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Foreign Gene Expression in Yeast: a Review

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INTRODUCTION

The yeast *Saccharomyces cerevisiae* has several properties which have established it as an important tool in the expression of foreign proteins for research, industrial or medical use. As a food organism, it is highly acceptable for the production of pharmaceutical proteins. In contrast, *Escherichia coli* has toxic cell wall pyrogens and mammalian cells may contain oncogenic or viral DNA, so that products from these organisms must be tested more extensively. Yeast can be grown rapidly on simple media and to high cell density, and its genetics are more advanced than any other eukaryote, so that it can be manipulated almost as readily as *E.coli*. As a eukaryote, yeast is a suitable host organism for the high-level production of secreted as well as soluble cytosolic proteins.

Most yeast expression vectors have been based on the multi-copy 2μ plasmid and contain sequences for propagation in *E.coli* and in yeast, as well as a yeast promoter and terminator for efficient transcription of the foreign gene (Figure 1). The recent

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rapid expansion in yeast molecular genetics has led to a great increase in our understanding of these components, and as a result there is now a bewildering choice of promoter systems and methods for propagating foreign DNA in yeast. In many cases ingenious new approaches have been employed, for example in increasing the strength of native promoters or the stability of expression vectors. We will attempt to review the choices now available and how they relate to different requirements.

Insertion of a foreign gene into an expression vector does not guarantee a high level of the foreign protein; gene expression is a complex multi-step process and problems can arise at numerous stages, from transcription through to protein stability. In the past, heterologous gene expression has often been treated empirically—a number of host organisms might be tested for successful expression and low yields or failures would be passed over. Frequently, inappropriate conclusions had been made, for example about relative promoter strengths, simply from knowledge of the input vector and the final steady-state level of the foreign protein. However there is now considerable accumulated experience on foreign gene expression in yeast. In many instances this has led to the identification of a particular problem at a specific stage in the chain of events, and then often to its solution. Among eukaryotes yeast offers unparalleled scope for solving such problems on account of the power of classical and molecular genetics combined. We have attempted to draw together examples of this from the literature, and from our own experience. These should be helpful in predicting and solving problems with new genes, and should be applicable, in many cases, to other expression systems, prokaryotic and eukaryotic.

The secretion of foreign proteins which are naturally secreted is often necessary for their correct folding, and is highly advantageous because of the initial purity of the product in the substantially protein-free culture medium. Although there have been several commercial successes using yeast, the area has frequently presented problems, especially for larger proteins. Despite increased understanding of the processes of secretion, the greatest success in improving yields in recent years has been with a classical random mutagenesis approach.

A number of other yeasts have become important host organisms for foreign gene expression because of advantages in promoter strength, secretion efficiency, or ease of growth to high cell density. In the future some of these will often be used in preference to *S.cerevisiae*. We have therefore devoted a substantial section to discussing other yeasts, particularly *Pichia pastoris* in which there are many examples of high-level expression.

An area which is frequently ignored by molecular biologists but which must be considered from the outset in vector design is the physiology of foreign gene expression. This includes the physiology of growth to high cell density and promoter induction, as well as the effect of expressing a foreign protein on host cell metabolism. We have gathered examples of the toxicity of foreign proteins and its effect in causing selection of genetic variants expressing lower yields; in many cases these effects can be controlled. Finally, we will discuss the considerations involved in industrial scale-up and fermenter optimization through examples with different promoter systems of *S.cerevisiae* and with *Kluyveromyces lactis* and *P.pastoris*.

TRANSFORMATION AND SELECTABLE MARKERS

Transformation

The first methods for the transformation of *S. cerevisiae* involved enzymatic removal of the cell wall to produce sphaeroplasts which could take up DNA on treatment with calcium and polyethylene glycol.[24,168] Transformants were then plated out in a selective, isotonic top agar for regeneration of the cell wall. A more convenient method was later developed in which intact yeast cells were made competent by treatment with lithium ions.[189] This method is now widely used despite the fact that it gives lower frequencies; a variation using DMSO increases frequency 25-fold.[167] More recently a third approach, electroporation, has been used, and a highly efficient method has been reported by Meilhoc *et al.*[252]

The process of transformation appears to be somewhat mutagenic, both for the host cell[342] and for the introduced DNA.[72] However the frequency of mutation is low enough that it should not be a major concern here. Also, Danhash *et al.*[83] have reported that transformation induces a heritable slow-growth phenotype in *S.cerevisiae*.

An important factor to consider in foreign gene expression is the frequent wide variation in the productivity of different transformants when 2μ vectors are used. This appears to be due to an unexplained stable variation in plasmid copy number between different transformants.[237,292] Clearly it is therefore important to analyse a number of transformants when optimizing expression.

Table 1. Selectable markers for *S.cerevisisae* transformation.

