Mol Gen Genet (1989) 220:95–101

MGG

Cauliflower mosaic virus promoters direct efficient expression of a bacterial G418 resistance gene in *Schizosaccharomyces pombe*

Hans Gmünder* and Jürg Kohli

Institute of General Microbiology, University of Bern, Baltzer-Strasse 4, CH-3012 Bern, Switzerland

Summary. A system is presented for transformation of the fission yeast *Schizosaccharomyces pombe* to resistance against the antibiotic G418. The bacterial resistance gene of the transposon Tn*5* is expressed under the control of promoters and transcription terminators from cauliflower mosaic virus (CaMV). The promoter of the *S. pombe* alcohol dehydrogenase gene has also been used. Transformants can be selected directly on medium containing G418 (up to 1 mg/ml) due to inactivation of G418 by the Tn*5* gene product, the aminoglycoside 3′-phosphotransferase (II). The plant viral promoter 35S confers higher resistance to G418 than the 19S promoter. This corresponds to the relative strengths of these promoters in plant cells. The strong plant promoter 35S yields resistance comparable to that obtained with the strong *S. pombe* promoter from the alcohol dehydrogenase gene. The constructions with the two plant promoters have been used on multicopy shuttle plasmids that replicate autonomously in *S. pombe* and *Escherichia coli*. In addition the 35S and the 19S constructions have been inserted into the *S. pombe* genome where they confer G418 resistance as single copy genes. Since vector sequences are excluded in this case, all the necessary signals for expression of G418 resistance are contained within the DNA fragments containing the plant promoters, the resistance gene and the plant terminators. This transformation system is independent of *S. pombe* mutants. It may be useful for the transformation of other lower eukaryotes. The activity of the CaMV promoters in *S. pombe* may be exploited for the expression of plant genes in fission yeast.

Key words: *Schizosaccharomyces pombe* – Cauliflower mosaic virus – Plant promoters – G418 resistance marker – Yeast transformation

Introduction

Transformation of the fission yeast *Schizosaccharomyces pombe* using marker genes that complement auxotrophic mutations has been reported. Various shuttle plasmids replicating autonomously in *S. pombe*, *Saccharomyces cerevisiae* and *Escherichia coli* have been described (Beach and Nurse 1981; Heyer et al. 1986; Wright et al. 1986b). Integrative transformation, gene disruption and gene replacement (all based on homologous recombination between transforming DNA and chromosomes) have also been described (Grimm et al. 1988; Grimm and Kohli 1989). For certain applications a marker gene conferring resistance to a drug is useful, because the recipient strain may then be free of any specific mutations.

G418 (Geneticin) is a 2-deoxystreptamine antibiotic with a wide range of inhibitory activity. It blocks protein synthesis in bacterial, fungal, plant and animal cells. G418 resistance determinants in bacteria are carried on transposable elements which encode aminoglycoside 3′-phosphotransferase. This enzyme inactivates a number of aminoglycoside antibiotics containing the 2-deoxystreptamine moiety at the 3′-hydroxyl position. Cotransformation of yeast vectors with other plasmids carrying the G418 resistance gene from the bacterial transposon Tn*903* has yielded antibiotic-resistant transformants containing recombined plasmids in *S. cerevisiae* (Jimenez and Davies 1980) and *S. pombe* (Sakai et al. 1984). But these results have not led to the construction of widely used vectors for yeast transformation.

Plants resistant to the related antibiotic kanamycin have been obtained by transformation with a combination of cauliflower mosaic virus (CaMV) promoters with the resistance genes from transposons Tn*5* (Paskowksi et al. 1984) and Tn*903* (Pietrzak et al. 1986). It was demonstrated that the promoter directing the synthesis of the viral 35S mRNA conferred higher resistance than that of the viral 19S mRNA.

For transformation of fission yeast we chose constructions that are proficient in plant transformation based on the resistance gene from transposon Tn*5*. The results indicate that the 35S and the 19S promoter from CaMV as well as the *S. pombe* alcohol dehydrogenase promoter confer resistance to G418. This is the case for autonomous, multicopy vectors carrying the constructions, and for single copy integrations into the genome.

Materials and methods

Strains and plasmids. The *E. coli* strains used in this study were JA221 (F⁻ *leuB6 trpE5 lacY hsdR hsdM*⁺ *recA1*) (Clarke et al. 1978) and BJ5183 (F⁻ *recBC sbcB endo-1 gal met str thi bio hsd*) (Losson et al. 1983). 972 h^- is the wild-type strain of *S. pombe*. Other strains carried the *ura4-294* and *leu1-32* point mutation alleles or the *ura4-D18* dele-

* *Present address:* F. Hoffmann-La Roche & Co Ltd, CH-4002 Basel, Switzerland

Offprint requests to: J. Kohli

tion (Grimm et al. 1988). Plasmids pABDI and pHP23 were obtained from J. Paszkowski (Paszkowski et al. 1984), pEVP11 from P. Russell (Russell and Hall 1983) and pURA3-1.1 was described earlier (Heyer et al. 1986).

