An efficient method using Cre recombinase-mediated cassette exchange for N-terminal tagging of Schizosaccharomyces pombe proteins

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Abstract

In this study we designed an N-terminus protein tagging protocol using a Cre recombinase-mediated cassette exchange method. A Schizosaccharomyces pombe strain that carried null allele of ura4 and leu1 genes was genomically modified by insertion of two lox sites, one original (loxP) and the other mutated (loxM3) preceded by ura4 gene, flanking the whole rad22 gene including the genes promoter. This base strain was transformed with the plasmid pAW8 carrying the sequence loxP-promoter-EGFP-rad22-loxM3, LEU2 selection marker, and the P1 bacteriophage Cre recombinase gene. Gene replacement between plasmid and chromosome occurred with high efficiency. We then assessed the phenotype of the strains expressing the N-terminal EGFP-Rad22 fusion protein and found no anomaly with respect to gamma and genotoxic sensitivity, foci formation and the dynamic in S-phase after hydroxyurea release. Together with other previously published protocols aiming genome modifications using Cre recombinase-mediated cassette exchange, this methodology may constitute a system for easy and accurate genome modifications for experimental studies.

Keywords: RMCE, epitope tag, Cre recombinase, linker

1. Introduction

An epitope tag attached to a gene is a useful tool for the characterization of proteins behaviour in various experimental applications. Tags can be attached to either their N or C termini; there are methods described for N-terminal tagging involving either two homologous integration steps or a one step process with the gene not under the original promoter (1, 2).

In this work we describe an N-terminal tagging protocol based on the recombination mediated cassette exchange (RMCE). Using this procedure a C-terminus tagging method has already been described in both essential and non-essential genes. Manipulating pAW8 a plasmid containing a multiple cloning site flanked by loxP and loxM3 heterospecific recognition sites for cre-recombinase and using Cre/lox site-specific recombination system, C-termini tags has been attached to essential and non-essential S. pombe genes. Genes tagging has been performed by RMCE between a chromosomal cassette termed loxP-ura4-loxM3 integrated at the 3’ end of the target gene from a "base strain" and a loxP-TAG-loxM3 cassette located on a pAW8 plasmid (3).

We adapted a variant of this protocol to place yEGFP (codon optimised EGFP) tag (4) at the N-terminal end of a gene in a one step procedure. In this case a loxP-yEGFP-linker-target gene-loxM3 cassette placed on the plasmid pAW8 was changed with a chromosomal loxP-target gene-ura4-loxM3 cassette from a S. pombe “base strain”. The base strain had...
loxP and ura4'-loxM3 inserted so as to flank the whole of the rad22 gene including its promoter (ura4' gene served as a selectable marker).

Rad22 – a multifunctional protein involved in various recombination processes and in DNA double-strand break repair whose functions are still under debate was chosen as the target protein for N-terminus tagging. Rad22 has been successfully tagged at its C terminus (5, 6, 7) but, to our knowledge, not at its N-terminus possibly due to the N-terminal tag adversely affecting the protein’s function. In this report we detail the protocol of Rad22 yEGFP N-terminal tagging and test the functionality of the tagged protein.

2. Materials and methods

Fig. 1 presents our strategy of EGFP-N-tagging of Rad22. We started with a previously created plasmid pAW8_rad22+ (rad22 promoter and rad22 gene sequence cloned in pAW8 between loxP/loxM3 sites). A Nhel restriction enzyme site was inserted directly upstream of the ATG of rad22 gene in the plasmid pAW8_rad22+ to create pAW8_Nhel_rad22+ (Fig. 1A). Separately, we cloned a yEGFP-linker as a Nhel fragment (EGFP sequence without STOP codon together with the linker sequence) in pAW8ENdel-cyEGFP and then subcloned EGFP-linker in the plasmid pAW8_Nhel_rad22+ (Fig. 1B). The rad22 base strain ((Nt) loxP-rad22-ura4'-loxM3 (Ct) ade6-704 leu1-32 ura4-D18 h-) was transformed with the plasmid pAW8_NyEGFP_Nhel_rad22+ and the N-terminally tagged Rad22 strain was obtained by RMCE (figure 1C).