Marker	Auxotrophic/ dominant	Comments
HIS3	A	
TRP1	A	Selection possible in CAA
LEU2	A	*LEU2*-d for high copy number selection
URA3	A	(a) Selection possible in CAA (b) *URA3*-d for high copy number selection[237] (c) Counter-selection using 5-FOA[37] (d) Autoselection in *fur1* (5-FU-resistant) strain[237,304]
LYS2	A	Counter-selection using α-amino adipate[17,61,121]
S.pombe POT	A	Used in *S.cerevisiae tpi*⁻ host; autoselection in glucose[208]
Tn903 kan^r	D	Active only in multiple copies unless yeast promoter used; selection using G418[154,200,400]
Cm^r	D	Only effective using yeast promoter; selection using chloramphenicol in glycerol medium only[153]
Hyg^r	D	Reference 150
CUP1	D	Level of Cu^{2+}-resistance dependent on gene dosage[159]
HSV *TK*	D	Thymidine/amethopterin/sulphanilamide selection; level of resistance dependent on gene dosage[418]
DHFR	D	Methotrexate/sulphanilamide selection; level of resistance dependent on gene dosage[256,419]

CAA, Casamino acids.

Auxotrophic selection markers

The first and most commonly-used markers for the selection of transformants were *LEU2, TRP1, URA3,* and *HIS3* used in corresponding mutant strains which are auxotrophic for leucine, tryptophan, uracil and histidine, respectively[24,308,370,382] (Table 1). Such strains are widely available, and some contain non-reverting mutant alleles constructed to give low background rates in transformations. Continued selection requires the use of minimal growth media lacking the relevant nutrient. It is worth noting that *TRP1* and *URA3* vectors can be selected in the presence of acid protein hydrolysates (which lack tryptophan and uracil, e.g. casamino acids) that are often added to semi-defined media in order to enhance growth rates.

A frequently-used variant of *LEU2*, *LEU2*-d,[24] has a truncated promoter and is poorly expressed, so that its selection gives rise to very high plasmid copy numbers.[109] Direct selection of transformants with this marker is inefficient and requires the sphaeroplast method, though low levels can be obtained using the lithium method, if cells are incubated overnight in non-selective medium prior to selection. Vectors which contain both *LEU2*-d and another marker, e.g. *URA3*, can be maintained at either high or low copy number depending on the selection used. Loison *et al.*[237] used an expression vector containing these markers for the schistosomal antigen P28-I and obtained product at 3% of total cell protein (t.c.p.) in uracil-deficient medium or 25% in leucine-deficient medium. Loison *et al.*[237] also constructed a promoter-defective *URA3* allele, *URA3*-d, which gives high copy number in uracil-deficient medium.

URA3 and *LYS2* are particularly versatile in that there are also methods for counter-selection of the marker. Thus *ura3*⁻ cells can be selected for their resistance to the toxic antimetabolite 5-fluoro-orotic acid,[37] and *lys2*⁻ cells can be selected for resistance to α-aminoadipic acid.[17,61,121] These methods can be

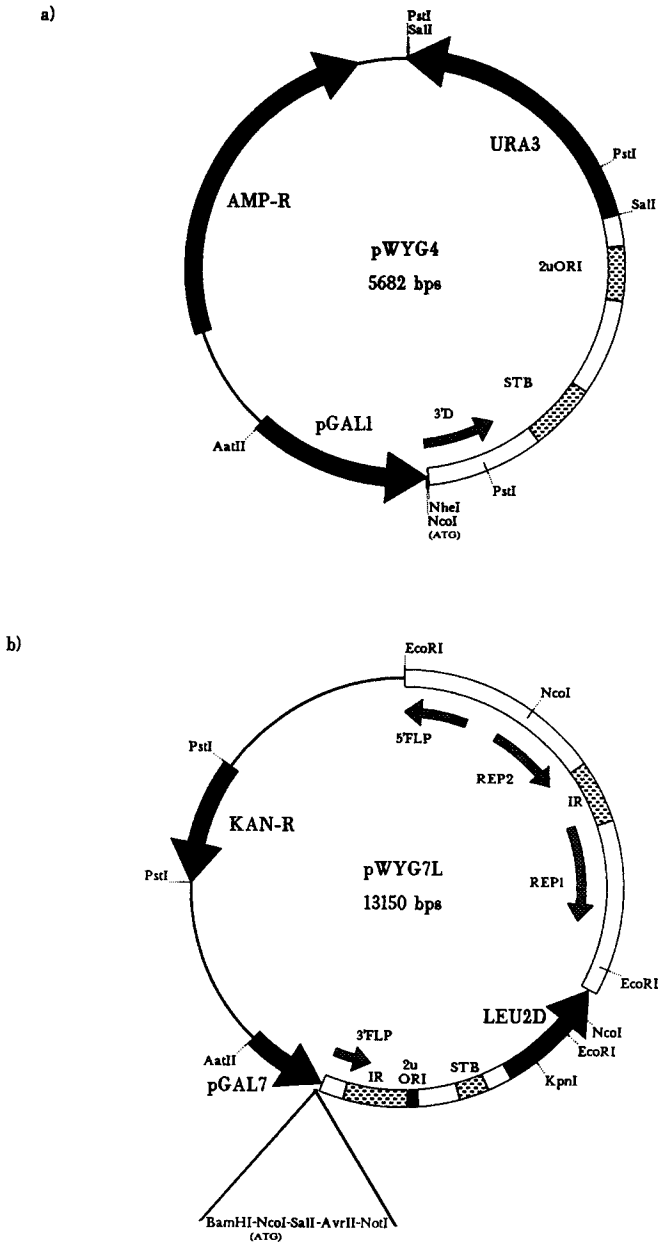

Figure 1. 2μ-based expression vectors for *S.cerevisiae* based on galactose-inducible promoters. (a) pWYG4 has the 2μ *ORI-STB* elements, the *GAL1* promoter, and the 2μ *D* gene terminator; an *Nco*I cloning site containing the ATG is used to insert foreign genes. (b) pWYG7L has intact 2μ *ORI, STB, REP1* and *REP2*, the *GAL7* promoter, and uses the *FLP* terminator; foreign genes are inserted in the polylinker with their 5' ends at the *Bam*HI or *Nco*I site.

