Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining

Yuuko Ninomiya, Keiichiro Suzuki, Chizu Ishii, and Hirokazu Inoue*

Department of Regulation Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama City 338-8570, Japan

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Gene disruption and overexpression play central roles in the analysis of gene function. Homologous recombination is, in principle, the most efficient method of disrupting, modifying, or replacing a target gene. Although homologous integration of exogenous DNA into the genome occurs readily in *Saccharomyces cerevisiae*, it is rare in many other organisms. We identified and disrupted *Neurospora crassa* genes homologous to human *KU70* and *KU80*, which encode proteins that function in nonhomologous end-joining of double-stranded DNA breaks. The resulting mutants, named *mus-51* and *mus-52*, showed higher sensitivity to methyl methanesulfonate, ethyl methanesulfonate, and bleomycin than wild type, but not to UV, 4-nitroquinoline 1-oxide, camptothecin, or hydroxyurea. Vegetative growth, conidiation, and ascospore production in homozygous crosses were normal. The frequency of integration of exogenous DNA into homologous sequences of the genome in the *KU* disruption strains of *N. crassa* was compared with that in wild type, *mei-3*, and *mus-11*. In *mei-3* and *mus-11*, which are defective in homologous recombination, none or few homologous integration events were observed under any conditions. When *mtr* target DNA with ≈2-kb 5′ and 3′ flanking regions was used for transformation of the *KU* disruption strains, 100% of transformants exhibited integration at the homologous site, compared to 10 to 30% for a wild-type recipient. Similar results were obtained when the *ad-3A* gene was targeted for disruption. These results indicate that *KU* disruption strains are efficient recipients for gene targeting.

Ku70 | Ku80 | gene targeting | *Neurospora crassa*

In eukaryotes, two main recombination pathways have been identified, differing as to whether double-strand break (DSB) repair depends on DNA sequence homology. The first, homologous recombination (HR), involves interaction between homologous sequences, whereas the second, nonhomologous end-joining (NHEJ), involves direct ligation of the strand ends independent of DNA homology. *Saccharomyces cerevisiae* mainly uses an HR system of DSB repair, in which exogenous DNA fragments are integrated at homologous sites in the genome if the DNA has a short region of homology at both ends (1, 2). The *RAD52* gene of *S. cerevisiae* is essential for this integration event (3). In contrast, many other organisms, including humans, plants, and insects, seem to use NHEJ preferentially in DSB repair. As a result, exogenous DNA can be integrated anywhere in the genome, even if it carries a long stretch of homologous sequence. The NHEJ process is mediated by the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the Ku70–Ku80 heterodimer, and the DNA ligase IV-Xrcc4 complex (4, 5). The Ku protein was originally found as an autoantigen in patients with polymyositis-scleroderma overlap syndrome (6). Human Ku is a heterodimer of 69- and 83-kDa polypeptides, Ku70 and Ku80, which bind tightly to the DNA ends. This is a key step in NHEJ repair. Homologs of Ku70 and Ku80 have been identified in vertebrates, insects, the worm *Caenorhabditis elegans*, yeasts, and plants (7, 8). However, NHEJ repair has not been reported in *Neurospora crassa*.

The *mei-3*, *mus-11*, and *mus-25* genes in *N. crassa* are homologs of *S. cerevisiae RAD51*, *RAD52*, and *RAD54*, which are specific for HR repair (9–11). The rate of homologous integration of plasmid pMTR (12) into the chromosomal *mtr* locus was assayed in the wild type and in mutants defective in these HR repair genes (11). In the wild type, 3–5% of transformants showed homologous integration, whereas <0.3% of transformants did so in *mei-3* and *mus-25* mutants. Because the homologous integration rate is so low in *N. crassa*, it is not easy to disrupt a particular gene by gene replacement. Repeat induced point mutation (RIP), which exploits the premeiotic inactivation of duplicated sequences (13), has been used as an alternative. Although this method is useful and effective, it is time consuming.

