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# New expression vectors for the fission yeast *Schizosaccharomyces pombe*

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A general expression vector (pMB332) for the fission yeast *Schizosaccharomyces pombe* was constructed. The heterologous gene expression is driven by the *S. pombe* alcohol dehydrogenase (adh) promoter. Transcription termination signals were isolated from the *S. pombe* actin gene. The vectors carry the *Saccharomyces cerevisiae Ura3* gene, which complements the *S. pombe ura4* mutation. The plasmid stability is conferred by the *S. pombe ars* and *stb* elements isolated from pFL120 [(1983) Cell 32, 371–377]. An 'ATG' vector (pMB340) was created, which allows the expression of protein fragments fused to a translational start codon downstream of the adh promoter. The function of this vector system is shown by the production of the human blood coagulation protein factor XIIIa.

Expression vector; Alcohol dehydrogenase; Gene promoter; Fission yeast; Factor XIIIa; (*Schizosaccharomyces pombe*, Human)

## 1. INTRODUCTION

The baker yeast *Saccharomyces cerevisiae* has gained popularity as a host for heterologous protein synthesis. This is due to the fact that *Sac. cerevisiae* is not a pathogen and is widely acceptable for use in the food industry. In addition, yeast exhibits the posttranslational modification systems (glycosylation, acetylation, phosphorylation etc.) which are lacking in prokaryotic microorganisms. The ability to introduce foreign DNA into the yeast cell and into the yeast genome, coupled with advantages in molecular cloning techniques has led to a number of eukaryotic proteins expressed in *Sac. cerevisiae*.

Among the yeast species studied so far, the fission yeast *Schizosaccharomyces pombe*, which is taxonomically and evolutionarily far distant from the baker yeast [1], has received less attention concerning genetic engineering. *S. pombe* probably provides an alternative host for heterologous gene expression. As *Sac. cerevisiae*, the fission yeast is also nonpathogenic and is genetically and physiologically well characterized (for review see [2,3]). The fission yeast has found interest in the alcohol industry [4] and is used as a means of deacidification of grape musts, because this yeast can metabolize malic acid [5,6].

So far, there are only some examples for expression of heterologous genes from higher eukaryotes in *S. pombe*: the polyoma virus middle-T antigen [7], the human antithrombin III [8] and the human liver epoxide hydrolase [9]. When promoters from *Sac. cerevisiae* genes were used [7,8], the expression rate was rather low, because transcription initiation signals recognized by *S. pombe* and *Sac. cerevisiae* are not identical [10,11]. The only reported homologous promoter for expression studies in *S. pombe* is the element from the alcohol dehydrogenase gene (*adh*), which has been cloned and sequenced by Russell and Hall [12]. For mitotic stability and partitioning of plasmids in the fission yeast, a DNA sequence termed *stb* [13] is needed in addition to an *S. pombe ars* element. So far, no expression vector for *S. pombe* is available, which combines (i) homologous transcription initiation and (ii) termination signals in combination with (iii) selection markers for *E. coli* and *S.*

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*pombe* in addition to (iv) DNA elements which function as *ori* and (v) elements which improve the symmetric segregation of the plasmid. This paper describes the construction of general expression vectors for *S. pombe* and its use to produce enzymatically active human blood coagulation protein factor XIIIa (FXIIIa).

## 2. MATERIALS AND METHODS

### 2.1. *Microbial strains and methods*

*E. coli* DH1 [14] was used for cloning in bacteria and cloning and expression in yeast was done in *S. pombe* 972 h-*ura4*-294 [13]. Transformed yeast cells were grown at 30°C in a shaker flask in selective YNBA medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, 0.2% asparagine). After two days, complex medium YPD (1% yeast extract, 2% peptone, 2% dextrose) was inoculated with 10% of a preculture grown in YNBA medium. Yeast cell extracts were prepared in a glass bead mill.

### 2.2. *DNA methods*

Isolation of plasmid DNA, preparation of DNA fragments, DNA synthesis reactions, DNA ligations, transformation in *E. coli* and screening of plasmid containing colonies were carried out essentially as described by Maniatis et al. [15]. Transformation of *S. pombe* was done as described previously [16]. Nucleotide sequence was determined by the dideoxy method [17] using [$^{35}$S]dATP$\alpha$S.

