A Family of Multifunctional Thiamine-Repressible Expression Vectors for Fission Yeast.

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Key words: Fission Yeast, Plasmids, Phagemids, Gene Expression, Thiamine-Repressible Expression, Regulatable Promoter, *nmt1* Promoter.

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SUMMARY

A series of thiamine-repressible shuttle vectors has been constructed to allow a more efficient DNA manipulation in *Schizosaccharomyces pombe*. These high-copy-number vectors with regulatable expression (pJR) are based on the backbone of the pREP-3X, pREP-41X and pREP-81X plasmids. The pJR vectors are all uniform in structure, containing i) sequences for replication (*ori*) and selection (Amp^R) in *Escherichia coli*, ii) the f1 *ori* sequence of the phage f1 for packaging of ssDNA, making them suitable for site-directed mutagenesis, and iii) the *ars1* sequence for replication in *S. pombe*. The pJR vectors differ among them in i) the selectable marker (*Saccharomyces cerevisiae LEU 2* gene, that complements *S. pombe leu1*$^-$ gene, and *S. pombe ura4*$^+$ and *his3*$^+$ genes); ii) the thiamine-repressible *nmt1* promoter (3X, 41X and 81X with extremely high, moderate or low transcription efficiency, respectively); and iii) the multiple cloning site (two multiple cloning sites, with twelve restriction sites each). The expression level of the pJR vectors has been analyzed using the β-galactosidase gene as reporter. Three levels of expression for each *nmt1* promoter version, with any selectable marker and for either repressed or induced conditions, have been found. The expression is dependent on the distance to the initiation codon, varying from 0.001 to 15 times the activity characterized for the pREP plasmids. Also, the gene expression has been found to be extremely sensitive to the nucleotide sequence prior to the initiation codon, being up to 50 fold higher with an A/T sequence than with a G/C sequence. Finally, the β-galactosidase mRNA levels were found to be similar in each *nmt1* series, suggesting a translational effect on gene expression. As a result, any of these eighteen new vectors allow performing gene expression in fission yeast as well as a more versatile cloning, sequencing and mutagenesis directly in the plasmid without the need for subcloning into intermediary vectors.

INTRODUCTION

Schizosaccharomyces pombe constitutes a very well established alternative to *Saccharomyces cerevisiae* as an experimental cellular model (Moreno, *et al*., 1991). Their remarkable differences make equally attractive both systems for a wide variety of studies. *S. cerevisiae* has shown an enormous progress in molecular genetics analysis, partly due to the broad variety of cloning vectors that have become available during the past years. On the contrary, the efforts in *S. pombe* to construct a diversity of cloning vectors have been much more reduced. The tools available in molecular genetics are largely limited, denoting a clear disadvantage for the fission yeast.

The investigation of a wide range of biological problems relies on the ability to regulate the expression of cloned genes under the control of heterologous promoters. In fission yeast, there are few expression plasmids available compared to *S. cerevisiae* and much less compared to *Escherichia coli*. Most of them contain constitutive promoters (Gmunder and Kohli, 1989; Kudla, *et al*., 1988; McLeod, *et al*., 1987; Russell, 1989; Russell and Nurse, 1986; Tohda, *et al*., 1994) and only some are based on regulatable promoters: the glucose-repressible fructose biphosphatase promoter (Hoffman and Winston, 1989), the glucocorticoid-inducible promoter that contains multiple glucocorticoid response elements (Picard, *et al*., 1990), the tetracycline-inducible cauliflower mosaic virus promoter (Faryar and Gatz, 1992), the glucose-repressed *inv1* promoter (Iacovoni, *et al*., 1999) or the powerful thiamine-repressible *nmt1* promoter (Maundrell, 1990).

Among all regulatable promoters, *nmt1* has been the most used and modified. It has been used to construct extrachromosomal (pREP) and integrative (pRIP) thiamine-regulated expression vectors (Maundrell, 1993). pREP-1 promoter was modified to create pREP-41 and pREP-81, each with a different reduced level of transcription efficiency (Basi, *et al*., 1993). These vectors contain an ATG sequence within their short polylinker region, reducing their capabilities for protein expression. This ATG sequence was destroyed and changed to a *Xho* I site in pREP-3, pREP-41 and pREP-81 to make pREP-3X, pREP-41X and pREP-81X,

respectively (Forsburg, 1993). Comparison of those last three vectors and a tetracycline-inducible vector showed the powerful capabilities of the thiamine-repressible system, with induction rates, comparing induced and repressed conditions, spanning from seven fold in 81X, to twenty five fold in 41X and to three hundred fold in 3X. On the contrary, the tetracycline-inducible system showed an induction rate of just ten fold, with a β-galactosidase activity after induction similar to that of 81X (Forsburg, 1993). The *nmt1* vectors have recently been modified to append HA, GST, GFP, Pk or myc-6His tags to the expressed proteins (Craven, *et al*., 1998; Forsburg and Sherman, 1997) or to create vectors for PCR-based manipulations of genes in their chromosomal locations, including overexpression and C- or N-terminal protein tagging with HA, GST or GFP (Bähler, *et al*., 1998). However, all the described *nmt1* expression vectors have the restriction of just one or two selectable markers and a limited multiple cloning site region, forcing sometimes to intermediate cloning of the DNA fragments into specialized vectors.

In this work we have constructed a series of eighteen plasmids, starting from the pREP-3X, -41X and -81X vectors. We have combined three different auxotrophic selectable markers and introduced two different multiple cloning sites that will help to perform direct cloning, mutagenesis and gene expression in the most commonly used regulatable expression vectors of *S. pombe*. All the constructs share identical cloning sites, allowing a quick replacement of the cloned sequence between plasmids with different promoters or different selectable markers. Also, using the β-galactosidase gene as reporter we have tested the reliability of the plasmids and have studied the requirements of the *nmt1* promoter for an efficient expression of the cloned gene.

MATERIALS AND METHODS

S. pombe strains used in this work are h^- *leu1-32* (Köhli, *et al*., 1977) and h^- *his3-Δ1 ura4-Δ18 leu1-32* (S. Moreno). *Escherichia coli* strains MV1190, CJ236 (Bio-Rad) and DH5α (Gibco BRL) were used. Yeast growth medium (YES) and selective minimal medium

supplemented with the appropriate amino acids (Moreno, *et al*., 1991), and bacterial LB and TB media (Sambrook, *et al*., 1989), have been described. Thiamine (5μg/ml) was added to minimal medium when necessary.

