentous fungi tested, including Ascomycetes, Basidiomycetes, and Zygomycetes. RAS3 may participate in a novel signaling pathway, and thus could serve as an antifungal drug target.

Regulation of the *Neurospora crassa* clock gene frequency. Susan K. Crosthwaite, Deanna L. Denault, Keith A. Johnson, Jay C. Dunlap and Jennifer J. Loros, Biochemistry Department, Dartmouth Medical School, Hanover 03755, New Hampshire

The circadian oscillator in *Neurospora crassa* requires that the products of the clock gene frequency (frq) cycle in a time of day dependent manner [Aronson et al. (1994) Science 263:1533]. To be able to describe what constitutes the clock at the molecular level, regulation of frq must be understood. We are interested in characterizing the factors that activate and repress frq expression to create the daily oscillation of frq transcript and protein, as well as understanding how this regulation is acheived.

The frq locus produces two sense transcripts of 4.5 and 5 kb which encode FRQ protein. FRQ negatively autoregulates levels of frq transcript so that frq mRNA cycles; FRQ also cycles in abundance and phosphorylation state [Garceau et al (1997) Cell 89:469]. Recently we showed that the products of the white collar genes are required for a functional oscillator. The finding that frq levels are repressed in the white collar mutants [Crosthwaite et al. (1997) Science 276:763] and the fact that WC-1 and WC-2 encode transcription factors [Ballario et al. (1996) EMBO J. 15:1650; Linden and Macino (1997) EMBO J. 16:98] suggests the WC proteins as candidates for the positive factors in the feedback loop, that bind to the frq promoter and activate transcription. The zinc-finger DNA-binding domains of these proteins have been expressed as GST fusions in BCL21 cells. The cell extracts and purified full length WC-2 have been incubated with fragments of the frq promoter. The results of gel mobility shift assays using these samples will be presented.

The possible regulatory role of two antisense (AS) transcripts is also under investigation. We have evidence that the frq locus produces two antisense transcripts of 5 and 5.5 kb. They are transcribed from within the frq locus and overlap the sense frq transcripts. These AS transcripts may affect the expression of the sense transcripts. The minimal region required for phenotypic rescue of rhythmic conidiation in a frq null background includes the AS promoter and constructs lacking this region show poor rescue of the overt rhythm.

Search for protein(s) that interact with the Neurospora clock protein FREQUENCY Christian Heintzen, Jennifer J. Loros and Jay C. Dunlap, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844

The protein FREQUENCY (FRQ) is a central component of the Neurospora circadian clock. A LexA interactor hunt, an implementation of the of the yeast two-hybrid screen, was conducted to identify proteins that may interact with the FRQ protein and hence might be part of the molecular feedback loop that comprises the circadian clock in Neurospora. Full length FRQ and various FRQ fragments were tested as baits. Western blot experiments show that all transformants produce LexA-FRQ fusions of the expected molecular size, however only FRQ fusions spanning the regions FRQ11-683 and FRQ11-262 were shown to be transcriptionally inert when fused to LexA. A repression assay confirmed that both proteins enter the nucleus. Transformants containing both constructs were used for the subsequent transformation of a Neurospora prey-library. 6×10^5 independent transformants (inserted as a prey behind the bacterial activation domain B42) were analyzed. 53 transformants that showed galactose dependent leucine

prototrophy and tested positive for beta-galactosidase activity were further analyzed. The majority of these clones encode for ribosomal proteins, ribosomal RNA's or mitochondrial proteins and are probably false positive. The remaining clones are currently being tested for the specificity of their interaction. The possibility of a FRQ-FRQ interaction was tested by using full length FRQ as well as different FRQ fragments as prey proteins which then were cotransformed with the transcriptionally inert FRQ-baits. Based on these experiments no indication for a FRQ-FRQ interaction has been found. These data are consistent with results of biochemical analyses previously reported (Garceau et al., Cell 89:469-476, 1997).

Roles in dimerization and blue light photoresponse of the PAS and BAT domains of *Neurospora crassa* White Collar proteins. P. Ballario, C. Talora, D. Galli, H. Linden, G. Macino, Universita di Roma La Sapienza, Roma, Italy

The genes coding for white collar-1 and white collar-2 (wc-l,wc-2) have been previously isolated and their products characterized as Zn-finger transcription factors involved in the control of blue light induced genes. The PAS dimerization domains present in both proteins enable the WC-1 and WC-2 proteins to dimerize in vitro. Homodimers and heterodimers are formed between the WC proteins. Inter-species heterodimers are formed between the WC proteins of Neurospora and the mammalian PAS- containing proteins AHR and ARNT. A computer analysis of WC-1 reveals a second PAS-like domain, called BAT a domain conserved in other redox-sensitive proteins such as NIFL and Bat in bacteria and NPH1, a putative blue light photoreceptor in plants. The WC-1 BAT domain has lost the ability to dimerize with canonical PAS domains, but it is able to self-dimerize. The isolation of three blind wc-1 strains, each with single amino acid substitutions only in the BAT domains, reveals that this region is essential for blue-light responses in Neurospora. The demonstration that the WC-1 proteins in these BAT mutants are still able to self-dimerize suggests that this domain plays an additional role, essential in blue light signal transduction.

Changes in Fatty Acid Composition of Neurospora crassa Accompany Sexual Development and Ascospore Germination
Kelly A. Howe, Marta Goodrich-Tanrikulu, Allan Stafford and Mary Anne Nelson
University of New Mexico

The sexual development of *Neurospora crassa* is complex involving the formation of several types of specialized sexual tissues and significant cell signaling. Several lines of evidence suggest that fatty acids play a critical role in the sexual development of filamentous fungi. Unsaturated fatty acids, particularly a-linoleate (18:2) and oleate (18:1), stimulate production of fruiting bodies in *N. crassa* and other filamentous fungi. Neurospora mutants impaired in fatty acid desaturation have impaired fertility, which can be improved by the addition of unsaturated fatty acids to the crossing medium. The metabolism of fatty acids appears to account in part for the requirement for fatty acids during sexual development. In *Podospora anserina*, a mutation affecting the assembly of peroxisomes (the organelle responsible for the metabolism of fatty acids in fungi) inhibits sexual development. Preliminary evidence suggests that an analogous mutation affects sexual development in *N. crassa*. The present study investigates the fatty acid and lipid composition of *N. crassa* during normal sexual development. The results show that the fatty acid composition of sexual tissue differs from that of asexual tissue. These results provide a

basis for study of the role of fatty acids and their metabolism on sexual development of *N. crassa*.

Characterizing a highly-expressed gene of the perithecial stage of *Neurospora crassa*
Harriett J. Bowannie Platero and Mary Anne Nelson University of New Mexico Albuquerque, New Mexico

We have identified a novel gene that is expressed almost exclusively during sexual development. This gene, which we have tentatively named *jun1*, is homologous to the mammalian transcription factor, c-Jun. c-Jun plays an important role in activating gene transcription; it is also a proto-oncogene and was the first transcription factor shown to induce cancer. Our hypothesis is that the corresponding *Neurospora crassa* gene is a novel transcription factor belonging to the same subfamily of transcription factors as c-Jun, the bZIP subfamily. The pattern of expression suggests that the protein encoded by *jun1* plays a specific role in control of gene expression during sexual development.

Session 4 - Metabolism

Regulation of sulfur metabolism in *Neurospora crassa*. John V. Paietta, Dept. of Biochemistry and Molecular Biology, Wright State University, Dayton, OH

The sulfur regulatory system of N. crassa consists of a group of sulfur- regulated structural genes (e.g., arylsulfatase, ars-l+) which are under coordinate control of the cys-3+ positive and several scon (sulfur controller) negative regulatory genes. The CYS3 regulator is a bZIP DNA-binding protein and transcriptional activator, while the SCON2 negative regulator is a F-box/WD-40 protein. F-box proteins are thought to act as receptors for ubiquination targets. The importance of the SCON2 F-box in sulfur gene regulation has been shown by site directed mutagenesis and the subsequent generation of a sulfur auxotrophic phenotype. For comparison, a Δ scon-2 strain was generated by transformation and homologous recombination. Deletion of the scon-2+ gene yielded constitutive expression of sulfur-regulated structural genes. The apparent functional role for the F-box in SCON2 implicates the involvement of proteolysis in the regulatory system, perhaps targeted at CYS3. Further support for this mechanism comes from the cloning of scon-3+, another sulfur negative regulatory gene, which may encode a component of the putative complex involved in the control of sulfur gene regulator activity.

Some pH regulatory mutations of A. nidulans can be complemented by a N. crassa genomic library. A.C. Aquino, G. Thedei Jr, W. Maccheroni Jr, S.R. Nozawa and A. Rossi, University of Sao Paulo, Ribeirao Preto, SP, Brazil

The pH regulation in the mold A. nidulans is mediated at least by genes pal (A, etc) and pacC. Mutants pal mimic growth at acid pH, increasing the level of the pH-dependent expressed proteins, and mutants pacC mimic growth at alkaline pH and lead, for example, to elevated levels of alkaline phosphatase and reduced levels of acid phosphatase. Mutants pal do not grow at pH 8.0 or in the presence of β< /ΦONT>-glycerolphosphate as the only source of Pi and mutants pacC do not grow at pH 3.0. This allowed us to transform these mutations by complementation with a genomic library of *N. crassa* (pCOSAX) and the self-replicating pAB4 plasmid, which

contains the *pyr*G gene of *A*. *nidulans*. Six colonies that were able to grow at pH 8.0, proved to be transformants of the *pal*A1 strain, and one was a transformant of the *pal*F15 strain. Four colonies that were able to grow at pH 3.0, were transformants of the *pac*C14 strain. The existence of *N*. *crassa* genes that may complement mutations in the *pac*C and *pal* genes of *A*. *nidulans* is puzzling because, according to models proposed in the literature, *A*. *nidulans* appear to control the transcription of the acid and alkaline phosphatases in response to the stimulus generated by the extracellular pH, whereas *N*. *crassa* synthesizes both enzymes irrespective of the extracellular pH, thus controling only their secretion to the external medium.