2.1. Introduction of Nhel restriction site. Insertion of restriction endonuclease site Nhel into pAW8-rad22+ plasmid directly upstream of the start codon of rad22 was carried out by mutational PCR using the following primers:

5’ TAGAAGGgcgtacATGTCTTTTGAGCAAAAACAGCA 3’ and
5’ CTAATATACTGCTTATATAAGCTAGAAGGgcgtacATGTCTTT 3’ (lower case represent the Nhel I sequence). The PCR reaction contained 10ng pAW8_rad22 DNA template, 0.25µM primers, 0.2mM dNTPs, 10x PfuTurbo Polymerase buffer, 2.5U PfuTurbo DNA Polymerase (Agilent Technologies). The PCR programme: 95°C for 2 minutes followed by 17 cycles of 95°C 30 seconds, 55°C 60 seconds, 68°C 20 minutes. Reaction mixture was incubated with 20U of DpnI restriction enzyme for 1 hour at 37°C and purified with PCR clean-up (QIAquick PCR Purification Kit) and transformed in competent DH5alpha cells. The resulting plasmid, termed pAW8_Nhel_rad22+ was sequenced to confirm insertion of the Nhel I site (figure 1A).

2.2. The construction of yEGFP-linker. In order to obtain the insert yEGFP-linker as Nhel fragment from the plasmid pAW8ENdel-cyEGFP the primers 5’-aaaGCTAGCATGCTAAGGTTGAAAGATTATTTCACTGGTGT-3’, 5’ GGTATACCATGCAGGTATGGATGAATT GTACAAAgctgttctacagatcacaacggtctccac gGCTAGCtttt-3’ were designed to introduce Nhel restriction site (outlined sequences for Nhel I site) and a sequence (lower case) for a T G S T G S T G S T translated linker. This hinge has to be structurally flexible assuring the independently moving of the chromatophore and the tagged protein not interfering with their three-dimensional shape (W. WRIGGERS & al. [8]). The PCR reaction contained 50ng pAW8_Ndel_cyEGFP DNA template, 0.3µM primers, 0.2mM dNTPs, 10x PfuTurbo Polymerase buffer, 2mM MgSO4, 0.02U/µl KOD HotStart DNA (Novagen). The PCR programme: 94°C for 3 minutes followed by 30 cycles of 94°C 15 seconds, 58°C 30 seconds, 72°C 30 seconds. The PCR product was run in a gel 1% and gel purified.

2.3. Restriction digests and ligation. The plasmid pAW8_Nhel_rad22+ and yEGFP-linker were restriction digested with Nhel restriction enzyme according to the manufacturer’s conditions (NEB, Ipswich, MA). The restricted vector pAW8_Nhel_rad22+ was treated with
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alkaline phosphatase (NEB, Ipswich, MA) and the vector and yEGFP-linker insert were ligated in a 1:2 ratio using T4 DNA Ligase (NEB, Ipswich, MA). The resulted plasmid, termed pAW8_NyEGFP _Nhe1 rad22+ was transformed in competent DH5alpha cells and the construct confirmed by sequencing (figure 1B).

### 2.4. *S. pombe* transformation and cassette exchange.
Rad22 base strain construction has been performed in two homologous integration events as described (3). In a first step it was introduced a loxP _ura4^-loxP sequence amplified from the pAW41 upstream of the promoter of rad22 gene. This strain has been transformed with Cre-expression pAW5 plasmid resulting only one loxP after cre recombinase removing of _ura4^+. In a second step an _ura4^-loxM3 cassette from the pAW12 has been integrated downstream of stop codon of rad22. This base strain was transformed with pAW8_NyEGFP _Nhe1 rad22+ using lithium acetate procedure as described by BAHLER & al. (1) (figure 1C).

**Figure 1.** Construction of plasmid pAW8_NyEGFP _Nhe1 rad22+ and NyEGFP-rad22 strain. (A) Introduction of _Nhe1_ site directly upstream of the _rad22_ start codon using site directed mutagenesis. _P rad22_=_rad22_ promoter sequence. (B) The yEGFP-linker fragment was cloned into the _Nhe1_ site of the pAW8_ _Nhe1 rad22+_ vector as a _Nhe1_ fragment. (C) The transformation of rad22 base strain with the plasmid pAW8_NyEGFP _Nhe1 rad22+ and cassette exchange following Cre expression. The _P rad22_ after loxP is not shown in Fig. 1C for simplicity. _Pnmt41_=_Saccharomyces pombe nmt41_ promoter sequence, _Tnmt_=_nmt_ terminator sequence, _LEU2_=_Saccharomyces cerevisiae_ leucine selectable marker, _CRE_ Cre recombinase coding sequence, _Cre_ Cre recombinase
Following transformation procedure the cells were plated onto EMM (Edinburgh minimal media) plates supplemented with adenine 10mg% and thiamine 15 µM. The colonies formed after 5 days at 30°C were re-streaked onto EMM+A+T plates. Colonies were grown in YE (0.5% yeast extract, 3% glucose) at 30°C to saturation and 10,000 cells plated onto 5-FOA to select the ura- cells. 5-FOA resistant colonies were replica plated onto EMM plates supplemented with adenine and uracil to confirm plasmid loss. The cells leu-, ura- were checked for EGFP tagged rad22 by PCR with primers upstream of yEGFP and from the rad22 gene sequence.