We simply hypothesized that the rate of homologous integration of exogenous DNA can be increased by blocking NHEJ function, and searched the *Neurospora* genome database (14) to find *N. crassa* genes homologous to human *KU70* and *KU80*. We disrupted these genes by a PCR-based method of direct replacement (15) and constructed two strains defective in *N. crassa KU70* and *KU80* homologs. These mutants showed mild sensitivity to mutagens such as methyl methanesulfonate, ethyl methanesulfonate, and bleomycin. The *Neurospora KU-70* and *KU-80* homologs were therefore named *mus-51* and *mus-52*, respectively. Targeting of two genes, *mtr* and *ad-3A*, on chromosomes IV and I, respectively, was conducted in these strains by a PCR-based method (16). In this study, exogenous DNA was introduced by electroporation (17, 18), which is more efficient and convenient than the spheroplast-polyethylene glycol (PEG) method (19, 20) used previously for transformation of *Neurospora*.

Materials and Methods

Strains and Plasmids. Table 1 shows *N. crassa* strains used in these experiments. C1-T10–37A and C1-T10–28a are wild-type strains closely related to the standard Oak Ridge wild type (21). *Escherichia coli* strains DH1 and XL-1 Blue were used for amplification of plasmids (22). Plasmids pBluescript SK$^+$ (Stratagene) and pGEM (Promega) were generally used for construction of new vectors. Plasmids pBARGEM7–1 (23) and pCSN43 (24) and cosmids G7H3 and G8B12 were obtained from the Fungal Genetics Stock Center, University of Kansas Medical School (Kansas City).

Genetic Studies in *N. crassa*. Genetic analysis was carried out as described by Davis and de Serres (25).

PCR Method. PCR amplification was carried out with the Expand High-Fidelity PCR system (Roche Diagnostics) following the manufacturer's protocol.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HR, homologous recombination; NHEJ, nonhomologous end-joining; MMS, methyl methanesulfonate; PFP, *p*-fluorophenylalanine.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AB177394 (*mus-51*) and AB177395 (*mus-52*)].

*To whom correspondence should be addressed. E-mail: hinoue@seitai.saitama-u.ac.jp.

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Table 1. *N. crassa* strains used in this study

Strain	Genotype	Source/ref.
C1-T10–37A	*A* (wild type)	Laboratory stock
C1-T10–28a	*a* (wild type)	Laboratory stock
74-OR256–14a	*a al-2 pan-2 cot-1*	Laboratory stock
C-P-1	*a pan-1 arg-2 leu-2*	Laboratory stock
C20–1	*A ad-2 ser-1*	Laboratory stock
54yo-728–5	*A mus-51*::*Hyg*^{r}	This study
54yo-728–7	*a mus-51*::*Hyg*^{r}	This study
54yo-828–3	*A mus-52*::*Hyg*^{r}	This study
54yo-828–4	*a mus-52*::*Hyg*^{r}	This study
FGSC#2764	*A mei-3*	FGSC
FGSC#6409	*A mus-11*	FGSC
FGSC#8341	*A mus-23 al-2 pan-2 cot-1*	FGSC

FGSC, Fungal Genetics Stock Center.

Construction of Plasmids for the Replacement of *mus-51* and *mus-52* by the *Hyg*^{r} Gene. The strategy for replacing the *mus-51* and *mus-52* genes by the hygromycin-resistance gene (*Hyg*^{r}) is presented in Fig. 1.

***Preparation of DNA for replacement of* mus-51.** The flanking DNA (2 kb 5′ and 3′) of the *mus-51* gene was amplified by PCR using cosmid G7H3 as a template. Primers for amplification of the 5′-flanking DNA were: (*p*-1) 5′-GTGCTGTAGCCGTTTT-GGGTATCGC-3′ and (*p*-2) 5′-GGCGTAATAGCGAAGA-GATAGTTGCTGGAAATAA-3′. Primers for the 3′-flanking DNA were: (*p*-3) 5′-AAGCATAAAGTGTAAAGGCTTGT-TGATGACCGT-3′ and (*p*-4) 5′-TTGGACGCCGCACACCT-CTCGCTCT-3′. The *Hyg*^{r} gene was amplified by PCR using plasmid pCSN43 as a template. The primers used were (*p*-5) 5′-TTATTTCCAGCAACTATCTCTTCGCTATTACGCC-3′ and (*p*-6) 5′-CACGGTCATCAACAAGCCTTTACACTTTA-TGCTT-3′. The three PCR-products were mixed and used as a template in a fusion PCR (15) that was carried out by using primers (*p*-1) and (*p*-4) under the following conditions: initial denaturation 94°C for 2 min, subsequent steps at 94°C for 15 sec, annealing at 60°C for 30 sec, extension at 68°C for 5 min, 10 cycles. Then, 94°C for 15 sec, 60°C for 30 sec, 68°C for 5 min (each cycle at 68°C 5 sec added), 20 cycles, final extension at 72°C for 7 min, hold at 4°C. The fusion PCR product was analyzed on an agarose gel (0.7%) and used for transformation of the *N. crassa* wild type.