### 2.3. *Protein analysis*

FXIIIa concentration was measured by an ELISA. Microtiter plates were coated with anti-FXIIIa immunoglobulins isolated from sera of rabbits immunized with human FXIIIa of 99% purity. Peroxidase-coupled anti-FXIIIa antibodies were used as conjugate. Western blots were done according to Burnette [18] using anti-FXIIIa rabbit serum and a second antibody coupled with alkaline phosphatase. Activity of FXIIIa was measured by the clot stability assay [19].

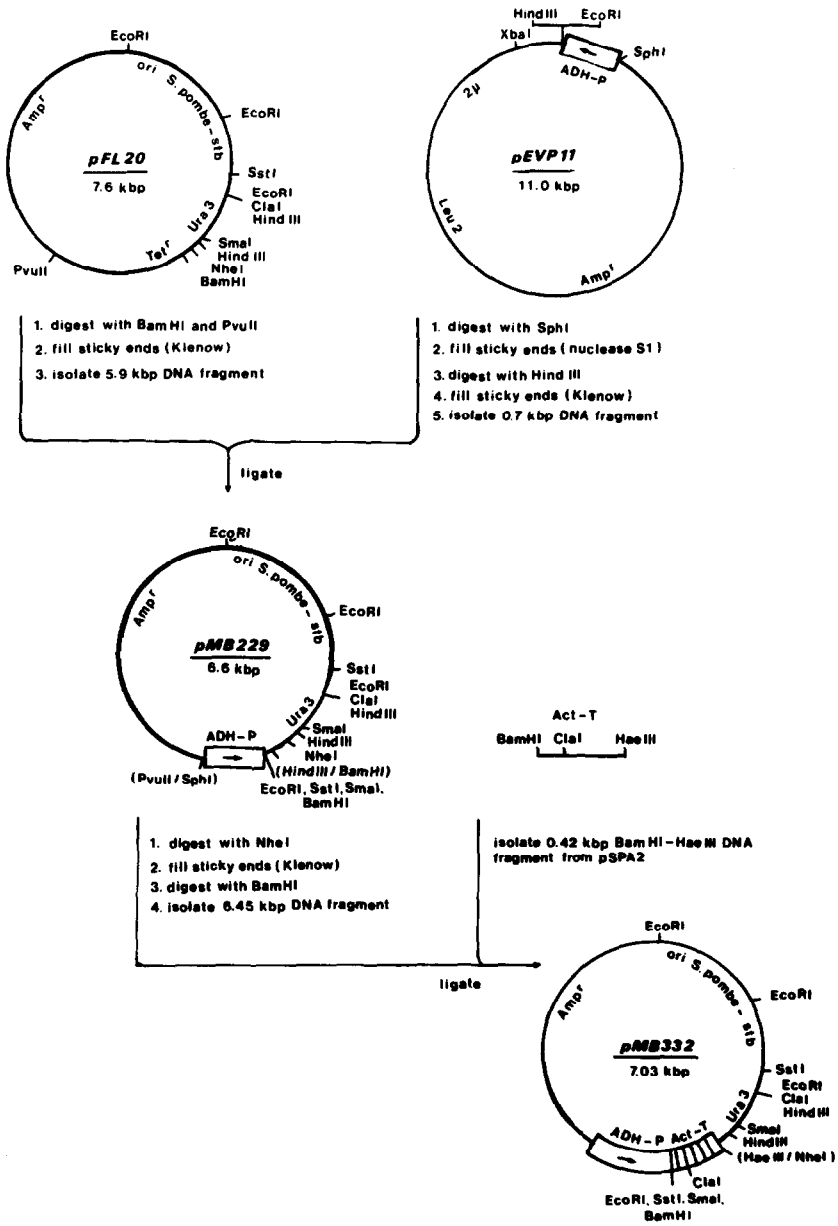
## 3. RESULTS

### 3.1. *Construction of an* S. pombe *expression vector*

The strategy to construct efficient expression vectors for the fission yeast was to combine characterized transcription and termination signals of *S. pombe* genes with elements that facilitate a high plasmid stability in this yeast. The ancestor for the promoter was the vector pEVP11 [20]. This plasmid is based on the *Sac. cerevisiae-E. coli* shuttle vector YEp13 [21] in which has been inserted the 700 bp *Eco*RI/SpH fragment containing the *S. pombe* adh promoter adjacent to the *Eco*RI/*Hin*dIII polylinker from pUC12. The *Eco*RI site is at position −45 to the translational start codon of the *adh* gene. The adh promoter fragment was isolated from pEVP11 and was ligated into the plasmid backbone of pFL20, from which the region which confers tetracycline resistance in *E. coli* has been deleted (for details see fig.1). The *Bam*HI/*Pvu*II DNA fragment from pFL20 confers the *ars* activity in *S. pombe* and the *stb* element promotes the mitotic stability of the vector in the fission yeast. In addition, this DNA fragment carries the *ori* and the *amp*$^{R}$ gene for selection in *E. coli*. Downstream of the adh promoter within pMB229, we inserted a DNA fragment from plasmid pSPA2 that carries efficient transcription termination signals originating from the *S. pombe* actin gene [22]. The *Bam*HI site is located in the carboxy-terminal region 96 bp upstream of the translational stop codon of the actin coding DNA. The transcription terminates at position +59/+60 downstream of the protein coding sequence of the actin gene.

