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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.

Keywords

Meiotic Recombination; Gene Conversion; Crossover; GFP; Octad; Tetrad

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

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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 (CCNCCNTNCCNC) found in recombination hotspots (Myers et al., 2008), and varies between *PRDM9* alleles. The 13-mer motif is also highly polymorphic (Jeffreys et al., 2002, 2005; Durbin et al., 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).

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; Catcheside, 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, *Neurospora 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 (1,200 spores) to find one octad with gene conversion at *his-3* (Yeadon et al., 2010) and at *am* over 6,000 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 the data obtained for wild type recombination at each locus can be used to screen for disturbances in crossing over and/or conversion due to deletion of a range of genes, both those known to be involved in recombination and for others with a role yet to be discovered, and to test for compliance with models of recombination mechanisms.

2. Materials and methods

2.1. Culture methods and media

As described by Bowring and Catcheside (1996) except that crosses were supplemented with, 200µg/ml histidine, 500µg/ml alanine, 200µg/ml adenine, and 400µg/ml lysine as required. Vegetative cultures were supplemented with, 200µg/ml histidine, 500µg/ml alanine, 400µg/ml adenosine, and 400µg/ml lysine as required. Cultures and crosses were incubated at 25° unless otherwise stated.

Crosses were performed in glass petri dishes on 50 ml solid crossing medium as described in Yeadon et al. (2010), except that medium was supplemented as required.

2.2. Generation of DNA constructs

The prototype for all constructs was pMF280 (Freitag et al., 2004), which contains a hH1-GFP fusion sandwiched between the right hand 70% of *his-3* and the adjacent ~1.6 kb of flanking sequence, both from the genome strain Oak Ridge 74A (Galagan et al., 2003; Fig. 4).

2.2.1. Vectors for targeting to his-3—GFP null mutants were generated using fusion PCR (Kuwayama et al., 2002) and confirmed by sequencing (not shown). The 5' allele is a substitution of T for G at nucleotide 26 of GFP (p. Glu6*). Four primers were used: L5' StopFwd (CACATGACGCATCATCTCC), L5' StopRev (ACAGCTCCTAGCCCTTGC), R5' Stopfwd (AGCAAGGGCTAGGAGCTG) and R5' StopRev (GGGAAATGGTGTCTTTTACG). Left and right components were amplified from pMF280 template using the L and R primer pairs (L5' StopFwd/ L5' StopRev and R5' Stopfwd/ R5' StopRev) respectively, yielding two amplicons with an 18 bp overlap containing the substitution (underlined in L5' StopRev and R5' StopFwd). L5' StopFwd and R5' StopRev were used to produce a single fusion amplicon containing the substitution (5' GFP). GFP was replaced with 5' GFP in pMF280 by digestion of the pMF280 plasmid and the 5' fusion amplicon with *SphI* and *BsmI*. The appropriate fragments were gel-purified and ligated together to yield phis-3GFP5'.

The 3' allele is a substitution of T for A at nucleotide 628 of GFP (p. Lys210*). 3' GFP was built using the same approach as the 5' allele, but with primers L3' StopFwd (AGCCCGCTGCCGAGAAGG), L3' StopRev (CGTTGGGGTCTTAGCTCAGG), R3' Stopfwd (CCTGAGCTAAGACCCCAACG) and R3' StopRev (AGCAAGAGCAACTAAACGG). Replacement of GFP in pMF280 with 3' GFP was by digestion of the plasmid and the 3' fusion amplicon with *PacI* and *BstBI*, yielding phis-3GFP3' (Genbank accession number JX156631).