Media and genetic methods. A description of standard genetic methods and media was published by Gutz et al. (1974). Complete medium contains 5 g/l yeast extract and 30 g/l glucose; 20 g/l agar is added for plates. Minimal medium contains 10 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids, and (when necessary) 100 mg/l uracil or leucine. G418-sulphate was dissolved in water and added to complete medium at 55° C to the desired final concentration.

The preparation of the medium for the selection of Ura$^-$ strains by 5-fluoro-orotic acid (5-FOA) and the transformation procedure used in this case have been described (Grimm et al. 1988). A 2× concentrated stock solution (1.34% yeast nitrogen base, 2% glucose, 100 µg/ml uracil) was mixed at 55° C with an equal volume of freshly autoclaved 4% agar. Then 1 mg/ml solid 5-FOA was dissolved at 55° C and the medium solidified in dishes.

DNA methods. Restriction endonucleases and DNA ligase were used according to the manufacturer's instructions. Dephosphorylation with calf intestinal alkaline phosphatase, conversion of fragments with protruding 5′ ends to blunt ends with Klenow fragment of DNA polymerase I and small-scale plasmid preparations were carried out as described (Maniatis et al. 1982). Large scale plasmid preparation was carried out according to Humphreys et al. (1975). Described procedures were followed for transformation of *E. coli* (Kushner 1978; Mandel and Higa 1970).

S. pombe was transformed by the lithium acetate protocol (Ito et al. 1983). With ars plasmids between 10^3 and 5×10^3 transformants were obtained. DNA was prepared according to Wright et al. (1986a). For physical analysis of plasmids and chromosomes, DNA electrophoresed in a 0.8% agarose gel was transferred to nitrocellulose membranes (Southern 1975) and hybridized with nick-translated probes.

For cloning, DNA fragments were isolated from low-melting agarose gels as described by Maniatis et al. (1982), or with DEAE-cellulose membranes from Schleicher & Schuell according to the manufacturer's instructions. For integrative transformation DNA fragments were purified by extraction with phenol, phenol/chloroform, chloroform, and finally on a Sephadex G-50 spin-column as described (Maniatis et al. 1982).

*Bam*HI linker, restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I and calf intestinal alkaline phosphatase were purchased from Boehringer. Antibiotic G418-sulphate (Geneticin) was from Gibco at a microbiological potency of 458 µg/mg of solid material. The amounts referred to herein are the actual concentrations of the antibiotic, not the amount of solid material.

Results

Sensitivity of S. pombe *cells to the antibiotic G418*

The sensitivity of *S. pombe* to the antibiotic G418 depends on the concentration of the antibiotic and the density of the plated cells. Wild-type cells of strain 972 were plated on complete medium containing G418 at concentrations varying from 50 µg/ml to 1 mg/ml. The number of cells inoculated per plate varied between 5×10^6 and 5×10^7. No colony formation at any cell density was observed, when the G418 concentration was 100 µg/ml or higher. At 50 µg/ml a few colonies formed at cell densities of 2×10^7 or higher. Restreaking of these growing colonies on medium with G418 showed that the cells were still sensitive to the antibiotic. Thus the minimum concentration of G418 should be 100 µg/ml in complete medium at cell densities between 5×10^6 and 5×10^7 per plate. Replica plating of colonies to G418-containing medium to distinguish between resistant and sensitive strains is not easily done. At high local cell concentrations growth can occur in the absence of genetically determined G418 resistance. In the experiments described below, usually fewer than 2×10^7 cells were plated on dishes containing G418. Throughout our studies we never observed spontaneous mutants with genetically stable resistance to G418 at the concentrations used.

Autonomously replicating plasmids conferring G418 resistance to S. pombe

To test the bacterial aminoglycoside 3′-phosphotransferase from transposon Tn*5* for inactivation of the antibiotic G418 in *S. pombe*, we relied on contructions used for transformation of plant cells. These contructions have the advantage that no out-of-frame AUG codons occur between the 5′ end of the mRNAs and the start AUG codon of the reading frame for the transferase, in contrast to the gene encoded by Tn*5* itself (Paszkowski et al. 1984). The resistance gene is under the control of the CaMV promoters and termina-