The new *S. pombe/E. coli* shuttle vector pMB332 has the following features: (i) due to the *S. pombe ars* and *stb* elements, it replicates episomally and has a high mitotic stability; (ii) the *Sac. cerevisiae Ura3* gene can complement *S. pombe ura4* strains to uracil prototrophy; (iii) it carries the strong transcription initiation signals from the *S. pombe adh* gene; and (iv) the transcription termination signals from the *S. pombe* actin gene. (v) The unique *Bam*HI site between the promoter and terminator elements can be used for insertion of foreign genes. The unique *Bam*HI site is 25 bp downstream of the transcription initiation site. A transcriptional hybrid would be synthesized, which has an *adh* specific leader of 13 bases, a

Fig.1. Genealogy of the *S. pombe* expression vector pMB332. For detailed cloning procedure and function of the various elements see text. At the bottom, the relevant DNA sequence around the unique *Bam*HI cloning site between the fusion of the adh promoter and actin terminator is shown. Restriction enzymes within the brackets indicate the fusion of two different DNA ends. The *Nhe*I site in pMB332 is regenerated by fusion to the *Hae*III site. The open arrow indicates the transcription start point within the *adh* gene, the two thin arrows indicate the transcription termination within the actin gene. A possible polyadenylation signal sequence is indicated by a broken line.


EcoRI
ori S. pombe - stb
EcoRI
Amp^r
pFL20
7.6 kbp
SstI
EcoRI
ClaI
Hind III
Ura 3
Tet^r
SmaI
Hind III
NheI
BamHI
PvuII
HindIII EcoRI
XbaI
SphI
ADH-P
2μ
pEVP11
11.0 kbp
Leu 2
Amp^r
1. digest with BamHI and PvuII
2. fill sticky ends (Klenow)
3. isolate 5.9 kbp DNA fragment
1. digest with SphI
2. fill sticky ends (nuclease S1)
3. digest with Hind III
4. fill sticky ends (Klenow)
5. isolate 0.7 kbp DNA fragment
ligate
EcoRI
ori S. pombe - stb
EcoRI
Amp^r
pMB229
6.6 kbp
SstI
EcoRI
ClaI
HindIII
Ura3
SmaI
HindIII
NheI
ADH-P
(HindIII / BamHI)
(PvuII / SphI)
EcoRI, SstI, SmaI, BamHI
Act-T
BamHI ClaI HaeIII
1. digest with NheI
2. fill sticky ends (Klenow)
3. digest with BamHI
4. isolate 6.45 kbp DNA fragment
isolate 0.42 kbp BamHI-HaeIII DNA fragment from pSPA2
ligate
EcoRI
ori S. pombe - stb
EcoRI
Amp^r
pMB332
7.03 kbp
SstI
EcoRI
ClaI
HindIII
Ura3
SmaI
HindIII
(HaeIII / NheI)
ADH-P
Act-T
ClaI
EcoRI, SstI, SmaI, BamHI
ADH-P
DNA linker
-54
5'-CTTTTCTTTTCTTTAAGCAAGAGAATTCGAGCTCGCCCGGGGATCC-
EcoRI SstI
SmaI
BamHI
coding
3'-noncoding region of Actin gene
+40
+70
--96bp--- --GAATAACATCGATTCTTCTGATATATATAAATTT-3'
ClaI

