# Homologous recombination following transformation in *Neurospora crassa* wild type and mutagen sensitive strains

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In filamentous fungi transformed with linear DNA, the frequency of gene replacement varies from a few percent in *Neurospora crassa*, where it depends on the extent of homology between the transforming DNA and the gene to be replaced, (Asch and Kinsey 1990 Mol. Gen. Genet. 22:37-43) to about 30% in Aspergillus (Miller et al. 1985 Mol. Cell Biol. 5:17-14). Yet, in the yeast, *Saccharomyces cerevisiae*, homologous gene replacement is the rule and ectopic integration of transforming DNA is extremely rare (Fincham 1989 Microbiol. Rev. 53:148-170). This variation could be the result of the action of one or a few genetic pathways and thus, be affected by single mutations. Mutants with altered ability to integrate transforming DNA may be sensitive to ionizing radiation, since double strand break repair is necessary for both recombination and survival after ionizing radiation damage. Therefore, we looked among the radiation-sensitive mutants, *uvs-2,3,6, mus-9,11*, and *mei-2,3*, for changes in the ability to integrate transforming DNA either ectopically or by homologous recombination. All mutant strains were extensively backcrossed to wild type strains of 74-OR23-1A background and the 74-OR23-1A strain served as a control.

A plasmid, pMTR::HYG (Figure 1), was designed to allow rapid distinction between transformants arising through homologous recombination events and those arrising through ectopic integration events. [Since we did not distinguish between homologous gene replacement and the other more complex changes that are occasionally found when exogenous DNA recombines with an endogenous homologous sequence (Miao et al. 1995 Genetics 139:1533-1544), these events will simply be called homologous events and ectopic events.] This plasmid carries the *amp* and *ori* segment and a 2.7 kb segment of *Neurospora crassa* DNA including the entire coding region of the *mtr* (methyl tryptophan resistance) gene. It was constructed by inserting a 2397 bp *Sal*I fragment containing the hygromycin resistance gene (*hyg* or *hph*) plus the *trpC* promoter from pCSN44 (Staben et al. 1989 Fungal Genet. Newslet. 36:79-81) into a *Xho*I site within the *mtr* coding sequence of pCVN2.9 (Koo and Stuart 1991 Genome 34:644-651). Hygromycin resistance is dominant, so all spheroplasts transformed with this construct will be hygromycin resistant (HYGr). The *mtr*- phenotype is recessive. The *mtr* gene controls the uptake of neutral amino acids including the toxic amino acids, 4-methyl tryptophan (leading to the *mtr* designation) and p-fluorophenylalanine (FPA). Transformants with an intact endogenous mtr gene will be sensitive to these compounds while transformants, in which the endogenous gene has been replaced with , or disrupted by the exogenous, disrupted gene, will be resistant (FPAr).

Spheroplasts were transformed as described by Vollmer and Yanofsky (1986 Proc. Natl. Acad. Sci. USA 83:4869-4873) with pMTR::HYG, cut with *Xho*I and *Sal*I in the *N. crassa* DNA outside the mtr coding regions (see Figure 1). They were plated in top agar on sorbose minimal medium containing 200 μg/ml HYG. After 3 - 6 days, pieces of colonies which had grown sufficiently to break through the top agar and produce conidial hyphae were transferred to 0.5 ml liquid minimal medium containing 200 μg/ml HYG. After 4 to 8 days, conidia or hyphae from

these tubes were suspended in water and spotted on sorbose minimal medium with no drug, 200 µg/ml HYG or 15 µg/ml FPA, and scored for growth after 36-48 h at 30°C. Isolates which grew on FPA were further tested by spreading a dilute conidial suspension from the HYG tube on FPA containing plates. Transformants in which recombination has occurred at the homologous *mtr* gene will produce many FPA resistant colonies. Transformants in which DNA has integrated at ectopic sites will be FPA sensitive unless they have acquired a spontaneous mutation in the *mtr* gene while growing in liquid medium or on the plate. Such mutants will have few, if any, FPA resistant colonies, unless the mutation occurred very early. To show further that the transformants identified as due to events at the *mtr* locus were due to the presence of exogenous DNA at the mtr gene, crosses to a *col-4- mtr*+ a strain were made. Single conidial isolates of the HYGr FPAr transformants were picked either from the FPA plates, or from HYG plates and shown to be HYGr FPAr. They were grown on minimal medium and then grown on minimal medium containing FPA to eliminate any FPAs nuclei before crossing. The *col-4*+ progeny from these crosses were scored for sensitivity to HYG and FPA. Because *col-4* is only 1 map unit from *mtr*, the *col-4*+ progeny of such a cross should be *hyg*+ *mtr*- with a HYGr FPAr phenotype, except for rare *col-4*+ *hyg*- *mtr*+ (HYGs FPAs) recombinants. Transformants which have an ectopic copy of the transforming DNA and an independent *mtr*- mutation should give *col-4*+ progeny with a ratio of the HYGr FPAr and HYGs FPAr phenotypes of 1:1.