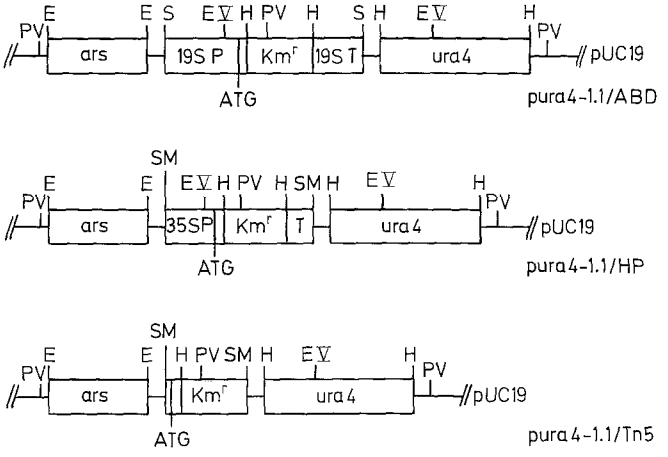

Fig. 1. Construction of replicating plasmids conferring G418 resistance. The *line* represents pUC19 sequences. The *Schizosaccharomyces pombe ars1* sequence (1.2 kb) and the *ura4* marker gene (1.7 kb) are *boxed. Boxes* marked 19S P, 19S T, 35S P and T represent the CaMV promoters and terminators derived from plasmids pABDI and pHP23 as described in the text. The bacterial kanamycin resistance gene (km^r) from transposon Tn*5* is *boxed.* This was introduced into the vectors together with promoter and terminator sequences from pABDI and pHP23. Only one orientation of the resistance gene cassette (promoter-Km^r-terminator) in the vectors in shown. The alternative orientations were also studied (see text). The recognition sites of restriction endonucleases are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; PV, *Pvu*II; S, *Sal*I; SM, *Sma*I; SP, *Sph*I

tors (polyadenylation sites). One series of constructions (ABD) contains a *SalI* fragment with promoter and terminator sequences that direct the synthesis of 19S RNA of CaMV (Paszkowski et al. 1984). As shown in Fig. 1 these control elements flank the antibiotic resistance gene. The other series of constructions (HP) is based on plasmid pHP23 (J. Paszkowski, unpublished). In this case an *Eco*RI fragment contains the promoter and terminator for the CaMV 35S RNA controlling the expression of the same antibiotic resistance gene. The protein products predicted from the nucleotide sequences of the hybrid genes are identical fusions of the bacterial phosphotransferase with 23 additional amino acid residues at the N-terminus. Beck et al. (1982) have shown that this fusion has little effect on the phosphorylation activity.

Initially it was planned to replace the viral promoters by the strong *S. pombe* promoter controlling the expression of the alcohol dehydrogenase gene (Russell and Hall 1983). But preliminary experiments with the unaltered ABD and HP contructions transferred into the replicating vector pURA3-1.1 demonstrated that both promoters from CaMV lead to reliable expression of G418 resistance in fission yeast. The vector pURA3-1.1 carries the gene *URA3* from *S. cerevisiae* as a selectable marker and the *ars1* sequence from the *S. pombe* genome promotes a high frequency of transformation (Heyer et al. 1986). The results obtained with these constructions are not shown.

Instead we present the results obtained with another series of constructions that confirm the initial data and allow the exclusion of effects of flanking vector sequences on the expression of the ABD and HP cassettes. The basis for the following constructions (Fig. 1) was plasmid pUC19 (Vieira and Messing 1982). The 1.1 kb *S. pombe ars1* sequence (Heyer et al. 1986; Maundrell et al. 1988) was inserted into the *Eco*RI site of the polylinker for promotion of high frequency of transformation and autonomous replication in fission yeast. The *S. pombe* marker gene *ura4* contained on a 1.8 kb fragment (Grimm et al. 1988) was inserted into the *Hin*dIII site of the plasmid. Then the *Sal*I fragment from plasmid pABDI (Paszkowski et al. 1984) containing the resistance gene under control of the 19S promoter was inserted in both orientations into the *Sal*I site of the polylinker. One orientation (pura4-1.1/ABD) is shown in Fig. 1. The vector with the other orientation was called pura4-1.1/DBA. From plasmid pHP23 (J. Paszkowski, unpublished) an *Eco*RI fragment containing the 35S promoter was cut and the overhanging ends filled in. It was then inserted in both orientations into the *Sma*I site. Plasmid pura4-1.1/HP is shown in Fig. 1. The version with an inverted insertion was called pura4-1.1/PH. As control vectors the plasmids pura4-1.1/Tn5 (Fig. 1) and pura4-1.1/5nT were obtained. They contain the *Eco*RV-*Bam*HI fragment (made blunt-ended by filling up with Klenow polymerase) of pABDI (Paszkoswki et al. 1984) inserted into the *Sma*I site. In comparison with the other plasmids they lack the CaMV promoter and terminator sequences.

Transformation of *S. pombe* with the newly constructed vectors was performed by the lithium acetate method (Ito et al. 1983). The recipient strain used throughout is of mating type h^- and carries a deletion of the chromosomal *ura4* gene, *ura4-D18* (Grimm et al. 1988). Two types of transformation experiments were carried out: firstly selection on minimal medium for uracil independence with subsequent analysis of the transformants for G418 resistance (Fig. 2);