Site-directed mutagenesis (Kunkel, 1985) was done with the Bio-Rad Muta-Gene kit, using the appropriate oligonucleotides and the corresponding U-containing ssDNA plasmid as template. DNA sequencing was done with a Sequenase kit (Amersham Pharmacia Biotech). Plasmid DNA was introduced into *S. pombe* as described (Valle and Wickner, 1993). All the expression plasmids were made by a simple ligation protocol. After restriction enzyme digestion, the DNA fragments were separated in low-melting temperature agarose. Vector and insert fragments were cut in sharp slides and 100-200 μl of water was added to the vector-containing tube. Both agarose fragments were melted at 65 ºC for 15 minutes and 1 μl of vector and 5 μl of insert were mixed in a 20 μl ligation mixture (final agarose concentration must be lower that 0.3% to avoid solidification). After one-hour incubation at room temperature the ligation mixture was used to transform *E. coli* cells. Other DNA manipulations were carried out essentially as described (Sambrook, *et al*., 1989).

Plasmid expression was done using the β-galactosidase gene as reporter (Casadaban, *et al*., 1983). The β-galactosidase gene was cloned from a 3.0 kb *Bam*H I-*Bam*H I fragment of pMC1871 (Casadaban, *et al*., 1983) into the *Bam*H I sites of pREP-3X and pJR1-3XL. This fragment contains a truncated form of the β-galactosidase gene, lacking the coding sequence of the first nine amino acids. The initiator ATG was inserted in frame in both plasmids by site-directed mutagenesis, changing the 5´*Bam*H I sequence GGATCC to GGATGC. The rest of expression vectors were made by cloning the modified β-galactosidase sequences into the corresponding pREP and pJR vectors. β-galactosidase activity was measure as Miller units (= OD_{420} x 1000 / OD_{600} x min x ml) according to standard protocols (Guarente, 1983).

For Northern blotting, h^- *leu1-32* cells transformed with the corresponding plasmids were grown in minimal medium in the absence of thiamine (induced conditions) to $O.D._{600}$ 0.5 -

0.75. Total RNA was prepared as described (Moreno, *et al*., 1991) and 10 μg RNA of each sample was denatured, electrophoresed through a 0.9% agarose gel containing formaldehyde and transferred to a nylon membrane as described (Sambrook, *et al*., 1989). The membrane was prehybridized in 50% deioniozed formamide, 10 X Denhardt´s (1 X contains 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyethylene glycol), 50 mM Tris-HCl pH 7.5, 1 M NaCl, 1% sodium dodecil sulfate (SDS), 0.1% Na pyrophosphate, 100 mg/ml dextran sulfate and 100 μg/ml denatured fish DNA at 42 °C for 4-6 hours. The hybridization was carried out in 66% deioniozed formamide, 13 X Denhardt´s, 66 mM Tris-HCl pH 7.5, 1.3% SDS, 0.13% Na pyrophosphate, 130 μg/ml denatured fish DNA and the ^{32}P-labelled probe at 42 °C for 18-24 hours. After hybridization the membrane was washed twice in 2 X SSC (1 X contains150 mM NaCl, 15 mM Na citrate pH 7.0) at room temperature for 5 minutes, twice in 2 X SSC, 1% SDS at 65 °C for 45 minutes and twice in 0.1 X SSC at room temperature for 30 minutes, and the label was detected by autoradiography. ^{32}P-labelled probes were made *in vitro* by the random prime labeling system followed by MicroSpin S-200 HR columns purification (Amersham Pharmacia Biotech). The β-galactosidase probe was made from a gel purified 3.0 kb *Bam*H I-*Bam*H I fragment of pSGS11 (Bluescript SK$^+$, Stratagene, containing the 3.0 kb *Bam*H I-*Bam*H I β-galactosidase fragment) and the actin probe was from a gel purified 1.3 kb *Sac* II-*Bam*H I fragment of pJR84 (Bluescript SK$^+$ containing the 1.3 kb *Sac* II-*Bam*H I *act1*$^+$ fragment of pA932 (Ishiguro and Kobayashi, 1996)).

RESULTS AND DISCUSSION

Starting from the vector pREP-3X (Forsburg, 1993), we analyzed a set of restriction enzymes in order to find out which sites are absent in the vector sequence. We used this method instead of the nucleotide sequence analysis for each fragment that takes part of the plasmid to avoid possible errors of absent sites in the sequence. We used the enzymes *Acc* I, *Apa* I,

Asc I, *Ava* I, *Avi* II, *Bal* I, *Ban* II, *Bcl* I, *Bgl* II, *Bst*B I, *Cla* I, *Dra* I, *Eag* I, *Eco*R I, *Fsp* I, *Hpa* I, *Hpa* II, *Kpn* I, *Mlu* I, *Nar* I, *Nco* I, *Nde* I, *Nhe* I, *Not* I, *Nru* I, *Nsi* I, *Pac* I, *Pml* I, *Pvu* I, *Pvu* II, *Sac* II, *Sfu* I, *Spe* I, *Sph* I, *Sty* I, *Xba* I and *Xmn* I. Among them, we found two blunt-end (*Bal* I and *Nru* I) and eleven cohesive-end enzymes (*Apa* I, *Asc* I, *Bgl* II, *Bcl* I, *Eag* I, *Nco* I, *Nhe* I, *Nsi* I, *Not* I, *Sac* II and *Spe* I) unable to cut the vector DNA. Discarding enzymes that produce the same protruding ends, we chose eight enzymes that originate one blunt and seven cohesive-ends. Two 61-mer oligonuclotides, complementary to each other except for the first and last four nucleotides, and containing the sequence of the eight restriction sites, were annealed. The resulting dsDNA oligonucleotide contained protruding ends compatible with *Sal* I and *Bam*H I. pREP-3X was cut with *Sal* I and *Bam*H I, and ligated with excess amount of the dsDNA oligonucleotide. The resulting vector, named pJR1-3XL, contained the multiple cloning site 1 (MCS 1) with twelve unique restriction sites (Figures 1 and 2). The 1 kb *Xho* I-*Sac* I fragment of pJR1-3XL, containing MCS 1 and *nmt1* terminator, was ligated to both pREP-41X and pREP-81X cut with *Xho* I and *Sac* I to make pJR1-41XL and pJR1-81XL, respectively (Figure 2).

The *ura4*$^+$ gene (Grimm, *et al*., 1988) was obtained from Bluescript SK$^+$-*ura4*$^+$, which was made from pP4 (*ura4*$^+$ gene cloned into *Hin*d III of pUC8 (Bach, 1987) and with the 5´*Hin*d III site destroyed) cut with *Pst* I-*Hin*d III and inserted into the same sites of Bluescript SK$^+$ (Stratagene). The *Pst* I site was removed with Klenow DNA polymerase and dNTPs to make pJR36. The *ura4*$^+$ sequence of pJR36 cut with *Sma* I-*Kpn* I was ligated into pREP-3X cut with *Eco*R V-*Kpn* I, to make pJR44. This plasmid was cut with *Sph* I and religated to make pREP-3XU. *nmt1* promoter and terminator sequences of pREP-3XU were replaced with the same *Pst* I-*Sac* I sequences of pJR1-3XL, pJR1-41XL and pJR1-81XL to make pJR1-3XU, pJR1-41XU and pJR1-81XU, respectively (Figure 2).