used either to select mutations in prototrophic strains or to select for plasmid loss in transformants.

Dominant selectable markers

Dominant markers are useful in that they increase the range of host strains that can be tested to include prototrophic and industrial strains of *S.cerevisiae*, and can be used for selection in rich medium. Since most strains are sensitive to the aminoglycoside antibiotic G418, the G418-resistance marker, encoded by the *E.coli Tn903* transposon, can be used, though it is inefficient in direct selection of transformants.[200,400] Frequencies are acceptable provided transformants are incubated overnight in non-selective medium prior to plating out on G418 agar.[23] Use of glycerol rather than glucose as the carbon source during selection reduces the number of untransformed G418-resistant mutant colonies that arise.[178] Multiple copies of the *Tn903* G418-resistance marker are needed to confer resistance in yeast cells unless a yeast promoter is used, in which case it is active in single copy.[154] It may be prudent to omit selection with antibiotics which affect ribosomes, such as G418, during induction of expression vectors due to the possibility of increased amino acid misincorporation. Other antibiotic-resistance markers that have been used successfully in yeast are hygromycin B[150] and chloramphenicol-resistance.[153]

Copper-resistance in yeast is conferred by multiple copies of the *CUP1* gene and can therefore be used as a dominant marker in sensitive (*CUP1*^{s}) strains.[122] It has proved useful on multi-copy vectors, particularly with industrial strains.[159] Two other markers which can be used for vector copy number amplification by increased drug selection are the herpes simplex virus thymidine kinase gene[418,419] and dihydrofolate reductase.[256]

Autoselection

A number of 'autoselection systems' have been devised to ensure that plasmid selection is maintained, irrespective of culture conditions. Bussey and Meaden[50] showed that expression of a cDNA encoding the yeast killer toxin and immunity gene could be used for self-selection of transformants of laboratory or industrial yeasts since plasmid-free cells are killed by plasmid-containing cells. Another system has used the *Schizosaccharomyces pombe* triose phosphate isomerase gene to stabilize plasmids in *S.cerevisiae* cells lacking the active gene, during growth on glucose.[208]

Loison *et al.*[236] used *ura3 fur1* strains as hosts for plasmids containing the *URA3* gene. These are non-viable since they are blocked both in the *de novo* and salvage pathways for uridine 5'-monophosphate synthesis; maintenance of a *URA3* plasmid is then obligatory for viability even in uracil-containing media. Since the double mutant without plasmid is non-viable, the transformant was obtained by mating a *fur1* strain with a *ura3* strain containing the *URA3* plasmid, and selecting the plasmid-containing *ura3 fur1* progeny. Subsequently, Loison *et al.*[237] were able to directly isolate spontaneous *fur1* mutants from *ura3* cells transformed with a *URA3* plasmid by selecting for resistance to 100 μg/ml 5-fluorouracil and then screening resistant colonies for resistance to 300 μg/ml 5-fluorouridine. We have found that it is possible to generate stable *URA3* transformants by a single direct selection on 1·3 mg/ml (10 mM) 5-fluorouracil to which only *fur1* mutants should be resistant[304] (Figure 2).

EPISOMAL VECTORS

Extra-chromosomal replicons are based either on plasmids containing yeast autonomous replication sequences (*ARS*),[54] which function as origins of replication, or on the native 2μ circle of *Saccharomyces*.[24] A comprehensive listing of both episomal and integrating vectors has been compiled by Parent *et al.*[281]

ARS *vectors*

ARS vectors are present in multiple copies per cell (1 to 20) but are mitotically highly unstable, plasmid-free cells accumulating at a rate of up to 20% per generation without selection, due to inefficient transmission to daughter cells during cell division.[264] Even when grown under selection the proportion of plasmid-containing cells can be very low, giving a correspondingly low average copy number. *ARS* vectors can be stabilized by the addition of yeast centromeric sequences (*CEN*), but the copy number is then reduced to 1 or 2 per cell.[75] In practice *ARS* vectors are hardly ever used for foreign gene expression, and *ARS/CEN* vectors are only used where low-level expression is desired.