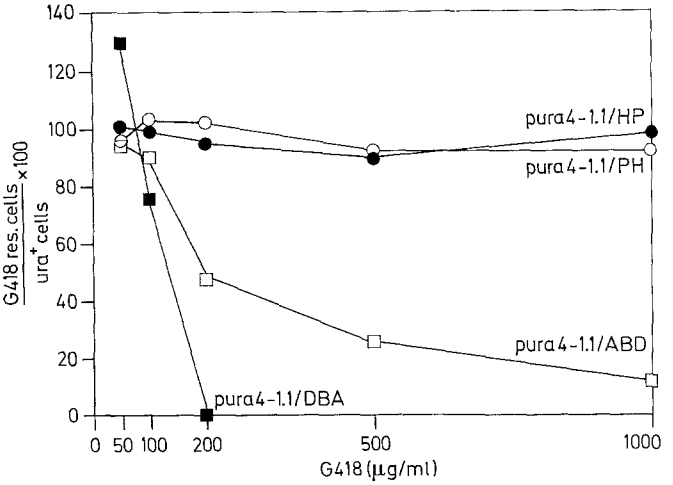

Fig. 2. G418 resistance of Ura^+ transformants. The plasmids shown in Fig. 1 were transformed into *Schizosaccharomyces pombe* h^- *ura4-D18* and Ura^+ transformants were selected. Five Ura^+ colonies from transformations with each of the six plasmids were studied. The antibiotic resistance of cells (colony forming ability) from Ura^+ colonies was examined on YEA plates containing G418. 100% is the number of colonies growing on MMA. The data for one transformant (typical of all five) for each plasmid are shown. The results for the control plasmids pura4-1.1/Tn5 and pura4-1.1/5nT, as well as the data points for the higher G418 concentrations for pura4-1.1/DBA (in all cases 0%) have been omitted for clarity

secondly, direct selection for antibiotic resistance of transformed cells (Fig. 3).

For the first analysis (Fig. 2) transformations were carried out with all six plasmids and Ura^+ colonies were selected on minimal medium. The frequency of transformants was in the order of $10^3 - 5 \times 10^3$/µg plasmid DNA for all vectors. Five transformants for each plasmid were then grown in liquid minimal medium for several hours and suitable dilutions plated on minimal medium (control for presence of plasmid), and also on complete medium containing G418 at various concentrations. Figure 2 shows the ratio of resistant cells divided by uracil prototrophs plotted against G418 concentration. Ura^+ cells containing either pura4-1.1/Tn5 or pura4-1.1/5nT (hence containing the G418 resistance gene without a promoter) could not grow on complete medium containing the antibiotic even at the lowest concentration. Cells containing plasmid pura4-1.1/ABD or pura4-1.1/DBA (19S promoter) were G418 resistant, but showed increasing sensitivity at high G418 concentrations (above 100 µg/ml). The pura4-1.1/DBA carrying cells were more sensitive to the antibiotic than pura4-1.1/ABD carrying cells. This indicates an effect of gene orientation on the strength of the 19S promoter. Cells transformed with pura4-1.1/HP or pura4-1.1/PH were resistant to G418 at concentrations as high as 1 mg/ml. The high strenght of the 35S promoter was also independent of the orientation of the cassette.

The results of direct selection for G418 resistance after transformation with the six plasmids (see Fig. 1) are given in Fig. 3. The transformed cells were grown for 3–5 generations in complete medium before plating on complete medium containing G418 at various concentrations. Control platings on minimal medium showed frequencies of approximately $10^3 - 5 \times 10^3$ Ura^+ transformants per µg plasmid DNA. Again no resistant colonies were obtained in the

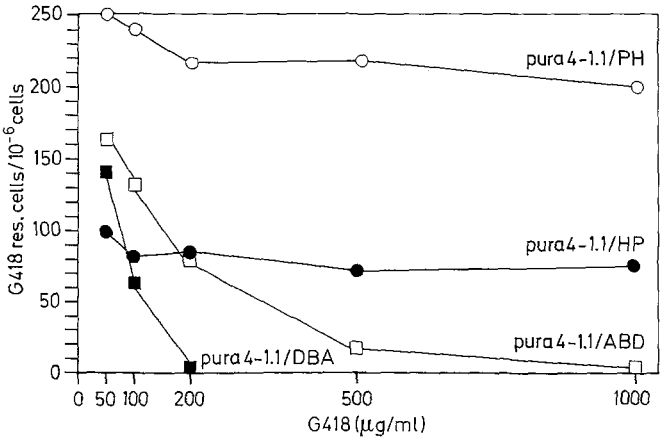

Fig. 3. Direct selection for G418 resistance after transformation. Transformation with each of the six plasmids shown in Fig. 1 was followed by growth for several generations before plating on YEA medium containing G418 at the concentrations indicated. The number of G418-resistant colony forming cells is shown. As in Fig. 2 the data for the control plasmids without promoter, and for pura4-1.1/DBA at higher G418 concentrations (no colonies formed) are omitted

experiments with the promoter-less plasmids. The constructions with the 19S promoter yielded resistant colonies only at low G418 concentrations. Again the ABD orientation of the gene allowed better resistance than the DBA orientation. Both constructions with the 35S promoter produced constant numbers of resistant colonies at all G418 concentrations. All the G418-resistant colonies were shown to be uracil independent by replica plating. This indicates that they still contain the plasmid with the *ura4*$^+$ marker gene.

