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Use of fluorescent protein to analyse recombination at three loci in *Neurospora crassa*

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ABSTRACT

We have inserted a histone H1-GFP fusion gene adjacent to three loci on different chromosomes of *Neurospora crassa* and made mating pairs in which a wild type version of GFP is crossed to one with a mutation in the 5′ end of GFP. The loci are *his-3*, *am* and *his-5*, chosen because recombination mechanisms appear to differ between *his-3* and *am*, and because crossing over adjacent to *his-5*, like *his-3*, is regulated by *rec-2*. At *his-3*, the frequencies of crossing over between GFP and the centromere and of conversion of 5′GFP to GFP$^+$ are comparable to those obtained by classical recombination assays, as is the effect of *rec-2* on these frequencies, suggesting that our system does not alter the process of recombination. At each locus we have obtained sufficient data, on both gene conversion and crossing over, to be able to assess the effect of deletion of any gene involved in recombination. In addition, crosses between a GFP$^+$ strain and one with normal sequence at all three loci have been used to measure the interval to the centromere and to show that GFP experiences gene conversion with this system. Since any gene expressed in meiosis is silenced in Neurospora if hemizygous, any of our GFP$^+$ strains can be used as a quick screen to determine if a gene deleted by the Neurospora Genome Project is involved in crossing over or gene conversion.

1. Introduction

Meiotic recombination is the exchange of information encoded in chromosomal DNA, and occurs during the pairing stage of meiosis prior to the Meiosis 1 division that separates homologous pairs of chromosomes into individuals in each daughter nucleus. Recombination exchanges long stretches of DNA in a reciprocal fashion, by crossing over (Muller, 1916), and shorter sequences can experience gene conversion, a form of recombination in which one allele increases in frequency at the expense of the other (Mitchell, 1955).

Chiasmata are the cytological manifestations of crossovers (McClintock, 1945). Chromosome disjunction is dependent on the formation of chiasmata during meiosis to ensure proper alignment at metaphase I and tethering of each centromere in a homologous chromosome pair to opposite ends of the cell (Carpenter, 1987). Both crossing over and gene conversion are thought to be a result of the same initiating event; a double strand break in the DNA made by the Spo11 protein (Keeney et al., 1997; Saito and Colaiácovo, 2011), but the pathways between a break in the DNA and the resolution of a recombination event can be quite different.

Current models of meiotic recombination, derived mostly from studies of *Saccharomyces cerevisiae*, suggest that at least two crossover pathways exist (Zalevsky et al., 1999; Getz et al., 2008; Stahl and Foss, 2010). One pathway requires MSH4/MSH5 proteins and the resultant crossovers are thought to ensure the correct segregation of chromosomes during meiosis (Ross-MacDonald and Roeder, 1994). Other crossovers proceed via a MUS81-dependent pathway and have a lesser role in disjunction (Argueso et al., 2004; de los Santos et al., 2001). MSH4-dependent crossovers reduce the chance of other crossovers occurring nearby, an effect called interference (Muller, 1916), while those that need MUS81 are interference-free (de los Santos et al., 2003). In the absence of MSH4 or MSH5, additional deleterious crossovers may occur, and suppression of these by MSH4/MSH5 is thought to ensure that chromosomes segregate correctly (Argueso et al., 2004).

Recombination frequency varies across the genome in all sexual species and the frequency of local recombination has also been found to vary between individuals of some species, including both mammals and *Neurospora crassa*. The mammalian system regulates meiotic recombination by use of *PRDM9*, a SET-domain histone H3 lysine 4 trimethyltransferase with zinc fingers that bind to DNA. The zinc finger array recognises a degenerate 13-mer motif (CCNCCNTNNCCNC) found in recombination hotspots (Myers et al., 2008), and varies between *PRDM9* alleles. The 13-mer motif is also highly polymorphic (Jeffreys and Neumann, 2002, 2005; Durbin, 2010), so the interaction between *PRDM9* alleles and motifs provides this mechanism of region-specific regulation of recombination (Baudat et al., 2010; Parvanov et al., 2010; Myers et al., 2010).

