Sidelights
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Are any fungal genes nucleus-limited?

1. Introduction

In long and illustrious research careers that ended only with their passing in 2007, David Perkins, Dorothy (Dot) Newmeyer Perkins and Robert (Bob) L Metzenberg made many important contributions in Neurospora genetics. Two 'Perspectives' articles in *Genetics* (Davis 2007; Selker 2008) and a 'Commentary' in this journal (Kasbekar 2007) have highlighted some of these contributions. Recently, I was privileged to read three letters exchanged by Bob, David and Dot in December 1971 (see *http://www.ias.ac.in/jbiosci/jun2014/supp/Kasbekar.pdf*). Bob Metzenberg described the apparent 'nucleus-limited' phenotype of the *Neurospora crassa scon-1*^{C} (*sulfur controller-1 constitutve*) mutant. He also wondered whether two other mutations – *pcon*^{C} (*phosphatase controller constitutive*), that caused dominant or co-dominant derepression of alkaline phosphatase, and *tol* (*tolerant*), were nucleus-limited. Subsequent research showed that *pcon*^{C} was, in fact, not nucleus-limited (Metzenberg *et al*. 1974). The question of whether *tol* has a nucleus-limited effect was not fully addressed (see below), and no further work was published on *scon-1*^{C} after the initial paper describing its nucleus-limited phenotype (Burton and Metzenberg 1972). Two other papers alluding to nucleus-limited effects have appeared since then, one in *Saccharomyces cerevisiae* (Demeter *et al*. 2000) and the other in *Aspergillus nidulans* (Czaja *et al*. 2013). Given that genotypes with putative nucleus-limited effects were described in fungi as diverse as Aspergillus, Neurospora and Saccharomyces, it was conceivable that a subset of fungal genes also might be nucleus-limited. A nucleus-limited gene may be envisaged as one in which nuclei bearing a wild-type allele (WT) fail to complement nuclei containing a null allele (Δ) in [(WT) + (Δ)] heterokaryons. As far as I know, such genes have not yet been reported. In this article I will first summarize the three sightings cited above, and then go on to suggest that it might be possible to establish whether such genes exist by introgressing *N. crassa* translocations into *N. tetrasperma*.

Animals and plants for the most part comprise uninucleate cells, whereas filamentous fungi have multinucleate hyphae. Hyphae are tubes of cytoplasm bounded by a plasma membrane and a cell wall, and they grow by extending at the tips. Lateral branching of hyphae can occur by formation of new tips at some distance proximal to pre-existing tips. The cytoplasm is continuous within the branched hyphae and contains the nuclei, mitochondria, endoplasmic reticulum, golgi, cytoskeleton, ribosomes and the other components of eukaryotic cells. Hyphae can cross-connect (anastomose) to form a web-like mycelium. Anastomosis with 'self' hyphae maintains the homokaryotic nature of the mycelium. That is, all the nuclei are mitotic derivatives of a single post-meiotic nucleus. But fusion of 'non-self' hyphae generates heterokaryotic mycelia whose nuclei are mitotic derivatives of more than one post-meiotic nucleus. Heterokaryons can be produced in the laboratory by selection for complementation between auxotrophic mutations on un-supplemented medium. The component nuclei of the heterokaryon must be genetically compatible for long-term hyphal viability (Smith and Lafontaine 2013).

2. Nucleus-limited genotypes

Neurospora. The *scon*^{c} mutation caused deregulation of arylsuphatase and other enzymes of sulphur metabolism (Burton and Metzenberg 1972). In the wild type, 'favoured' sulphur sources (e.g. methionine, potassium sulphate) repressed these enzymes, whereas 'un-favoured' sources (e.g. cysteic acid) derepressed them, but in the mutant the enzymes were nonrepressible. The two *ars* (*arylsulfatase*) alleles in the [(*scon*^{c}

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ars^{+}) + (*scon*$^{+}$ *ars(UFC)*)] heterokaryon encode electrophoretically distinguishable forms of arylsulphatase. Under sulphur limitation both forms were produced, but only the wild-type (ars+) form was produced under sulphur abundance, and in heterokaryons in which the *ars* and *scon* alleles were in the opposite relationship, only the ars(UFC-220) form was produced under conditions of sulphur abundance. Thus, the derepressive effect of *scon*^{c} was limited to its own nucleus.