From several G418-resistant transformants total DNA was isolated, digested with restriction endonucleases and analysed by hybridisation according to Southern (1975). In all cases the fragments expected from the different plasmids were detected. No additional rearranged fragments were found (data not shown). Thus the majority of transformants carry unrearranged autonomously replicating vectors. Copy number of plasmids was not determined, but may be in the order of similar vectors carrying the *ars1* sequence (25 per plasmid-carrying cell, see Heyer et al. 1986).

Russell and Hall (1983) have reported efficient transcription of the alcohol dehydrogenase gene *adh1* of *S. pombe* and constructed plasmid pEVP11, derived from the vector YEp13 (Broach et al. 1979), carrying the *adh1* promoter. The *Eco*RV-*Bam*HI fragment from pABDI (Paszkowski et al. 1984) was placed downstream of the *adh1* promoter in pEVP11. The resulting plasmid was used for transformation of an *S. pombe* strain carrying *leu1-32* that is complemented by the *LEU2* marker gene present on the vector. The transformation frequency was low as is usual for YEp13-derived plasmids, but all leucine-independent colonies displayed resistance to G418 at the high antibiotic concentration of 1 mg/ml (data not shown). Control pEVP11 transformants were sensitive at all G418 concentrations. G418-resistant transformants could also be obtained directly by selection on medium containing the drug after growth of transformed cells for 3–5 generations (data not shown). These results suggest that constructions with the CaMV 35S promoter (but not with the 19S promoter) result in the expression of the resistance gene at a similar high level to constructions with the strong promoter from the *adh1* gene of fission yeast.

The cassettes with 35S and 19S promoters in combination with the G418 resistance gene derived from Tn*5* were also placed into different vectors replicating autonomously in the budding yeast *S. cerevisiae*. No clear-cut G418-resistant transformants of budding yeast could be obtained, either by direct selection, or by preselection for amino acid independence and subsequent testing for resistance (J. Gafner, personal communication).

Single-copy integration of G418 resistance genes into chromosome III

The number of resistance genes per cell is high (approx. 25) and fluctuates depending on plasmid stability, when replicating plasmids are used for transformation. A better estimate of promoter strength can be obtained by single copy integration into the genome. In addition effects of flanking plasmid sequences on resistance gene expression can be excluded. For this reason constructions with both promoters from CaMV were introduced into chromosome III by replacement of the wild-type *ura4* gene following published procedures (Grimm et al. 1988).

From a pUC19 plasmid carrying the *ura4* gene with flanking sequences a 1.8 kb fragment was excised that contains the open reading frame and expression control sequences of *ura4* (Grimm et al. 1988). As shown in Fig. 4 the resulting plasmid pUC19-ura4-D18 retains a *Hind*III recognition sequence at the site of excision. The various constructs with the G418 resistance gene and CaMV promoters were inserted into this *Hind*III site that separates 5′ and 3′ chromosomal sequences on the plasmid. The plas-

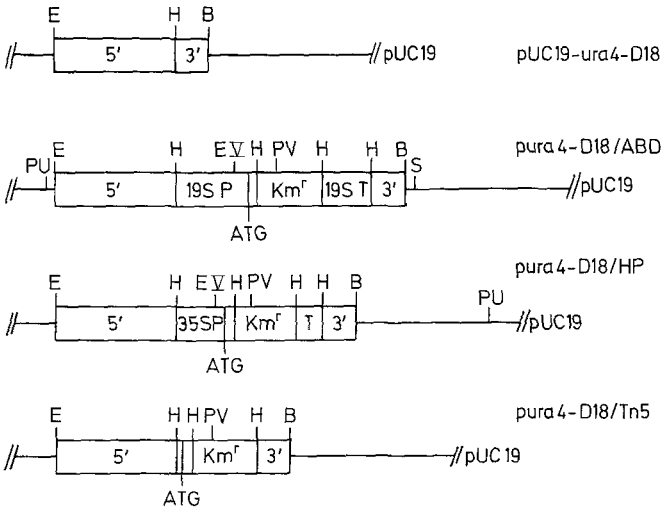

Fig. 4. Construction of plasmids for integrative transformations. The *line* represents pUC19 sequences. 5′ and 3′ represent the 5′ and 3′ flanking regions of the genomic *ura4* gene. The other *boxes* represent promoter – resistance gene – terminator sequences as explained in Fig. 1. The cutting sites S (*Sal*I) and PV (*Pvu*I) in the pUC19 vector are indicated in addition to the restriction sites explained in Fig. 1. The sites used for preparation of linear plasmid fragments are described in the text. The linear fragments were used for replacement of the *ura4*$^+$ gene by transformation. Plasmids with the resistance gene cassettes in the opposite orientation were also constructed (pura4-D18/DBA, pura4-D18/PH and pura4-D18/5nT) and used for gene replacement

mid pUC19-ura4-D18 was digested with *Hind*III, the cohesive ends filled in and the blunt-ended *Sal*I fragment from pABDI, the *Eco*RI fragment from pHP23 or the *Eco*RV-*Bam*HI fragment from pABDI was inserted. This led to plasmids pura4-D18/ABD and pura4-D18/DBA with the CaMV 19S promoter and terminator, plasmids pura4-D18/HP and pura4-D18/PH with the 35S promoter and polyadenylation site, and plasmids pura4-D18/Tn5 and pura4-D18/5nT with the resistance gene alone (no promoter). The fragments were inserted in both orientations in all cases (Fig. 4).