Alleles adjacent to and *in cis* to the recombination hotspot *cog*⁺ are preferentially converted at high frequency (Catcheside and Angel, 1974; Yeadon and Catcheside, 1998; Yeadon et al., 2001, 2010). *cog*⁺ is to the right of *his-3* (Bowring and Catcheside, 1991; Fig. 1) in strains of Lindegren background and the intervening sequence is quite different to that from Oak Ridge (OR), the source of the corresponding region in pMF280. So that gene-conversion would primarily be of 5' GFP to GFP⁺, it was necessary to place the 5' GFP allele in a *cog*⁺ background. To facilitate homologous integration of the 5' GFP targeting vector, we replaced the OR flank with the corresponding Lindegren flank in phis-3GFP5'. The primers his3FlankF (TCCAATGCGGATGGATTTCG) and NSS_N_V2_H3F_R (GACGATTGCGGCGGCCGCGGTACCCTCGTCCGACTG) were used to amplify the Lindegren flank, using DNA from strain T12335 (Table 1) as template. NSS_N_V2_H3F_R contains a *NotI* site, and there is a *DraII* site 26 bp from the other end of the amplicon, such that the sites bracket the Lindegren and the corresponding OR sequence in pMF280. pMF280 and the Lindegren flank amplicon were digested with *NotI*/*DraII*. The appropriate fragments were gel-purified and ligated together to yield pGFP5'*cog*⁺ (Fig. 4; Genbank accession number JX156630).

2.2.2. Vectors for targeting to am and his-5—All targeting vectors have the same basic structure as pMF280: a truncated gene that can complement a mutation only when the plasmid integrates at the homologous site in the recipient strain, ~1.5 kb of flank and the hH1::GFP fusion sandwiched between these two components. For each of the *am* and *his-5* loci, a plasmid was built containing a truncated version of the respective gene and the flank, bisected by sites for *AscI* and *BstBI*.

Truncated *am* and *am* flank were amplified from cosmid pMOcosX (Orbach, 1994) G9:A10 using the primer pairs *am_truncF* (GCAGGCCTACAAGGGTACGTC)/*Asc_NSSRC_BB1_amtR* (GGCGCGCCGACGATTGCGTTCGAAATTGGGAGCACGCAGCCA) and *am_flankF_PP* (TTCGAACGCAATCGTCGGCGCGCCGCTGTTACAAAATCCCGAACC)/*am_FlankR* (AGTGACACCAACATCGCATC) respectively. Amplicons were joined using fusion PCR and the resulting product TA-cloned into pCR2.1 (Invitrogen), yielding plasmid *pamtrunc*.

Similarly, truncated *his-5* and *his-5* flank were amplified from genomic DNA of strain F7448 (Table 1) using the primer pairs *His5tF* (CAAACTTCTCTGTCTATGACC)/*Asc_NSSRC_BB1_H5tR* (GGCGCGCCGACGATTGCGTTCGAAAGGAACATCTCCCTCGACACC) and *His5FlankF_PP* (TTCGAACGCAATCGTCGGCGCGCCCGTATTCGGTGCTGGCA)/*His5FlankR* (AGCGCTATTGGCGATGTTCA) respectively. Amplicons were joined using fusion PCR and the resulting product TA-cloned into pCR2.1 (Invitrogen), yielding plasmid *phis-5*.

Both GFP⁺ and GFP5⁺ cassettes were excised from pMF280 and *phis-3GFP5⁺* by digestion with *NotI/XbaI*. The smaller fragment including the GFP cassette was excised from agarose and end-filled. *phis-5* and *pamtrunc* were digested with *AscI/BstBI*, end-filled using a Blunt-ending kit (NEB) and treated with shrimp alkaline phosphatase (Promega). The components were combined by ligation and the orientation of the resultant plasmids (*phis-5GFP5⁺*, *phis-5GFP⁺*, *pamGFP5⁺* and *pamGFP⁺*; Genbank accession numbers JX156633, JX156632, JX156629 and JX156628 respectively) was determined by restriction analysis.

2.3 Construction of strains

For strains with GFP inserted at *his-3*, the *his-3^{K480} cog⁺* strain T12437 was transformed with *pGFP5⁺ cog⁺* and the *his-3^{K480} cog* strain T11759 with pMF280 and separately with *phis-3GFP3⁺*, selecting for growth without histidine, to give T12498, T12511 and T12515 respectively (Table 1). After separation of heterokaryons (Ebbole and Sachs, 1990), the homokaryotic transformants were confirmed by Southern analysis.