Metzenberg also noted that the [(*mat A; tol*$^{+}$) + (*mat a; tol*)] heterokaryon was more viable than [(*mat A; tol*) + (*mat a; tol*$^{+}$)], suggesting that the role of *tol*$^{+}$ was limited primarily to *mat a* nuclei. In *N. crassa*, the presence of both *mat A* and *mat a* idiomorphs (nonhomologous alleles) in a common vegetative cytoplasm triggers 'mat-mediated heterokaryon incompatibility', and makes [(*mat A*) + (*mat a*)] heterokaryons inviable. Mat-mediated heterokaryon incompatibility abates in the sexual cycle to enable fertilization to produce the sexual dikaryon. Newmeyer (1970) had found that the *tol* (*tolerant*) mutation suppressed this incompatibility and allowed [(*mat A; tol*) + (*mat a; tol*)] heterokaryons to thrive, provided that the nuclei were not incompatible at other *het* (*heterokaryon incompatibility*) loci. Metzenberg's observation prompted Newmeyer to re-check her strains and she wrote that [(*mat A; tol*$^{+}$) + (*mat a; tol*)] heterokaryons indeed initially grew as well as the wild type but eventually they broke down (see online supplementary material), thus suggesting that *tol* was at least partly dominant when it was coupled to *mat a*. But it remained possible that the greater inviability of the [(*mat A; tol*) + (*mat a; tol*$^{+}$)] heterokaryon was due to the action of some other *het* gene. Years later, Chang and Staben (1994) replaced the *mat A* idiomorph by *mat a* to produce an isogenic *mat A*/*mat a* strain pair. These strains would have been ideal to test for viability difference between [(*mat A; tol*$^{+}$) + (*mat a; tol*)] and [(*mat A; tol*) + (*mat a; tol*$^{+}$)]. But as far as I know this experiment has not been done.

Saccharomyces cerevisiae. Demeter *et al*. (2000) made binucleate yeast cells by mating *mat a* with *mat α* strains bearing the *kar1-Δ15* mutation that prevents nuclear fusion. The *mat a* nucleus also induced an irreparable double strand break (DSB) in the *mat α* nucleus via expression of HO endonuclease from a galactose-inducible promoter, but the *mat a* nucleus itself was made HO-insensitive by deletion of the HO cleavage site from its *mat* allele. Finally, the *mat a* and *mat α* nuclei were visually distinguished by expressing a lacI-GFP fusion protein from a transgene in the *mat a* nucleus that bound to tandem lacO sites in the *mat α* nucleus. Thus, the binucleate cell contained one nucleus with a single green-fluorescent dot, which became two green dots upon segregation of sister chromatids in mitosis. Mitosis was visualized by tubulin immunofluorescence of the elongating mitotic spindle. Under DSB non-inducing conditions ~ 85% of the cells showed binuclear divisions, whereas under DSB inducing conditions 68% of the cells showed mononuclear division in which the undamaged nucleus divided while the GFP-marked damaged nucleus did not. Thus, the signal to arrest division appeared not to diffuse from the damaged to the undamaged nucleus. The authors suggested that the DSB-containing nucleus might block entry of a mitosis-activating protein, or exit of a mitosis-inhibiting protein.

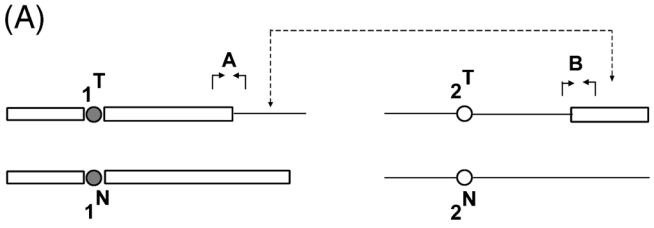
Aspergillus nidulans. The *Aspergillus nidulans matA*^{HMG} gene is on chromosome III. Czaja *et al.* (2013) found that insertion of another copy of this gene into either a linked or unlinked location induced an RNA-mediated silencing of both the endogenous and transgenic *matA* alleles and thereby blocked the sexual-phase-specific increase in *matA* expression and rendered the fruiting bodies (cleistothecia) barren and devoid of asci or ascospores. This silencing, called MatIS (Mat-induced silencing), was restricted to the nucleus bearing the duplicated gene, because in a heterokaryon it did not spread to another nucleus bearing an unduplicated *matA* gene. The authors suggested that MatIS nucleus-limitation might reflect a new type of post-transcriptional gene silencing in which the *matA* RNA does not diffuse and is degraded in a sub-cellular compartment close to the nucleus of origin.