***DNA preparation for replacement of* mus-52.** Amplification of the 2 kb 5′ and 3′ flanking DNA of *mus-52* was done by using cosmid G8B12 as a template. The method was as described above.

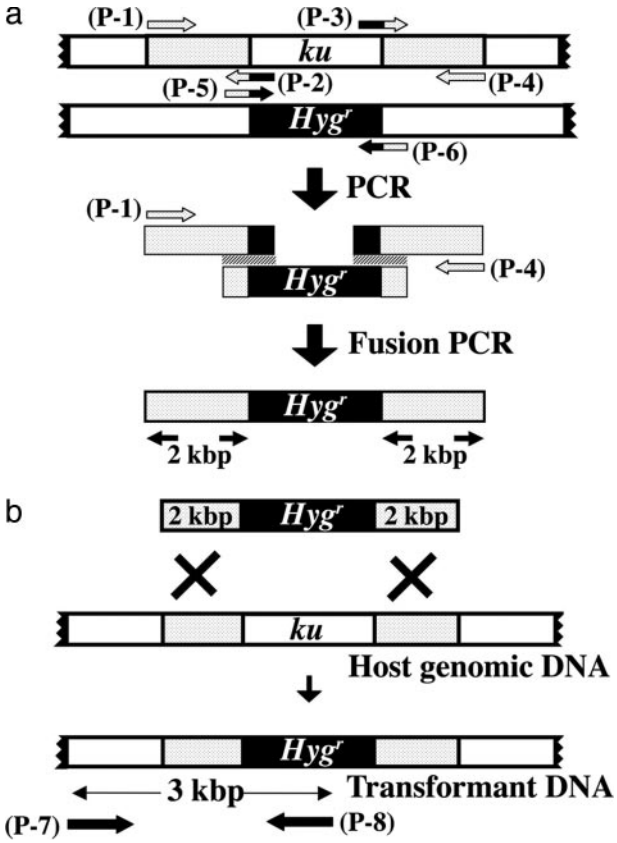

Fig. 1. Strategy for replacing *mus-51* and *mus-52* by *Hyg*^{r}. (*a*) The construction of a *ku*-ortholog gene-targeting vector. The 5′ region of the *ku*-ortholog gene was amplified by using primers (*p*-1) and (*p*-2), and the 3′ region was amplified with primers (*p*-3) and (*p*-4). The size of both products was ≈2 kb. The *Hyg*^{r} gene was amplified with primers (*p*-5) and (*p*-6). These three PCR products were used as a template in fusion PCR using primers (*p*-1) and (*p*-4), and the fusion product was used for transformation. (*b*) Homologous integration of a fusion PCR product into a target gene. This product was introduced into recipient cells by electroporation, and homologous replacement of the *ku*-ortholog gene by the *Hyg*^{r} gene was confirmed by PCR using primers (*p*-7) and (*p*-8).