The *his3*$^+$ gene was obtained from pJB1 (Burke and Gould, 1994). This gene was found placed, respect to the *Sac* I and *Pst* I sites, in opposite orientation to that published (Burke and Gould, 1994). The 1782 bp *Eco*R V-*Dra* I *his3*$^+$ fragment was cloned into *Eco*R V of Bluescript SK$^+$ and the clone with *Pst* I and *Eco*R V located at opposite sites of the *his3*$^+$

gene was selected, making pJR45. The *Pst* I-*Eco*R V fragment was inserted into pREP-3X cut with *Pst* I-*Eco*R V to make pREP-3XH. The *his3*$^{+}$ fragment contains the *Xho* I and *Bam*H I sites, which are also present in MCS 1. To make the *his3*$^{+}$-containing vectors more useful, both *Xho* I and *Bam*H I sites from the *his3*$^{+}$ sequence were destroyed without altering the amino acid sequence, by site-directed mutagenesis, making pREP-3XH2. As it was done with pREP-3XU, *nmt1* promoter and terminator sequences of pREP-3XH2 were replaced with the *Pst* I-*Sac* I sequences of pJR1-3XL, pJR1-41XL and pJR1-81XL to make pJR1-3XH, pJR1-41XH and pJR1-81XH, respectively (Figure 2).

The *Nsi* I site of MCS 1 is unique only in the pJR1-L series. Also, *Nsi* I and *Nco* I sites of MCS 1 contain an ATG codon that may be used to express truncated proteins or to attenuate downstream expression of deleterious proteins. A more adaptable multiple cloning site was designed substituting *Nsi* I and *Nco* I for *Pst* I and *Kpn* I, respectively (Figure 1). First, the unique *Pst* I site of the nine-pJR1 vectors was Klenow removed, making pJR37-39 (Leu series), pJR51-53 (Ura series) and pJR54-56 (His series). In order to make the MCS substitution, site-directed mutagenesis was performed. The available literature does not show the f1 *ori* in any pREP plasmid. The f1-containing fragment is displayed in the pREP-originating vectors as a sequence with both possible orientations, keeping the pREP series of little interest for mutagenesis experiments. pREPs were described containing the pUC119 plasmid as backbone and, at the same time, referred to the *S. pombe* shuttle vector pIRT2 as the plasmid where *nmt1* promoter and terminator sequences were cloned into (Maundrell, 1990). On the contrary, pIRT2 was described based on pUC118 as backbone (Hindley, *et al*., 1987). pUC118 and pUC119 differ in the MCS orientation and therefore, the originated REP plasmids would contain opposite f1 *ori* orientation. That made us to design two different 62-mer oligonucleotides whose sequences, complementary to each other, are both strands of MCS 1 and contain changes to introduce *Pst* I and *Kpn* I restriction sites (MCS 2). Site-directed mutagenesis was done using pJR1-3XL ssDNA and both oligonucletides. MCS 2-containing clones were obtained only using the (-)ssDNA sequence of MCS 2. Those clones were named pJR2-3XL (Figures 1 and 2). Therefore, the pJR1-3XL ssDNA used as template

contains the (+) strand of the *nmt1*-MCS sequence. That means the pREP plasmids are based on pUC119.

The *Asc* I-*Sac* I polylinker-*nmt1* terminator fragment from pJR2-3XL was used to replace the equivalent sequences of the other eight pJR1 vectors, making pJR2-41XL, -81XL, -3XU, -41XU, -81XU, -3XH, -41XH and -81XH (Figures 1 and 2).

The new plasmids were tested for efficient expression of cloned genes using the β-galactosidase gene as reporter (Casadaban, *et al*., 1983). The 3.0 kb *Bam*H I-*Bam*H I fragment of the β-galactosidase gene from pMC1871 was cloned into the *Bam*H I site of pREP-3X and pJR1-3XL. This fragment lacks the coding sequence of the first nine amino acids. The initiator ATG was inserted in frame in both pREP-3X and pJR1-3XL, changing the 5´*Bam*H I sequence GGATCC to GGATGC.

In order to test the expression of different deletions between cloning sites, a second *Sac* II site was created by removing the A residue between *Not* I and *Bam*H I sites, making CCGCGG instead of CCGCAGG (Figure 1). The series of reporter plasmids were made by cloning the modified *Sal* I-*Sma* I β-galactosidase fragment from pREP-3X and *Not* I-*Sma* I β-galactosidase fragment from pJR1-3XL into the same sites of pREP and pJR plasmids, respectively. Surprisingly, the β-galactosidase activity found in pJR1-3XL was one thousand fold lower than that of pREP-3X (Table 1, left columns). We did not expect to affect gene expression in that manner by adding 61 bases between initiation of transcription and translation. In *S. pombe,* transcription generally starts within 200 nucleotides upstream of the open reading frame (Russell, 1989) and in the *nmt1* mRNA transcription initiates on the A residue at position -69 (Maundrell, 1990).

In order to study the effect of added cloning sites on gene expression we performed a series of deletions between different sites of MCS 1 or 2 and the *Not* I site (Figure 1). Even deleting *Xho* I-*Not* I, that leaves 13 bases between *nmt1* promoter and initiation codon, the expression was ten times lower than that of the control (Table 1, left columns), which contains 20 bases between *nmt1* promoter and initiation codon. The *Not* I deletions-series leave 8 G/C residues before the initiator ATG sequence that may inhibit the expression.

However, the *Sac* II deletions-series, that use the new 3´end *Sac* II site, leave only the GG sequence. The analysis of the *Sac* II deletions-series showed that the activity of *Xho* I-*Sac* IIΔ is two fold higher and the activity of *Sal* I-*Sac* IIΔ is almost like that of the control (Table 1, left columns). The *Nru* I-*Sac* II deletion resembles the position of the β-galactosidase gene in pREP-3X, but in this construct the activity was four times lower. *Nru* I is closer to the promoter than the *Bam*H I site of pREP, as it is located 6 bases downstream *Sal* I while *Nru* I follows *Sal* I in the pJR vectors. Consequently, the decrease of activity is not only due to the distance between promoter and open reading frame. Those pREP 6-bases between *Sal* I and *Bam*H I form a *Xba* I site containing 4 A/T residues that may be important to enhance the expression of distant genes. The A/T region may be needed to avoid a strong structure close to the initiation codon that would explain the lower expression of *Nru* I-*Sac* II deletion.