3. *N. crassa* vs *N. tetrasperma*

Ascospore germination provides a convenient point to begin a description of the *N. crassa* life cycle. Ascospores are the products of a sexual cross between two strains, one of mating type *mat A*, and the other *mat a*, and when induced to germinate by heat, or chemicals produced by heating the substrate, they send out a germ tube that becomes the first hypha. Ascospores also serve as units of dispersal, although strain disperal also occurs during vegetative growth via the budding off of vegetative spores, called conidia, from the tips of aerial hyphae. Conidia are spheroid cells with 2 to about 10 nuclei, and can live from a few days to up to several weeks, whereas ascospores are more resistant to stress and longer lived than conidia. Under favourable conditions a conidium sends out a germ tube to produce a new hypha. Conidia (and hyphal fragments) can,

additionally, function as the paternal fertilizing element during a sexual cross. The protoperithecium, a specialized knot of hyphae that forms upon nutrient deprivation, is the maternal element. Specialized hyphae called trichogynes emanate from the protoperithecia and grow towards conidia of the opposite mating type in response to conidially derived mating-type-specific sex hormone. Fertilization of protoprithecia by conidia of opposite mating type is the prelude to their differentiation into perithecia. Within the perithecia, the *mat A* and *mat a* nuclei undergo karyogamy (nuclear fusion) in the ascogenous hyphae, and the diploid zygote nucleus produced by each nuclear fusion immediately undergoes meiosis in a sac-like cell called the penultimate cell that differentiates to become an ascus. The four haploid products of meiosis undergo mitosis, and the resultant eight nuclei are then partitioned into the eight ascospores that form within each ascus. Additional mitotic divisions in each ascospore produce numerous identical haploid nuclei before the ascospores enter dormancy.

(A)



(B)

Meiosis 1	Centromeres to poles	
	I	II
Alternate	1^T 2^T	1^N 2^N
Genotype	*T*	*N*
Ascospore phenotype (*Nc*)	viable	viable
Ascospore phenotype (*Nt*)	viable, self-fertile produces homokaryons of both mating types	
Adjacent 1	1^T 2^N	1^N 2^T
Genotype	*Df1/Dp2*	*Dp1/Df2*
Ascospore phenotype (*Nc*)	inviable	inviable
Ascospore phenotype (*Nt*)	viable, self-fertile * produces no homokaryons	

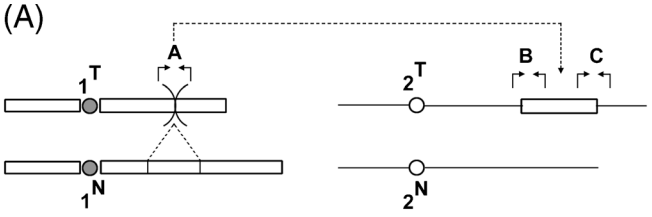
* Unless *Df1* or *Df2* deletes a nucleus-limited gene.

Figure 1. (**A**) Reciprocal translocation interchanges terminal segments of two chromosomes (1 and 2, shown as box and line). Centromeres 1^N and 2^N identify the normal sequence chromosomes; centromeres 1^T and 2^T identify the reciprocal translocation. (**B**) Consequences of alternate and adjacent 1 segregation in *Neurospora crassa* (*Nc*) and *N. tetrasperma* (*Nt*). *N*, *T*, *Dp* and *Df* stand for normal sequence, translocation, duplication and deficiency. The translocation is defined by the A and B junctions and the bent arrows indicate the oligonucleotide primers used to PCR amplify across the junctions.