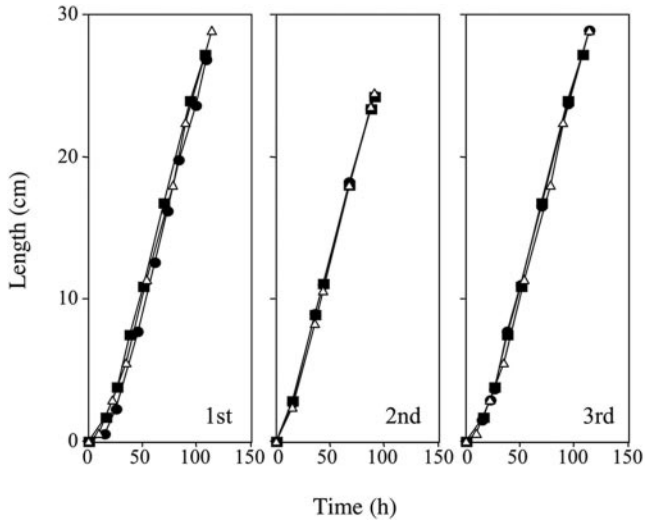

Fig. 2. Measurement of linear growth at 25°C race tubes (30 cm length × 1.5 cm) containing minimal medium (sucrose 0.5%) were used. Distance from inoculated point to front of elongating tip was measured at various times. 1st, 2nd, and 3rd indicate order of consecutive transfers of mycelia. Open triangles, wild type; filled circles, *mus-51*; filled squares, *mus-52*.

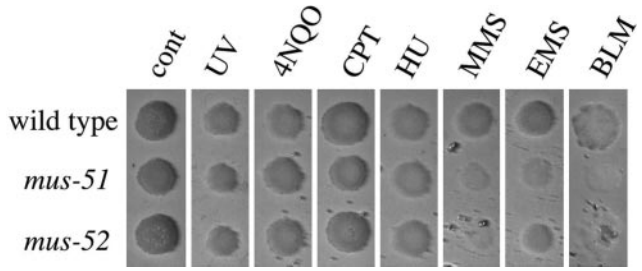

Fig. 3. Sensitivity of wild type, *mus-51*, and *mus-52* to UV and chemical agents. A conidial suspension was spotted on the agar surface of plates containing 4-nitroquinoline 1-oxide (4NQO, 75 ng/ml), camptothecin (CPT, 0.4 μg/ml), hydroxyurea (HU, 1.5 mg/ml), MMS (0.175 μl/ml), ethyl methanesulfonate (EMS, 6 μl/ml), and bleomycin (BLM, 5 μg/ml). UV irradiation was 375 J/m^2.

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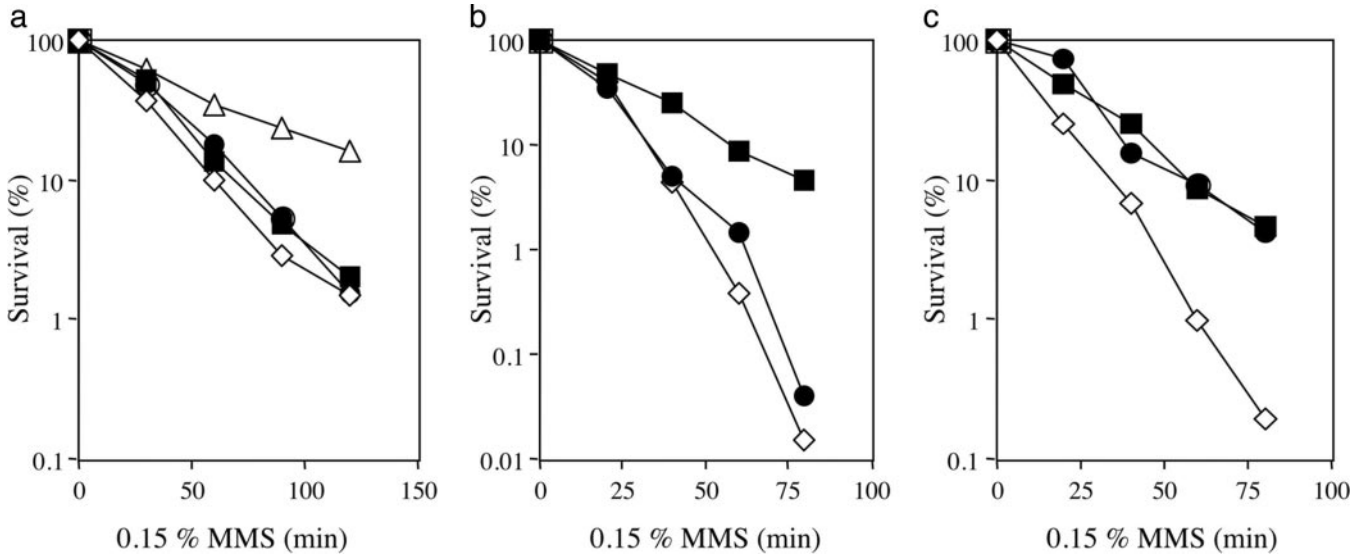

Fig. 4. Comparison of sensitivity to MMS in the double mutants and their parental single mutants. A conidial suspension was treated by MMS (1.5 μl/ml) for the indicated time. (*a*) Wild type (open triangles), *mus-51* (filled circles), *mus-52* (filled squares), and *mus-51 mus-52* (open diamonds). (*b*) *mus-52* (filled squares), *mus-23* (filled circles), and *mus-52 mus-23* (open diamonds). (*c*) *mus-52* (filled squares), *mei-3* (filled circles), and *mus-52 mei-3* (open diamonds).