In order to prevent RIP (Selker, 1990), a *rid* mutation (Freitag et al., 2002) was crossed into the His⁺ GFP⁺ and GFP5⁺ strains. Thus T12520 was extracted from a cross between T12498 and FGSC #9015 and T12529, T12530 and T12634 from a cross between T12515 and FGSC #9014 (Table 1).

For strains with GFP inserted at *his-5*, T12603 and T12607/T12608, construction was similar, by transformation of T12487 with *phis-5GFP5⁺* and of T12488 with *phis-5GFP⁺*, followed by separation of homokaryons and Southern analysis.

For strains with GFP inserted at *am*, T12482 and T12483 were respectively transformed with *pamGFP5⁺* and *pamGFP⁺*, followed by separation of homokaryons and Southern analysis. Thus T12598 is a transformant of T12482. T12632 is progeny of T12595, a GFP⁺ transformant of T12483, crossed to T12464. T12633 was extracted from a cross between T12588, a GFP⁺ transformant of T12483, and T12463.

2.4 Microscopy and data collection

Rosettes were collected by dissection of perithecia in 10% glycerol on a glass slide, using needles sharpened in molten sodium nitrite. The contents of 25–30 perithecia were spread on each slide, and the rosettes of asci allowed to unravel prior to application of cover slips. Because we occasionally observed spore displacement in the centre of asci on spreads

prepared by tapping the cover slip, rosettes were spread by capillary action drawing excess mounting medium from under the cover slip with a tissue. Cover slips were sealed with melted dental wax and slides examined on a Nikon Diaphot.

2.5 Data analysis

As it is not possible to tell whether crossing over has occurred in an ascus that has experienced conversion, crossover frequencies were calculated by the formula $(SD/2(SD + FD)) \times 100\%$ (where FD = first division segregation frequency and SD = second division segregation frequency). Conversion frequencies were calculated as conversion events per 10^5 asci, where an ascus that has experienced a single conversion event (6+:2M, for example) is considered as one, while if two events have happened in the same ascus (8+:0M or 7+:1M), that ascus is counted as two. So for example, if the total number of asci is 1000, of which there were four 6+:2M asci and three 8+:0M asci, the frequency of conversion is $((4 + (2 \times 3))/1000) \times 10^5$, or $100/10^5$.

Since some spores do not survive to maturity, we could not be confident that 2+:6M asci were due to conversion, so these asci were ignored.

3. Results

3.1 Use of GFP to measure recombination on three chromosomes

In crosses between strains carrying GFP⁺ and mutant GFP (5'GFP) constructs, crossing over between GFP and the centromere was measured by the frequency of second division segregation of GFP and conversion of 5'GFP to GFP⁺ was measured by the frequency of 6+:2M asci (Fig. 5). Both crossing over and gene conversion occur at different frequencies for each locus studied (Table 2). *his-3* is closest to the LGI centromere at 5.8 cM, followed by *his-5* at 10.3 cM from the LGIV centromere and *am* is furthest away from the LGV centromere at 20 cM. In contrast, conversion is least frequent at *his-5* (50–90/ 10^5 asci), a little more at *am* (90–130/ 10^5) and most frequent at *his-3* (370–480/ 10^5). The conversion frequencies per 10^5 viable spores, which are one fourth of the frequency per ascus (25/ 10^5 , 17/ 10^5 and 106/ 10^5 spores at *am*, *his-5* and *his-3* respectively), are consistent with those measured by classical recombination assays (Bowring and Catcheside, 1996; Smith, 1965; Yeadon et al., 2004).

3.2 Gene conversion is detectable in crosses between two mutant GFP alleles

In a cross in which one strain has a 5'GFP and the other a 3'GFP mutant construct, each inserted at *his-3*, conversion of either allele to GFP⁺ is relatively frequent and readily detectable. Of 13490 asci scored, there were 34 in which fluorescence was seen in two spores (Fig. 6), eight where only a single spore was fluorescing, and four with an equal number of fluorescent and non-fluorescent spores (Table 3). This suggests a conversion frequency of 320–420/ 10^5 asci, very similar to the frequency of conversion of 5'GFP to GFP⁺.