To create linear DNA fragments with recombinogenic ends for integrative transformation the plasmids were cut as follows: pura4-D18/ABD and pura4-D18/DBA with *Sal*I and *Pvu*I, leaving at the 3′ flank 12 bp and at the 5′ flank 117 bp from pUC19; pura4-D18/HP and pura4-D18/PH were cut with *Eco*RI and *Pvu*I leaving at the 3′ flank 1652 bp from pUC19; and pura4-D18/Tn5 and pura4-D18/5nT were cut with *Eco*RI and *Bam*HI. The fragments were not separated on gels, but the digests were treated with phenol and purified as described in Materials and methods. The DNA was then used directly for transformation of the *S. pombe* wild-type strain 972.

Transformation with each of the six constructions was carried out with several micrograms of DNA fragment. Then the cells were grown for approximately 10 generations in complete medium (Gutz et al. 1974). This leads to elimination of the *ura4* gene product, a condition for the selection of Ura$^-$ transformants by 5-FOA. In addition a sufficient level of the G418 resistance protein can be built up in the cells. Two different experiments were then performed: firstly, selection for Ura$^-$ transformants with subsequent testing for G418 resistance; secondly, direct selection for G418 resistant transformants.

With all six constructions between 20 and 50 Ura$^-$ transformants were obtained per µg fragment. From each transformation 30 colonies were streaked on minimal medium and minimal medium supplemented with uracil. Replacement of the *ura4* gene by the G418 construction is expected to lead to uracil dependence. This was probably the case in almost all of the colonies tested: they grew on minimal medium supplemented with uracil. Of the only two exceptions one showed slow growth on minimal and supplemented medium. The other grew neither on minimal nor on supplemented minimal medium. Both exceptions are probably due to the rarely occurring illegitimate integration of transforming DNA at locations other than the *ura4* locus (Grimm and Kohli 1988).

Five Ura$^-$ transformants from each of six constructs and additional control strains were analysed further. After growth in complete medium for 20 h, suitable dilutions were plated on minimal medium, minimal medium with uracil, and complete medium containing G418 at various concentrations. The results are presented in Fig. 5. As expected, cells carrying the ura4-D18 deletion (Grimm et al. 1988) and untransformed 972 cells could not grow in the presence of G418. Transformants containing only the resistance gene without promoter sequences were also G418 sensitive at the lowest concentration. Ura$^-$ cells carrying the 19S promoter constructions could form colonies at low G418 concentrations. The degree of resistance was dependent on the orientation of the cassette in the chromosome. However 35S promoter constructions led to resistance to G418 concentrations of up to 1 mg/ml independently of the orientation in the chromosome. From the 20 Ura$^-$ colonies derived from transformations with CaMV promoter constructions only two showed no G418 resistance at all. They may represent irregular integration events leading to *ura4* gene inactivation without creation of a functional G418 resistance gene.

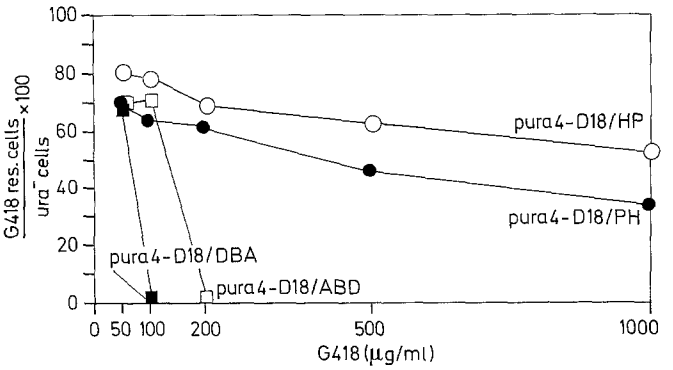

Fig. 5. G418 resistance of Ura$^-$ cells resulting from gene replacement. A wild-type strain was transformed with linear DNA cut from the plasmids described in Fig. 4 (see text). Ura$^-$ transformants were selected using 5-FOA (see text). Cells from five transformants chosen at random from experiments with each linear DNA were assayed for G418 resistance. The cells were plated on medium containing G418 at different concentrations and on control medium supplemented with uracil. The number of G418-resistant cells which formed colonies is given (100% is the number of colonies formed on G418-free medium). Plating efficiency for G418 resistance is maximal at low G418 concentrations (approximately 70% for all successful constructions). The data shown are typical of all five Ura$^-$ transformants studied for each construction. The results for the control constructions, as well as constructions pura4-D18/DBA and pura4-D18/ABD at higher G418 concentrations, are omitted in the figure. They yielded no G418-resistant colonies