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Distribution, nature, and possible significance of the base changes in 25 well characterized mutants of the trp-3 (tryptophan synthase) gene of *Neurospora crassa.* Ann M. Lacy, Goucher College, Baltimore; Mary E. Case and William Nelson, University of Georgia, Athens

The DNAs from 25 well characterized CRM+ mutants altered in the trp-3 gene in *Neurospora* were amplified and partially sequenced to determine the precise location and alteration in each mutant. These mutants were representative of different physiological groups with respect to partial enzyme activities, osmotic remediability, and complementation. Many of these mutants had been previously mapped by genetic recombination. Their map locations were, in almost all cases, confirmed by the sequence data.

Of the 25 mutants, 19 represent unique alterations. Of these, one has a two codon insertion, one has two base changes in a single codon, and one has a substitution of an entire codon. The 19 sites are distributed over roughly 85% of the coding region. None occur before the first intron; however, four sites fall within the relatively short region between introns 1 and 2. None are in the connector region; however a mutant with unique characteristics is located just upstream of it and another one just downstream of it. Correlation of all these findings with the physiological data will be discussed and, where appropriate, the properties of the *Neurospora* enzyme will be compared with those of the *E. coli* enzyme.

Subunit structure, substrate selection and nucleotide hydrolysis activities of HSP80 and 70 of *Neurospora crassa*.

M. Kapoor, Department of Biological Sciences, University of Calgary, Canada

The cytosolic molecular chaperones of *Neurospora crassa*, HSP80 and HSP70, interact directly to form a hetero-oligomeric complex. In mammalian systems, multiprotein complexes containing HSP70, HSP90 (equivalent of *N. crassa* HSP80) and other proteins in conjunction with hormone receptors have been documented. However, a direct physical interaction between HSP70 and 90 has not been observed. *N. crassa* HSP80 is a tetramer in its native state while HSP70 is predominantly monomeric, forming higher-order oligomers in the presence of certain nucleotides. Formation of the [HSP80:70] complex was demonstrated by crosslinking of purified HSP80 and 70 with bifunctional reagents, gel filtration, Western blotting and ELISA using specific antibodies. Ligand-induced conformational changes of HSP80, promoted by nucleotides--CTP, UTP and NAD--were monitored by following the intrinsic tryptophan

fluorescence and by the use of environmentally sensitive fluorescent probes: ANS and TNS. Although both HSP80 and HSP70 hydrolyse ATP and GTP, significant differences in the kinetics of hydrolysis were evident. The molecular chaperoning potential of HSP80 and HSP70 was assessed by comparing the peptides selected by these proteins from M13 Phage Display libraries. HSP80 predominantly selected peptides with a common structural motif defined by an invariant hydrophobic core and flanking variable sequences containing aromatic and charged residues. Peptides containing charged residues were selected by HSP70 but the substrate selectivity of [HSP80:70] was distinct from that of either protein.

Construction of a *Neurospora crassa* mutant lacking the mitochondrial outer membrane translocase protein TOM40. Frank E. Nargang, Bryan McHale, Lara Corrigan, and Rebecca D. Taylor, Department of Biological Sciences, University of Alberta, Edmonton, Alberta

Most of the proteins found in mitochondria are encoded by nuclear genes, synthesized as mitochondrial preproteins on cytosolic ribosomes, and imported into mitochondria. The initial steps in the import process are mediated by the Tom complex (Translocase of the outer mitochondrial membrane). The complex consists of receptor proteins that extend into the cytosol and integral membrane proteins that form the pore through which preproteins traverse the outer mitochondrial membrane. The TOM40 protein is thought to play the major role in the formation of the pore. We have created a *N. crassa* mutant of TOM40 using the procedure of "sheltered RIP". The technique allows the isolation of a mutant gene in one nucleus, even if that gene is essential for the survival of the organism, by sheltering the nucleus carrying the mutant allele in a heterokaryon with an unaffected nucleus. Genetic and biochemical manipulation of the heterokaryon allows the study of the mutant phenotype. When the nucleus containing the RIPed allele of tom40 is forced to predominate the heterokaryon, growth of the cells virtually ceases as TOM40 levels decrease to a critical low level. Genetic analysis has shown that tom40 is an essential gene in *N. crassa*. The mutant *tom40* allele contains a stop codon at position 35 of the 349 residue protein suggesting that it represents a null allele of the gene.

Isolation and characterization of a new gene, *sre*, which encodes a GATA- tyge regulatory protein that controls iron transport in *Neurospora crassa.* Liwei Zhou, Hubertus Haas, and George A. Marzluf, Ohio State University, Columbus, Ohio

Multiple GATA factors, regulatory proteins with consensus zinc finger motifs that bind to DNA elements containing a GATA core sequence, exist in the filamentous fungus, *Neurospora crassa*. The gene encoding a new GATA factor, named SRE, was isolated from *Neurospora* using a PCR-mediated method. Sequence analysis of the new GATA factor gene revealed an open reading frame specifying 587 amino acids, which is interrupted by two small introns. Unlike all previously known *Neurospora* GATA factors which possess a single zinc finger DNA binding motif, SRE contains two GATA-type zinc fingers. The deduced amino acid sequence of SRE shows significant similarity with URBS1 of *Ustilago* and SREP of *Penicillium*. A loss of function mutation was created by the rip procedure. Analysis of *sre*+ and *sre*- strains revealed that SRE acts as a negative regulator of iron uptake in *Neurospora* by controlling the synthesis of siderophores. The *sre* promoter contains a number of GATA sequences; however, expression of *sre* mRNA occurs in a constitutive fashion and is not regulated by the concentration of iron available to the cells.

Invited Lecture

Genetic and molecular analysis of the Neurospora circadian system. Jay C. Dunlap, Allan C, Froehlich, Chenghua Luo, Yi Liu, Susan Crosthwaite and Jennifer J. Loros, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

The circadian system in Neurospora is among the best understood of any organism (Dunlap, Ann. Rev. Genetics 30:579, 1996). The oscillator is an autoregulatory feedback cycle, wherein the *frequency* gene encodes two forms of the FRQ protein; both *frq* RNA and protein cycle in abundance, and FRQ is rhythmically phosphorylated (Aronson *et al.*, Science 263:1578, 1994; Garceau *et al*. Cell 89:469, 1997; Merrow *et al.*, PNAS 94:3877, 1997). Light resets the clock by rapidly inducing *frq* (Crosthwaite *et al*., Cell 81:1003, 1995). Ambient temperature influences the clock by determining both the absolute amount of FRQ and the ratio between the two forms (Liu *et al*., Cell 89:477, 1997). The WC-1 and WC-2 proteins, required for light-induction of *frq* and for sustained cycling in the dark, are clock proteins having sequences similar to clock-associated proteins from the mouse and fly (Crosthwaite *et al.* Science 276:763, 1997). FRQ is a nuclear protein and nuclear localization is essential for its function (Luo *et al.*, EMBO J., in press). Temperature steps reset the clock in essence by changing the set points for the oscillation, and in this way changing the subjective time to which different absolute levels of *frq* and FRQ correspond.

Efforts are also underway to develop luciferase and GFP as in vivo circadian reporters in Neurospora.

Session Abstracts

Friday evening, June 19

7:00 - 10:00 pm

1. Sequence analysis of a cosmid clone of *Neurospora crassa*. Laura Bean[1], Mara Giles[1], Edward L. Braun[1,2], Mary Anne Nelson[1], and Donald O. Natvig[1]. [1]Department of Biology, University of New Mexico, Albuquerque, NM 87131 and [2]National Center for Genome Resources, 1800 Old Pecos Trail, Santa Fe, NM 87505

We have nearly completed sequencing a 35 kbp region of genomic DNA from *N. crassa*. This region of the *N. crassa* genome was chosen because it contains homologs of stationary phase genes (*snz-1* and *sno-1*) from the budding yeast, *Saccharomyces cerevisiae*. In addition to these two genes, we have identified at least eight additional genes. These include a homolog of the *ALG3* gene from *S. cerevisiae*, a serine/threonine protein phosphatase gene, a 3

hydroxyisobutyrate dehydrogenase gene, and genes encoding D-amino acid oxidase and thioredoxin. Based upon our current analysis, the gene density in this region of the *N. crassa* genome appears to be 1 gene for every 3 kb, a value consistent with recent estimates of 10,000 to 13,000 genes in *N. crassa*.