3.3 GFP null alleles can be used to investigate the timing of recombination

In a cross heteroallelic for 5'GFP and 3'GFP mutants, we expect to see fluorescent nuclei only when recombination has converted a mutant allele to GFP⁺. We have observed that fluorescence first appears early in meiosis (not shown), indicating that recombination occurs with normal timing in this cross. This result can thus be used as a comparison with data from mutant crosses in which timing may be abnormal.

3.4 Reversion of either 5'GFP or 3'GFP to GFP⁺ is very infrequent

In crosses between a mutant GFP strain and a strain lacking GFP sequence, we saw no evidence of reversion as measured by visible fluorescence at any stage of sporulation. Thus, the frequency of gene conversion in a 5'GFP × 3'GFP cross (Table 3) is much higher than the frequency of reversion (3'GFP $\chi^2 = 91$; $p < 0.0001$; 5'GFP $\chi^2 = 54$; $p < 0.0001$), as is the frequency of conversion of 5'GFP to GFP⁺ (Table 2; $\chi^2 = 57$; $p < 0.0001$).

3.5 Ectopic gene conversion is very infrequent

In a cross in which one strain has a 3'GFP construct inserted at *his-3* and the other a 5'GFP mutant construct inserted at *his-5*, we saw no evidence of fluorescence in 16720 asci, scanned after spores had formed and therefore after the stage at which genes in unpaired DNA are silenced. Thus, the frequency of gene conversion when the alleles of GFP are on different chromosomes is much less than the frequency of conversion of 5'GFP to GFP⁺ when both are inserted at *his-5* ($\chi^2 = 8.3$; $p = 0.0041$), and strikingly less than the frequency of fluorescence in a 5'GFP × 3'GFP cross, when both are inserted at *his-3*.

3.6 Recombination of GFP alleles inserted at *his-3* is regulated by *rec-2*

We analysed a cross between a GFP⁺ *rec-2*⁺ strain (T12634; Table 1) and the same 5'GFP strain (T12520) used to obtain the *rec-2* data (Table 2), scanning 6804 asci from this cross. We detected 201 asci with second division segregation of GFP and the centromere (crossover asci), a crossover frequency of 1.5%, which is less than the 5.8% measured in the cross homozygous for *rec-2* ($\chi^2 = 430$; $p < 0.0001$). We saw five asci that exhibited conversion of 5'GFP to GFP⁺, a lower frequency than that seen in the *rec-2* cross ($\chi^2 = 13$; $p = 0.0003$). These data confirm that *rec-2*⁺ regulation of *cog*-initiated recombination is retained in the fluorescent recombination system, evidence that insertion of this foreign DNA does not perturb normal recombination.

3.7 Gene conversion and crossing over can be measured in crosses hemizygous for GFP⁺

In crosses between a GFP⁺ strain and a strain lacking GFP sequence, second division segregation and gene conversion were detected at all three loci (Table 4). The conversion frequencies at *his-3* and *am* are lower than those measured in a crosses heterozygous GFP⁺/5'GFP ($\chi^2 = 10.5$; $p = 0.001$ and $\chi^2 = 4.9$; $p = 0.027$ respectively) but still substantial (Tables 2 and 4). Although the frequency of crossing over between GFP and the centromere is higher in the hemizygous *his-3* cross, increasing from 6 to 14 % ($\chi^2 = 833$; $p < 0.0001$), crossing over between *am* and the LGV centromere increases only slightly, from 20 to 23%. It seems likely that the difference in crossing over at *his-3* reflects differences in strain background between the two crosses as the native sequence strain (T12456; Table 1) is substantially of Lindegren provenance, while the GFP strains are mostly of Emerson background. In contrast, the hemizygous and heterozygous *am* crosses are of near identical background.