Both transient and stable transformants are produced when spheroplasts are transformed with pMTR::HYG. It was not possible to quantify the total number of transformants obtained. However, no large, i.e. >5x, difference in transformation frequency was observed between 74-OR23-1A and the mutant strains. Of transformants which had grown sufficiently to break the agar surface (see Table I), greater than 90% proved stable for HYGr in all strains except *mus-9* (80%) and *uvs-6* (56%). We have observed a similar instability of benomyl resistance in *uvs-6* conidia transformed with *bml* carrying plasmids.

As shown in Table I, the mutagen sensitive strains appear to fall into two classes when the frequencies of homologous recombination events among total transforming events are compared. One group, *mei-2, mus-9*, and *mus-11*, has frequencies of homologous events similar to wild type. A second group, *uvs-2, uvs-3, uvs-6*, and *mei-3*, has lower frequencies of homologous events. In *uvs-6* and *mei-3*, where sufficient isolates have been examined, this frequency is significantly lower than in 74-OR23-1A.

Table I. Transformation of Neurospora by the pMTR::HYG plasmid.

| Strain | # of isolates | HYGr trans-formants | HYGr FPAr transformants | Frequency of HYGr FPAr transformants | Homologous events confirmed by crosses |
|---|---|---|---|---|---|
| 74-OR23-1A | 705 | 669 | 16 | 0.024 | 16/16 |
| 74-OR23-1A(a) | 200 | 200 | 4 | 0.02 | 1/4 |
| mei-2 | 200 | 200 | 4 | 0.02 | 1/3 |
| mus-9 | 250 | 201 | 3 | 0.015 | 0/1 |
| mus-11 | 291 | 274 | 6 | 0.022 | -- |
| uvs-2 | 300 | 297 | 2 | 0.0067(b) | 0/2 |
| uvs-3 | 250 | 233 | 1 | 0.0043(b) | -- |
| uvs-6 | 1101 | 619 | 3 | 0.0048(c) | 1/1 |
| mei-3 | 550 | 527 | 3 | 0.0057(c) | -- |

(a)The *Xho*I and *Sal*I cut plasmid used in these transformations was blunt ended with the Klenow fragment of DNA polymerase I, dCTP, dTTP, dGTP and dideoxyATP.

(b)Chi square; $p<0.1$.

(c)These values are significantly different by chi square from transformation in 74-OR23-1A at $p<0.01$

When the 16 HYGr FPAr transformants in 74-OR23-1A were crossed to the *col-4- mtr*+ strain, the *col-4*+ progeny were primarily the HYGr FPAr phenotype expected if these transformants had arisen through an homologous gene replacement or insertion event. About 1.5% of the *col-4*+ progeny were HYGs FPAs recombinant progeny. Thus, all 16 of these transformants arose through recombination events at the *mtr* locus (rightmost column, Table I). A few (3 each) unexpected FPAr HYGs and FPAs HYGr progeny were seen. The FPAr HYGs progeny could be the result of the presence of an additional, ectopic insertion of the pMTR::HYG plasmid allowing RIP during the cross which would inactivate the hyg+ DNA but would have no effect on the FPAr phenotype, since the *mtr* gene is already inactive.

When the HYGr FPAr isolates from the mutagen sensitive strains were crossed to *col-4 - mtr*+, crosses from 7 such transformants produced ascospores. Two, a *mei-2* isolate and a *uvs-6* isolate, gave only HYGr FPAr progeny, indicating that they arose through homologous recombination events. A *uvs-2* isolate gave 13 HYGr FPAr : 4 HYGs FPAr progeny and thus probably occurred via an ectopic event with a subsequent mutation in the *mtr* gene or had both homologous and ectopic integration of DNA followed by RIP of the *hyg* gene during the cross. In each of the remaining 4 strains, substantial numbers of *col- 4*+,FPAs progeny occurred. It is possible that quiescence of the *mtr* gene caused by multiple ectopic inserts of the plasmid led to the initial FPAr classification. Another possibility is that occasionally FPAs nuclei can survive growth on FPA in strains with many FPAr nuclei. These nuclei would then have a selective advantage in crosses when present in the mutagen sensitive strains which are less fertile than wild type, even in heterozygous crosses.

In mammalian cells, it has been reported that transformation with DNA that has been blunt ended with dideoxynucleotides at the 3' end of each strand, gives higher percentages of homologous targeting of transforming DNA. Consequently, the 74-OR23-1A strain was also transformed with *Xho*I and *Sal*I cut plasmid blunt ended with the Klenow fragment of DNA polymerase I and dCTP, dTTP, dGTP and dideoxyATP. Four of 200 isolates were HYGr FPAr. In crosses, only 1 gave *col-4*+ progeny with mostly HYGr FPAr phenotypes, while the other three gave a variety of progeny (Table I, row 2). Thus in Neurospora, we see no increase in the frequency of homologous recombination events when blunt ended DNA is used and there may be some decrease.