short sequence determined by the *Sst*I/*Sma*I/*Bam*HI DNA linker region and possibly stretches of the foreign mRNA leader. The transcription termination signals of the actin gene are located 154 bp 3′ to the unique *Bam*HI site. In case that the foreign gene carries no signals for transcription termination in the fission yeast, the 3′-nontranslated region of the expressed mRNA thus would additionally contain a stretch of 96 bases determined by the carboxy-terminal portion of the actin coding DNA and the adjacent sequence of the nontranslated actin mRNA stretch. (vi) A further advantage of pMB332 is its relatively small size compared to other yeast cloning vectors.

### 3.2. *Construction of an* S. pombe *'ATG vector'*

In order to facilitate the expression of foreign DNA sequences coding for protein fragments, but missing a translational start codon, we broadened pMB332 to an 'ATG vector'. The plasmid was opened with *Bam*HI, the single-stranded overhangs were digested with nuclease $S_1$ and a synthetic DNA linker was inserted such that a translational start codon is located 31 bp downstream of the *adh* transcription initiation site. For translational in frame fusion of foreign DNA, one can either use the first *Bam*HI site or the unique *Bgl*II site which follow the ATG codon. Many eukaryotic proteins are subjected to a variety of co- or posttranslational modifications. The structural alterations resulting from these modifications can influence translocation, biological activity or turnover. Two enzymes, methionine aminopeptidase and $N^{\alpha}$-acetyltransferase can act in concert to remove the initiator methionine residue and add an *N*-acetyl group [23]. Several lines of evidence suggest that the N-terminal amino acid of proteins may play a major role in determining intracellular stability, which led to the N-end rule [24]. Serine in position +2 can cause a high stability of intracellular proteins. In addition, a compilation of the distribution of amino acids at position +2 showed, that in total, 28% of baker yeast genes favor serine at this position [25]. Therefore we positioned a triplet coding for serine at position +2 sandwiched between the translational start codon and the cloning sites. Once the foreign DNA has been inserted into the *Bam*HI or *Bgl*II site, it can be isolated in its modified form with its translational start codon on a transportable *Dra*I fragment and can be transferred to other expression vectors.

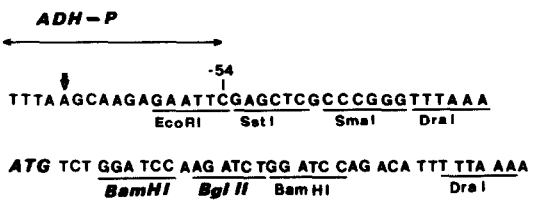



Fig.2. Relevant DNA sequence of the 'ATG vector' pMB340. The translational ATG start codon and the possible *Bam*HI and *Bgl*II cloning sites for translational in frame fusion are marked in bold letters.

### 3.3. *Expression of human FXIIIa in* S. pombe

The function of the expression vector was analyzed by cloning a prokaryotic and an eukaryotic test gene into the plasmid pMB332.

The prokaryotic marker gene was the *E. coli lacZ* gene, coding for β-galactosidase. *S. pombe* cells were transformed with pMB333, a modified pMB332, in which the 3000 bp *Hin*dIII/*Nco*I *lacZ* bearing DNA fragment from pLG400 [26] had been cloned. These cells produced enzymatically active β-galactosidase. Colonies turned blue on agar plates containing the enzyme substrate X-Gal and cell free extracts hydrolyzed the substrate ONPG (data not shown).

For more detailed analysis, we tested the new expression system to direct the synthesis of the human protein factor XIIIa (FXIIIa). FXIIIa is a blood coagulation protein that is distributed in plasma, platelets and placenta. The plasma protein consists of a tetramer ($a_2b_2$) containing two copies of each an a and b subunit, while the platelet and the placenta-derived material is an $a_2$ dimer [27]. The a subunit contains the active sites. FXIIIa (84 kDa) is activated by thrombin to FXIIIa′ to yield the enzymatically active transglutaminase (80 kDa) and the activation peptide of 4 kDa from the amino-terminus (for enzymatic and clinical aspects see review [28]). The cDNA coding for FXIIIa has been cloned and sequenced [29] and FXIIIa has been recently expressed in *E. coli* [30,31]. But most of the material is found in unsoluble inclusion bodies and therefore has no biological activity.