Primers were: (*p*-1) 5′-GCGCCGGGAGGTTGTTCGTAAGCTG-3′, (*p*-2) 5′-GGCGTAATAGCGAAGAGGCTTTTCGGCTTTGCTG-3′, (*p*-3) 5′-AAGCATAAAGTGTAAAGCAGGGTTGGAGACAGGT-3′, and (*p*-4) 5′-AAGGCGGAGTTGTTGGCTGCGAAGG-3′. Primers (*p*-1) and (*p*-2) were used for amplification of 2 kb of 5′ flanking DNA of *mus-52*. Primers (*p*-3) and (*p*-4) were used for amplification of 2 kb of 3′ flanking DNA of *mus-52*. The primers for amplification of the *Hyg*^{r} gene were (*p*-5) 5′-CAGCAAAGCCGAAAAGCCTCTTCGCTATTACGCC-3′ and (*p*-6) 5′-ACCTGTCTCCAACCCTGCTTTACACTTTATGCTT-3′. The fusion PCR was carried out with primers (*p*-1) and (*p*-4) as described above.

Table 2. Rate of homologous integration at the *mtr* locus in the wild type and in *mus-51, mus-52, mei-3,* and *mus-11* mutant strains

Strains	Exp. no.	Bla-resist	PFP-resist	Homologous integration, %
Wild type	1	9	3	
	2	22	3	
	3	11	3	
	4	16	3	
	Total	58	12	21
mus-51	1	41	41	
	2	18	18	
	Total	59	59	100
mus-52	1	12	12	
	2	23	23	
	3	12	12	
	4	26	26	
	Total	73	73	100
mei-3	1	58	2	
	2	35	1	
	Total	93	3	3*
mus-11	1	45	0	
	2	20	0	
	Total	65	0	0

Bla-resist, blastcitidin resistance; PFP-resist, PFP resistance; homologous integration rate = number of PFP-resist/number of Bla-resist.
*Confirmation of homologous integration was not obtained by PCR.

Electroporation. A suspension of conidia (2.0×10^9 per ml) was prepared in 1 M sorbitol. A solution (5 μl) containing 10 μg DNA of the fusion PCR product was mixed with 50 μl of the conidial suspension, incubated on ice for 5 min, and 40 μl of the