2. Osmotic-sensitive mutants of *Neurospora crassa* with atypical morphology. Sara Neville Bennett and Wayne A. Krissinger, Georgia Southern University, Statesboro, GA 30460

The osmotic-sensitive (*os*) mutants fail to grow on medium containing elevated concentrations of NaCl. The first of these mutants were mapped to LG I and LG IV and had a characteristic altered morphology which allowed them to be scored easily on the basis of this typical *os* morphology. Both osmotic sensitivity and morphology were thought to be pleiotropic effects brought about by an altered cell wall. In our laboratory we have isolated a number of osmotic-sensitive mutants following UV irradiation and filtration-concentration in medium with 3% NaCl. Among our isolates are alleles of *os-1* and *os-4* in LG I. In addition, several new osmotic-sensitive mutants were isolated and in crosses to wild type, each mutant exhibited 1 : 1 segregation of osmotic sensitivity, indicating involvement of single genes under Mendelian control. Mapping of these mutants placed *os-8* (SS-931) in LG III between *ad-2* and *trp-1*; *os9* (SS-788) in LG VI distal to *del*; SS-1018 and AR3-34 both in LG IV and linked to the osmotic-sensitive mutant, *cut*. Thus, osmotic mutants have now been located in two new linkage groups, LG III and LG VI. These new osmotic-sensitive mutants all grossly resemble the wild type strain when the cultures are grown on slants and therefore exhibit atypical *os* morphology. This suggests that there is a difference in the degree of any alteration of the cell wall and that osmotic sensitivity in these mutants might be accomplished by a different route or routes.

3. *Neurospora crassa* as a heterologous system for study of mammalian Gα proteins. Jennifer A. Bieszke, Qi Yang, and Katherine A. Borkovich, University of Texas Medical School - Houston

Heterotrimeric (αβγ) GTP binding proteins interact with sensory receptors to transduce a signal to downstream effectors. Gα subunits have been classified according to the sequence identity. The mammalian system has four classes of Gα subunits, Gαi, Gαs, Gαq, Gα12. Previously, we reported that *Neurospora crassa* contains a Gα protein, GNA-1, that is a member of the mammalian Gαi family based on amino acid sequence identity and its ability to serve as a substrate for pertussis toxin. Deletion of *gna-1* in *N. crassa* leads to multiple phenotypic defects including female infertility and a slower growth rate on solid medium which is more pronounced under hyperosmotic conditions. In this report, we tested the ability of mammalian Gα $_{i}$ subfamily genes to complement a D*gna-1* mutation. We have successfully expressed the following Gαi subfamily members in *N. crassa*: Gαz, Gαo, Gαt, Gαi. As a control, Gαs of the stimulatory G protein family was also expressed. The order of complementation for growth on hyperosmotic solid medium by the mammalian Gα genes is as follows: Gαz> Gαo> Gαs> Gαi. Gαt gave no complementation. Gαo was the only mammalian Gα to rescue the sexual defects of a Δ*gna-1* strain. Biochemical analysis was also used to determine the levels of complementation by the mammalian Gα proteins. Total cAMP was measured under vegetative conditions and complementation was similar to that observed for hyperosmotic growth rates. However, cAMP levels in the mammalian Gα transformants during the sexual cycle do not correlate with

complementation of Δ*gna-1* defect. The above results suggest that GNA-1 regulates the vegetative and sexual cycles using two different pathways. This study also demonstrates *N. crassa* is a valuable heterologous system for study of mammalian G proteins.

4. Characterization of con-11, a gene which is highly expressed during conidiation. Kristin Black and Charles Yanofsky, Department of Biological Sciences, Stanford University, Stanford CA 94305-9020

con-11 was identified and isolated in a screen for highly expressed genes that could be used to study regulation of conidiation (Mol. Cell. Biol., 1985, 5:849-855). con-11 is the last of these con genes to be expressed during asexual development. con-11 is not essential to conidiation or growth; its function is not known.

Sequence comparisons demonstrate that genes encoding similar proteins exist in S. pombe and in A. nidulans. Interestingly, no similar gene sequence can be found in the S. cerevisiae genome. The S. pombe homolog is induced by many stresses including nitrogen or carbon starvation, heat shock and stationary phase (Mol. Gen. Genet., 1995, 248:439-445). Of these stresses, only stationary phase induces con-11 expression in *N. crassa*. However, there appear to be many potential stress-regulated sequence elements upstream of the con-11 ORF (heat shock, nitrogen, phosphate, pH, and general stress response elements).

Like con-10, con-11 is repressed during vegetative growth by Rco1, a negative regulator. con-11 appears to be transcribed shortly after con-10, the most thoroughly characterized con gene. Unlike con-10, translation of con-11 mRNA during conidiation is delayed by several hours after the message appears. Thus, regulation of con-11 expression appears to be distinct from regulation of con-10 and, thus, analysis of con-11 regulation may provide new insights into the relationship between regulation of gene expression by stress vs. conidiation. Finally, the sequence similarity of Con11 to a stress-regulated protein of S. pombe, its dispensability, and the late appearance of Con11 during conidiation, are suggestive of a protective role for this gene product.

5. Cloning of rec-2, two contigs on LG V. F.J. Bowring and D.E.A. Catcheside, Flinders University, Adelaide, Australia

We have established two contigs on linkage group V. The largest of these extends from 350kb proximal to 150kb distal of am while the other is 100kb and centred across leu-5. Mapping of crossovers either side of rec-2 suggests that this locus, situated 3 centi-Morgans (cM) proximal of am, is about 200kb downstream. The genetic to physical distance ratio is lower distal of am where the 1cM to ure-1 corresponds to 150kb.

6. Molecular analysis of recombination events at am. F.J. Bowring and D.E.A. Catcheside, Flinders University, Adelaide, Australia

We have used naturally occurring polymorphism in and near the Neurospora am locus to examine the genesis of chromatids recombinant in this region. The analysis indicates that here crossing over may be only loosely associated with gene conversion and has provided a high

resolution picture of the pattern of gene conversion at this locus. However, as chromatid data do not always allow the unambiguous distinction between gene conversion and crossing over, we are examining tetrads in order to extend the power of the analysis.

7. Centromere structure in *Neurospora crassa*: degenerate transposons and simple repeats. E.B. Cambareri, R. Aisner, and J. Carbon, Molec. Cell., and Devel. Biology, University of California, Santa Barbara, CA 93106

DNA from the centromere region of LG VII of Neurospora was cloned previously from a YAC library, and was found to be atypical of Neurospora DNA in both composition (AT-rich) and complexity (repetitive). We have determined the DNA sequence of a small portion (-16. 1 kb) of this region, and have identified a cluster of three new retrotransposon-like elements, as well as degenerate fragments from the 3' end of Tad, a previously identified LINE-like retrotransposon. This region contains a novel full length but nonmobile copia-like element, designated Teen, that is only associated with centromere regions. Adjacent DNA contains portions of a gypsy-like element designated Tgl1. A third new element, Tg12, shows similarity to the Ty3 transposon of Saccharomyces cerevisiae. All three of these elements appear to be degenerate, containing predominantly transition mutations suggestive of the RIP (Repeat-Induced Point mutation) process. Three new simple repeated DNAs have also been identified in the LG VII centromere region. While Tcen elements map exclusively to centromere regions by RFLP analysis, the defective Tad elements appear to occur most frequently within centromeres, but also are found at other loci including telomeres. The characteristics and arrangement of these elements are similar to that seen in the Drosophila centromere, but the relative abundance of each class of repeats, as well as the sequence degeneracy of the transposon-like elements is unique to Neurospora. These results suggest that the Neurospora centromere is heterochromatic and regional in character, more similar to centromeres of Drosophila than to those in most single-cell yeasts.

8. Characterization of arg-6 mutants affecting two unlinked genes. Jessica Y. Chung[1], Suhn-Kee Chae[2] and Richard L. Weiss[1], [1]University of California, Los Angeles and [2]Pai Chai University

N-acetyl glutamate synthase (AGS) and N-acetyl glutamate kinase (AGK) are the first two enzymes in the arginine biosynthetic pathway. AGS and AGK are encoded by two unlinked genes, arg-14 and arg-6, respectively. Some arg-6 mutants lack AGS activity. Interaction between AGS and AGK has been demonstrated using the yeast Two-Hybrid system. The interaction domain of AGS resides at the N-terminus and the interaction domain of AGK is at the C-terminus. AGS and AGK are regulated via feedback inhibition by arginine. Mutations linked to the arg-6 locus, su(pro-3), are feedback resistant and suppress proline auxotrphic mutations (pro-3). We have mapped the su(pro-3) mutations to the N-terminus of AGK, and all su(pro-3) genes have a single amino acid mutation from phenylalanine (F) to leucine (L) at amino acid 81. The mutant AGK failed to show interaction with AGS. To further investigate the regulation of feedback inhibition by arginine, we have characterized representatives of the various arg-6 mutant classes. Here we report results of sequencing and yeast Two-Hybrid analysis. Two $K^- S^+$ strains have point mutations L289F and N231N. A $K^+ S^+$ strain has a point mutation, L102P. A

K^- S^- strain has a F135V mutation. Another K^- S^- strain has a frame-shift mutation generating a premature stop codon at 143. In general, all mutations, including su(pro-3) mutations, mapped to the N-terminus of AGK. When interaction with AGS was tested, none of arg-6 mutant AGKs showed interaction with AGS. The implication of these observations for the mechanism of feedback inhibition will be described.

9. A membrane skeleton is present in *Neurospora crassa*. Norbert Degousee, Gagan Gupta and I. Brent Heath. York University Biology Department, North York, Ontario, Canada M3J 1P3

An F-actin based membrane skeleton has been proposed to play a crucial role in the tip growth of hyphae, but the relevancy of this model has not been investigated in the dominant group of hyphal organisms - the true fungi. Plasma membranes (PM) were isolated from growing *Neurospora crassa* hyphae by discontinous sucrose density gradient centrifugation and subjected to SDS-PAGE and Western blot analysis. The PM isolate was enriched in actin and also contained other cytoskeletal proteins such as spectrin, tubulin and possibly an integrin homologue. Consistent with this, indirect immunofluorescence showed that all these antigens were present (in varying proportions) at the peripheries of Neurospora hyphae. Strikingly, the actin, spectrin and integrin antigens were all concentrated at and were colocalized to hyphal apices, yet their precise distributions at the extreme apex were different. Together, these results are best explained by the presence of a functional membrane skeleton in Neurospora hyphal tips.

