An Appropriate Increase in the Transcription of *Aspergillus nidulans uvsC* Improved Gene Targeting Efficiency

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Gene targeting to knock out the activity of specific genes has become important due to recent progress in genomics research. But this technique is still unavailable for many organisms, including economically important microorganisms, due to the high background of ectopic integration during genetic transformation. Strategies to improve targeting efficiency have included manipulating the expression of genes that are involved in homologous recombination. In this study, transcription of *Aspergillus nidulans uvsC* was elevated using the promoter sequences of the glyceraldehyde-3-phosphate dehydrogenase and Taka-amylase A genes from *A. nidulans* and *A. oryzae* respectively. Although a several-fold increase in the efficiency of targeting was observed at 3 loci, mycelial growth was suppressed in strains that had higher levels of *uvsC* transcription. These results suggest that *uvsC* is a rate-limiting factor in gene targeting, and that the increased efficiency of this targeting is hindered by a negative effect of increased transcription on cell proliferation.

Key words: *Aspergillus nidulans*; *uvsC*; gene targeting efficiency; genetic transformation

Foreign DNA fragments can be incorporated into a cell and further integrated into a chromosome when subjected to relatively simple conditions.1,2 In principle the integration of extracellular DNA fragments is classified as homologous and ectopic (or illegitimate if the incoming DNA does not have a sequence homologous to the targeting genome). In the budding yeast *Saccharomyces cerevisiae*, homologous integration predominates over ectopic integration.3,4 Gene targeting depends on this fact and is a powerful tool for studying the role and function of genes of interest. The importance of this technique has therefore been increasing with the recent completion of genome sequencing projects. In *S. cerevisiae*, the Yeast Genome Deletion Project created sets of mutants in which nonessential genes were deleted through efficient gene targeting.4 But in most plants and animals, including human, ectopic integration occurs almost exclusively.5,6 Hence creation of knock out lines using a gene targeting approach in these organisms is generally limited, with some exceptions such as mouse ES, chicken B cell lines, and *Physcomitrella patens*.7–9 Because of this, great efforts have been made to improve targeting efficiency in these recalcitrant organisms.3,10,11

One of the strategies for improving gene targeting efficiency is to control the expression of genes involved in this process when homologous recombination between extracellular DNA and homologous sequences on a chromosome occurs. The molecular mechanisms of homologous recombination have generally been explained by the double-strand break (DSB) repair model.12 In *Escherichia coli* and *S. cerevisiae*, recA and RAD51 have been shown to be critical factors in homologous recombination and DSB repair respectively.13,14 The RecA and Rad51 proteins have been shown to be responsible for at least two steps during homologous recombination, namely homology search and strand exchange between two DNA strands.15 In *S. cerevisiae*, mutations in RAD51 caused a 10-fold decrease in both illegitimate and homologous integration.16 In addition, site specific disruption of the RAD51 ortholog from the filamentous fungus *Penicillium paxilli* resulted in a less frequent occurrence of direct repeat formation during illegitimate integration.17 Further-
more, a null mutant of the *Aspergillus nidulans* ortholog of *RAD51*, *uvsC*\(^{18,19}\) showed deficiency in homologous integration.\(^{20}\) These results indicated that *RAD51* orthologs are involved in homologous recombination during DNA integration. Yanez and Porter\(^{21}\) reported that increased expression of human Rad51 protein in a human cell line enhanced both the efficiency of gene targeting and resistance to ionizing radiation by 2- to 3-fold and 6-fold respectively. The introduction of RecA protein, fused with a nuclear localization signal, to mouse cells increased targeting efficiency by 10-fold.\(^{22}\) But these successes have been tempered by the recent report that overexpression of human Rad51 protein in a human cell line decreased cell viability and growth rate and increased the proportion of cells undergoing apoptosis.\(^{23}\) In addition, overexpression of Rhp51, the *A. nidulans* orthologs on DNA integration. In this paper, we describe for investigating the effects of overexpression of *RAD51* orthologs are involved in homologous recombination. We have investigated the effects of overexpression of *RAD51* on DNA integration. In this paper, we describe the overexpression of *uvsC* transcription on the efficiency of gene targeting and cell proliferation are described.