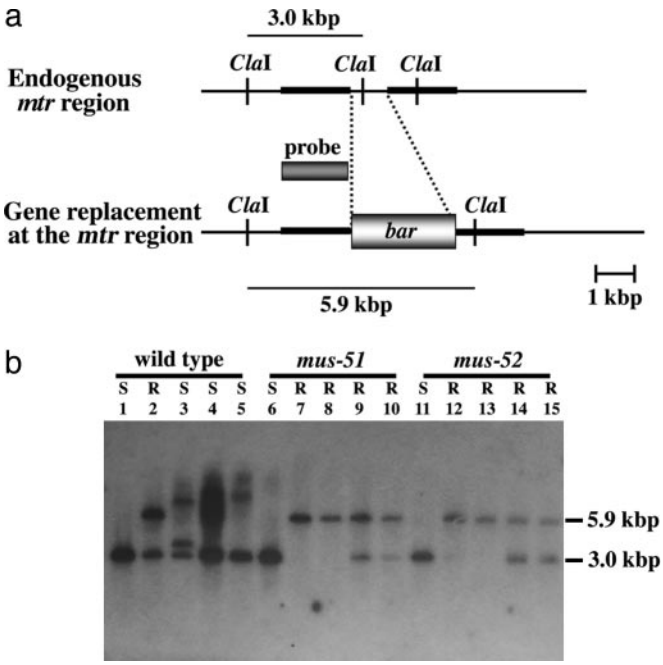

Fig. 5. Southern hybridization analysis in the wild type, *mus-51*, *mus-52*, and their transformants. (*a*) Diagram of the relevant region of the endogenous *mtr* gene and the homologously integrated *mtr*::*bar* gene. The thick lines show 2-kb *mtr* segments attached to the *bar* gene. The target DNA was introduced into the recipient strain, and blastocidin-resistant transformants were isolated. (*b*) Results of Southern analysis. Lanes 1, 6, and 11 are nontransformants, showing the 3-kb *Cla*I fragment of *mtr*. All other lanes are transformants. Homologous integration produces a 5.9-kb *Cla*I fragment. Because transformants are frequently heterokaryons carrying both transformed and nontransformed nuclei, both 3- and 5.9-kb bands may be observed. Ectopic integration produces different-sized bands, as shown in lanes 3–5. S, sensitive to PFP; R, resistant to PFP.

Table 3. Rate of homologous integration at the *ad-3A* locus in the wild type and in *mus-51* and *mus-52* mutant strains

Strains	Bla-resist	Adenine-requiring	Homologous integration, %
Wild type	85	34	40
mus-51	46	46	100
mus-52	36	36	100

Bla-resist, blasticitidin resistance.

mixture was transferred to an electroporater cell (BTX Electro Cell Manipulation 600, Genetronics) and shocked by using a charging voltage of 1.5 kV and a resistance of 186 ohm.

After the electroshock, 1 ml of Vogel's minimal medium containing 1.2% sucrose was added, and the conidia were incubated at 30°C for 2 h. The solution (200 μl) was spread on agar medium containing hygromycin B (500 μg/ml). Colonies resistant to hygromycin were isolated and tested by PCR using primers (*p*-7) and (*p*-8) to determine whether replacement occurred at the target gene (Fig. 1*b*). Southern blotting was used to confirm whether the colonies contained extra copies of the *Hyg*^{r} gene. The targeted strain was crossed to wild type to test for normal segregation of the markers.

Mutagen Sensitivity. Sensitivity to UV and various chemicals was tested by spot test (26). Survival to methyl methanesulfonate (MMS) killing was determined as described (27).

Results

Replacement of *mus-51* and *mus-52*. We searched the *Neurospora* genome database (www.broad.mit.edu/annotation/fungi/neurospora) to find *N. crassa* genes homologous to human *KU70* and *KU80*. The candidate genes encode proteins of 645 and 661 aa, respectively. Alignment of the amino acid sequences of human Ku70 and *Neurospora* Ku70 homolog, and human Ku80 and *Neurospora* Ku80 homolog is shown in Fig. 6, which is published as supporting information on the PNAS web site. Cosmids G7H3 and G8B12 from the Orbach/Sachs libraries (28) contain *Neurospora KU70* homolog gene *mus-51* and *KU80* homolog gene *mus-52*, respectively. These cosmids were used as templates to amplify *mus-51* and *mus-52* by PCR. As described in *Materials and Methods*, DNA fragments carrying *Hyg*^{r} with 2-kb 5′ and 3′ flanking DNAs from the *Neurospora KU* homolog gene were produced by fusion PCR. The fusion PCR products were introduced into wild type, and hygromycin-resistant colonies were isolated. About 200 transformants were subcultured, and genomic DNA was extracted to determine by PCR whether *mus-51* or *mus-52* was replaced by *Hyg*^{r}. Primers in this PCR were set so that one is outside the targeted gene and the other is inside. About 10% of hygromycin-resistant colonies contained *Hyg*^{r} in place of the *KU* ortholog. Strains 54yo-728 carrying *mus-51*::*Hyg*^{r} and 54yo-828 carrying *mus-52*::*Hyg*^{r} were then used as *mus-51* and *mus-52* mutants.

Characterization of *mus-51* and *mus-52* Mutants. ***Conventional mapping.*** The genome database indicates that *mus-51* and *mus-52* are located in linkage groups IV and III, respectively. To prove genetic linkage, we crossed *mus-51* to strain C-P-1 (*a pan-1 arg-2 leu-2*) and *mus-52* to strain C20–1 (*A ad-2 ser-1*). The *mus*-gene was scored as hygromycin-resistant. *mus-51* was near *arg-2* (<1%) and *mus-52* was near *ser-1* (1.2%), as expected.