10. The mating pheromones of *Neurospora crassa*. Daniel J. Ebbole, Deborah Bell-Pedersen, Neal Van Alfen, Piotr Bobrowicz, Wei-Chiang Shen, Lori A. Shrode, Patricia McCabe, and Pam Kazmierczak. Texas A&M University, College Station.

The mating type loci of *N. crassa* encode regulators that control expression of genes involved in sexual fertility and development. We have begun to analyze the genes encoding the sex pheromones of *N. crassa*. One gene, from Mat A strains, express a pheromone precursor gene with a structure resembling the yeast alpha-factor precursor. The predicted sequence of the mature pheromone is remarkably similar to that of the chestnut blight fungus, Cryphonectria parasitica. Mat a strains express a pheromone precursor gene that resembles yeast a-factor that is predicted to be isoprenylated. Mating type-specific, nutritional, developmental and circadian regulation of the pheromone genes will be presented. Experiments to produce pheromone gene mutants are in progress.

11. The effects of mutations in the *Neurospora crassa* arg-2 arginine attenuator peptide on arginine-specific translational attenuation. Peng Fang, Zhong Wang, Anthony Gaba and Matthew S. Sachs, Oregon Graduate Institute, Portland OR.

The mRNA transcript encoding the *Neurospora crassa* arg-2 gene product contains an evolutionarily conserved upstream open reading frame (uORF). Translation of the arginine attenuator peptide (AAP) specified by this uORF in the presence of a high level of arginine causes ribosomes to stall, resulting in reduced translation of the mRNA. To further investigate the role of the AAP coding region in this translational attenuation phenomenon, we have used a variety of techniques to mutate the AAP coding region and tested the effects of these mutations on translational control using an *N. crassa* cell-free translation system. Mutations that change

highly conserved amino acid residues can eliminate regulation. Silent mutations that place new, unique restriction sites near the beginning, middle and end of the uORF coding region do not reduce regulation. Deletion of the N-terminal region of the AAP, which is not evolutionarily conserved, does not eliminate regulation, nor does the introduction of a His_6 affinity-tag in this region. The available data indicate that the sequence of the nascent peptide, and not the mRNA from which it is synthesized, is of primary importance for arginine-specific translational attenuation.

12. Protein-protein interactions mediate the functional interactions between the global positive regulator NIT2, the global negative regulator NMR and the pathway-specific regulator NIT4. Bo Feng, H.G. Pan and George. A. Marzluf, Department of Biochemistry, The Ohio State University, USA

In *Neurospora crassa*, nitrogen metabolism is controlled by NIT2 and the negative regulator NMR. Activation of structural genes in different pathways also requires pathway-specific regulators, such as NIT4. The major feature of this regulatory system is that the expression of catabolic enzymes depends on both the lifting of nitrogen repression and substrate induction. In the nitrate assimilation pathway, the pathway-specific regulator NIT4 mediates nitrate induction. Both NIT2 and NIT4 are required to turn on the nit-3 and nit-6 genes in this pathway. Neither one alone is sufficient. This stringent requirement for both positive regulators to turn on transcription is different from the promiscuous cooperation between transcription factors, and appears to represent a distinct regulatory mechanism, possibly by protein-protein interaction. Data presented here reveal that NIT2-NIT4 interaction indeed occurs and is critical for the integrated control of NIT2 and NIT4 over the nitrate assimilation pathway. Another GATA factor in Neurospora, which shares similar DNA-binding specificity with NIT2 but functions differently, did not show any detectable interaction with NIT4 protein. These protein-protein interactions appear to play an important role in the integration of regulatory signals at the transcriptional level and in achieving the functional specificity of different GATA factors in *N. crassa*. This work may have implications in other regulatory processes.

13. Isolation of a putative methyltransferase (Mtase) gene from Aspergillus nidulans. Michael Freitag, Alejandro Correa*, Rodolfo Aramayo* and Eric U. Selker, University of Oregon, Eugene, OR and *Texas A&M University, College Station, TX.

DNA of many eukaryotes is modified by methylation of cytosines. This process is essential for proper development and cell maintenance in some eukaryotes. Numerous proteins are likely involved in the establishment and regulation of DNA methylation. One of the essential components of any methylation machinery is the methyltransferase itself. We have previously attempted to identify methyltransferase genes in *Neurospora crassa* either by functional, biochemical means or by homology-based approaches that rely on conserved amino acid sequences among eukaryotic Mtase genes. Here we report the isolation and preliminary characterization of an Aspergillus nidulans gene that we identified based on sequence similarity of an Aspergillus EST clone to pro- and eukaryotic Mtases. This gene contains all conserved motifs commonly found in eukaryotic Mtases including a conserved active site. Curiously,

Aspergillus - like Saccharomyces cerevisiae and Schizosaccharomyces pombe - has been reported to lack detectable DNA methylation in vegetative tissue. The Aspergillus Mtase gene is most similar to the Ascobolus immersus masc1 gene that is required for efficient silencing by MIP (methylation induced premeiotically) but is not essential for methylation in vegetative tissue (Malagnac, F. et al., 1997, Cell, 91:281-290). Sequence similarities among these putative fungal Mtases are being exploited to search for the Neurospora homologue(s).

14. Genetic and molecular analysis of the Neurospora circadian system. Allan C. Froehlich, Chenghua Luo, Yi Liu, Susan Crosthwaite, Jennifer J. Loros, and Jay C, Dunlap Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

The circadian system in Neurospora is among the best understood of any organism (Dunlap, Ann. Rev. Genetics 30:579, 1996). The oscillator is an autoregulatory feedback cycle, wherein the frequency gene encodes two forms of the FRQ protein; both frq RNA and protein cycle in abundance, and FRQ is rhythmically phosphorylated (Aronson et al. Science 263:1578, 1994; Garceau et al. Cell 89:469, 1997; Merrow et al. PNAS 94:3877, 1997). Light resets the clock by rapidly inducing frq (Crosthwaite et al. Cell 81:1003, 1995). Ambient temperature influences the clock by determining both the absolute amount of FRQ and the ratio between the two forms (Liu et al. Cell 89:477, 1997). The WC-1 and WC- 2 proteins, required for light-induction of frq and for sustained cycling in the dark, are proteins having sequences similar to clock-associated proteins from the mouse and fly (Crosthwaite et al. Science 276:763, 1997). FRQ is a nuclear protein and nuclear localization is essential for its function (Luo et al. EMBO J., in press). Temperature steps reset the clock in essence by changing the set points for the oscillation, and in this way changing the subjective time to which different levels of frq and FRQ correspond.

Efforts are also underway to develop luciferase and GFP as in vivo circadian reporters in Neurospora.

15. Recombination on the mating-type chromosome of Neurospora tetrasperma. Alena Gallegos[1], David J. Jacobson[2], Marian P. Skupski[3], Gregory S. Saenz[1] and Donald O. Natvig[1]. [1]Department of Biology, University of New Mexico, Albuquerque, NM 87131, [2]Department of Biological Sciences, Stanford University, Stanford, CA and [3]National Center for Genome Resources, Santa Fe, NM 87505.

Unlike *N. crassa*, which produces eight homokaryotic self-sterile ascospores per ascus, N. tetrasperma produces four self-fertile ascospores per ascus each containing nuclei of both mating types (A and a). The self-fertile ascospores of N. tetrasperma result from programmed nuclear movement during ascus development, which includes first division segregation of mating type and nuclear spindle overlap at the second meiotic division. Merino et al. presented population-genetic evidence that crossing over is suppressed on the mating-type chromosome of N. tetrasperma (Genetics 143:789-799, 1996). Given the pattern of ascus development in this species, crossovers between the centromere and the mating locus would allow some second-division segregation of mating type resulting in a proportion of self-sterile ascospores. It appears, therefore, that in reducing crossing over natural selection has worked to prevent the occurrence of such self-sterile ascospores. We have experimentally confirmed suppressed crossing over for most of the mating type chromosome of N. tetrasperma by examining segregation of molecular

markers in crosses of wild strains. Surprisingly, our study also revealed a region, located on the far left arm, where recombination is obligatory. This result suggests at least one region, analogous to the pseudoautosomal regions of animal sex chromosomes, that is crossover competent as a result of a need to maintain pairing during meiosis.

16. Disruption of the gene for Hsp30, an α -crystallin-related heat shock protein of *Neurospora crassa*, causes defects in import of proteins into mitochondria. Nancy Gardner, Nora Plesofsky-Vig, Roland Lill* and Robert Brambl, The University of Minnesota, Saint Paul; and the *Philipps-Universitat Marburg, Germany.

Hsp30 is the only α -crystallin-related heat shock protein in Neurospora. It is not synthesized at normal temperatures, but it is strongly induced at high temperature (45° C). This hsp associates with membranes, mainly the mitochondrial (mt) membrane, at high temperature, but it dissociates from the membranes as the cells are returned to normal temperature. This reversible association with mitochondria suggests that Hsp30 is associated with the mt outer membrane. Furthermore, anti-bodies against the outer mt membrane proteins block binding of Hsp30 to mito- chondria. Hsp30, which has chaperone activity, may interact with and stabilize components of the mt protein import complex in the outer mt membrane. In the present study we examined the effect of Hsp30 disruption upon import of proteins into mitochondria of Neurospora during exposure to high temperature. By use of immunoprecipitation analysis of radiolabeled mt proteins, we found that import of most proteins into mt internal subcompartments was sharply reduced in an Hsp30- disrupted mutant strain. Assembly of proteins into the outer mt membrane was not affected, however, except for Tom20, which was sharply reduced. We conclude that in wild type cells Hsp30 may help stabilize the protein conducting channel of the outer mt membrane at high temperature, possibly by interacting with Tom20 or with Tom22, a protein that is essential for protein import into mitochondria.