**Materials and Methods**

**Fungal strains, media and genetic transformation.*** A. *nidulans* strains DC1 (*pyrG89*/*argB2*), ATr-61 (*pyrG89*/*argB2*; *pyr-4*), and ATr-60cd1 (*argB2*/*pyrG89*; *uvsC*/*pyr-4*) were as described in Ichioka et al.\(^{20}\) ATr-61 and ATr-N143 (used as control strains) were obtained by illegitimately inserting the *Neurospora crassa* *pyr-4* gene by genetic transformation. Standard *Aspergillus* minimal medium (MM) was prepared as described.\(^{26}\) Minimal medium was supplemented with arginine and pyrimidine (uridine and uracil) at 7.5 mM and 5 mM respectively. Genetic transformation was performed using the method described by Ichikawa et al.\(^{20}\) which was modified essentially from Yelton et al.,\(^{25}\) except that Lysing enzyme (Sigma, catalog no. #L1412) was replaced by Tatalase (Takara) and used at 20 mg/ml. When ptrA was used as the selectable marker, pyrithiamine was added at a final plate concentration of 0.1 µg/ml. Transformants are indicated by the prefix ATr-. All strains used and analyzed in this study were conidium-purified as described by Itoh et al.\(^{27}\) and maintained on Silica-gel (Nacalai) at 4°C.

**Nucleic acid manipulations.** Standard procedures were employed for restriction and modification enzyme reactions, PCR, cloning, and Southern blot analysis.\(^{28}\) Chromosomal DNA of *A. nidulans* was extracted from lyophilized mycelia obtained from shaking cultures as described by Yoder,\(^{29}\) with minor modifications.\(^{27}\) Restriction and modification enzymes were purchased from Takara, Toyobo, and Nippon gene. Oligonucleotide primers, used for PCR reactions, were purchased from Sigma Genosys. Unless otherwise noted, PCR products were cloned into pGEM-T Easy vector (Promega). The DIG DNA labeling and detection kit (Roche Diagnostics) was used to identify fragments that hybridized to digoxigenin (DIG)-dUTP labeled DNA probes.

**Plasmid constructions.** Media for routine growth and maintenance of *E. coli* were as in Sambrook and Russell.\(^{20}\) Plasmid DNA was isolated by either the Wizard MiniPreps DNA Purification System (Promega) or the Quantum Prep Plasmids Midiprep Kit (Bio-Rad) and used according to the manufacturer’s instructions. Plasmid pMS12 (*argB*), pDHG25 (AMA1 plus *argB*), and pFB6 (*N. crassa pyr-4*) were obtained from the Fungal Genetics Stock Center. Plasmid pTN2 that had *pyr-4* and *Pgd* fused to *uvsC* (*Pgd/uvsC*) was constructed as follows. A 2.6-kb *Pgd* fragment was amplified from pAN7-1\(^{30}\) with a set of primers (TN#020: 5'-CCCTGATTCTGTGGATAACCGTATT-3' and TN#021: 5'-GTCACHTGAGTGTGATGTTGTCGTC-3'), digested by *Pst* and the resulting 2.2-kb fragment was subcloned into pUC18 to give rise to pTN3-1. A 3.2-kb *BglII* fragment containing *pyr-4* from pFB6 was subcloned into pTN3-1 to give pTN4. A 4.6-kb *PstI* fragment that contained *pyr-4* and *Pgd* was excised from pTN4 and inserted into the *PstI* site of pMRC1\(^{20}\) to create pTN2. A 0.6-kb fragment containing *PtrA* was amplified from pCAME-3 with a set of primers (TN#109: 5'-CTAGATGATCTTCAAAAATGCCTCT-3' and TN#110: 5'-CATGGGATTCTTGATCATTCTATT-3') and subcloned into pGEM-T Easy to give rise to pTN5a. A 0.7-kb *NsiI* fragment containing *PtrA* was subcloned into pTN5a and subcloned into the *PtrA* site of pMRC1 to give pTN6a. A 2.4-kb *PstI* and *BglII* fragment containing *pyr-4* was excised from pFB6, blunt-ended, and subcloned into the blunt-ended *NotI* and *SpeI* site of pTN6a to give pTN7a. *PtrA*, which induces the transcription of downstream genes through maltose induction, was used to replace *Pgd* of pTN2 to construct pTN7a. An end-in event with these plasmids results in an in-frame insertion of 4.6- and 3.0-kb fragments, containing *pyr-4* and either *Pgd* or *PtrA* respectively, into the *PtrA* site that resides 2-bp downstream of the start codon of *uvsC*. Transformants were first screened for such insertion events by PCR with a set of primers flanking the targeted *PtrA* site (TN#018: 5'-
Construction of A. nidulans strains that had increased transcription of \textit{uvsC}