Vegetative growth, conidiation, sporulation, and telomere length. Race tubes were used to measure linear growth rate (Fig. 2). Even after three consecutive transfers, growth rate of *mus-51* or *mus-52* was identical to wild type. Conidiation was also normal. Homozygous crosses of *mus-51* or *mus-52* were fertile and produced normal ascospores. There was no apparent elongation or shortening of telomeres (data not shown).

Table 4. Relationship between the frequency of integration into a homologous site in the genome and the length of the homologous sequence

Strain	Length of homology, bp	Exp. no.	Bla-resist	PFP-resist	Homologous integration, %
Wild type	50	1	22	0	
		2	29	1	
		Total	51	1	2
mus-51	50	1	30	0	
		2	29	1	
		Total	59	1	2
mus-52	50	1	29	0	
		2	30	0	
		Total	59	0	0
Wild type	100	1	20	0	
		2	30	1	
		Total	50	1	2
mus-51	100	1	8	2	
		2	13	0	
		Total	21	2	10
mus-52	100	1	14	0	
		2	32	2	
		Total	46	2	4
Wild type	500	1	40	4	
		2	40	3	
		Total	80	7	9
mus-51	500	1	40	35	
		2	39	37	
		Total	79	72	91
mus-52	500	1	30	28	
		2	39	36	
		Total	69	64	93
Wild type	1,000	1	9	3	
		2	22	7	
		3	40	6	
		4	40	7	
		Total	111	23	21
mus-51	1,000	1	4	4	
		2	8	8	
		3	10	10	
		4	8	8	
		Total	30	30	100
mus-52	1,000	1	31	31	
		2	19	19	
		Total	50	50	100

Bla-resist, blasticidin resistance; PFP-resist, PFP resistance.

Mutagen sensitivity. Mutagen sensitivity of the *mus-51* and *mus-52* was determined with spot tests using conidial suspensions. Camptothecin, 4-nitroquinoline 1-oxide, hydroxyurea, MMS, ethyl methanesulfonate (EMS), and bleomycin (BLM) were tested, as was sensitivity to UV. The mutants showed mild sensitivity to MMS, EMS, and BLM, but they were not sensitive to other chemical agents or to UV (Fig. 3).

Epistasis relationship. Double mutants were constructed by combining *mus-51* and *mus-52* with DNA repair mutants representing various epistasis groups. MMS was used to compare sensitivity of each strain. The *mus-51 mus-52* double mutant was identical in sensitivity to the single-mutant parents (Fig. 4*a*). The *mus-23* mutation (which is homologous to *S. cerevisiae MRE11*) (29) was epistatic to *mus-52* (Fig. 4*b*), but the double mutant carrying *mei-3* and *mus-52* was more sensitive than either

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parental single mutant (Fig. 4*c*). Therefore, *mus-51* and *mus-52* belong to the NHEJ group of recombination repair mutants, not to the HR group.

Targeting the *mtr* and *ad-3A* Genes by Using Wild Type, *mus-51*, *mus-52*, *mei-3*, and *mus-11* as Recipients of Transforming DNA. The *mtr* and *ad-3A* genes on chromosome IV and I, respectively, were selected as targets for gene disruption experiments. *mtr* mutants are resistant to the amino acid analog *p*-fluorophenylalanine (PFP). *ad-3A* mutants accumulate purple pigment (30). Targeting vectors were constructed by replacing the *mtr* or *ad-3A* ORF's with the blasticidin-resistance gene *bar*. A 2.7-kb DNA fragment containing the *bar* gene was cut from pBARGEM7–1 with restriction enzymes *Sca*I and *Sma*I.

In the case of *mtr*, the construction and introduction of targeting vectors was carried out as follows: pMTR, containing the *mtr* gene, was digested by *Msc*I to delete ≈1 kb containing the promoter and a part of the *mtr* ORF. A 2.7-kb *bar* fragment was inserted to make plasmid pGS1 (9.5 kb). This plasmid was digested by *Not*I, and the resulting 6.7-kb linear fragment carrying 1.8-kb 5′ and 1.9-kb 3′ of the *mtr* gene on both sites of *bar* was introduced into host strains of different genetic background by electroporation. Transformants resistant to blasticidin (200 μg/ml) were isolated and tested to determine whether they are resistant to PFP (20 μg/ml). If *bar* DNA is integrated into the *mtr* locus, the strain should be resistant to PFP. We further investigated by PCR whether the integration is through homologous replacement. Table 2 shows that 10–30% of the blasticidin-resistant transformants in the wild-type strain resulted from homologous integration. All transformants in the *mus-51* and *mus-52* strains resulted from homologous integration and replacement. There was little homologous integration in *mei-3* and *mus-11* strains, consistent with their defect in HR repair.