4. Discussion

We have devised a fluorescent reporter system to investigate genes involved in recombination in *Neurospora crassa*. Crosses heterozygous for GFP⁺/5'GFP insertions at specific locations on three chromosomes have been shown to allow estimation of the distance between the insertion site and centromere and measurement of gene conversion at each locus. Diploids hemizygous for GFP not only allow measurement of crossing over between the insertion site and centromere but also yield substantial frequencies of conversion of the native sequence to GFP⁺ at both *his-3* and *his-5* (Tables 2 and 4).

The high frequency of conversion in hemizygous diploids may seem surprising but is consistent with previous data. In crosses hemizygous for an *arg2-TK* construct inserted between *his-3* and *cog* (Yeadon et al., 2001), conversion of a *his-3* allele was accompanied by complete transfer of the sequence from the other homologue. Thus if the chromosome in which *his-3* was converted to His⁺ carried *TK*, the His⁺ progeny would be *TK*⁻ and vice versa. This was the case regardless of the nature or length of the sequence between *his-3* and *cog* (Yeadon et al., 2001), suggesting that non-homologous sequence can be converted to *TK*⁺ at high frequency. The data in this report showing 6+:2M segregation of GFP⁺ to non-homologous native sequence are confirmation of the previous results, which were based only on chromatid data.

This hemizygous recombination system provides a convenient way to screen any *Neurospora* deletion mutant for a role in recombination. Many of these mutants, made as part of the project to annotate the *Neurospora* Genome (for example, Galagan et al., 2003; Hood et al., 2005; Colot et al., 2006), currently have no known function. In *Neurospora*, unpaired DNA sequences are silenced during meiosis (Aramayo and Metzenberg, 1996; Shiu et al., 2001; Shiu and Metzenberg, 2002), so genes whose expression is mostly limited to meiosis experience meiotic silencing. Thus a deletion of a meiotic gene will behave as a dominant in such a screen, allowing assessment of an effect on meiosis and/or recombination. We envisage our screen as fuel for multiple undergraduate projects across the USA analysing *Neurospora* deletion mutants, and have made the strains available through the Fungal Genetics Stock Centre for this purpose.

In crosses between strains carrying 5' GFP and 3' GFP alleles, each inserted at *his-3*, the frequency of conversion, as measured by the appearance of fluorescence, is similar to that in GFP⁺/5' GFP crosses (Table 2). Since fluorescence is only seen if recombination has occurred, this system can be used to measure recombination frequency in mutants that severely disrupt meiosis and to determine whether such recombination occurs with normal timing. Indeed, we have used a 5' GFP/3' GFP heterozygote to investigate residual recombination in a cross lacking the *spo11* gene, that encodes the protein responsible for making double strand breaks in DNA and thus initiating recombination (Bowring et al., unpublished; manuscript in preparation).

We have shown that reversion of either GFP allele is so infrequent that it is unlikely to have a detectable effect on the measurement of recombination frequencies in either the GFP⁺/5' GFP or the 5' GFP/3' GFP analyses. Ectopic conversion, as measured by appearance of fluorescence in spores from a cross with a 5' GFP allele at *his-5* and a 3' GFP allele at *his-3*, is also very infrequent, but may conceivably be increased in certain mutant backgrounds, so our system allows a screen for this.

At *his-3*, recombination is initiated by *cog* (Angel et al., 1970; Yeadon and Catcheside, 1998; Yeadon et al., 2001, 2004) and is regulated by the unlinked gene *rec-2* (Catcheside, 1975; Catcheside, 1986). The dominant allele, *rec-2*⁺, reduces recombination initiated by *cog*. Crossing over between *lys-4* and *ad-3*, loci on either side of *his-3* (Fig. 1), is less than 2% in the presence of *rec-2*⁺ but can be up to 12% in crosses homozygous *rec-2* (Yeadon et al., 2004). *rec-2*⁺ has a similar but more dramatic effect on allelic recombination in *his-3*, as a cross heterozygous for *his-3* K1201 and K874 mutants can yield up to 1% His⁺ spores but, 200-fold fewer in the presence of *rec-2*⁺ (Yeadon et al., 2004; Yeadon unpublished). For the GFP construct inserted between *cog* and *his-3*, we therefore expect that conversion of 5' GFP to GFP⁺ and crossing over between GFP and the centromere would likewise be regulated by *rec-2*⁺. We found this to be the case, as crossing over is reduced from 6% in a cross homozygous for *rec-2* to 1.5% in a cross where the GFP⁺ strain is *rec-2*⁺ and conversion is reduced by *rec-2*⁺ to ~25% of the *rec-2* frequency.