The Kozak consensus sequence for initiation of translation by eukaryotic ribosomes points out that an initiation codon can be designed strong by considering only positions -3 and +4 (where +1 is the A of ATG) (Kozak, 1989). The only important position is -3 and it must be a purine (A or G). When it fails, a G at +4 is essential for efficient translation. The β-galactosidase gene contains a C at +4 in all the tested vectors. pREP contains an A at -3 while all the pJR *Not* I deletions-series contain a C residue. However, in the *Sac* II deletions-series *Xho* I-*Sac* IIΔ and *Sal* I-*Sac* IIΔ contain at -3 an A residue, and *Nru* I-*Sac* IIΔ, *Asc* I-*Sac* IIΔ and *Apa* I-*Sac* IIΔ a G residue. Therefore, it seems the decrease of activity of the *Nru* I-*Sac* II deletion is due to a sequence other than the Kozak consensus.

To check the possibility that the pREP 6-bases between *Sal* I and *Bam*H I must be an A/T-rich region to enhance translation initiation, we made a new β-galactosidase expression vector from pJR1-3XL. The new vector contained the same changes as the previous one and included an eight-base sequence TTTAAAAT between the new 3´end-*Sac* II site and the initiation codon (changing GCGGCCGCGGATG to GCGGCCGCGGTTTAAAATATG). The expression of the new vector was just a little bit higher than that of the previous vector, indicating the long distance is still critical for expression (Table1, right columns). This eight-

base increase made the expression of *Kpn* I-*Not* I deletion to fall 100 times. Deletion constructs from *Sac* II-*Sac* IIΔ to *Asc* I-*Sac* IIΔ and from *Apa* I-*Not* IΔ to *Asc* I-*Not* IΔ of the *Sac* II and *Not* I deletions-series, respectively, increased the previous activity but it still was 5-10 times lower than that of the control plasmid pREP3X (Table 1, right columns). The presence of one or two ATG sequences from MCS 1 had little effect as equivalent deletions in MCS 2 showed only a slight increase of activity. Finally, *Nru* I-*Not* I, *Sal* I-*Not* I or *Xho* I-*Not* I deletions gave acceptable expression levels. Therefore, the presence of 8 G/C residues before the initiation codon in the initial *Not* I deletions-series is responsible for the reduced activity of *Nru* I-*Not* IΔ, *Sal* I-*Not* IΔ or *Xho* I-*Not* IΔ, while insertion of 8 A/T between the *Not* I G/C-rich sequence and the initiation codon is sufficient to increase their activity to levels similar to that of the control pREP3X (Table 1, compare left and right columns). On the other hand, the expression of *Sac* II deletions-series containing the 8 A/T insertion showed that the activity of any of the three *Nru* I-*Sac* IIΔ, *Sal* I-*Sac* IIΔ or *Xho* I-*Sac* IIΔ was higher than that of the control pREP3X and even remarkably higher in the last two cases (Table 1). That suggests an additive effect in gene expression by both the presence of A/T nucleotides prior the initiation codon and the absence of a G/C sequence before the A/T nucleotides.

The properties of the 3X-*nmt1* promoter were also analyzed in other expression vectors, comparing the activity of pJR2-3XL, pJR2-3XU and pJR2-3XH to that of pJR1-3XL. Vectors with deletions from *Apa* I, *Sal* I or *Xho* I to either *Not* I or *Sac* II, with or without the 8 A/T insertion before the initiation codon, were analyzed. In all cases, the vectors showed the same pattern as that described for pJR1-3XL (Table 2). The activities detected in pJR2-U constructs were half-activity those of pJR2-L and pJR2-H. This may be due to the plasmid-copy number. The heterologous *LEU2* gene may be less efficient to complement its auxotrophy than the *ura3*$^{+}$ gene and consequently, the plasmid-copy number would need to be higher with the *LEU2* gene. The *his3*$^{+}$ gene was cloned using the minimal sequence needed to complement its auxotrophy (Burke and Gould, 1994) and it may contain a truncated promoter, being necessary a higher plasmid-copy number to complement the His^{-} auxotrophy.

Next, the other two *nmt1* promoter versions 41X and 81X, from the pJR1-L series, were analyzed for β-galactosidase expression as the previous vectors. The expression from the three promoter versions, 3X, 41X and 81X, and from either induced (absence of thiamine) or repressed conditions (+5 μg/ml thiamine), was analyzed. The three-*nmt1* promoter versions showed the same expression pattern, dependent on the type of deletion and on the G/C or A/T region prior to the initiation codon, as shown before (Table 3). That means all sites of both MCS may be used for 3´end cloning, but only the first three sites are suitable for direct 5´end cloning. The third site produces a blunt-end, increasing enormously the possibilities of cloning any fragment cut with a blunt-end generating enzyme. Also, it will be possible for 5´end cloning to use any other site by a two-step process, deleting previously the region between *Xho* I and the restriction site preceding the site chosen for cloning.

Finally, in order to know if this strong effect on gene expression is promoted at transcriptional or at translational level, a Northern analysis was performed. RNA of twelve strains, each four containing one of the three-*nmt1* promoter versions and the β-galactosidase gene, was analyzed. Strains with the -3X, -41X and -81X REP plasmids expressing the β-galactosidase gene were used as control in each series. Three strains containing the pJR1 expression vector with the intact, the *Asc* I-*Sac* II Δ or the *Xho* I-*Sac* II Δ multiple cloning site, were analyzed for each series. The pJR1 expression vectors showed a β-galactosidase activity spanning from a thousand-fold lower to five-fold higher than that of the corresponding REP vector. However, the amount of β-galactosidase mRNA was the same in each of the four strains of each series (Figure 3). Therefore, the described differences of gene expression seem to be promoted at a translational, rather than transcriptional level.

In summary, *nmt1* expression may vary extremely, depending on the distance to and on the nucleotide sequence previous to the initiation codon. Extra ATG sequences before the initiation codon have weak deleterious effects. Depending on the distance there are several levels of expression, from low (0.001-0.005x control activity) for distances longer than 59 residues, to medium (0.1-0.2x control activity) for distances longer than 26 residues, and to

high (0.5-3.5x control activity) for distances closer than 25 residues (Table 1). A G/C sequence adjacent to the initiation codon only produces medium expression, up to 50 times lower than the high expression obtained when A/T residues precede the initiation codon. The expression activity obtained from vectors with preceding A/T sequences, measured from any of the three expression-level *nmt1* promoter and from either induced or repressed conditions, may be much higher than that reported for control pREP vectors (Forsburg, 1993), spanning from 2.5 to 14.9 times that of the corresponding control (Table 3). The new vector series present several advantages respect to the existing vectors as i) the possibility of using a wide set of cloning sites. This reduces the probability of finding all the sites in the fragment to be cloned. ii) the estimation of the expression level that will be obtained for each construct; iii) the use of the same multiple cloning site in all the series. The first cloning site of the pREP vectors can be *Xho* I, *Nde* I or *Bal* I; iv) the use of three different selectable markers, *LEU2*, *ura4*$^{+}$ and *his3*$^{+}$.