To elucidate the effect of increased transcription of \textit{uvsC} on homologous integration, \textit{A. nidulans} strain DC1 was transformed with pTN2 and pTN7a. Both plasmids were linearized with \textit{EcoRI} and used for genetic transformation. Four positive candidates were obtained from 33 transformants with pTN2 and were further examined by Southern blot analysis. Three of these showed the integration profiles expected for the insertion event by gene conversion (Fig. 1B and C). One transformant was designated ATr-N22 and used in further experiments. Similarly, 7 out of 27 transformants with pTN7a were positive during PCR screening. But none of these showed the integration profile by Southern blot analysis that was expected for a simple insertion event. Two such representative transformants, ATr-N112 and -N114, are shown in Fig. 1C. Further analysis with different restriction digests and probes indicated that multiplecopies of the 5.5-kb fragment containing \textit{P\textsubscript{taa}}/\textit{uvsC} together with the backbone pUC18 sequence were integrated in tandem array in these transformants. When conidia of these transformants were inoculated onto inducible plate, colony formation was suppressed, though not completely inhibited (Fig. 2). The colonies showed a thinner layer of mycelia than the wild type, and the conidiation of the transformants was also affected under both inducible and non-inducible conditions. Furthermore, revertants that grew comparably to the wild type were obtained from these transformants by prolonged growth on inducible plates (Fig. 2). These revertants (ATr-N112r1 and -N114r1) lost most of the copies that integrated originally.

Expression analysis was carried out by extracting total RNA from mycelia prepared from liquid shaking cultures that were treated with maltose or MMS. Genes for \textit{\beta}-tubulin, \textit{benA} and \textit{tubC}, were used as internal controls. Since \textit{tubC} is more specifically transcribed during conidiation\textsuperscript{31} and the ratio of \textit{benA} to \textit{tubC} was not significantly different between strains and treatments, only \textit{benA} was used to normalize \textit{uvsC} transcript levels. To compare the relative transcription level of \textit{uvsC}, real-time PCR analysis was performed. Since the melting curves of \textit{benA}, \textit{tubC}, and \textit{uvsC} exhibited a single peak, it was considered that the specific PCR products were amplified so that real-time plots were reliable. The results of quantifying the \textit{uvsC} transcript are shown in Table 1. Transcription of \textit{uvsC} was induced by MMS as previously reported.\textsuperscript{19} In ATr-N22, an increase of up to 150-fold in transcript levels was observed compared to ATr-61 in the absence of MMS. In ATr-N112, transcription of \textit{uvsC} was increased 70-fold by maltose induction. This corresponded to an increase of about 400-fold compared to ATr-61 without any treatment.
Effect of increased transcription of uvsC on homologous integration