Plating of transformed cells directly on G418-containing complete medium after growth for 10 generations in com-

Table 1. Direct selection for G418 resistance after integrative transformation of the wild-type strain 972

Fragments[a]	G418-resistant colonies/2×10^7 cells[b]				
	Concentration of G418 (µg/ml)				
	1000	500	200	100	50
pura4-D18/Tn5	–	–	–	–	–
pura4-D18/5nT	–	–	–	–	–
pura4-D18/ABD	–	–	–	11	99(3)
pura4-D18/DBA	–	–	–	1(1)	7(1)
pura4-D18/HP	42	44	38(1)	41(2)	50
pura4-D18/PH	89	140(3)	185(1)	144(2)	134(4)
pUC19-ura4-D18	–	–	–	–	–
None	–	–	–	–	–

[a] The plasmids shown in Fig. 4 were cut and purified before transformation as described in the text

[b] Cells were grown after transformation for 10 generations in complete medium and 2×10^7 cells were then plated on medium with G418. The numbers in parentheses are the few G418 transformants that were able to grow on minimal medium after replica plating (uracil-indepenent colonies). – indicates that no G418-resistant transformants were obtained. Repetition of this experiment led to very similar results

plete medium led to the results presented in Table 1. Colonies were only observed after transformation with promoter carrying fragments. Again, the G418 gene under the control of the 35S promoter confers resistance to high G418 concentrations. As observed in the previous experiments the ABD orientation of the 19S construct gives higher resistance than the DBA orientation. Overall the 19S promoter is again weaker than the 35S counterpart. The G418-resistant colonies were replicated on the appropriate media to check for uracil dependence. Only a few transformants (1%–2%) turned out to be uracil independent. They are assumed to be irregular integrations at the *ura4* locus or illegitimate integrations of the resistance gene eleswhere in the *S. pombe* genome.

Several G418-resistant (and uracil-dependent) clones derived from integrative transformations with the constructions shown in Fig. 4 were subjected to Southern blot analysis of their genome (data not shown). The pattern of hybridization with probes diagnostic for the *ura4* locus on chromosome III confirmed in all cases the expected structure of integration of the contruction by recombination between the homologous flanking sequences. No tandem amplifications or other rearrangements were observed. Thus the degree of G418 resistance indicated by the results in Table 1 can be correlated with the promoter strength of single copy genes.

Discussion

The experiments described demonstrate that the combination of transcription promotion and termination sequences from CaMV with the aminoglycoside 3′-phosphotransferase gene from the bacterial transposon Tn*5* leads to resistance to antibiotic G418, when the constructions are transferred into the fission yeast *S. pombe*. The identical combination of viral transcription signals with the antibiotic resistance gene has been used successfully in plants (Paszkowski et al. 1984). It was demonstrated that the constructions work on autonomously replicating multi-copy plasmids and also when they are integrated as single copies into the yeast genome. The results with different orientations of the G418 resistance gene constructions on replicating vectors and in the chromosome indicate that the CaMV and Tn*5* sequences are sufficient for expression of the resistance protein. No flanking sequences of the vector or chomosome are critically involved. This is also confirmed by the observation that the constructions with the 35S promoter consistently yield resistance to higher G418 doses than the constructions with the 19S promoter. The relative strength of the two promoters in *S. pombe* is thus the same as in plants (Paszkowski et al. 1984). The 35S promoter can be considered as a strong promoter for fission yeast: as a single copy it confers resistance to G418 concentrations of up to 1 mg/ml. It also yields resistance levels comparable to constructions with the strong *S. pombe* promoter of the alcohol dehydrogenase gene (Russell and Hall 1983).

No experiments have been carried out on the mRNA levels produced by the different constructions or to determine the transcription start and termination sites. But recently the 35S promoter has been combined with a bacterial β-glucuronidase gene. Efficient synthesis of the enzyme in *S. pombe* was observed and the S1 mapping of the 5′ end of the mRNA indicated the start of transcription in *S. pombe* to be at exactly the same position as usually observed in plant cells (N. Käufer, personal communication).

An interesting detail of integrative transformation by homologous recombination of the linear G418 constructions with chromosome III deserves discussion. At the ends of the homologous flanking sequences of the linear transforming DNA non-homologous vector sequences of 12, 117 and 1652 bp remained (Fig. 4). Nevertheless the same frequency of correct integrations was observed with these linear DNAs compared with other linear constructions that had no non-homologous sequences added to their ends. It has been shown before that integration of linear DNA by homologous recombination occurs efficiently in *S. pombe* (Grimm et al. 1988). The present results indicate that rather long non-homologous sequences added to the ends of homologous linear DNA are no severe obstacle to integration by homology.