The combination of three selectable markers with three different-strength *nmt1* promoters and a broad spectrum of common cloning sites, combined with the possibility of performing site-directed mutagenesis in all those vectors will provide a tool of valuable flexibility for direct cloning, sequence replacement and gene expression.

ACKNOWLEDGMENTS

We wish to thank Dr. P. Pérez (CSIC/Univ. of Salamanca) for a critical reading of the manuscript. Dr. H. Levin (NIH, USA) and Dr. S. Moreno (CSIC/Univ. of Salamanca) for their comments and S. García and Dr. A. Domínguez (CSIC/Univ. of Salamanca) for providing the β-galactosidase plasmid.

This work was supported by grant BIO98-0814-C02 from the "Comisión Interministerial de Ciencia y Tecnología", Spain.

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FIGURE LEGENDS

Figure 1. Sequence and orientation of the multiple cloning sites (MCS 1 and MCS 2) of pJR-L, pJR-U and pJR-H vectors. The nucleotide sequences shown in bold are the (+) and (-) ssDNA 61-mers used to insert the MCS 1 between the previous *Sal* I and *Bam*H I restriction sites of pREP-3X. Insertion of MCS 2 was by site-directed mutagenesis using the MCS 1-containing vector pJR1-3XL as ssDNA template. The ssDNA strand produced upon M13 phage-infection of *E. coli* harboring the plasmid corresponds to the (+) strand of the sequence. The nucleotide sequence shown in normal characters is that of the restriction sites available in the starting vectors pREP-3X, -41X and -81X. Unique restriction sites within MCS 1 and MCS 2 are shown below the nucleotide sequences. The restriction enzymes listed in brackets produce compatible protruding ends and can be used to clone fragments into the sites of MCS 1 and 2. Available unique restriction sites for each series of vectors is shown as (+). (-) denotes not available site due to its absence in the MCS (pREP series) or to additional sites along the vector sequence (pJR series). The original *his3*$^{+}$ sequence contains *Xho* I and *Bam*H I restriction sites. To make more versatile the pJR-H series, both *Xho* I and *Bam*H I sites were removed by site-directed mutagenesis from the *his3*$^{+}$ sequence, making them unique in the MCS. A second *Sac* II site was created by removing the A residue between *Not* I and *Bam*H I sites, making CCGCGG. The new 3´end *Sac* II site was used to make deletions series for expression analysis.

Figure 2. Restriction and genetic map of pJR-L, pJR-U and pJR-H vectors. The *E. coli ori*, the *S. pombe* autonomously replicative sequence (*ars1*) and the *ori* region of the phage f1 (f1 *ori*) are represented. Position and direction of transcription of the *S. cerevisiae LEU2* gene, the *S. pombe ura4*$^{+}$ and *his3*$^{+}$ genes and the β-lactamase gene (Amp^{R}) are shown. Also, position and direction of the *nmt1* gene promoter (as 3X or its mutated forms with reduced transcription efficiency, 41X and 81X) and position of the *nmt1* 3´ stop sequence are depicted. Restriction sites within the plasmid used for manipulation or as control are shown.

(*Pst* I)* means the presence of this site in the series pJR1-L, pJR1-U and pJR1-H (plasmids with MCS 1), and its absence in the series pJR2-L, pJR2-U and pJR2-H (plasmids with MCS 2) to make unique the *Pst* I site from MCS 2. Orientation of f1 *ori* indicates the (+) ssDNA produced upon phage-infection of *E. coli* harboring the plasmid. MCS 1 and MCS 2 are shown and oriented respect to the *nmt1* gene promoter. Not unique restriction sites for each MCS and for each series of vectors is shown in Figure 1.

Figure 3. Northern blot analysis of β-galactosidase mRNA levels in transformants harboring different 3X-*lacZ*, 41X-*lacZ* and 81X-*lacZ* plasmids. h^- *leu1-32* cells were transformed with plasmids containing the 3X (lanes 1-4), 41X (lanes 5-8) and 81X (lanes 9-12) versions of the *nmt1* promoter and the *lacZ* gene or with pREP3X alone as control (lane 13). PREP3X-*lacZ*, pREP41X-*lacZ* and pREP81X-*lacZ* (lanes 1, 5 and 9) were used as mRNA control in each series. The mRNA expression of (8 A/T)-*lacZ* constructs from pJR1-3XL, pJR1-41XL and pJR1-81XL, with the entire (lanes 2, 6, and 10), the *Asc* I-*Sac* IIΔ (lanes 3, 7 and 11) or the *Xho* I-*Sac* IIΔ (lanes 4, 8 and 12) multiple cloning site, was analyzed. Cells were grown in minimal medium in the absence of thiamine (induced conditions) and RNA was extracted and analyzed as described in Materials and Methods. Actin mRNA was used as control of total RNA loaded in each lane.

TABLE 1. β-galactosidase activity[a] of *S. pombe* expression plasmids pREP3X and derivatives of pJR1-3XL and pJR2-3XL.

Vector	-*lacZ*[b]			-(8 A/T)-*lacZ*[c]		
	Control	...-*Not* I Δ[d]	...-*Sac* II Δ[d]	Control	...-*Not* I Δ	...-*Sac* II Δ
pREP3X	1920.3 ± 6.2[e]	-	-	-	-	-
pJR1-3XL(1)[f]	1.94 ± 0.01	-	-	10.54 ± 0.91	-	-
pJR2-3XL(2)	1.51 ± 0.02	-	-	3.23 ± 0.24	-	-
(1)*Nco* I-...	-	1.76 ± 0.14	-	-	0.66 ± 0.01	-
(2)*Kpn* I-...	-	106.67 ± 7.41	-	-	1.25 ± 0.07	-
(1)*Sac* II-...	-	-	32.89 ± 1.44	-	-	177.39 ± 6.60
(2)*Sac* II-...	-	-	31.76 ± 4.12	-	-	288.26 ± 0.09
(1)*Apa* I-...	-	110.39 ± 7.83	109.06 ± 11.91	-	175.11 ± 3.87	155.78 ± 6.53
(2)*Apa* I-...	-	147.91 ± 2.44	332.45 ± 25.56	-	190.94 ± 5.27	561.95 ± 18.18
(1)*Asc* I-...	-	88.65 ± 10.76	201.53 ± 12.33	-	99.02 ± 3.04	246.54 ± 3.97
(1)*Nru* I-...	-	62.17 ± 0.50	556.20 ± 2.87	-	735.18 ± 1.42	2514.9 ± 23.7
(1)*Sal* I-...	-	167.71 ± 13.75	1325.1 ± 9.2	-	896.54 ± 29.88	3726.2 ± 144.7
(1)*Xho* I-...	-	133.12 ± 10.68	3924.1 ± 127.8	-	1860.0 ± 112.0	6558.9 ± 39.1

[a]*S. pombe* h⁻ *leu1-32* cells were grown in the absence of thiamine (induced conditions) and the activity was measured as Miller Units (Guarente, 1983) = OD_{420} x 1000 / OD_{600} x min x ml.