In conclusion, we have shown that insertion of GFP alleles at a several locations in the *Neurospora* genome provides all of the advantages of tetrad analysis without the usual prohibitive labour overhead. Recombination frequencies can be measured and the spectrum of recombination events documented easily and rapidly. This system can be used to determine the effect of genes known to be involved in recombination, to detect additional genes whose involvement is not yet suspected, and to test predictions of recombination models.

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- built and tested *Neurospora crassa* fluorescent recombination reporter system
- measured gene conversion and crossing over in three locations
- shown that recombination is not perturbed by the system
- reporter system can be used to anywhere
- hemizygous cross of use as screen for novel meiosis or recombination genes

Figure 1. A partial linkage map of *Neurospora 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 1966), while *rec-3* regulates recombination within *am* with no detectable effect on crossing over in the vicinity (Smyth, 1973).

Figure 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 & 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.

Figure 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, B & C). However, occasionally recombination will generate a wild-type GFP allele and fluorescence will become visible (2 in A, B & 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 & C). With this assay, recombination can also be detected in asci lacking a full complement of spores (C).

Figure 4. Two of the constructs for targeting GFP to *his-3*

The layout is the same in all constructs; a hH1::GFP fusion gene sandwiched between part of the gene to the left and the flanking sequence of the gene to the right. The construct below (B) illustrates sequences present in pMF280 (Freitag et al., 2004) while that above (A) shows pGFP5' cog⁺.

Figure 5. Detection of gene conversion and crossing over

From left to right, the asci illustrated have experienced conversion of 5' GFP to wild-type (6+:2M), crossing over between GFP and the centromere (second division segregation 4+:4M) and no recombination (first division segregation 4+:4M). This photomicrograph is of asci from a cross heterozygous for 5' GFP and GFP⁺, inserted at *his-3*.

Figure 6. Recombination in a cross heterozygous for 5'GFP and 3'GFP mutant alleles
 In this spread of two rosettes, two asci have experienced conversion of mutant GFP to wild-type, resulting in two fluorescent spores in each ascus (2+:6M). The asci in which recombination has occurred have been enlarged to assist the reader. This photomicrograph is of rosettes from a cross heterozygous for 5'GFP and 3'GFP, inserted at *his-3*.