17. RIP of a homolog of the *Saccharomyces cerevisiae* gene for the beta subunit of fatty acid synthase yields cel-like mutants. M. Goodrich-Tanrikulu∗ , D. Jacobson∗ ∗ , S. Schwartz#, W. D. Stuart#, A. Stafford∗ , J.- T. Lin∗ and T. McKeon∗ , ∗ USDA/Agricultural Research Service/Western Regional I Research Center, 800 Buchanan Street, Albany, CA 94710; ∗ ∗ Department of Biological Sciences, Stanford University, Stanford, CA 94305; #Genetics and Molecular Biology, University of Hawaii School of Medicine, Honolulu, HA 96822

A *Neurospora crassa* clone identified as homologous to the *S. cerevisiae* gene encoding the beta subunit of fatty acid synthase was used to RIP the Neurospora gene. Five ascospore-derived lines showed a strict requirement for fatty acid (palmitate) supplementation. Test crosses showed linkage to the cot-1 marker. Phenotypically, these mutants resemble the cel mutant of Neurospora. The cel mutant has a defective alpha subunit. Heterokaryon tests of the mutants showed expected complementation with cel. The new mutants have therefore been designated cel-2. Both cel and cel-2 mutants require supplementation with palmitate for normal growth. Fatty acid synthase activity in cel was 1.2% of wild-type, and it was lower in cel-2. Differences were observed between cel-type mutants and wild type in fatty acid and lipid metabolism. For example, cel and cel-2 utilize supplemental palmitate more efficiently than wild type for the synthesis of unsaturated fatty acids. The cel mutant has been used extensively in studies of membrane properties and physiological effects of particular fatty acids, because its membranes

can be heavily enriched in supplemental fatty acids. Because the cel-2 mutants synthesize even less fatty acid de novo than does cel, cel-2 should be even more useful for such studies.

18. Isolation of a new *Neurospora crassa* mutant defective in methylation, *dim-4*. Ann Hagemann, Michael Freitag and Eric U. Selker, University of Oregon, Eugene, OR

Repeat-induced point mutation (RIP) is a homology-based genome-wide search mechanism that detects gene-size sequence duplications during premeiosis and results in G:C to A:T point mutations of duplicated sequences. We conducted a screen for mutants that have reduced levels of RIP. A duplication of the pan-2 gene served as the substrate for RIP; inactivation of pan-2 confers an ascospore color phenotype which we utilized in a visual screen of the progenies of 106 separate crosses. Six putative mutants satisfied additional criteria for mutations affecting RIP: 1. passage through a cross with segregation of the defect among the progeny; 2. absence of a detectable rearrangement of the pan-2 duplication; and 3. inability to support normal RIP of an unmutated duplication. The putative RIP mutants were also tested by Southern analysis for levels of DNA methylation. One strain showed an approximately threefold reduction in methylation levels of numerous assay sites. Allelism tests against the three known DNA methylation mutants (dim-1, dim-2 and dim-3) showed the new mutant, now called dim-4, to be distinct. We have initiated a chromosome "walk" to isolate the dim-4 gene and are in the process of characterizing the RIP defect(s).

19. Altered cAMP levels, adenylyl cyclase and cAMP phosphodiesterase activities associated with mutation of G protein α subunits in Neurospora crassa. F. Douglas Ivey*, Qi Yang, and Katherine A. Borkovich, University of Texas Medical School, Houston, TX

Heterotrimeric GTP-binding proteins are key components of signal transduction in eukaryotic organisms. Previously, we reported that the filamentous fungus Neurospora crassa possesses two G protein α subunits, GNA-1 and GNA-2. Loss of *gna-1* leads to multiple phenotypes, while Δ gna-2 strains do not exhibit visible defects. However, Δ gna-1; Δ gna-2 mutants are more affected in all Δ gna-1 phenotypes. This report presents results of a biochemical investigation of the roles of GNA-l and GNA-2 in cAMP metabolism. Comparison of Mn^{2+}ATP-dependent adenylyl cyclase activities indicated that the levels of enzyme were normal in Δ gna-2 mutants, but reduced 40% in Δ gna-1 and Δ gna-1; Δ gna-2 mutants. Assays of Mg^{2+}ATP-dependent adenylyl cyclase activity (± GppNHp) indicated that Δ gna-2 strains were normal, whereas Δ gna-1 and Δ gna-1; Δ gna-2 strains had only 10-15% the activity of the wild-type control. Mg^{2+}ATP-adenylyl cyclase activity in wild-type cell extracts could be inhibited using anti-GNA-l IgG, suggesting a direct interaction between GNA-1 and adenylyl cyclase in *N. crassa*. The intracellular levels of cAMP in Δ gna-1 and Δ gna-1; Δ gna-2 mutants were reduced relative to wild-type under conditions that result in morphological abnormalities. cAMP phosphodiesterase activities in Δ gna-1 and/or Δ gna-2 strains were lower than in wild-type controls; the individual deletions were additive in decreasing phosphodiesterase activity in the Δ gna-1; Δ gna-2 double mutant. Thus, G protein α subunits differentially influence adenylyl cyclase and cAMP phosphodiesterase activity in *N. crassa*.

20. Isolation and identification of Neurospora RAD1 and RAD1 homologs: evidence for two excision repair systems in *Neurospora crassa*. Hatakeyama, S., Y. Ito, C. Ishii, and H. Inoue, Saitama University, JAPAN

We cloned two genes homologous to yeast RAD1 and RAD2 genes from *N. crassa*, using degenerate PCR method. These genes were mapped to the left arm of Linkage group I. Several lines of evidence showed RAD1nc gene is mus-38. RAD2nc gene is a newly identified gene and named mus-40. To isolate mus-40 mutant, we used RIP and the obtained strain was characterized. Genetic analysis of mus-38 and mus-40 mutations showed that these belonged to same DNA repair group, but did not to other repair groups such as mus-18 (UV-damaged excision repair), mei-3 (recombinational repair) and uvs-2 (postreplication repair). Assays using antibodies against UV damages showed that double mutants mus-18 mus-38 and mus-18 mus-40 were completely defective in release of tymine dimer and TC(6-4) photoproduct from UV-irradiated DNA. These data indicate that there are two UV damage excision repair systems in *N. crassa*.

21. Suppressor mutants of an adenylyl cyclase mutant. Akihito Kana-uchi, National Institute of Bioscience and Human-Technology, Tsukuba city and Tadako Murayama, College of Engineering, Kanto-Gakuin University. Yokohama, Japan

The wild type grew to be filamentous, whereas an adenylyl cyclase mutant, cr-1, grew to be colonial on the solid medium. However, the wild type also grew to be colonial on the solid medium containing sorbose. The cr-1 strain grew to be much smaller colony than the wild type on this medium. High number of large colonies appeared in single colonies from 2-weeks old conidia of cr-l mutant. The strains from these large colonies grew to be filamentous on the medium without sorbose, though they still showed some form of morphological abnormality. Genetic analysis showed that all 5 strains from the large colonies had original cr-l mutation and suppressor mutations. The suppressor mutants had the mutations in the different genes and different types of morphological abnormality. Some of the suppressor single mutants contained the increased amount of cyclic AMP (cAMP). These mutants did not or scarcely formed conidia and had elevated activity of trehalase. One of the suppressor mutants did not produce conidia and trehalase. Some of them showed abnormality in carotenoid formation. They may be related to the signal transduction pathway mediated by cAMP. The role of these suppressor genes and the regulation of trehalase, carotenoid production, conidial formation through the cAMP cascade will be discussed.

22. Characterization of a *Neurospora crassa* serine carboxypeptidase gene homologous to the *S. cerevisiae* KEX1. Elie Kato and W. Dorsey, Stuart University of Hawaii

With the aid of the Neurospora Genome Project, we have cloned and characterized the *Neurospora crassa* kex-1 gene. A Blast search of the NGP EST database using the active sites of the A. saitoi pepF gene identified a cDNA with homology to a serine carboxypeptidase. Sequence analysis revealed greatest homology to the KEX1 gene of *S. cerevisae*. Inactivation by RIP resulted in progeny deficient in pheromone activity, reduced fertility as a male parent but completely fertile when used as the female parent.