Several conclusions may be drawn from the above studies:

1. Wild-type *Neurospora crassa* gave about 2% recombination of endogenous mtr sequences with the homologous sequences of the transforming DNA of the plasmid, pMTR::HYG designed

to measure such recombination. Similar frequencies were seen by Asch and Kinsey (1990 Mol. Gen. Genet. 221:37-43) at the *am* gene with small linear DNAs.

2. Wild-type transformed with dideoxynucleotide blunt ended DNA, and three mutagen sensitive strains, *mei-2, mus-9*, and *mus-11*, gave transformation frequencies due to apparent homologous events (HYGr FPAr transformants) similar to those for wild type transformed with *Sal*I, *Xho*I cut DNA. However, the actual frequency may be lower, as most of the isolates tested in crosses gave ratios of progeny not expected for strains with homologous recombination events.

3. Four mutagen sensitive mutant strains, *uvs-2, uvs-3, uvs-6* and *mei-3* appeared to give lower percentages of homologous recombination events among HYG resistant transformants.

4. Two of the mutants, *mus-9* and especially *uvs-6*, gave much higher frequencies of unstable transformants than did the wild type.

5. Blunt ending the transforming DNA with a dideoxynucleotide did not give higher frequencies of homologous recombination events when studied in wild type *N. crassa*.

6. Transformation with pMTR::HYG may be useful in that homologous recombination at the mtr locus provides two dominant selectable markers at the *mtr* locus. A cross between *mtr*+ and *mtr::hyg* strains both carrying the same neutral amino acid auxotrophs allows the selection for disomics of chromosome IV (Ann Hagemann and Eric Selker, personal communication). Strains carrying the *mtr::hyg* mutation are available from the Fungal Genetics Stock Center as is the pMTR::HYG plasmid.

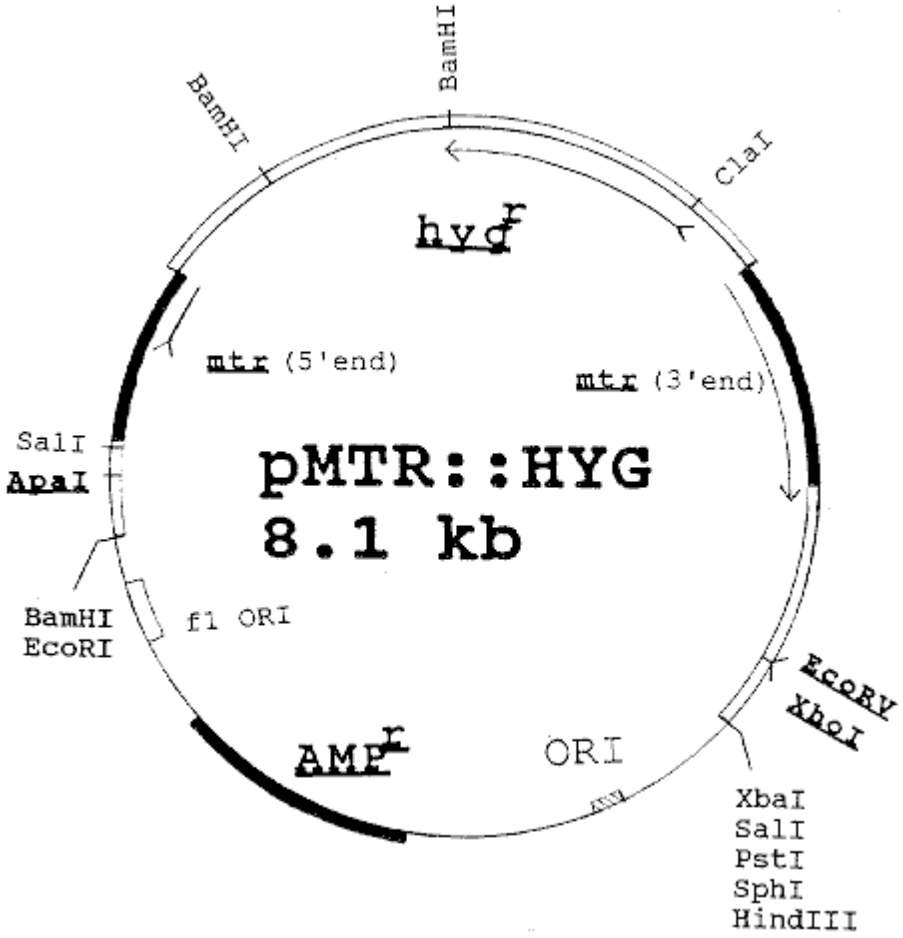


**Figure 1**. pMTR:HYG, a plasmid designed to differentiate between transformants arising through homologous recombination events and those arising through ectopic integration events, was constructed by inserting the hygromycin B resistance gene (designated *hyg*r) into the coding sequence of the *Neurospora crassa mtr* gene, disrupting the mtr function. Known unique sites are underlined. Apparent coding sequences of *mtr* and *AMP*r are filled in.