Finally, octets of ascospores are shot out of the perithecium. Since the strain (mycelium) produced by germination of an individual ascospore is homokaryotic, it cannot complete the sexual cycle on its own, but needs mycelium of the opposite mating type to do so. That is, in *N. crassa* the products of two ascospores are required to complete the sexual cycle, and therefore *N. crassa* is a heterothallic species.

In contrast, in *N. tetrasperma* the eight haploid nuclei produced by meiosis and the post-meiotic mitosis are packaged into each of four ascospores as four non-sister pairs (1 *mat A* + 1 *mat a*). Each ascospore contains nuclei of both mating types, and since the resulting dikaryotic mycelium produced from a single ascospore is competent to complete the sexual cycle without the need for a mycelium from another ascospore, *N. tetrasperma* is a pseudohomothallic species. During vegetative growth a subset of the conidia produced by the heterokaryotic mycelium can have all nuclei of the same mating type; that is, it can be homokaryotic. Such conidia give rise to

(A)



(B)

Meiosis 1	Centromeres to poles I	Centromeres to poles II
Alternate	1^T 2^T	1^N 2^N
Genotype	*T*	*N*
Ascospore phenotype (Nc)	viable	viable
Ascospore phenotype (Nt)	viable, self-fertile produces homokaryons of both mating types	
Adjacent 1	1^T 2^N	1^N 2^T
Genotype	*Df*	*Dp*
Ascospore phenotype (Nc)	inviable	viable
Ascospore phenotype (Nt)	viable, self-fertile * produces homokaryon of mating type of *Dp* nucleus	

* Unless Df deletes a nucleus-limited gene.

Figure 2. (**A**) Insertional translocation transfers a segment from one chromosome (1, box) to another chromosome (2, line) without any reciprocal exchange. Centromeres 1^N and 2^N identify the normal sequence chromosomes; centromeres 1^T and 2^T identify the reciprocal translocation. (**B**) Consequences of alternate and adjacent 1 segregation in *Neurospora crassa* (*Nc*) and *N. tetrasperma* (*Nt*). *N*, *T*, *Dp* and *Df* stand for normal sequence, translocation, duplication and deficiency. The translocation is defined by the A, B and C junctions and the bent arrows indicate the oligonucleotide primers used to PCR amplify across the junctions. *T* possesses all three breakpoints; *N* lacks all three breakpoints and *Dp* contains only the B and C breakpoints.

single-mating-type derivative strains that are self-sterile. Homokaryotic derivative strains can cross with like strains of the opposite mating type. In addition, *N. tetrasperma* asci can occasionally produce five or more (upto eight) ascospores instead of the normal four. This happens when a dikaryotic ascospore is replaced by a pair of homokaryotic ascospores, each of which is slightly smaller in size than a dikaryotic ascospore. The mycelium generated from the small ascospores is self-sterile. Homokaryotic mycelia can cross with a homokaryon of the opposite mating type. The dominant *Eight-spore* (*E*) mutation substantially increases replacement of dikaryotic ascospores by pairs of smaller homokaryotic ascospores. For figures and a more comprehensive account of the *N. tetrasperma* life cycle, see Raju and Perkins (1994).

4. *N. crassa* translocations

Hundreds of chromosome rearrangements in *N. crassa* were described by Perkins (1997). They included reciprocal translocations, insertional translocations, quasiterminal translocations, intrachromosomal transpositions, inversions and other more complex rearrangements such as linked reciprocal and insertional translocations, etc. Reciprocal translocations interchange the terminal segments of two chromosomes (figure 1A). Quasiterminal translocations move a distal segment of a donor chromosome to the tip region of another chromosome, distal to any essential gene, and presumably cap the breakpoint on the donor chromosome with the tip of the recipient chromosome, whereas insertional translocations transfer a segment from one chromosome (donor) to another chromosome (recipient) without any reciprocal exchange (figure 2A). An intrachromosomal transposition is essentially an insertional translocation in which the same chromosome is both 'donor' and 'recipient' and inversions are essentially insertional translocations in which the translocated segment is re-inserted into the same site but in opposite orientation.