23. A G protein α subunit, gna-3, regulates cAMP metabolism and aerial hyphae formation in *Neurospora crassa*. Ann M. Kays, Patricia S. Rowley, Rudeina Baasiri, and Katherine A. Borkovich, University of Texas Medical School - Houston

In eukaryotic systems, heterotrimeric αβγ guanine nucleotide proteins are used to convey extracellular signals. Signals such as light, pheromone, and neurotransmitters activate G protein coupled signal transduction pathways. Cellular responses are generated by the conversion of the initial signal to production of intracellular secondary messengers. Our lab previously identified and characterized two G protein α subunits, *gna-1* and *gna-2* . A third novel G protein α subunit, *gna-3*, has been cloned and found to be 70% and 67% similar to *gna-1* and *gna-2* , respectively, in *Neurospora crassa* . A deletion of *gna-3* was marked by resistance to the bacterial hygromycin B phosphotransferase *(gna-3::hph)*. Western blot analysis indicated that GNA-1 and GNA-2 were present at wild-type levels in *Δgna-3* strains. On solid medium, Δgna-3 strains exhibited denser conidiation and stunted aerial hyphae growth, but were not significantly affected in their apical extension rate. The level of cAMP in Δgna-3 mutants was only 20% of wild-type on solid medium. The reduced cAMP levels suggest a role for GNA-3 in the metabolism of cAMP in Neurospora crassa

24. Uptake, efflux and distribution of calcium and magnesium in the vacuole of *Neurospora crassa*. Kelly A. Keenan, T. Kirn, T. Wisniewski and T. Zielinski, Richard Stockton College of New Jersey

Using the cupric ion permeabilization system, vacuolar distribution of calcium and magnesium was characterized. It was shown that 43.6% of calcium and 79.5% of magnesium are associated with the vacuole; the result was confirmed by a cellular fractionation experiment. Distribution did not change with altered nutritional conditions including starvation for nitrogen or phosphorous or addition of calcium and magnesium. Specificity of uptake and kinetics were characterized. Uptake was consistent with Michaelis Menten kinetics and Michaelis constants were 1.7 mM and 1.3 mM respectively for calcium and magnesium. Uptake of calcium was inhibited by magnesium and the converse was true; the metals acted as non-competitive inhibitors. Efflux was characterized. Calcium showed a low basal level of efflux that was increased by the addition of inositol triphosphate. Magnesium showed a basal level of efflux nearly five times that of calcium and the efflux was decreased by the addition of other metals. The results suggest the possibility of an electrical gradient involved in magnesium efflux. The effect of phosphate pool size on uptake and efflux was also characterized.

25. Biochemical and genetic charawcterization of vacuolar basic amino acid permease and vATPase mutants in *Neurospora crassa*. Kelly A. Keenan, Marc Mancinelli, Thomas K. Zielinski and Brian Swanke, Richard Stockton College of New Jersey

N. crassa stores large amounts of the basic amino acids in the vacuole. Arginine, lysine and ornithine are transported into the vacuole via three distinct permeases. Strains that showed a basic amino acid-sensitive phenotype were isolated by mutation of wild type followed by filtration enrichment procedure. The ability of the strains to accumulate basic amino acids was characterized. Some strains failed to accumulate only one amino acid while others failed to accumulate more than one. These strains were tested for amino acid uptake using the cupric ion

permeabilization system to measure vacuolar uptake. Of 20 mutants tested to date, nine showed the same behavior: a decreased uptake of all three amino acids. Since such a phenotype might be expected of a vacuolar ATPase mutant, the mutants are currently being tested for vATPase activity. To date, six mutants have been tested and two show a significantly decreased vATPase activity (RSC-41 and RSC-44). Two mutants failed to accumulate and transport ornithine only. vATPase activity was normal and both mutants appear to be defective in an ornithine permease (RSC-39 and RSC-63). Other mutants had decreased uptake of two amino acids and are being characterized. Complementation group analysis has been done on the strains.

26. Analysis of Tad elements that are essential for transposition. John A. Kinsey and Yi Zhou, University of Kansas Medical Center, Kansas City, KS

Tad is a LINE-like retrotransposon from *Neurospora crassa*. Using a combination of in-vitro mutagenesis and targeted transformation we have determined elements that are essential for Tad transcription and/or transposition. Both ORF1 and ORF2 are required for transposition but not transcription. ORF 3 which is a minus strand ORF from the atypical 3' tail is not essential for either process. Either ORF4, another 3'tail ORF, or the sequences that encode it are essential for Tad transposition. We have also found that Tad contains two elements in the 5' end that are essential. The promoter is located more than 150 bp internal to the element and is essential for both transcription and transposition. In addition we have found a new element that is approximately 24 bp internal that is essential for transposition, but not for transcription.

27. Methyl-DNA binding proteins in Neurospora. Gregory O. Kothe and Eric Selker, Institute of Molecular Biology, University of Oregon, Eugene, OR, 97403

Numerous studies directed at understanding DNA methylation in eukaryotic organisms have addressed how methylation patterns are established and propagated. One strategy for determining the role of methylation in controlling cellular phenomena in Neurospora is to isolate factors that specifically associate with methylated DNA, and then to determine their physiological function. Using gel-mobility-shift assays we have identified two factors in Neurospora that bind methylated DNA sequences. A low-mobility factor was identified that binds methylated sequences preferentially, but also binds RIPed, unmethylated sequences, although not as strongly. This factor was found to bind RIPed, methylated sequences most strongly, and not to bind to unRIPed, unmethylated sequences at all. We refer to this factor as M/R-BP1 (Methyl/RIP Binding Protein 1). A higher-mobility factor was also identified that is specific for methylated DNA. This factor is referred to as M-BP1 (Methyl Binding Protein 1). M/R-BP1 and M-BP1 may be involved in establishing and/or maintaining methylation patterns in Neurospora. It is also possible that these proteins function "downstream", exerting their effects after methylation has been set up (ex. regulating gene expression). Our goal is to test these possibilities by purifying M/R-BP1 and M-BP1 to homogeneity, cloning the genes that encode them, and then generating and characterizing mutants affected in these genes.

28. Reverse genetic analysis of signal transduction loci of *N. crassa*. Peter Margolis and Charles Yanofsky, Stanford University, Stanford, CA

We have been employing a reverse genetic approach to identify genes capable of specifying proteins known, in other eukaryotes, to participate in signal transduction pathways. Using degenerate PCR primers, we amplified fragments corresponding to eleven loci encoding putative signal transduction proteins of *N. crassa*. We assigned the corresponding genes to specific Orbach/Sachs library cosmids or subclones thereof. These plasmids are appropriate for mutagenesis of these genes by either RIP or insertional mutagenesis (e.g., Tn5-hyg mutagenesis has been initiated for three of the genes). The clones and corresponding sequence information will be available through the FGSC.

The genes that we have isolated could encode the following proteins: 5 potential members of mitogen-activated protein kinase (MAPK) cascades; 2 cell-division kinases (CDK, one of which may be pgov); a homolog of yeast Ste20, a p21 activated kinase (PAK); a homolog of yeast Mcm1, a transcription factor known to control mating type genes in other fungi; a highly conserved (from bacteria through vertebrates) GTP-binding protein; and a glycogen synthase kinase (GSK). Thus far, only the GSK gene has been isolated as a cDNA in the Neurospora Genome Project. Our ability to isolate and characterize new potentially important loci implies that reverse genetic analysis could form a valuable adjunct to existing molecular genetic dissection of the Neurospora genome.

29. A Comparison of the efficiency of RIPing of three similar proteins, two amino acid permeases and a calcium transporter. Emilio Margolles-Clark, Stephen Abreu, Ian Hunt, and Barry J. Bowman, University of California, Santa Cruz, CA 95064

We have characterized three genes which encode transport proteins. The gap-1 gene appears to encode a general amino acid permease; aap-2 encodes a protein that may be homologous to GABA transporters; and cax-1 encodes a calcium transporter. The chromosomal location of each has been determined. To generate mutations in these genes we constructed plasmids in which 2-4 kb of DNA was inserted into the pBM61 vector and then targeted to the his-3 locus. Homokaryotic transformants were identified and crossed to marked strains. Analysis of the progeny showed that the cax-1 gene was RIPed at a relatively high rate (30%). No RIPped progeny have yet been identified in gap-1 (40 analyzed) or in aap-2 (20 analyzed). It is unlikely that gap-1 and aap-2 are essential genes, but they appear to be difficult to RIP.

30. The vacuolar ATPase: Identifying the parts of a complex machine. Emilio Margolles-Clark, Karen Tenney, Ian Hunt, June Pounder, and Barry J. Bowman, University of California, Santa Cruz

The vacuolar ATPase is a proton pump which generates an electrochemical gradient across the vacuolar membrane. The enzyme is composed of at least 13 different types of subunits and probably functions as a rotary motor. We have previously identified the genes that encode six subunits. With the assistance of the Neurospora Genome Project at the U. of New Mexico we have now characterized the genes for four additional subunits, vma-7, 8, 10,11. The possible roles of these subunits in the function of the ATPase will be described.

31. ro-4(E8): an allele of Neurospora ARP1 that affects dynein and dynactin localization. Peter Minke1 and Mike Plamann2, 1Texas A&M University, Department of Biology, College

Station, TX 77843, 2University of Missouri-Kansas City, School of Biological Sciences, 5100 Rockhill Road, Kansas City, MO 64110-2499

We continue to use the filamentous fungus *Neurospora crassa* as a model organism in which to genetically analyze the intracellular motor cytoplasmic dynein and the associated dynactin. Dynactin is thought to mediate interaction of dynein with membranous cargo. Previously, ro-3 and ro-4 were shown to encode p150Glued and actin related protein 1 (ARP1) which represent the largest subunit and the most abundant subunit of dynactin, respectively. Western analysis reveals that RO3 is absent in a deletion strain of ro-4 and suggests that an incomplete dynactin complex is not permitted within the cell. Alleles of ro-4 which retain RO3 may identify specific mutations which allow formation of a dynactin complex but affect dynactin function. Examination of 13 independent ro-4 alleles reveals one allele, ro-4(E8), which retains the presence of RO3. A ro-4(E8) colony is less dense and radial extension is slower than the deletion strain. Sequencing reveals a single base-pair change resulting in an amino acid substitution. The Y to S change occurs in a region that is highly conserved with conventional actin. In conventional actin, this region is responsible for myosin and gelsolin interaction. Immunolocalization of RO1 (cytoplasmic dynein heavy chain) and RO3 in ro-4(E8) reveals an accumulation of both complexes in heavy streaks at the hyphal tip.