2μ-based vectors

By far the most commonly-used expression vectors are *E.coli*-yeast shuttle vectors based on 2μ.[8,44,281] 2μ is a 6·3 kb plasmid present in most

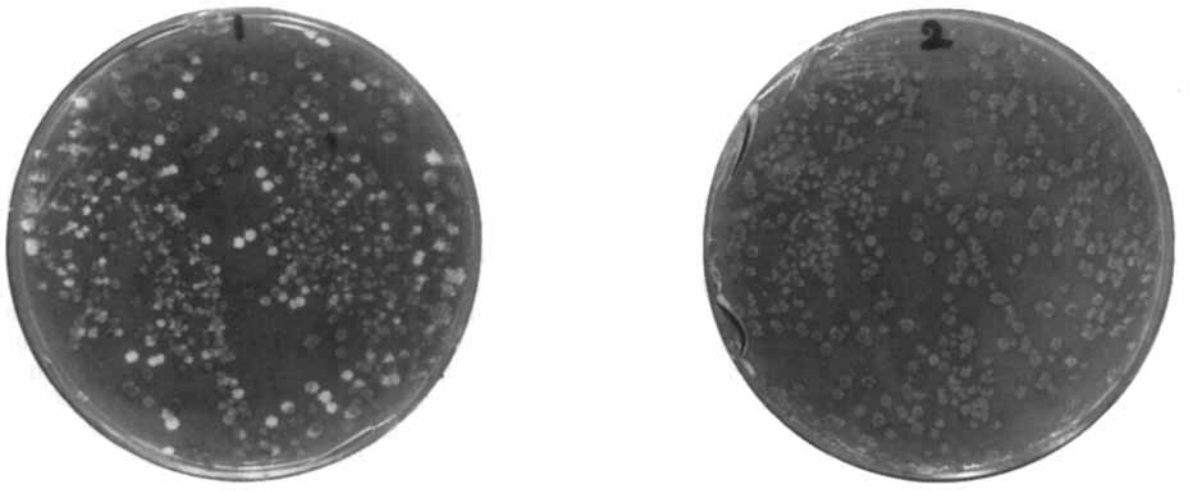

Figure 2. Stability of a *URA3* vector in wild-type and 5-fluorouracil-resistant strains. A β-galactosidase expression vector (pWYG4-*lacZ*) was introduced into strain KY117 and spontaneous mutants resistant to 10 mM-5-fluorouracil were selected.[304] Wild-type (1) and mutant (2) transformants were grown for ten generations in non-selective inducing medium, then plated out on non-selective plates containing XGal and galactose to assay for β-galactosidase expression. White colonies, indicating plasmid-loss, were not present with the mutant strain. This indicates autoselection of the vector in the 5-fluorouracil-resistant strain.

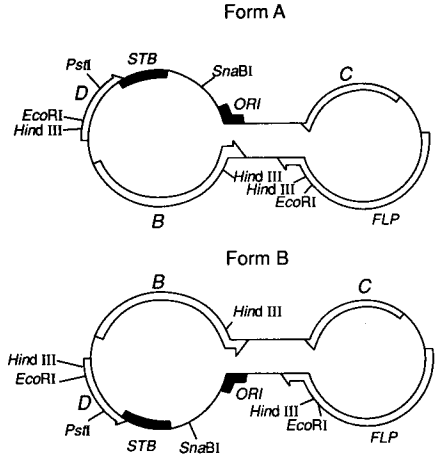

Figure 3. The two forms of the 2μ plasmid. *Cis*-elements are shown as filled boxes and genes by open boxes, inverted repeat regions are aligned; see text for a detailed description.

Saccharomyces strains at about 100 copies per haploid genome[128,265,395] (Figure 3). The plasmid encodes four genes: *FLP* (or *A*), *REP1* (or *B*), *REP2* (or *C*) and *D*. In addition, 2μ contains an origin of replication (*ORI*, which behaves as a typical *ARS* element), the *STB* locus (required *in cis* for stabilization), and two 599 bp inverted repeat sequences. *FLP* encodes a site-specific recombinase which promotes flipping about the FLP recombination targets (FRT) within the inverted repeats, so that cells contain two forms of 2μ, A and B.

Despite the fact that it confers no known phenotype and may indeed be slightly disadvantageous to the host cell,[129,251,397] 2μ is stably inherited; plasmid-free cells arise at the rate of 1 in 10^4 per generation.[129] It achieves this using two mechanisms: (1) by partly overcoming the strong maternal bias in plasmid transmission, and (2) by amplification to correct fluctuations in copy number caused by inefficient transmission.

Efficient segregation depends on having the *STB* locus *in cis* and the *REP1* and *REP2* gene products.[211] Amplification overcomes the host regulation which restricts each replication origin to one initiation per cell cycle; it appears to depend on the inverted repeat sequences and the *FLP* gene product. According to the model proposed by Futcher,[128] FLP promotes recombination between replicated and unreplicated DNA so that inversion occurs and two replication forks can follow each other around the circle. Replication terminates after a second recombination, and further recombination generates multiple 2μ monomers.