The efficiency of homologous integration in strains overproducing uvsC transcript was examined at three loci, \textit{argB}, \textit{wA}, and \textit{yA}. The transformant ATr-N22 was chosen for the subsequent analysis, because the colony formation of the pTN7a transformants (ATr-N112 and -N114) was severely suppressed (Fig. 2). The \textit{wA} and \textit{yA} loci are required for the production of a dark green pigment in the conidial cell wall of \textit{A. nidulans}. Mutants

![Diagram](https://example.com/diagram.png)

**Table 1.** Quantification of the Relative Transcript Levels of uvsC

<table>
<thead>
<tr>
<th>Strain Treatment</th>
<th>Relevant characteristics</th>
<th>Relative transcript level(^a) (uvsC/benA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATr-61</td>
<td>Control strain</td>
<td>1.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose(^b)</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>MMS(^b)</td>
<td></td>
<td>14.7</td>
</tr>
<tr>
<td>ATr-N22</td>
<td>uvsC under control of Pgpd</td>
<td>148.2</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATr-N112</td>
<td>uvsC under control of Ptaa</td>
<td>5.7</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>402.2</td>
</tr>
</tbody>
</table>

\(^a\) Copy number of uvsC and benA cDNA per 1 \(\mu\)g of total RNA was calculated using standard curves that were obtained from known amounts of the standard templates. Numbers are shown as ratios relative to the control strain, ATr-61.

\(^b\) Refer to “Materials and Methods” for detailed procedures.
at the \( yA \) locus produce yellow conidia, while \( wA \) mutants produce white conidia. \( wA \) mutations are epistatic to \( yA \) mutations.\(^{32,33}\) Transforming plasmids were constructed so that transformants that had homologous integration events could be scored by observing colony color (Fig. 3). An ends-in event with pTN40, linearized with \( SmaI \) (pTN40/\( SmaI \)), results in the insertion of a 6.3-kb pTN40 fragment into the \( SmaI \) site of the \( yA \) ORF. Similarly an ends-in event of pTN43, linearized with \( EcoRV \) (pTN43/\( EcoRV \)), results in the insertion of a 7.1-kb pTN43 fragment into the \( EcoRV \) site of the \( wA \) ORF (Fig. 3). These events yield transformants with yellow and white conidia respectively, although some transformants contained both wild-type (green) and mutant populations due to the multi-nucleate nature of protoplasts and the uninucleate nature of conidia. In order to limit the possibility of under-estimating targeting efficiency because of ambiguous colonies, such colonies were re-inoculated onto new selective plates to confirm their phenotype.

To determine the effect of \( uvsC \) overexpression on gene targeting, ATr-61 and ATr-N22 were transformed with 2 pmol of targeting plasmids. In the case of pTN40/\( SmaI \), a higher targeting frequency was observed in ATr-N22 with a 4.6-fold difference (Table 2). A higher targeting frequency was also obtained with pTN43/\( EcoRV \), but the difference was less: 2-fold. Because the number of transformants obtained with pPTR1 linearized with \( KpnI \) and the viability of protoplasts did not differ, increased transcription of \( uvsC \) clearly resulted in an improvement of targeting efficiency.

Homologous integration was further evaluated at the \( argB \) locus. The \( argB2 \) mutation was identified as a deletion of a single cytosine nucleotide at position 248 from the start codon, resulting in the generation of a stop signal at codon 115.\(^{20}\) A system in which only homologous integration events were detectable was constructed (Fig. 4). In brief, DNA fragments that contained the \( argB \) sequence but not the complete ORF were prepared. Using these fragments, only trans-

### Table 2. Effect of Increased Transcription of \( uvsC \) on Homologous Integration at the \( yA \) and \( wA \) Loci

<table>
<thead>
<tr>
<th>Colony color</th>
<th>ATr-61</th>
<th>ATr-N22</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTN40</td>
<td>Yellow</td>
<td>1.7(^a)</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>27.0</td>
</tr>
<tr>
<td>Targeting frequency (%)(^b)</td>
<td>5.9</td>
<td>26.9</td>
</tr>
<tr>
<td>pTN43</td>
<td>White</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>47.3</td>
</tr>
<tr>
<td>Targeting frequency (%)(^b)</td>
<td>9.0</td>
<td>17.3</td>
</tr>
<tr>
<td>pPTR1</td>
<td>Green</td>
<td>21.0</td>
</tr>
</tbody>
</table>

\(^a\) Average number of transformants per \( 5 \times 10^6 \) protoplasts from three transformation experiments with independently prepared protoplasts is shown.