To express FXIIIa in *S. pombe*, we ligated the

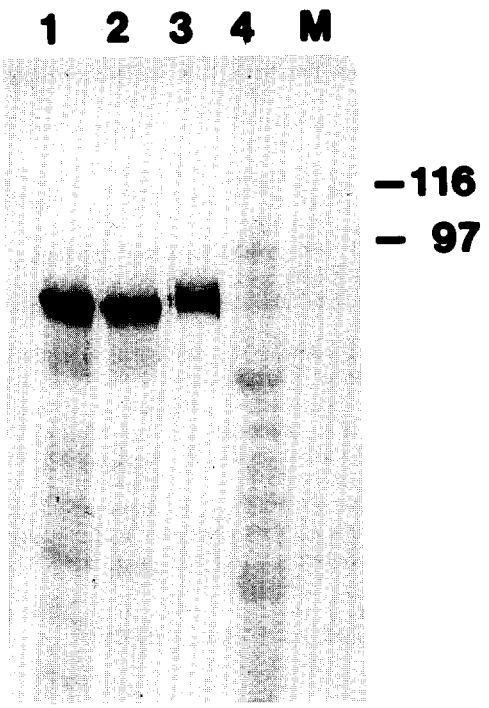



Fig.3. Western blot analysis of *S. pombe* cell extracts with anti-FXIIIa serum. *S. pombe* [pMB334] cells expressing FXIIIa and harvested after 70 h growing in complex medium (lane 1) and after 90 h (lane 2); purified placental FXIIIa (lane 3); extract of *S. pombe* cells without plasmid; molecular mass marker proteins (lane M): only β-galactosidase (116 kDa) and phosphorylase (97 kDa) can be identified in the original blot due to a faint unspecific immunoreaction.

2327 bp *Eco*RI/*Hin*dIII fragment from pTrc99A-FXIIIa [31] into the unique *Bam*HI site of pMB332. The resulting plasmid pMB334 was transformed into *S. pombe ura4* strains, and uracil prototrophic colonies were analyzed for synthesis of FXIIIa. In extracts of *S. pombe* [pMB334] cells, grown in complex YPD medium, up to 2 mg FXIIIa/l culture broth were detected by a specific ELISA. Western blot analyses show that FXIIIa synthesized in *S. pombe* has the same apparent molecular mass as placenta-derived FXIIIa and no degradation products of recombinant FXIIIa could be identified (fig.3). The biological activity of recombinant FXIIIa from the fission yeast was verified in the clot stability assay.

## 4. DISCUSSION

Here we present new expression vectors for the fission yeast *S. pombe*, which allow the construction of transcriptional or translational fusions. To facilitate effective production rates, homologous signals for initiation and termination of transcription were combined with elements that confer a high mitotic stability of the vectors. The production yield of the human placenta protein was 2 mg/l, when the transformants were grown in shaker flasks. Even higher yields should be obtained under optimized conditions in fed batch fermentations. It seems likely that several factors are responsible for the yield of heterologous products. These could be the stability of the protein, the efficiency of translation of the heterologous mRNA, the stability of the transcripts and the efficiency of the mRNA synthesis as well as the copy number of the expression vector [32]. Therefore, the production yield will differ from one case to the next and the expression efficacy has to be individually tested for each protein.

So far, FXIIIa is stably expressed in *S. pombe*, has the same molecular mass and can be converted to enzymatically active FXIIIa′ by the action of thrombin. Deduced from the cDNA sequence [29], FXIIIa has six potential glycosylation sites, but no sugar residues were detected when the protein sequence was analyzed [33]. As the migration pattern of FXIIIa from yeast is identical to FXIIIa from placenta, we conclude that the recombinant FXIIIa is not glycosylated or it contains only minor carbohydrate residues.

Based on the expression system described above, *S. pombe* might be an appropriate and alternative host for production and functional analysis of complex eukaryotic foreign proteins.

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