The use of a G418 resistance gene coupled with expression signals from a plant virus has led to the development of a novel and efficient transformation system for fission yeast. In contrast to the marker genes described so far for *S. pombe* transformation (Beach and Nurse 1981; Heyer et al. 1986; Wright et al. 1986) the selection for G418 resistance in transformants is independent of the genetic background of the recipient strain. The described constructions may also be helpful in the development of vectors for other yeasts and fungi. But neither the 35S or the 19S promoter of CaMV seems to be active in *S. cerevisiae* (J. Gafner, personal communication).

We have demonstrated the activity of plant virus expression signals for gene expression in fission yeast. This should allow the construction of shuttle vector systems for alternative expression of genes in *S. pombe* and plants using the same DNA construction. This situation resembles the correct expression of SV40 transcription signals in *S. pombe* (Jones et al. 1988) that have allowed the expression cloning of mammalian genes by complementation of the corresponding *S. pombe* mutants (Lee and Nurse 1987). Also plant genes can now be cloned by expression of function and studied in the model eukaryotic system represented by the unicellular fission yeast.

Acknowledgements. We thank J. Paszkowski and P. Russell for discussions and the plasmids pABDI, pHP23 and pEVP11. Thanks also to J. Gafner and N. Käufer for the communication of results prior to publication. This work was supported by a grant from the Nestlé Research Center, Nestec Ltd, Vevey, Switzerland.

References

Beach D, Nurse P (1981) High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature 290:140–142

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Broach JR, Strathern JN, Hicks JB (1979) Transformation in yeast: Development of a hybrid cloning vector and isolation of the *can1* gene. Gene 8:121–133

Clarke L, Carbon J (1978) Functional expression of cloned yeast DNA in *Escherichia coli*: specific complementation of argino succinate lyase (argH) mutations. J Mol Biol 120:517–532

Grimm C, Kohli J (1988) Observations on integrative transformation in *Schizosaccharomyces pombe*. Mol Gen Genet 215:87–93

Grimm C, Kohli J, Murray J, Maundrell K (1988) Genetic engineering of *Schizosaccharomyces pombe*: a system for gene dis-

ruption and replacement using the ura4 gene as a selectable marker. Mol Gen Genet 215:81–86
Gutz H, Heslot H, Leupold U, Loprieno N (1974) *Schizosaccharomyces pombe*. In: King RD (ed) Handbook of genetics vol 1. Plenum, NY, pp 395–446
Heyer WD, Sipiczki M, Kohli J (1986) Replicating plasmids in *Schizosaccharomyces pombe*: Improvement of symmetric segregation by a new genetic element. Mol Cell Biol 6:80–89
Humphreys GO, Willshaw GA, Anderson ES (1975) A simple method for the preparation of large quantities of pure plasmid DNA. Biochim Biophys Acta 383:457–463
Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact cells treated with alkali cations. J Bacteriol 153:163–168
Jimenez A, Davies J (1980) Expression of a transposable antibiotic resistance elment in *Saccharomyces*. Nature 287:869–871
Jones RH, Moreno S, Nurse P, Jones NC (1988) Expression of the SV40 promoter in fission yeast: Identification and characterization of an AP-1-like factor. Cell 53:659–667
Käufer N, Simanis V, Nurse P (1985) *Schizosaccharomyces pombe* correctly exises a mammalian RNA transcript intervening sequence. Nature 318:78–80
Kushner SR (1978) An improved method for transformation of *Escherichia coli* with Co1E1-derived plasmids. In: Boyer HB, Nicosia S (eds) Genetic engineering. Elsevier/North-Holland, Amsterdam, p 17
Lee MG, Nurse P (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. Nature 327:31–35
Losson R, Lacroute F (1983) Plasmids carrying the yeast OMP decarboxylase structural and regulatory genes: transcription regulation in a foreign environment. Cell 32:371–377
Mandel M, Higa A (1970) Calcium dependent bacteriophage DNA infection. J Mol Biol 53:154
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manuel. Cold Spring Harbor Laboratory, NY
Maundrell K, Hutchinson A, Shall S (1988) Sequence analysis of ARS elements in fission yeast. EMBO J 7:2203–2210
Paszkowski J, Shilito RD, Saul M, Mandak I, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722
Pietrzak M, Shilito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res 14:5857–5868
Russell PR, Hall BD (1983) The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. J Biol Chem 258:143–149
Sakai K, Sakaguchi J, Yamamoto M (1984) High-frequency cotransformation by copolymerization of plasmids in the fission yeast *Schizosaccharomyces pombe*. Mol Cell Biol 4:651–656
Southern EM (1975) Detection of Specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268
Wright APH, Maundrell K, Shall S (1986a) Transformation of *Schizosaccharomyces pombe* by non-homologous unstable integration of plasmids in the genome. Curr Genet 10:503–508
Wright A, Maundrell K, Heyer WD, Beach D, Nurse P (1986b) Vectors for the construction of gene banks and the integration of cloned genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Plasmid 15:156–158

Communicated by W. Gajewski

Received August 14, 1989