[b]The β-galactosidase gene was inserted at the *Bam*H I site. The GGATCC sequence was changed by site-directed mutagenesis to GGATGC, to make it the initiation codon of the *lacZ* gene. A second change was introduced in the pJR series, removing the A residue between *Not* I and *Bam*H I sites, to make a second *Sac* II site, CCGCGG, to be used for deletion analyses.

[c]The pJR series were modified as above and a sequence of eight A/T bases (TTTAAAAT) was inserted between the second *Sac* II site and the initiation codon.

[d]Deletions between different sites and *Not* I or the 3´end *Sac* II site of the polylinker were analyzed.

[e]Values are means and standard deviations, calculated from three to four independent experiments.

[f]pJR1-3XL and pJR2-3XL are abbreviated as (1) and (2), respectively.

TABLE 2. β-galactosidase activity[a] of *S. pombe* expression plasmids pJR2-3XL, pJR2-3XU, pJR2-3XH and derivatives.

Vector	-*lacZ*[b]			-(8 A/T)-*lacZ*[b]		
	Control	...-*Not* I Δ	...-*Sac* II Δ	Control	...-*Not* I Δ	...-*Sac* II Δ
pREP3X	2028.5 ± 13.7	-	-	-	-	-
pJR2-3XL(1)[c]	1.72 ± 0.03	-	-	4.50 ± 0.23	-	-
(1)*Apa* I-...	-	130.81 ± 7.59	296.23 ± 0.81	-	201.29 ± 3.04	557.46 ± 17.53
(1)*Sal* I-...	-	149.71 ± 8.52	1325.0 ± 9.3	-	852.18 ± 4.96	4136.3 ± 51.9
(1)*Xho* I-...	-	130.42 ± 7.82	5271.0 ± 13.7	-	1533.5 ± 1.4	6769.5 ± 248.4
pJR2-3XU(2)	0.497 ± 0.067	-	-	1.30 ± 0.08	-	-
(2)*Apa* I-...	-	72.53 ± 1.76	134.75 ± 12.23	-	93.41 ± 1.39	314.95 ± 0.94
(2)*Sal* I-...	-	93.56 ± 4.70	743.90 ± 25.0	-	568.29 ± 20.48	2647.3 ± 119.4
(2)*Xho* I-...	-	59.39 ± 0.77	3272.3 ± 76.1	-	961.89 ± 30.76	4048.9 ± 13.6
pJR2-3XH(3)	1.11 ± 0.01	-	-	3.06 ± 0.08	-	-
(3)*Apa* I-...	-	115.17 ± 5.27	249.05 ± 1.32	-	227.86 ± 5.87	534.08 ± 6.55
(3)*Sal* I-...	-	129.87 ± 6.12	1197.5 ± 70.9	-	852.17 ± 32.55	4857.9 ± 64.6
(3)*Xho* I-...	-	156.79 ± 0.47	6037.1 ± 69.2	-	1783.4 ± 128.6	7324.1 ± 46.3

[a]*S. pombe* h⁻ *his3-Δ1 ura4-Δ18 leu1-32* cells were grown in the absence of thiamine (induced conditions) and the β-galactosidase activity was measured as in Table 1.

[b]Expression plasmids were made and tested as described in the legend of Table 1.

[c]pJR2-3XL, pJR2-3XU and pJR2-3XH are abbreviated as (1), (2) and (3), respectively.

TABLE 3. β-galactosidase activity[a] of *S. pombe* expression plasmids pJR1-3XL, pJR1-41XL, pJR1-81XL and derivatives.