\(^b\) Frequency of gene targeting is shown as the ratio of the number of colored (yellow or white) colonies to the total number of transformants.

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**Aspergillus nidulans**

GenBank (M19158). The numbers in parentheses indicate lengths of homologous sequences. The effects of increased transcription of \( uvsC \) on gene targeting are shown as relative values to the control strain, ATr-61. The competency of host strains was normalized by dividing the number of transformants obtained with these fragments to that obtained with pDHP25 containing autonomously replicating sequences. These numbers were compared between ATr-61 and ATr-N22 by dividing the number of transformants obtained from ATr-N22 by that of the control strain (ATr-61). Averages of three independent experiments are shown. The oligonucleotide primers used for obtaining these fragments by PCR are indicated by arrowheads with their positions. The filled circles at the left end of fragments are derived from pMS12 and indicate artificial sequences that are not homologous to chromosomal DNA. The gray arrow, black box, and open box indicate the \( argB2 \) ORF, \( argB \) sequence, and \( 5\prime \)-flank sequence of \( argB \) respectively. Abbreviations for restriction enzymes are: \( H \), \( HinI \); \( BglI \); \( EcoRI \); \( KpnI \); \( SalI \); \( SphI \); \( SmaI \); \( SpI\); \( SpH\).
formants that had homologous integration at the argB locus with ends-out events were scored. No transformant was obtained with these fragments from ATr-60cd1 in which uvsC was disrupted and homologous integration was not observed, indicating that the background of spontaneous mutation was insignificant. In addition to the argB containing fragments prepared by PCR (fragments 1 to 3), cloned fragments of this locus that were subcloned from pMS12 were also used (fragments 4 and 5). It should be noted that a short region of non-homologous sequence derived from the multiple cloning site of the vector remained at one end. Both strains, ATr-61 and -N22, were transformed with 1 pmol of each fragment and 0.1 pmol of pDHG25 which was used for normalizing the competency of protoplasts. A level of increase in the efficiency of homologous integration similar to that at the yA and wA loci was observed in ATr-N22 compared to ATr-61: 2-fold for fragment 2 (771-bp of PCR product), and 3- or 2.4-fold for fragments 4 and 5 (the Sall and HindIII fragments of pMS12) respectively. In addition an increase in targeting efficiency was seen for fragment 3. But a decrease in efficiency was observed for fragment 1 (0.6-fold). Therefore, with the exception of the shortest fragment examined, elevation of uvsC transcript levels led to improvements in targeting efficiency during homologous recombination.

Discussion

In many eukaryote cells, from fungi to human, ectopic integration of extracellular DNA dominates over homologous integration. Hence improving the efficiency of gene targeting is of great interest to both basic and applied researchers. To achieve this purpose, two strategies can be considered. One is either to increase the efficiency of homologous integration or to prevent ectopic integration, and the other is to develop a screening system in which only homologous recombination events are detected. In this study, improvement in targeting efficiency was attempted by increasing the level of transcription of A. nidulans uvsC, the ortholog of S. cerevisiae RAD51.