The simplest 2μ vectors contain the 2μ *ORI-STB*, a yeast selectable marker, and bacterial plasmid sequences (e.g. pWYG4, Figure 1), and are used in a $2\mu^+$ host strain which supplies REP1 and REP2 proteins.[211] *ORI-STB* expression vectors are the most convenient to use routinely in the laboratory

due to their small size and ease of manipulation. They are tenfold more stable than *ARS* plasmids, being lost in 1 to 3% of cells per generation in non-selective conditions, and are present in 10 to 40 copies per cell. In 2μ-free cells such plasmids behave as *ARS* vectors. Commonly-used *ORI-STB* vectors contain a 2·2 kb *Eco*RI fragment or a 2·1 kb *Hin*dIII fragment from the B form of 2μ, each having one inverted repeat. In order to limit recombination with 2μ, the inverted repeat can be removed, but it is important not to remove adjacent *STB*-distal sequences since these appear to have an important role in protecting *STB* from transcriptional inactivation.[266] It should be noted that shuttle vectors containing inverted repeats will exist as a variety of recombinants with the native 2μ.[139] In addition, there appears to be some competition between exogenous 2μ vectors and native 2μ such that the copy number of both is depressed.[130]

More complex 2μ-based shuttle vectors contain the *REP1* and *REP2* genes in addition to *ORI-STB* and can therefore be used in 2μ-free host strains.[8] They are more cumbersome than *ORI-STB* vectors but stabler and more suitable for scale-up. Many examples of this type of vector are disrupted in *D* (e.g. pJDB248), and some in *FLP* (e.g. pJDB219) so that they cannot flip or amplify in 2μ-free cells. Nevertheless *FLP*$^-$ plasmids can reach high copy numbers in 2μ-free cells, presumably through asymmetric segregation. In 2μ$^+$ strains they can amplify independently if they have two inverted repeats, or following integration into 2μ if they have only one repeat. Recently, vectors have been developed which have all functional regions of 2μ intact, by insertion of the foreign DNA at the unique *Sna*BI site between *ORI* and *STB*.[32] These are highly stable, but still 20- to 80-fold less stable than native 2μ, possibly due to low-level transcription through *STB*. In future similar vectors should find wide use in foreign gene expression.

Vectors free of bacterial DNA may be advantageous in foreign gene expression in relation to food and drug regulatory authorities. They can be made in one of two ways: (1) by targetted integration of an expression cassette into native 2μ, or (2) by use of a shuttle vector which can remove bacterial sequences *in vivo* by excisional recombination. Using the latter approach, Chinery and Hinchcliffe[67] constructed 'disintegration vectors' which used the FLP/FRT system to excise bacterial DNA inserted at the *Xba*I site of one of the inverted repeats. Vectors with inserts at the unique *Pst*I site in *D* or the *Sna*BI site between *STB* and *ORI* were extremely stable, though it is not clear whether stability would be retained upon insertion of a high-level expression cassette.

A number of ultra-high-copy number vectors (e.g. pWYG7L, Figure 1) are based on pJDB219 which contains the entire 2μ B form cloned in the bacterial plasmid pMB9 with disruption of *FLP*.[24] The selectable marker is *LEU2*-d whose use results in very high copy numbers (200–400 copies per haploid genome); the fraction of cells with the highest plasmid copy number are constantly selected, resulting in reduced growth rate. Selective growth or use of other markers gives a more normal copy number of about 50 per cell.[109] pJDB219 is best used in 2μ-free cells since it can undergo FLP-mediated recombination with resident 2μ, leading to loss of the foreign DNA but retention of the *LEU2*-d marker.[98] In 2μ-free cells pJDB219 is very stable due to its high copy number, making it suitable for large-scale culture.[130,397] *FLP*$^+$ versions of pJDB219, e.g. pXY, have greater stability in non-selective medium.[130] Since the entire sequence of pMB9 has not been determined, it may be more convenient to use a variant of pJDB219 based on pBR322, e.g. pC1/1.[385]

Vectors can have very high copy number even in non-selective conditions if extra FLP recombinase is supplied. For example, induction of FLP from a single integrated copy of the gene under control of the *GAL1* promoter leads to a shift in plasmid copy number from <50 to 200–400.[8] Alternatively, the autoselective high-copy *URA3*-d marker can be used in a *fur1* host strain.[237]

Regulated copy number vectors

Foreign gene expression could be regulated by inducing an increase in vector copy number. Two types of yeast episomal vectors with regulated copy number have been described: (1) vectors with regulatable centromeres, and (2) 2μ vectors in cells with inducible FLP (see above). The first type depends on the observation that *CEN* elements can be inactivated by transcription. Chlebowicz-Sledziewska and Sledziewski[69] constructed vectors containing the glucose-repressible *ADH2* promoter adjacent to *CEN3* and either an *ARS* or 2μ *STB-ORI*. In the *ARS* vectors copy number could be increased from 1–2 to 5–10 by a switch from glucose to ethanol as the carbon source. In the *ORI-STB* vectors copy number could be increased from 1–2 to about 100 and the vector was very stable. Such vectors could be used to increase the degree of regulation in expressing toxic proteins.

INTEGRATING VECTORS

YIp vectors

Chromosomal integration offers a more stable alternative to episomal maintenance of foreign DNA. In *Saccharomyces* integration normally occurs by homologous recombination.[278] Integrating vectors (YIp) contain yeast chromosomal DNA to target integration, as well as a selectable marker and bacterial replicon. Vectors are usually digested at a unique restriction site in the homologous DNA as this promotes high efficiency transformation and targets integration. Such single cross-over integration results in a duplication of the chromosomal target sequence, so that the vector can subsequently 'pop out' by excisional recombination. Nevertheless the integrants are quite stable, the typical rate of vector loss being $<1\%$ per generation in the absence of selection.[168]

For convenience vector integration can be targetted to the chromosomal mutant allele of the selection marker used. However, continued selection of the resulting transformants is ineffective, since the duplicated DNA can be excised and a wild-type marker retained. In contrast, continued selection is effective where integration is targetted elsewhere.