Vector	-*lacZ*[b]			-(8 A/T)-*lacZ*[b]		
	Control	...-*Not* I Δ	...-*Sac* II Δ	Control	...-*Not* I Δ	...-*Sac* II Δ
Induced conditions (- Thiamine)						
pREP3X	1920.3 ± 6.2	-	-	-	-	-
pJR1-3XL(1)[c]	1.94 ± 0.01	-	-	10.54 ± 0.91	-	-
(1)*Apa* I-...	-	110.39 ± 7.83	109.06 ± 11.91	-	175.11 ± 3.87	155.78 ± 6.53
(1)*Asc* I-...	-	88.75 ± 10.76	201.53 ± 12.33	-	99.02 ± 3.04	246.54 ± 3.97
(1)*Sal* I-...	-	167.71 ± 13.75	1325.1 ± 9.2	-	896.54 ± 29.88	3726.3 ± 144.7
(1)*Xho* I-...	-	133.12 ± 10.68	3924.1 ± 127.8	-	1860.03 ± 111.97	6558.9 ± 39.1
pREP41X	98.70 ± 2.78	-	-	-	-	-
pJR1-41XL(2)	0.0102 ± 0.0001	-	-	0.0722 ± 0.0051	-	-
(2)*Apa* I-...	-	0.905 ± 0.065	2.16 ± 0.19	-	1.63 ± 0.01	2.35 ± 0.01
(2)*Asc* I-...	-	0.720 ± 0.001	1.72 ± 0.06	-	2.71 ± 0.09	4.53 ± 0.03
(2)*Sal* I-...	-	2.21 ± 0.09	120.25 ± 3.92	-	51.27 ± 1.68	150.28 ± 12.14
(2)*Xho* I-...	-	16.29 ± 1.07	257.65 ± 31.29	-	158.30 ± 8.99	515.43 ± 25.71
pREP81X	2.68 ± 0.03	-	-	-	-	-
pJR1-81XL(3)	0.0022 ± 0.0004	-	-	0.00700 ± 0.00032	-	-
(3)*Apa* I-...	-	0.0173 ± 0.0009	0.084 ± 0.002	-	0.0553 ± 0.0019	0.0801 ± 0.0015
(3)*Asc* I-...	-	0.0103 ± 0.0006	0.081 ± 0.002	-	0.0828 ± 0.0086	0.175 ± 0.006
(3)*Sal* I-...	-	0.145 ± 0.003	1.95 ± 0.01	-	1.01 ± 0.02	2.60 ± 0.09
(3)*Xho* I-...	-	0.383 ± 0.012	8.27 ± 0.44	-	3.31 ± 0.45	11.38 ± 0.31
Repressed conditions (+ Thiamine)						
pREP3X	7.72 ± 0.08	-	-	-	-	-
pJR1-3XL(1)[c]	0.0032 ± 0.0008	-	-	0.0142 ± 0.0007	-	-
(1)*Apa* I-...	-	0.326 ± 0.020	0.297 ± 0.012	-	0.417 ± 0.010	0.387 ± 0.001
(1)*Asc* I-...	-	0.128 ± 0.014	0.693 ± 0.003	-	0.257 ± 0.005	1.064 ± 0.046
(1)*Sal* I-...	-	0.318 ± 0.017	3.30 ± 0.07	-	3.96 ± 0.20	41.14 ± 0.12
(1)*Xho* I-...	-	0.304 ± 0.012	61.27 ± 0.70	-	7.96 ± 0.17	115.52 ± 1.43
pREP41X	2.54 ± 0.06	-	-	-	-	-
pJR1-41XL(2)	0.00082 ± 0.00034	-	-	0.00543 ± 0.00100	-	-
(2)*Apa* I-...	-	0.0232 ± 0.0008	0.134 ± 0.001	-	0.0579 ± 0.0007	0.0619 ± 0.0021
(2)*Asc* I-...	-	0.0377 ± 0.0020	0.162 ± 0.001	-	0.0832 ± 0.0004	0.302 ± 0.004
(2)*Sal* I-...	-	0.166 ± 0.001	1.77 ± 0.04	-	0.704 ± 0.01	2.60 ± 0.03
(2)*Xho* I-...	-	0.541 ± 0.007	4.47 ± 0.07	-	1.57 ± 0.03	9.60 ± 0.08
pREP81X	0.143 ± 0.002	-	-	-	-	-
pJR1-81XL(3)	<0.00006 ± 0.00001	-	-	0.00062 ± 0.00008	-	-
(3)*Apa* I-...	-	0.00147 ± 0.00036	0.00585 ± 0.00107	-	0.00728 ± 0.00047	0.00907 ± 0.00040
(3)*Asc* I-...	-	0.00124 ± 0.00013	0.00905 ± 0.00065	-	0.00836 ± 0.00138	0.0167 ± 0.0004
(3)*Sal* I-...	-	0.0111 ± 0.0002	0.116 ± 0.004	-	0.0857 ± 0.0030	0.169 ± 0.010
(3)*Xho* I-...	-	0.0281 ± 0.0001	0.230 ± 0.024	-	0.137 ± 0.015	0.341 ± 0.005

[a] *S. pombe* h⁻ *leu1-32* cells were grown in the absence or in the presence of thiamine (induced or repressed conditions, respectively) and the β-galactosidase activity was measured as in Table 1.

[b] Expression plasmids were made and tested as described in the legend of Table 1.

[c] pJR1-3XL, pJR1-41XL and pJR1-81XL are abbreviated as (1), (2) and (3), respectively.

M C S 1

GTTAAATGGCCTCGAGG**TCGACTCGCGAGGCGCGCCATGCATTAGGGCCCCCGCGGCCATGGACTAGTGCGGCCGCAG**GATCCCCGGGTAAAAGG
CAATTTACCGGAGCTCCAGCT**GAGCGCTCCGCGCGGTACGTAATCCCGGGGGGCGCCGGTACCTGATCACGCCGGCGTCCTAG**GGGCCCTTTTTCC

***Xho* I**	***Sal* I**	***Nru* I**	***Asc* I**	***Nsi* I**	***Apa* I**	***Sac* II**	***Nco* I**	***Spe* I**	***Not* I**	***Bam*H I**	***Sma* I**
(*Sal* I)	(*Xho* I)	(Blunt)	(*Bss*H II) (*Mlu* I)	(*Pst* I)			(*Bsp*LU11 I) (*Bsp*H I)	(*Avr* II) (*Nhe* I) (*Xba* I)	(*Bsp*120 I) (*Eag* I)	(*Bcl* I) (*Bgl* II) (*Sau*3A I)	(Blunt)

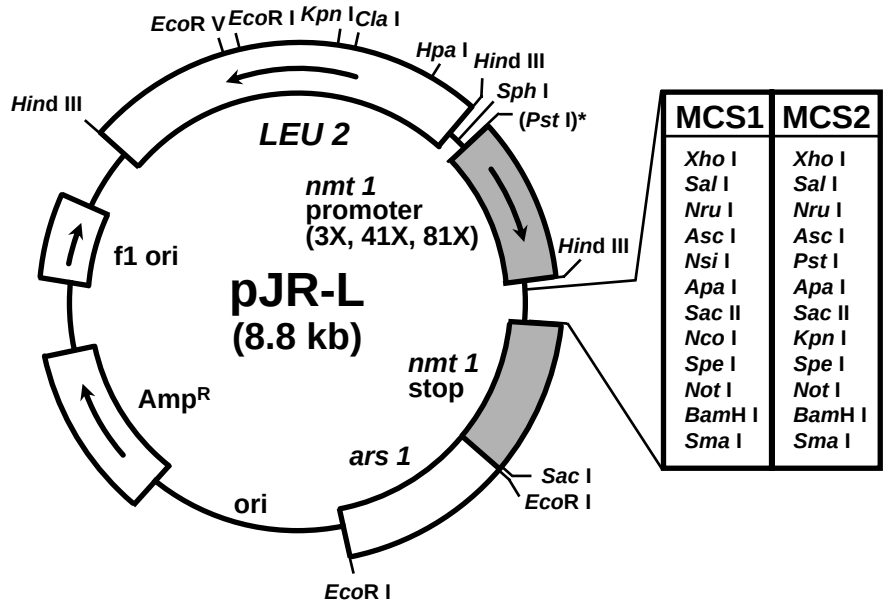
pREP-3X, -41X, -81X	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**+**	**+**
pJR1-3XL, -41XL, -81XL	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
pJR1-3XU, -41XU, -81XU	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
pJR1-3XH, -41XH, -81XH	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**

M C S 2

GTTAAATGGCCTCGAGG**TCGACTCGCGAGGCGCGCCCTGCAGTAGGGCCCCCGCGGGGTACCACTAGTGCGGCCGCAG**GATCCCCGGGTAAAAGG
CAATTTACCGGAGCTCCAGCT**GAGCGCTCCGCGCGGGACGTCATCCCGGGGGCGCCCCATGGTGATCACGCCGGCGTCCTAG**GGGCCCTTTTTCC