32. Extracellular enzymes in a ras mutant of Neurospora. Tadako Murayama[1], Yuko Mochizuki[1], and Lena Suzuki[2], Kanto-Gakuin Univ. Yokohama, Japan[1], and Vanderbilt Univ., Nashville, TN[2]

The smco7 mutant grew to be semicolonial on solid medium in a petri dish. Transformation of the smco7 mutant with the plasmid carrying NC-ras2, a ras gene homologue of Neurospora, resulted in the recovery of the wild type morphology and a deletion of one nucleotide was detected in the region corresponding to the N-terminal region of the putative ras protein homologue encoded by NC-ras2 of the smco7 mutant.

Colonial morphology depends on hyphal growth, which is limited to the apex of the hypha. Many apical vesicles which contain materials necessary for cell wall and cell membrane synthesis are clustered at the apical region of hyphae and fuse with the hyphal tip. Vesicles appear to originate from Golgi cisternae. The apical cells of the smco7 mutant were shorter and thiner than those of the wild type. The results that the apex of the hyphae of the smco7 mutant was fragile suggested that the smco7 mutation causes defects in cell wall synthesis at the apex.

The extracellular enzymes may be transported to the apex and secreted there through the common mechanism in the transportation and exocytosis of the cell membrane and cell wall precursors. The secreted invertase and trehalase activities were much lower in smco7 than in the wild type. The activities of mycelial invertase and trehalase which may be located in the vacuole or periplasm was also lower in smco7 than in the wild type. The NC-ras2 protein was thought to play a role similar to Rsr1 protein of Saccharomyces cerevisiae.

33. Incomplete chromosome pairing is correlated with a recombination block on the mating-type chromosome of Neurospora tetrasperma. N. B. Raju and D. J. Jacobson, Stanford University, Stanford, CA

In N. tetrasperma, each ascospore encloses nuclei of opposite mating type. The dual-mating-type ascospores are the direct result of segregation of mating types at the first division of meiosis and overlapping spindles at the second and third divisions (Dev. Genet. 15: 104). Earlier genetic studies clearly show that there is little or no recombination between various markers on the mating-type chromosome (linkage group I), whereas markers on the remaining six chromosomes recombine as expected (Genetics 54: 293; Genetics 143: 789). We have examined meiotic chromosome pairing in various laboratory and wild-collected N. tetrasperma strains to see whether the recombination block is correlated with any cytologically detectable chromosome pairing anomalies. Three- to four-day-old perithecia were stained with the DNA-specific fluorochrome acriflavin and the young, developing asci were observed under a fluorescence microscope (Mycologia 78: 901). All but the longest chromosome showed intimate homologous pairing along the entire length. In contrast, the longest chromosome, which bears the mating type locus, usually showed a long unpaired segment in the middle, with normal pairing at both ends. In the sibling species N. crassa, all seven chromosomes, including the mating-type chromosome, show complete homologous pairing. We have not yet established a cause and effect relationship between the recombination block and incomplete pairing on the longest chromosome in N. tetrasperma. However, our observations are consistent with the notion that the recombination block between mating type and centromere (possibly due to impaired pairing) is a significant correlate of normal production of self-fertile ascospores in this pseudohomothallic species.

34. A quick silver-staining method for conidial nuclei, and its usefulness for studying the behavior of the nucleolus organizer in Neurospora. N. B. Raju, Stanford University

The silver-staining procedure described by Lu (Chromosoma 102: 464) for surface-spreading synaptonemal complexes of Neurospora is also suitable for staining expelled paraphysal nuclei. Each nucleus shows a darkly-stained prominent nucleolus. I have adapted the staining method for routine observation of conidial nuclei. Conidia were fixed in a few drops of 4% paraformaldehyde for 3 or 4 min at 4^{o} C. A drop of conidial suspension was then placed on a glass slide and squashed under a cover glass with blows from a rubber hammer to break open the conidia.The cover glass was lifted off after freezing the slide on a Cold Plate and the nuclei were flooded in a few drops of 4% paraformaldehyde + 0.04% SDS for 15 to 20 min. The slide was rinsed in distilled water, dipped in 0.2% Kodak Photoflo, and air-dried. For silver staining, one drop of 50% silver nitrate and one drop of gel developer (2% gelatin+1% formic acid) were mixed together and added on to the slide and covered with a cover glass. The slide was heated on a hot plate at 45^{o} C for 2-3 min during which the nuclei were stained brownish to dark brown. The cover glass was promptly floated off and the slide was rinsed in distilled water and mounted under a new cover glass for microscopic examination. I find the silver staining particularly useful for examining nucleolus behavior in the segmental duplication strain Dp(AR33) that contains two normal nucleolus organizers (Chromosoma 76: 255), and in the translocation strain T(OY321) that contains a split nucleolus organizer (Chromosoma 89: 8). In both strains, up to 10% of nuclei show two nucleoli (slightly smaller than normal) while the remaining nuclei contain a single larger nucleolus, interpreted as resulting from fusion. The extracted conidial nuclei may also be used for in situ hybridization studies.

35. Characterization of the un-25 locus of *Neurospora crassa*. Thomas J. Schmidhauser and Dan Chen, The University of Southwestern Louisiana

The unknown-25 (un-25) mutant of *Neurospora crassa* is a heat-sensitive conditional mutant. Strain un(T51M54) was previously mapped to Linkage Group VI (LGVI), but unassigned to locus. We used complementation analysis to isolate a cosmid complementing the mutation and demonstrated that the mutation was not an allele of one of the three un genes mapped to LGVI;un-4, un-13 or un-23. Genetic mapping places un-25 between AP31a.7/R15.7 and Bml on the left arm of LGVI. Un-25 was subcloned to a 4.8 kbp fragment. Sequence analysis and analysis of expression of un-25 is presented.

36. Expression and regulation of the fluffy gene of *Neurospora crassa.* Lori A. Shrode and Daniel J. Ebbole, Texas A&M University, College Station

The fluffy gene of *N. crassa* encodes a C6 Zinc cluster transcription factor that is required for macroconidiophore morphogenesis. FL is most similar to NIT4 of *N. crassa* and NirA of A. nidulans, transcriptional activators of genes for nitrate utilization. Nitrogen starvation or exposure to air induces macroconidiation and these treatments cause elevated expression of fl. We examined the expression of fl and several conidiation-specific genes during nitrogen and air induced conidiation in wild type and several conidiation mutants. Epistatic relationships between fld, acon-2, acon-3 and fl were examined.

37. Molecular cloning of the genes for the morphological mutants frost and spray of *Neurospora crassa*. Teruo SONE*,***, Jin-Woo Bok*, Frederick J. Bowring**, David E. A. Catheside** and Anthony J. F. Griffiths* *Department of Botany, University of British Columbia, Canada. **School of Biological Sciences, Flinders University, Australia. ***Present address: Faculty of Agriculture, Hokkaido University, Japan

Ca++ is thought to be an important factor for controlling tip growth and branching in fungi but its role at the molecular level is still unknown. Products of fr and sp genes of *N. crassa* are considered to be related to Ca++-dependent growth because phenotypes of these mutants are corrected at high [Ca++]1). We attempted to clone and analyze them to use as molecular probes for figuring out the mechanism of Ca++-dependent tip growth. A sib selection was performed to clone the fr gene using pMOcosX genomic library. Although fr is considered to be semi-aconidial, we found that the mutant produces enough numbers of arthroconidia for the transformation, on complete media supplemented with 0.5M CaCl2, at 37C. A cosmid clone, named X18:G8, was found to contain fr gene. We subcloned 3.0 kb KpnI-XhoI fragment which contains fr gene and its DNA sequence was determined. Deduced amino acid sequence from the DNA showed some homology to S. cerevisiae CDC1 protein. The sp gene has been found in a cosmid clone X11:G10 by chromosomal walking from am locus and complementation of the mutant. Results of sequence analysis will be presented.
1)Dicker and Turian, JGM, 136, 1413-1420 (1990).

38. Senescence associated with the over-replication of a mitochondrial retroplasmid is suppressed by nuclear mutations. Charles B. Stevenson, A. Nicole Fox and John C. Kennell, Dept. of Biological Sciences, Southern Methodist University, Dallas, TX 75275

The Mauriceville and Varkud retroplasmids are autonomously replicating circular plasmids that reside in mitochondria of certain isolates of Neurospora spp. Variant forms of the plasmids

containing DNA copies of mitochondrial RNAs can be recovered from growth-impaired and senescent cultures. Senescence is generally associated with deletions or rearrangements of the mitochondrial genome caused by the integration of the variant plasmids. Here, we describe the characterization of a novel variant of the Mauriceville retroplasmid which impairs growth without integrating into mtDNA and leads to highly predictable frequencies of senescence. Vegetative growth decline correlates with an increase in the level of the variant plasmid and a concomitant decrease in cytochromes b and aa_3, suggesting that mitochondrial gene expression is inhibited by plasmid over-replication. We also report the isolation of a mutant strain that escapes senescence. This long-lived derivative strain shows vigorous and indefinite growth yet still tolerates high levels of the variant plasmid. New forms of variant plasmids arise and replace existing plasmids and inheritance studies show that the long-lived trait is controlled by recessive nuclear genes. The characterization of these genes could reveal mechanisms involved in the suppression of retroplasmid over-replication or pathways that respond to mitochondrial dysfunction.