Table 1

Neurospora strains

Stock no.	Genotype
F7448	<i>A, his-3^{K874}, cog, ad-3^{K118}; rec-2</i>
T11759	<i>a, his-3^{K480}, cog; am^{K314}, rec-2</i>
T12335	<i>a, lys-4^{STL4}, his-3^{K480}, cog⁺; am^{K314}, rec-2</i>
T12385	<i>A, rid^{ip1}, his-3^{Δ234-732}; rec-2⁺ (FGSC #9014)</i>
T12386	<i>a, rid^{ip4}, his-3^{Δ234-732}; rec-2⁺ (FGSC #9015)</i>
T12437	<i>A, his-3^{K480}, cog⁺; rec-2</i>
T12456	<i>a, his-3^{K26}, cog⁺; am^{K314}, rec-2</i>
T12457	<i>a, his-3^{K26}, cog⁺; am^{K314}, rec-2</i>
T12463	<i>rec-3, A; am^{B501}</i>
T12464	<i>rec-3, a; am^{B501}</i>
T12482	<i>rid^{ip4}, a; am^{B501}</i>
T12483	<i>rid^{ip1}, A; am^{B501}</i>
T12487	<i>rid^{ip4}, a; his-3^{K451}</i>
T12488	<i>rid^{ip1}, A; his-3^{K451}</i>
T12498	<i>A, his-3⁺::pccg-1::hH1::5' GFP, cog⁺; rec-2</i>
T12511	<i>a, his-3⁺::pccg-1::hH1::GFP⁺, cog; am^{K314}, rec-2</i>
T12515	<i>a, his-3⁺::pccg-1::hH1::3' GFP, cog; am^{K314}, rec-2</i>
T12520	<i>rid^{ip4}, a, his-3⁺::pccg-1::hH1::5' GFP, cog⁺; rec-2</i>
T12529	<i>rid^{ip1}, A, his-3⁺::pccg-1::hH1::GFP⁺, cog; am^{K314}, rec-2</i>
T12530	<i>rid^{ip4}, A, his-3⁺::pccg-1::hH1::GFP⁺, cog; am^{K314}, rec-2</i>
T12588	<i>rid^{ip4}, a; am⁺::pccg-1::hH1::GFP⁺</i>
T12595	<i>rid^{ip1}, A; am⁺</i>
T12598	<i>rid^{ip1}, A; am⁺::pccg-1::hH1::5' GFP</i>
T12603	<i>rid^{ip4}, a; his-5⁺::pccg-1::hH1::5' GFP</i>
T12607	<i>rid^{ip1}, A; his-5⁺::pccg-1::hH1::GFP⁺</i>
T12608	<i>rid^{ip1}, A; his-5⁺::pccg-1::hH1::GFP⁺</i>
T12632	<i>rid^{ip1}, A; am⁺</i>
T12633	<i>rid^{ip4}, a; am⁺::pccg-1::hH1::GFP⁺</i>
T12634	<i>rid^{ip1}, A, his-3⁺::pccg-1::hH1::GFP⁺, cog; rec-2⁺</i>

Recombination at three Neurospora loci, using crosses in which one chromosome carries a GFP⁺ and the other a 5' GFP construct. *T12634 is *rec-2⁺*, showing that recombination in GFP inserted between *his-3* and *cog* is regulated as in the native *his-3* gene. **Co** represents the percentage crossing over between GFP and the centromere while **C** indicates the frequency of conversion events per 10⁵ asci.

Table 2

Locus	Cross	FD	SD	6:2	5:3	8:0	7:1	Total	Co	C
<i>am</i>	T12598 × T12633	11628	7769	10	3	3	1	19393	20	108 ± 22
<i>his-5</i>	T12608 × T12603	14077	3669	10	0	1	0	17757	10.3	68 ± 20
<i>his-3</i>	T12529 × T12520	13225	1735	44	0	9	1	15013	5.8	426 ± 53
<i>his-3</i>	*T12634 × T12520	6598	201	3	0	2	0	6804	1.5	103 ± 15

Table 3

Reversion of either the 5' GFP or 3' GFP construct inserted at *his-3* is very rare compared to allelic recombination frequency. Where fluorescence was not detected, the frequency given is the upper estimate at the 95% confidence level. FI indicates the frequency of fluorescent events per 10^5 asci.

Cross	Construct	Asci	GFP ⁺	FI
T12437 × T12515	3' GFP	27455	0	<1.36
T12498 × T12759	5' GFP	16446	0	<2.28
T12498 × T12515	5' GFP × 3' GFP	13490	* ₄₆	371 ± 50

* Asci with GFP⁺ spores were as shown:

- 2+:6M - 34
- 1+:7M - 8
- 4+:4M - 4

Table 4

Recombination at three Neurospora loci, using hemizygous crosses in which only one chromosome carries a GFP⁺ construct. **C_o** represents the percentage crossing over between GFP and the centromere while **C** indicates the frequency of conversion events per 10⁵ asci.

Locus	Cross	FD	SD	6:2	8:0	Total	C _o	C
<i>am</i>	T12632 × T12633	18554	15436	10	3	34993	22.7	46 ± 11
<i>his-3</i>	T12456 × T12530	4654	1769	6	0	6429	13.7	93 ± 38
<i>his-5</i>	T12456/7 × T12607	560	271	1	0	832	16.2	120 ± 120