***Xho* I**	***Sal* I**	***Nru* I**	***Asc* I**	***Pst* I**	***Apa* I**	***Sac* II**	***Kpn* I**	***Spe* I**	***Not* I**	***Bam*H I**	***Sma* I**
(*Sal* I)	(*Xho* I)	(Blunt)	(*Bss*H II) (*Mlu* I)	(*Nsi* I)				(*Avr* II) (*Nhe* I) (*Xba* I)	(*Bsp*120 I) (*Eag* I)	(*Bcl* I) (*Bgl* II) (*Sau*3A I)	(Blunt)

pREP-3X, -41X, -81X	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**+**	**+**
pJR2-3XL, -41XL, -81XL	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**
pJR2-3XU, -41XU, -81XU	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
pJR2-3XH, -41XH, -81XH	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**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EcoR I
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Hind III
Sph I
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ars 1
ori
Sac I
EcoR I
EcoR I
MCS1
MCS2
Xho I
Sal I
Nru I
Asc I
Nsi I
Apa I
Sac II
Nco I
Spe I
Not I
BamH I
Sma I
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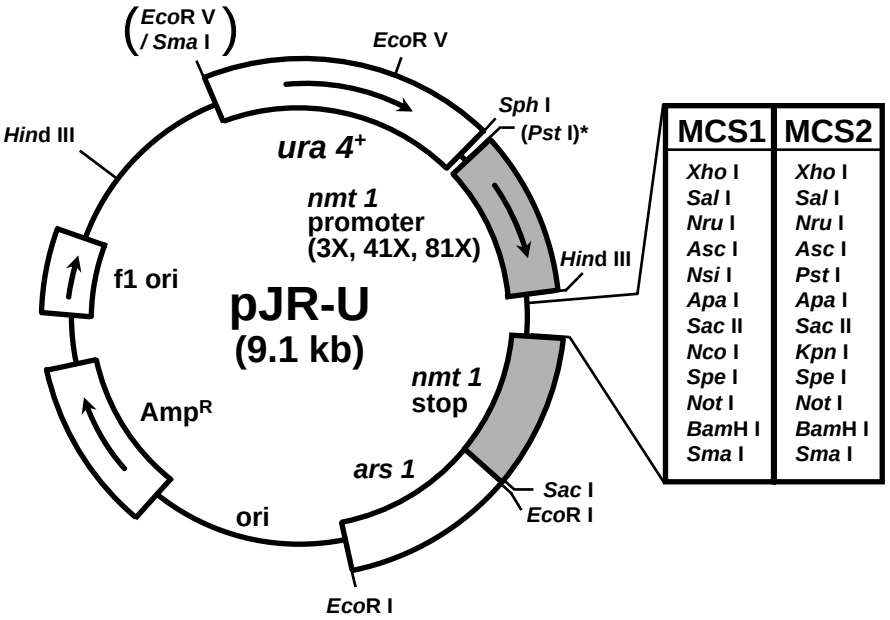
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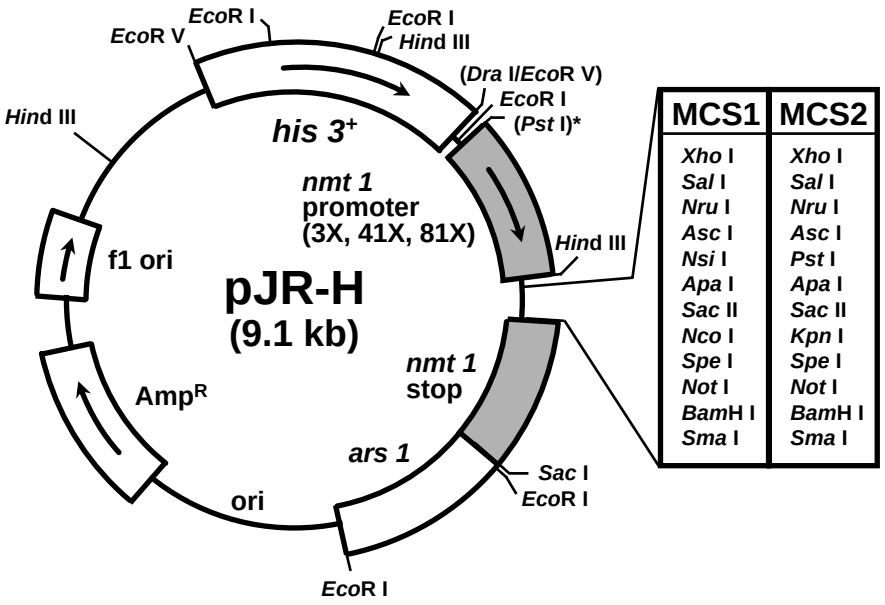
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MCS1
MCS2
Xho I
Sal I
Nru I
Asc I
Nsi I
Apa I
Sac II
Nco I
Spe I
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Sma I
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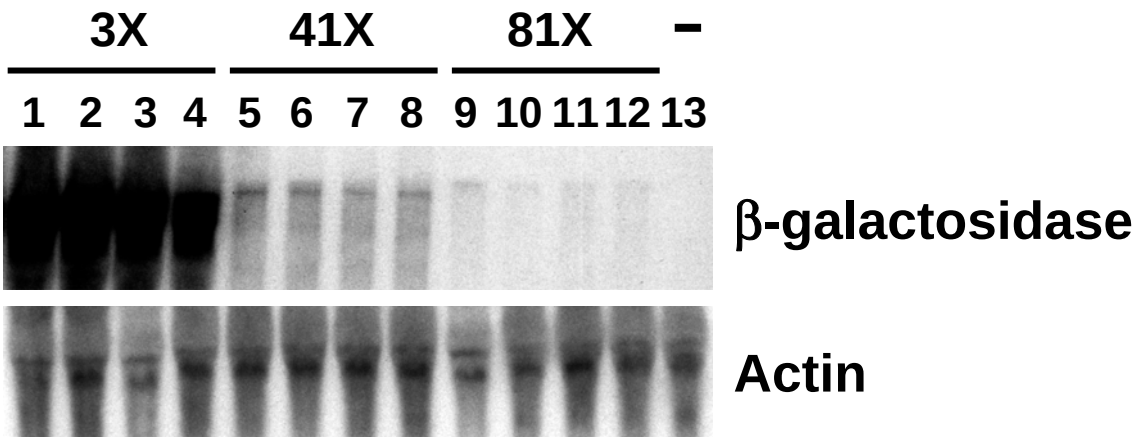
BamH I
Sma I


3X
41X
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1 2 3 4 5 6 7 8 9 10 11 12 13
β-galactosidase
Actin