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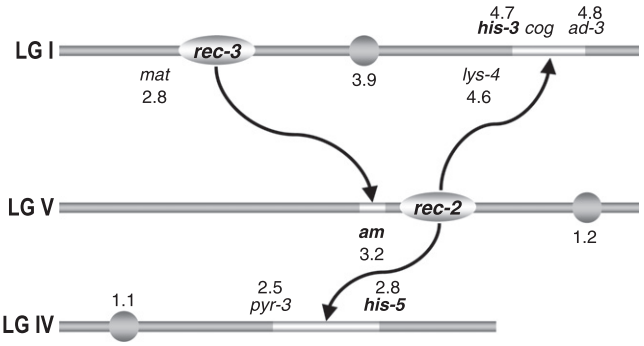

Fig. 1. A partial linkage map of *N. crassa*. The discs represent the centromeres and positions are in Mbases from the left end of linkage group I. By convention, *his-3* is on the right arm of LGI, *am* on the left arm of LGV and *his-5* on the right arm of LGIV. Recombination within *his-3* and crossing over between *his-3* and *ad-3* are regulated by *rec-2* on LGV. *rec-2* also regulates crossing over between *his-5* and *pyr-3* but has no known effect within *his-5* (Smith, 1965), while *rec-3* regulates recombination within *am* with no detectable effect on crossing over in the vicinity (Smyth, 1973).

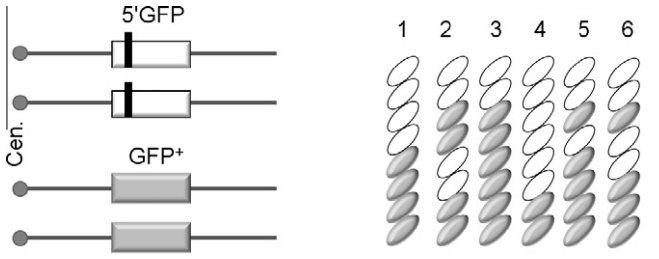

Fig. 2. Schematic of the fluorescence-based recombination reporter system. Wild type green fluorescent protein (GFP) or a mutant variant (5′GFP) are fused to histone H1. (Cen. = centromere; Grey oval = green fluorescence; white oval = no fluorescence). Some of the expected octad types are illustrated: 1 – normal Mendelian segregation with no recombination (first division segregation); 2 – normal Mendelian segregation with a crossover between GFP and the centromere (second division segregation); 3 – conversion of 5′GFP to wild-type; 4 – conversion of wild-type GFP to 5′GFP; 5 and 6 – post-meiotic segregation of uncorrected heteroduplex. 5 and 6 show some of the unusual patterns characteristic of a failure of mismatch repair; 5+:3M in 5 and aberrant 4+:4M in 6.

However, analysis of sperm from men of the same *PRDM9* and hotspot genotypes shows that substantial variation in recombination frequency remains (Berg et al., 2010), suggesting that *PRDM9*-independent regulation exists even in mammals. The Neurospora regulatory system, which appears to have no homology to PRDM9, comprises at least three naturally polymorphic loci (*rec-1*, *rec-2* and *rec-3*), each acting at more than one position in the Neurospora genome (reviewed in Catcheside, 1975). For example, the *rec-2* gene product regulates crossing over in part of Linkage Group (LG) 4 and in separate regions on either arm of LG 1 (Fig. 1). Thus each *rec* gene must make a product that can act on specific recombination hotspots at a distance from the gene (Catcheside, 1975, 1986). The nature of the *rec* gene products is currently unknown but could theoretically be either protein or RNA. *cog*, a hotspot regulated by *rec-2* (Fig. 1), is also polymorphic, with alleles capable of initiating high (*cog*$^+$) or low (*cog*) frequencies of recombination (Angel et al., 1970).