When high DNA concentrations of integrating vectors are used in transformations, tandem multi-copy inserts can result, presumably due to repeated recombination events.[277] Multi-copy integrants are relatively stable and have been used, for example, in gene dosage studies.[57]

Transplacement

An alternative type of integration, transplacement, makes use of double homologous recombination to replace yeast chromosomal DNA, resulting in a stable structure without duplications.[313] Where a stable single-copy transformant is required this is the method of choice. Transplacement vectors contain the exogenous DNA and selection marker flanked by yeast DNA homologous to 5' and 3' regions of the chromosomal DNA to be replaced. Prior to transformation the vector is digested with restriction enzymes which liberate the transplacing fragment with 5' and 3' homologous ends. The frequency of transformation is low so that the sphaeroplast method is usually used, and the chromosomal structure of the transformants must be checked phenotypically and by Southern blot analysis.

Integration into reiterated DNA

A number of strategies based on integration into reiterated chromosomal DNA have been used to generate stable multi-copy integrants. At present the best results in terms of copy number and expression appear to be using integration into the ribosomal DNA (rDNA) cluster. The rDNA cluster consists of about 140 tandem repeats of a 9·1 kb unit on chromosome XII. Lopes *et al.*[238,239] have constructed an integrating vector, pMIRY2, containing a portion of the rDNA unit and the *LEU2*-d marker. Transformation with pMIRY2 digested at *Sma*I or *Hpa*I gave Leu^+ transformants with 100–200 copies integrated at a non-transcribed spacer of the rDNA locus. Use of the *LEU2*-d or other promoter-defective markers was important for isolation of high copy integrants. The transformants were highly stable, 80–100% of the integrated copies being retained after 70 generations, and the levels of foreign protein produced using the *PGK* promoter were as high as from 2μ vectors. This approach could also be used as an alternative to episomal vectors in species where none has been found.

Another reiterated DNA that can be used as a target for integration is the transposable element *Ty* which is present in 30–40 copies per genome in most *Saccharomyces* strains. Kingsman *et al.*[216] described the use of a transplacement vector targetted to replace *Ty*, whose copy number could be amplified using the *LEU2*-d selection marker. Levels of interferon produced from such amplified transformants were several times higher than from single-copy *ARS/CEN* vectors but almost ten-fold less than with 2μ vectors. Jacobs *et al.*[193] used similar vectors in order to co-express the different forms of hepatitis B surface antigen (HBsAg). They obtained transformants containing from one to several copies of the vector without the need for amplification. The multi-copy transformants consisted mainly of the transplacing DNA at a single *Ty1* locus and arose by an unexplained mechanism. In order to co-express the different forms of HBsAg in the desired ratio, **a** and α strains were transformed with each vector, multi-copy integrants selected, and diploids made. Stable diploids with a total of 10 copies were made but expression levels appeared to be low.

More recently Shuster *et al.*[345] have used vectors that integrate by single cross-over into δ elements, which exist either alone or as part of *Ty* throughout the *S.cerevisiae* genome. They constructed a vector expressing the *E.coli lacZ* gene with the *LEU2* and *CUP1* markers. Leu^+ transformants were selected

following transformation with vector digested by *Xho*I, which cuts in the δ element within the *LEU2* marker fragment. These were then selected for copper-resistance to isolate multi-copy integrants which yielded up to ten-fold the β-galactosidase level of single-copy strains. Integration into δ, coupled with crossing of haploid integrants, has been used to generate a 20-copy strain for efficient secretion of nerve growth factor.[322]

Transposition vectors

A different approach to multi-copy integration is the use of *Ty* transposition vectors analogous to retroviral vectors for mammalian cells. *Ty* transposes *via* a full-length transcript which is encapsidated into virus-like particles and reverse transcribed to DNA, which can then integrate at multiple sites. In transposition vectors a regulated promoter, e.g. *GAL1*, is used in place of the *Ty* δ promoter to generate a transcript encompassing the foreign gene and selectable marker. Transcription termination signals must be removed from the marker gene for the full-length transcript to be produced. The whole unit is placed on an episomal vector for the initial transformation, but can be lost following induction of transposition. Boeke *et al.*[38] obtained relatively low copy number integrants (1–10) by this method, but it may prove feasible to use the *LEU2*-d gene for amplification. Although this method is not fully developed it could be useful in a variety of yeasts, since *Ty* encodes all the functions required for transposition.