Strains were constructed by inserting either a constitutive or an inducible promoter, Pgpd or Ptaa respectively, upstream of uvsC from its ORF. The simple gene conversion with Ptaa construct, pTN7a, was not obtained. Lesser activity of pyr-4 that was caused by the negative effect of the downstream sequence in pTN7a was a possible interpretation. This result, however, demonstrated more clearly the suppressive effect of an increased level of uvsC transcription in pTN7a transformants on colony growth (Fig. 2 and Table 1). Such a suppressive effect was an important observation, as it highlights an intrinsic limitation of modifying the activity of the fundamental cellular pathway, as in recombinational repair. Possible explanations for the observed suppressive effect considered in relation to the role of RAD51 orthologs in vivo are two-fold. First, aberrant recombination might occur in these transformants. Since Rad51 protein plays a critical role in homology search and the strand invasion reaction, high levels of UVSC protein might promote homologous recombination between sequences in which recombination is usually repressed, for example, between homologous sequences that reside on different chromosomes. Such events should result in instability of the genome. Secondly, recombinational repair by interacting with proteins that belong to the RAD52 epistasis group. Increased levels of UVSC protein might sequester the repair complex from genomic DSB sites that must be repaired. Which of these scenarios is true cannot be answered from the present study, but there should be a threshold between ATr-N22 and pTN7a transformants for suppression of colony formation, since ATr-N22 did not show any visible abnormality (Fig. 2 and Table 1).

The effect of increased transcription of uvsC on homologous integration was assayed with a constitutively overexpressing Pgpd/uvsC strain. At 3 loci examined, with different vector constructs, the targeting efficiency was improved several-fold (Fig. 4 and Table 2). This level of improvement was in the range of attempts in other organisms. In human cells overexpressing Rad51 protein, a 2- to 3-fold increase in the ratio of gene targeting has been reported. Introduction of the RecA protein fused with a nuclear localization signal increased the ratio of targeting by 10-fold in mouse cells. These modest improvements suggest that there might be an intrinsic limit to improving targeting efficiency by increasing the activity of RecA/Rad51 homologs. This could be reasoned as follows. First, the negative effect of increased transcription and expression of RAD51 orthologs on cell proliferation might mask the increase in the efficiency of homologous integration. This notion is compatible with the results obtained in this study and in others. The frequency of genetic transformation by illegitimate integration can be increased by the addition of restriction enzymes, owing to additional DSBs. But this improvement is hampered by the lethality of excess amounts of enzyme. Although the mechanisms operating here should be different to those in which UVSC is increased, the two are similar with respect to modification of extracellular DNA integration and cell lethality, by changing the amounts of cellular enzymes. The notion that this negative effect might mask dramatic increases in targeting efficiency can be applied to the results obtained for mammals. In the human cell, a 10-fold expression of human Rad51 protein resulted in suppression of cell proliferation accompanying various abnormal phenotypes. In contrast, a 4-fold overexpression of human Rad51 protein increased targeting efficiency by about 2-fold without any detectable negative effect. Secondly, although
Rad51 protein is certainly a physically rate-limiting factor during homologous integration, another factor might become limiting when excess amounts of Rad51 protein are provided. Because Rad51 protein is involved in the early steps of homologous recombination, initiation of recombination might become a rate-limiting step. It has been reported that introduction of DSBs into a target locus by inducible expression of I-SceI resulted in significant increase in targeting efficiency in tobacco,\(^7\) indicating that DSB at the target locus might be a limiting factor.

In conclusion, the results shown in this study suggest that *uvsC* is, at least to some degree, a rate-limiting factor in homologous integration. Although the increase in targeting efficiency was comparable to levels reported in mammals, a negative effect of increased transcription of *uvsC* on cell proliferation was also observed. Therefore, such manipulations must concentrate on better control of the transcription and expression of the gene(s) of interest, without affecting normal cellular processes. A further implication of this study is the role of *uvsC* in the competency of cells for genetic transformation. The nature of nuclei determines the fate of DNA integration in *N. crassa*,\(^38\) but the molecular mechanisms remain to be resolved. If a competency for integration exists, attention should be given to the difference between normal and competent cells, because such differences are likely to have effects on the cell. Clearly, *uvsC* has an effect on both the efficiency of extracellular DNA integration, especially for gene targeting, and cell proliferation.

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**References**