To confirm that transformants showing resistance to both blasticidin and PFP carry the *mtr* gene integrated homologously into genomic DNA, Southern hybridization analysis was carried out by using *mtr* as a probe (Fig. 5). Four transformants from each strain, wild type, *mus-51*, and *mus-52*, were isolated by random sampling. Genomic DNAs from these were digested by *Cla*I and electrophoresed for Southern analysis. In the wild type, one transformant (Fig. 5, lane 2) was resistant to both chemicals, indicating homologous integration, whereas the other three were resistant to blasticidin but sensitive to PFP, indicating ectopic integration. In *mus-51* and *mus-52*, all four transformants were resistant to both chemicals, indicating homologous integration.

Construction of an *ad-3A* targeting vector carrying 1.6 kb 5′ and 2.0 kb 3′ of the *ad-3A* gene on both sites of the *bar* was similar to the *mtr* construction, and the vector was introduced into host strains. Transformants resistant to blasticidin were isolated and subcultured in minimal medium supplemented with adenine for 10 days to confirm the pigmentation. The numbers of colonies with purple mycelia, indicating loss of *ad-3A* function, were scored. As shown in Table 3, 40% of blasticidin-resistant transformants from the wild-type strain had purple mycelia, whereas 100% of transformants in the *mus-51* and *mus-52* backgrounds were purple. PCR was used to confirm that the strains with purple mycelia were disruptants resulting from homologous replacement.

Relationship Between Targeting Frequency and Length of Homology. To investigate the relationship between the targeting frequency and the length of the homologous sequence, 50, 100, 500, and 1,000 base pairs homologous to 5′ and 3′ flanking DNA's of the *mtr* gene were amplified by PCR and attached to both sides of the *bar* gene. Blasticidin-resistant transformants were tested for resistance to PFP (Table 4). Few blasticidin-resistant transformants into which DNA had been introduced with 50- and 100-bp homology showed resistance to PFP, either in the *mus-51* and *mus-52* strains or in wild type. With 500-bp homology, <10% of blasticidin-resistant transformants in the wild-type strain showed resistance to PFP, whereas ≈90% in the *mus-51* and *mus-52* strains were resistant to PFP. With 1,000-bp homology, ≈20% of blasticidin-resistant transformants in the wild type were resistant to PFP, and all blasticidin-resistant transformants in the *mus-51* and *mus-52* strains were resistant. Greater than 1,000-bp homology is apparently required for completely efficient replacement of the target gene when NHEJ is not functional.

Discussion

We demonstrated that exogenous DNA is integrated into homologous sequences in the genome of *N. crassa* at a frequency of 100% in *mus-51* and *mus-52* strains, indicating that suppression of NHEJ increases the frequency of HR. This finding has strong implications for recombination technology, because it is easy to make genetic alterations in the *mus-51* or *mus-52* mutants as recipients for transformation, and because these mutants do not show severe defects, but differ from wild type only in being moderately sensitive to mutagens that produce double strand breaks.

Systematic gene disruption is important for determining the functions of target genes and for adding new markers and reporters in studies of gene expression. The virtually complete *Neurospora* genome sequence is now available for searching (14, 31). Sequences of potential target genes are easily amplified by PCR even if they are >1 kb long. The *mus-51* and *mus-52* mutations are marked with Hyg^r, and once a targeted gene has been disrupted, the *mus* mutation can easily be eliminated by crossing to a wild type strain and isolating hygromycin-sensitive progeny.

Until now, ability to achieve 100% homologous integration of transforming DNA has been a unique advantage of *S. cerevisiae*. Knocking out targeted genes in other eukaryotes has been far less efficient. Our findings with *Neurospora* change this situation. They should be widely applicable to other systems.

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