In a cross of a translocation by normal sequence (i.e. $T \times N$) either alternate or adjacent 1 segregation can occur in the first division of meiosis. Alternate segregation restores the parental *T* and *N* types in the progeny (figures 1B and 2B), whereas adjacent 1 segregation generates non-parental types (figures 1B and 2B). If the translocation is insertional or quasiterminal type, adjacent 1 segregation results in the production of duplication (*Dp*) and deficiency (*Df*) progeny (figure 2B). The *Dp* progeny are viable, and depending on the translocation, the duplicated segment can be hundreds of kilobase pairs (kbp) in size and can include many genes. The *Df* progeny are inviable, and the ascus produced following an adjacent 1 segregation contains four viable and four inviable ascospores. The viable ascospores blacken (B), whereas the inviable ones remain white (W); therefore the ascus is 4B:4W type. If the translocation is reciprocal type, adjacent 1 segregation results in all eight progeny receiving complementary duplications and deficiencies (figure 1B). Since all eight ascospores contain one or another deficiency, they are inviable and remain white, and the ascus is 0B:8W type. Regardless of the type of translocation, alternate segregation produces 8B:0W asci (figures 1B and 2B). Since both alternate and adjacent 1 segregation can occur at equal frequencies, if 8B:0W and 4B:4W asci are produced in equal numbers, the translocation is insertional or quasiterminal, and if 8B:0W = 0B:8W, then it is reciprocal (Perkins 1997). Isosequential crosses (e.g. $N \times N$ or $T \times T$) produce mostly (> 95%) 8:0 asci.

Insertional translocations are defined by three breakpoint junctions. One, designated 'A', created by deletion of the translocated segment from the donor chromosome, and two, 'B' and 'C' (proximal and distal), created by insertion of the translocated segment into the recipient chromosome (figure 2A). Quasiterminal and simple reciprocal translocations are defined by two junctions: 'A', at the boundary between the breakpoint-proximal sequence on the 'donor' chromosome and the tip from the 'recipient' chromosome, and 'B', at the boundary between the breakpoint-proximal sequence on the recipient chromosome and the 'donor' segment grafted onto it (figure 1A). *T* progeny possess all the breakpoints; *N* progeny lack all the breakpoints; and *Dp* progeny contain the B and C breakpoints but not A (mnemonic: *A absent* in *Dp*). My laboratory defined the breakpoints of 13 translocations onto the genome sequence (Singh *et al*. 2010), including *T(IIR > IL)NM177* that was used by Metzenberg *et al*. (1974) to test for nucleus-limitation of $pcon^{C}$.

5. Proposal: Introgression of *N. crassa* translocations into *N. tetrasperma*

In setting up crosses to introgress *N. crassa* translocations (*T*) into *N. tetrasperma* (*N*) we need to be able to unambiguously distinguish *T* progeny from the *N* and *Dp* progeny produced in each round of $T \times N$. Knowing

the breakpoints of translocation allows us to design breakpoint-specific PCR primers. Primers for all three breakpoints A, B and C will amplify PCR products with *T* DNA as template; primers for B and C but not A will amplify with *Dp* DNA template; and none of the primers will amplify products with *N* DNA template. Therefore, in principle, it should become possible to introgress these 13 translocations into *N. tetrasperma*.

The desired end-point of the introgressions is the creation of self-fertile [*T* + *N*] heterokaryon strains. Self-cross of a [*T* + *N*] strain should generate [*T* + *N*] and [*Dp* + *Df*] type progeny from alternate and adjacent 1 segregation (figures 1B and 2B). In [*Dp* + *Df*] progeny the *Df* nucleus will be rescued by the *Dp* nuclei. [*T* + *N*] progeny can potentially yield homokaryotic conidia of both mating types, whereas [*Dp* + *Df*] progeny can yield homokaryons of only the mating type of the *Dp* nucleus, since the *Df* homokaryons are inviable. But both [*T* + *N*] and [*Dp* + *Df*] types should be self-fertile, and when self-crossed, each will again produce [*T* + *N*] and [*Dp* + *Df*] progeny. However, if the *Df* removes any nucleus-limited gene required for completion of a cross, then the [*Dp* + *Df*] type would effectively regress to a self-sterile [*Dp*] type. A [*T* + *N*] strain 50% of whose self-cross progeny are self-sterile would putatively signal a nucleus-limited gene.

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