2.5. Spot test assay. The cells were grown to 1x10^7 cells/ml in YE and 10-fold serial dilutions were spotted on plates with YEA and YEA containing DNA damaging agents: MMS (0.005%), CPT (5µM) and HU (6mM). For gamma sensitivity the cells were irradiated with 50Gy, 100Gy, 200Gy in YE liquid, diluted as above and spotted onto YEA plates. Plates were grown at 30°C until colonies had formed.

2.6. HU treatment and FACS analysis. For the synchronous HU arrest experiments S. pombe cells were grown in YE media to a density of approximately 4-5 x 10^6/ml, and HU was added to a final concentration of 12 mM. The culture was collected by centrifugation after 4 h at 30°C washed once in YE, and re-suspended in YE. Samples were taken immediately after washing and at 20 minute intervals up to 120 mins and fixed with ethanol 70%. The DNA content of propidium iodide stained cells was then analyzed using a FACSscan machine (Becton Dickinson FACSscan) and CELLQuest software according to the protocol described by E.B. GOMEZ & S.L. FORSBURG (9).

2.7. Microscopy. Microscopy was performed using the Delta Vision microscope system (Applied Precision, Issaquah, WA). For fixed cells, 10 Z-axis sections at 0.4µm intervals were combined using the softWoRx quick projection program. Cells that were grown to 1X10^7/ml in YE at 30°C collected by centrifugation and suspended in 100µl of 1 µg/ml Hoechst 33342 and incubated for 5 min at room temperature in the dark. After incubation 1ml methanol was added and the tubes incubated on ice for 10 minutes. The cells were pelleted and washed with 1 ml of acetone and pelleted again. 10 µl of the pellet was spotted onto 0.6% low melting agarose in 0.1 M Tris-acetate pH 8.5. Each set of images for EGFP and Hoechst 33342 were obtained at 25–26°C.

3. Results and Discussion

The plasmid pAW8_NyEGFP NheI rad22+ was created in which yEGFP followed by the linker sequence was fused in-frame with the rad22 coding sequence. This plasmid, also containing the Cre recombinase gene and the S. cervisiae LEU2 selection marker, was transformed into S.pombe rad22 base strain where the rad22 gene is flanked by loxP site and a ura4+-loxM3 sequences.

3.1. In MMS challenged cells EGFP-Rad22 delocalizes from a diffuse nuclear distribution to distinct foci. NyEGFP-Rad22 was visualized in unsynchronized mitotic cells using epifluorescence microscopy. As described previously for Rad22 with fluorescent tags at the C-terminus, in asynchronous cells the yEGFP Rad22 signal had a diffuse nucleoplasmic distribution in most of the cells, with occasional observation of spontaneous nuclear foci (10). In our experiments too, the simultaneous staining of the DNA with Hoechst showed that in most cells NyEGFP-Rad22 displays a diffuse nuclear localization throughout the mitotic cell cycle with foci formation in a few cells (figure 2A).

Similar to C-terminal tagging (10), yEGFP signal appears to delocalize from a diffuse nuclear distribution to distinct foci (majority of cells has 2-3 foci) following MMS genotoxic exposure (figure 2B).
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Figure 2. The NyEGFP-rad22 signal (green) and chromosomal DNA (stained with Hoechst 33342-blue) of exponentially growing NyEGFP-Rad22 cells observed using a Delta Vision microscope system attached to an inverted Olympus IX70 microscope. Visualization of NyEGFP tagged Rad22 in asynchronous cells (A) and in cells treated 4 hours with MMS 0.005% (B). The scale bar, 5 µm.