TRANSCRIPTIONAL PROMOTERS AND TERMINATORS

Foreign versus *yeast promoters*

The expression of foreign genes in yeast was examined as soon as transformation procedures became available. The first study was of the rabbit β-globin gene which was found to give rise to aberrant transcripts in which the introns were not spliced.[25] In a few cases the foreign transcript was correctly initiated, e.g. with zein,[228] but in general foreign transcriptional promoters were found to give aberrant initiation, e.g. *Drosophila ADE8*,[161] or were totally inactive, e.g. herpes simplex virus thymidine kinase.[217] Thus for efficient transcription of foreign genes the use of yeast promoters with cDNAs was soon found to be essential. The first published example was the use of a 1500 bp fragment 5' of the *ADH1* gene for efficient intracellular expression of leukocyte α-interferon.[172]

Yeast mRNA promoters consist of at least three elements which regulate the efficiency and accuracy of initiation of transcription:[369] upstream activation sequences (UASs), TATA elements, and initiator elements. Many also contain elements involved in repression of transcription. UASs, which have some similarities to mammalian enhancers, determine the activity and regulation of the promoter through specific binding to transcriptional activators (e.g. *GAL4* and *GCN4*), and act at variable distances 5' to the initiation site. Some UASs have been mapped to short regions of DNA, e.g. the *GAL1-GAL10* UAS to a 108 bp intergenic region containing four short sequences of dyad symmetry. These sequences (17–21 bp) are necessary and sufficient for binding of the *GAL4 trans*-activator and for galactose-regulation, and act synergistically. UASs of some constitutively-expressed genes contain poly(dA-dT) tracts which probably activate transcription by affecting nucleosome structure.[369] TATA elements (consensus TATAA) are found 40 to 120 bp upstream of the initiation site, in contrast to the more rigid distance of 25 to 30 bp in higher eukaryotes, and provide a window within which initiation can occur.[369] The initiator element, which is poorly defined, directs mRNA initiation at closely adjacent sites. Yeast promoters may be highly complex, extending over 500 bp, containing multiple UASs and negative regulatory sites, and multiple TATA elements associated with different initiation sites.[369]

Most promoters are regulated to some extent, but the most powerful, glycolytic promoters are poorly regulated. This makes them undesirable for use in large-scale culture, where there is more opportunity for the selection of non-expressing cells, and unsuitable for expressing toxic proteins. In such cases it is preferable to use a tightly-regulated promoter so that the growth stage can be separated from the expression stage. Despite a severe limitation in efficiency with multiple copies, *GAL1* has been the most commonly-used regulated promoter. However, there is now a large variety of native or engineered promoters (Table 3); the right choice is critical for any one application, especially where a process is to be scaled-up.

Glycolytic promoters

The first promoters used were from genes encoding abundant glycolytic enzymes, e.g. alcohol dehydrogenase I (*ADH1*),[172] phosphoglycerate

Table 2. *S.cerevisiae* vector systems.

Vector	Yeast sequences	Transformation frequency[a]	Copy no. /cell	Mitotic instability[b]	Reference
A. Integrating					
YIp	Homologous DNA	10^2	≥1	0·1%	168
Transplacement	Homologous DNA	10	1	stable	313
rDNA integrating	rDNA	n/a[c]	100–200	stable	239
Tyδ	*Tyδ* DNA	n/a	≤20	stable	322, 345
B. Episomal					
Replicating (YRp)	*ARS*	10^4	1–20	20%	264
Centromere (YCp)	*ARS/CEN*	10^4	1–2	1%	75
2μ-based (YEp)	*ORI, STB*, in $2\mu^+$ host (YEp13)	10^4	25	2·8%	130
	ORI, STB, REP1, REP2, FLP in $2\mu^0$ host (pJDB248)	10^4	50–100	0·6–1·8%	130
	ORI, STB, REP1, REP2, in $2\mu^0$ host (pJDB219)[d]	n/a	200	0·26%	130
	ORI, STB, REP1, REP2, D, FLP in $2\mu^0$ host (pJB205)	n/a	50–100	0·20%	32
Regulated copy no.	*ORI, STB, ADH2-CEN3*	10^4	3–5 to 100[e]	Stable[f]	69

[a]Transformants per μg DNA using sphaeroplast method.
[b]Plasmid-free cells arising per generation during non-selective growth.
[c]Data not available.
[d]Increased copy number using *LEU2*-d selection.
[e]On change from glucose to ethanol.
[f]85% after 30h non-selective growth in log-phase.

kinase (*PGK*),[173,383] or glyceraldehyde-3-phosphate dehydrogenase (*GAP*).[177] These were at first thought to be constitutive but were later found to be induced by addition of glucose, e.g. expression of α-interferon using the *PGK* promoter was induced 20- to 30-fold by addition of glucose to a culture grown on acetate as carbon-source.[383] Glycolytic promoters are the most powerful of *S.cerevisiae*, for example *PGK* mRNA accumulates to 5% of total. Despite their poor induction ratio, *ADH1*, *PGK* and *GAP* vectors have been used extensively in the laboratory, and in some cases industrially.[250]

The *PGK* promoter has been studied in some detail: it extends over 500 bp, contains a complex UAS at -473 to-422 relative to the initiation site, a heat shock regulatory element, and other features contributing to accurate and efficient initiation.[215] In contrast, less is known about other glycolytic promoters, such as *GAP*. There are three *GAP* genes, of which *GAP491* or *TDH3* is the most highly-expressed and whose promoter has been used successfully to express a number of proteins.[311] The full-length promoter extends over approx. 700 bp,[33] but there have been a number of reports of smaller fragments having full promoter activity, e.g. a 198 bp fragment.[169] However, it appears that the activity of shorter *GAP491* promoters is dependent on bacterial vector sequences.[311]

Table 3. *S.cerevisiae* promoter systems.