39. Characterization of DNA de novo methylation signals in vegetative cells of *Neurospora crassa*. Hisashi Tamaru, Michael Freitag, Vivian Miao and Eric Selker, University of Oregon, Eugene, OR 97403.

DNA is modified by conversion of cytosines to 5-methyl-cytosines in many eukaryotes including Neurospora. Our goal is to understand how certain cytosines are targeted for methylation. Most DNA sequences that are subject to de novo methylation in *N. crassa* have undergone RIP (repeat-induced point mutation) and are therefore relatively rich in A and T nucleotides and are enriched for TpA dinucleotides. We have carried out a detailed dissection of a relic of RIP, the z-h region, to elucidate which mutations induced by RIP and/or which general features of mutated DNA create methylation signals. We report results from tests of hybrid constructs involving segments of h and its unmutated homologue, q. We also report creation of methylation signals by in vitro mutagenesis. For part of our study, we developed an assay to test the capacity of short random sequences to trigger methylation. We constructed a his-3 -targeting vector that carries a 100 bp z-h fragment surrounded by a lightly mutated allele of the am gene (am^{RIP4}). The mosaic am^{RIP4}:: z-h construct does not establish methylation by itself at the his-3 locus but provides a sensitive background to test various small (e.g. 25 bp) segments for their capacity to trigger methylation in conjugation with the am^{RIP4}:: z-h sequences. Our results show that increases in A+T content and TpA density of DNA contribute differentially, but additively to create signals for de novo methylation.

40. Proteins important for vacuolar function: Two possible vacuolar proteases and the calcium transporter. Karen Tenney, Emilio Margolles-Clark, Ian Hunt, and Barry J. Bowman, University of California, Santa Cruz

We have identified a gene, named vac-5, which encodes one of the most abundant proteins in the *N. crassa* vacuole. Analysis of the sequence of this 20 kDa protein suggests that it may be a metaloprotease. A second gene, cpy-1, has been found which encodes a ~45 kDa vacuolar protein. The gene product is similar to a carboxypeptidase described in human lysosomes. A third gene, cax-1, appears to encode a 45 kDa protein which transports calcium across the vacuolar membrane. Mutations in the cax-1 gene have been produced by RIPing and the

phenotype of these mutant strains will be described. The assistance of the Neurospora Genome Project at the University of New Mexico is gratefully acknowledged.

41. Regulation and properties of the major forms of arginase in *Neurospora crassa*. Gloria Turner, Christine Palmier and Richard L. Weiss, University of California, Los Angeles

Multiple arginases (L-arginine amidinohydrolase, EC 3.5.3.1), encoded by the complex aga locus, are found in *N. crassa*. Two differentially transcribed mRNAs, 1.4 kb and 1.7 kb, produce 36-kDa and 41-kDa proteins, respectively. The larger transcript and protein are made only in the presence of exogenous arginine. In order to understand the function of these two proteins, strains were constructed that make only one form: Tm1 makes the 36-kDa form and Tm3 makes the 41-kDa protein. Interestingly, the ability to utilize arginine as a sole nitrogen source is impaired in these strains. Nomarski microscopy revealed a hyphal branching defect for the two mutant strains. Tm1 appears to have normal apical extension, but branching aborts after a short extension of the branch. Tm3, which makes only the 41-kDa protein, has difficulty extending hyphae and has a more pronounced branching defect – only branch buds are seen in this strain. Enzyme inhibition and kinetic studies have been performed on crude extracts from these strains. It appears that the two forms share some features, their affininty for arginine and the mode of inhibition by ornithine. However, the two arginases differ in the concentration of ornithine required for 50% inhibition and in the amount of manganese needed for 50% activity. These vegetative growth defects and catalytic properties support a model where the two forms of arginase work together for maximal activity.

42. The *Neurospora crassa* arg-2 arginine attenuator peptide regulates the movement of ribosomes that have translated it. Zhong Wang, Peng Fang and Matthew S. Sachs, Oregon Graduate Institute, Portland OR

Translation of the upstream open reading frame (uORF) in the 5′ leader segment of the *Neurospora crassa* arg-2 mRNA causes reduced initiation at a downstream start codon when arginine is plentiful. Previous examination of this regulatory mechanism using a primer-extension inhibition (toeprint) assay in a homologous *N. crassa* cell-free translation system showed that arginine causes ribosomes to stall at the uORF termination codon. This stalling apparently regulates translation by preventing a scanning ribosome from reaching the downstream start codon. Here we provide evidence that neither the distance between the uORF stop codon and the downstream initiation codon, nor the nature of the stop codon used to terminate uORF translation, are important for regulation. Furthermore, the arginine attenuator peptide (AAP) coding region regulates synthesis of the firefly luciferase polypeptide when it is fused directly at the N-terminus of that polypeptide. In this case, the elongating ribosome stalls in response to Arg soon after it translates the AAP coding region. Regulation by this eukaryotic leader peptide thus appears to be exerted through a novel mechanism of cis-acting translational control.

43. Isolation and characterization of *Neurospora crassa* genes encoding homologs of the bacterial MutS and MutL mismatch repair proteins. K. Yamamoto, *D. H. Huber, *H. Bertrand, C. Ishii and H. Inoue, Saitama University and *Michigan State University

Using PCR with degenerate primers, we cloned two *N. crassa* genes (mlh-1, msh2), and a fragment of a third gene (msh-X), all encoding homologs of the bacterial MutS and MutL proteins. We obtained the msh-2 homolog and msh-X fragment with the same pair of primers in a single experiment. A deduced MLH1 polypeptide is 751 a.a. long and shared 34.0% and 45.1% identity with Escherichia coli MutL and Saccharomyces cerevisiae Mlh1p proteins, respectively. The MSH2 polypeptide is 937 a.a. long and shows identity of 28.4% and 46.8% with E. coli MutS and S. cerevisiae Msh2p, respectively. A deduced partial amino-acid sequence of MSHX showed almost the same levels of similarity to S. cerevisiae Msh1, Msh6 and mouse Rep-3 (Msh-3). Mutants of mlh-1 and msh-2 were made by repeat-induced point mutations and characterized. These mutants were not sensitive to UV, MMS, NG or cis-Platinum. The msh-2 mutant was fertile in a homozygous cross. As expected, the spontaneous mutation frequency in the msh-2 mutant was higher than in wild type, and similar experiments with mlh-1 mutants are in progress. We are also constructing strains for the characterization of the RIP phenomenon in these mutants. RFLP mapping showed that mlh-1, msh-2 and msh-X are located in LGIV, LGVII and LGI, respectively.

44. Phenotypic consequences and subcellular distribution of an activated Gα i protein in *Neurospora crassa*. Qi Yang, F. Douglas Ivey, Katherine A. Borkovich, University of texas Medical School-Houston

Heterotrimeric G proteins, consisting of α , β and γ subunits, transduce environmental signals through coupling to plasma membrane-localized receptors. Activation of G proteins occurs through stimulation of GDP-GTP exchange on the α protein and dissociation of the trimer into α and β γ units. Hydrolysis of GTP to GDP by the α subunit leads to inactivation of signaling and reassociation of the α with β γ; hence, mutations which inhibit the GTPase activity of Gα proteins lead to constitutive signaling in several systems. We previously reported that the filamentous fungus *Neurospora crassa* possesses a Gα protein, GNA-1, that is a member of the Gα i family. Deletion of *gna-1* leads to defects in apical growth rate, differentiation of asexual spores, female fertility and sensitivity to hyperosmotic media. In this study we examine properties of *N. crassa* strains with mutationally-activated *gna-1* alleles (R178C and Q204L) as the only source of GNA-1 protein. GNA-1 protein from wild-type, *gna-1*R178C and *gna-1*Q204L strains was particulate in the presence or absence of GTP analogue. *gna-1*R178C and *gna-1*Q204L strains exhibit normal apical extension growth rates on normal and hyperosmotic media; however, teh mutants produce abundant, long aerial hyphae in comparison to wild-type controls. *gna-1*R178C and *gna-1*Q204L strains are female fertile, but produce fewer, larger perithecia than wild-type strains. These results support a positive role for GNA-1 in signal transduction in *N. crassa*, independent of β γ dimers.

45. Recombination events at the *cog* recombinator. P. Jane Yeadon and David E. A. Catcheside, Flinders University, Adelaide, Australia

Multiple polymorphisms distinguish Emerson *a* and Lindegren *25a* strains of *Neurospora crassa* within the *histidine-3* gene and in its distal flank. Restriction site and sequence length polymorphisms in an overlapping set of PCR products covering this region have been used to identify the parental origin of DNA in histidine-prototrophic recombinant progeny of crosses between the strains. 41% of conversion tracts are interrupted. In progeny from *rec-2*$^{+}$ crosses, where the recombination hotspot *cog* is inactive, conversion tracts are short, most are not initiated at *cog* and either chromosome seems equally likely to be converted. Where the absence of *rec-2*$^{+}$ permits activity of *cog*, conversion appears to originate at *cog* and conversion tracts are up to 5.9 kb long. The chromosome bearing *cog*^{La}, the dominant allele which confers a high frequency of recombination, is almost invariably the recipient of information. 29% of conversion events have a crossover sufficiently close to the conversion tract for association to be likely.

We are currently investigating *his*$^{+}$ progeny from a translocation heterozygote (*his-3* TM429/ K874) and tetrads from crosses heteroallellic *his-3* and *his-3/his*$^{+}$.