3.2 The EGFP-Rad22 showed normal DNA repair capacity and lack of thermosensitivity. There are reports describing alterations of folding properties by using GFP as a tag (11), resulting in slow maturation. We investigated this possibility by comparing growth of cells at 30°C and 37°C by a spot assay. The NyEGFP-Rad22 strain did not exhibit thermosensitivity at 37°C; colony growth is similar to that at 30°C (figure 3A). This demonstrates that Rad22 function is unaffected by N-terminal yEGFP tag. We also investigated the function of the new strain NyEGFP-Rad22 in DNA repair processes and compared to WT ((Nt) loxP-rad22-\textit{loxM3} (Ct) \textit{ade6-704 leu1-32 ura4-D18 h-}) by exposure to γ radiation and genotoxins such as MMS, HU and CPT. Because the Rad22 mutants are very sensitive to gamma irradiation (12) we chose 50, 100, 200 Gy doses of ionizing radiation. The NyEGFP-Rad22 strain showed similar sensitivity to WT after gamma radiation (50Gy, 100Gy, 200Gy) (figure 3B) 0.005% MMS 5uM CPT and 6mM HU (data not shown). Our data does not show significant strain differences in terms of genotoxic sensitivity or temperature influence (figure 3).

Figure 3. A. Wild type and NyEGFP-Rad22 cells in serial dilution were grown at 30°C (A1) and 37°C (A2). The pictures are taken after 4 days of incubation. B. Pictures taken at 5 days after exposing the cells at 50Gy (B1), 100Gy (B2) and 200Gy (B3) of gamma irradiation.
3.3. The N-terminal tagging of Rad22 does not cause a delay in replication. We synchronised NyEGFP-rad22 and WT strains in S-phase using HU and monitored the release using flow cytometry. In our experiment we compared the strains regarding the ability to recover after HU treatment (13) considering the role of Rad22 in replication fork restarting (14). After 4 hours of HU treatment no differences were observed between NyEGFP-rad22 and WT cells after release (figure 4).

![Figure 4](image)

Figure 4. Cell-cycle profile of WT (A) and EGFP-rad22 (B) cells stained with propidium iodide before and after HU block release. After HU block release, cells were collected at time intervals indicated.

The protocol that we propose completes a previous protocol (3, 7) aiming the C-end protein tagging and gene replacement by using RMCE. The completion consists of an N-end tagging of the gene product. Other protocols for N-end tagging have been published (1, 2). Both previous protocols propose construction of linear double-stranded DNA fragments consisting of the tag, a selectable gene marker and a \textit{nmt} promoter, all flanked with two long sequences homologous to the ones at which the fragment have to be inserted in the chromosome. The linear fragment is then transformed into a \textit{S. pombe} strain and insertion at the appropriate site occurs with low frequency at the targeted place by homologous recombination. Subsequently, the strain with the insert is selected due to the phenotype conferred by the selection marker. The function of the gene is assured by the \textit{nmt} thiamine-regulatable promoter from the insert. In one of the protocols (3) the tagged gene is placed under the control of its natural promoter by a second step in which the selectable marker and the \textit{nmt1} promoter are excised from the chromosome. These methods, although efficient, have drawbacks such as the low frequency of insertion by homologous recombination and the possibility of wrong insertion, the correct integration ranging between 6 and 63 \% (1). This makes the checking of the correct insertion necessary, sometimes in an important number of transformants.

Our described method like all the methods based on insertions by RMCE assures a frequent and accurate insertion of a fragment from a plasmid which makes the insertion step of a tag or other gene constructs much more rapid and needs no systematic checking of correct insertion. The most difficult part of our protocol, the construction of base strains, has to be carried out only once for a gene locus (3). An increasing collection of base strains modified in different genes exists already in our laboratory at the Genome Damage and Stability Center, Sussex University UK. The basic plasmid AW8 is a versatile tool by which genomic constructs-tagged or mutated genes-placed between loxP and loxM3 may be transferred to the base strain by a simple one-step transformation.
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4. Conclusion

We adapted a protocol initially design for C-terminal proteins tagging using the recombination mediated cassette exchange (RMCE) in order to attach an EGFP tag at N termini of Rad22 protein. The experimental model was the fusion yeast and the protocol used a S. pombe “base strain” and the pAW8 plasmid. After obtaining N-terminal EGFP-Rad22 fusion protein we assessed the phenotype of the new strain. Our data showed a good functionality regarding gamma and genotoxic sensitivity, foci formation and the dynamic in S-phase after hydroxyurea release of the strain with Rad22 N-termini tagged.

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