Fungi are particularly useful to the student of recombination because in many fungi a single meiosis yields a discreet package (the ascus) that holds the products of meiosis (the spores). Every spore in an ascus provides information about each of the DNA strands involved in recombination in a single meiosis, making it possible to differentiate between crossovers and conversions. This feature has been exploited previously, using spore colour mutations in *Sordaria fimicola* (Olive, 1959) and *Ascobolus immersus* (Lissouba et al., 1962) to study recombination. More recently, artificial "spore colour mutants" have been generated by genomic insertion of various fluorescent protein gene sequences. When such a histone H1 construct tagged with green fluorescent protein (GFP) was generated in *N. crassa* to examine meiotic silencing (Freitag et al., 2004), we were impressed by the ease of detection and enumeration of recombinant asci and so began to develop our current system.

Subsequently, an elegant system was developed in *Arabidopsis thaliana* (Francis et al., 2007) using a *qrt* mutant that prevents separation of pollen grains and yields pollen tetrads. Shotgun transformation of seeds with ECFP (enhanced cyan fluorescent protein), EYFP (enhanced yellow fluorescent protein) or DsRed (Discosoma red) constructs yielded plants with fluorescent genes in multiple locations, allowing study of crossing over and gene conversion. In addition, CFP (m-Cerulean), RFP (tdTomato), and GFP markers have recently been used to make a set of portable constructs for insertion into *S. cerevisiae* chromosomes (Thacker et al., 2011). The system has been tested by analysis of recombination at *arg4* and of the effect of *spo11* deletion on crossover homeostasis in two intervals (Thacker et al., 2011).

However, *N. crassa* has an advantage over both *S. cerevisiae* and *A. thaliana*, as Neurospora has an additional mitotic division after meiosis is complete giving an ascus of eight ordered spores, while yeast asci and pollen tetrads have only four "spores" in no particular order. Because of this, one can also readily identify failure of mismatch repair by post-meiotic segregation of alleles in a Neurospora octad. Octad analysis has been largely avoided because of the massive amount of work involved in obtaining data. As an example, we picked, grew and genotyped 150 octads (1200 spores) to find one octad with gene conversion at *his-3* (Yeadon et al., 2010) and at *am* over 6000 octads were examined to find seven that were exceptional (Bowring, Stadler and Catcheside, unpublished).

Because of this, we devised the fluorescence-based reporter system described in this study to greatly accelerate the rate of collecting octad data. We have targeted histone H1 constructs tagged with GFP$^+$ or mutant 5′GFP to the *his-3*, *am* and *his-5* loci (Fig. 1) in three separate mating pairs, and an additional mutant, 3′GFP, at *his-3*. This system allows an assay for crossovers, conversions and rarer recombination events simply by scanning meiotic products under the fluorescence microscope and looking for asci in which segregation of spores with fluorescing nuclei is atypical (Fig. 2). In addition, crosses between two different GFP null mutants can be used to determine the timing of recombination, by identifying the stage of sporogenesis at which fluorescence first appears (Fig. 3).

We have measured gene conversion (6+:2M segregation of GFP) at *his-3*, *his-5* and *am*, and crossing over between each GFP insertion and the respective centromere (Fig. 1). We suggest that

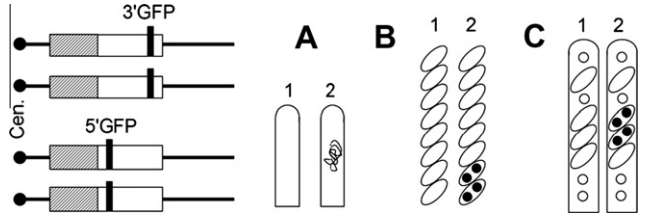

Fig. 3. The system can be used to determine the timing of recombination. In crosses of strains carrying different mutants of hH1::GFP (3′GFP and 5′GFP) as alleles, chromosomes in most asci will not fluoresce (1 in A–C). However, occasionally recombination will generate a wild-type GFP allele and fluorescence will become visible (2 in A–C). If recombination occurs with normal timing, fluorescence should be observed in young asci (A) and in all subsequent stages. Where the timing of recombination is abnormal, fluorescence will be restricted to a later stage (B and C). With this assay, recombination can also be detected in asci lacking a full complement of spores (C).