Promoter	Host genotype	Strength	Regulation[a]	Reference
A. Native				
PGK[b], *GAP*, *TPI*	wt	++++	≤20-fold induction by glucose (*PGK*)	311, 383
GAL1	wt	+++[c]	1000-fold induction by galactose	202
ADH2	wt	++[d]	100-fold repression by glucose	291
PHO5	wt	+/++	200-fold repression by P_i	169
PHO5	*pho4*^{ts} *pho80*	+	50-fold induction by shift 37°C to 24°C	223
CUP1	wt	+	20-fold induction by Cu^{2+}	113
*MF*α1	wt	+	Constitutive in α cells	42
*MF*α1	*sir3*^{ts}	+	10^5-fold induction by shift 37°C to 24°C	42
B. Engineered				
PGK/α2 operator	*sir3*^{ts}	++++	100-fold induction by shift 37°C to 24°C	398
TPI/α2 operator	*sir3*^{ts}	+++++?	>50-fold induction by shift 37°C to 24°C	352
GAP/GAL	wt	+++?	150–200-fold induction by galactose	35
PGK/GAL	wt	+++?	Induction by galactose	77
GAP/ADH2	wt	+++[d]?	Repressed by glucose	78
GAP/PHO5	wt	++++?	Two- to five-fold repressed by P_i	169
CYC1/GRE[e]	Expressing glucocorticoid receptor	+++?	50–100-fold by deoxycorticosterone	332
PGK/ARE[f]	Expressing androgen receptor	++++	Several 100-fold by dihydrotestosterone	293

[a]Induction ratios are reporter-dependent.
[b]5% of mRNA in single copy.
[c]1% of mRNA in single copy but severely limited in multiple copies. Improved by *GAL4* over-expression.
[d]Improved by *ADR1* over-expression.
[e]Glucocorticoid responsive element.
[f]Androgen responsive element.

Galactose-regulated promoters

The most powerful tightly-regulated promoters of *S.cerevisiae* are those of the galactose-regulated genes *GAL1*, *GAL7*, and *GAL10*, involved in metabolizing galactose. Galactose-regulation in yeast is now extremely well-studied and has become a key model system for eukaryotic transcriptional regulation (reviewed in reference 201). Many genes are involved in regulation of *GAL* promoters but the central interaction is between the *trans*-activator encoded by *GAL4*, the repressor encoded by *GAL80*, and the *GAL* UAS (Figure 4). Binding of GAL4 protein to the UAS is necessary for induction; GAL80 protein binds GAL4 and acts as a repressor unless galactose is added. The regions of GAL4 protein that bind GAL80 and the UAS have been defined, as have the structural features of the different *GAL* promoters.

GAL1, *GAL7* and *GAL10* mRNAs are rapidly induced >1000-fold to approx. 1% of total mRNA on addition of galactose.[363] The promoters are

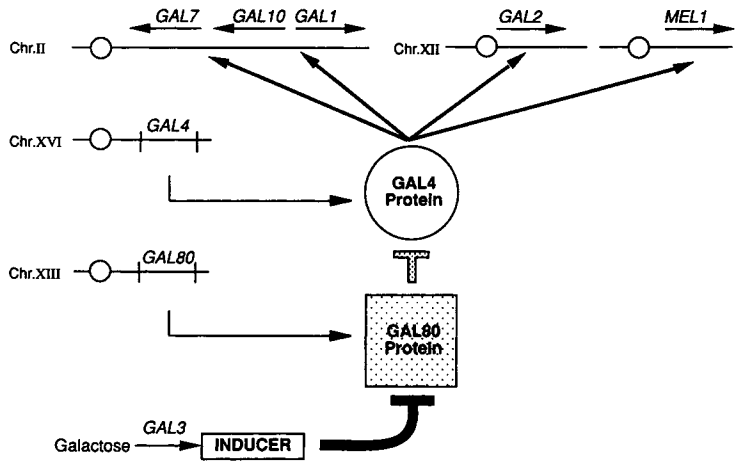

Figure 4. Galactose regulation in yeast. The genes involved in regulation and metabolism and their chromosomal location are shown. Stimulation is indicated by bold lines with arrows and inhibition by lines with bars.

strongly repressed by glucose, so that in glucose-grown cultures maximal induction can only be achieved following depletion of glucose. Galactose-inductions can be carried out in one of three ways: (1) by growing the culture on a non-repressing, non-inducing ('neutral') sugar, when very rapid induction follows addition of galactose; (2) by growing the culture in glucose medium but removing the glucose by centrifugation and washing the cells before resuspension in galactose medium (this leads to a lag of 3 to 5 h in induction[1]); and (3) by growing the cells in medium containing both glucose and galactose, when the glucose is preferentially metabolized before galactose-induction can occur. The first two methods are frequently used for small-scale but are impractical in large-scale inductions. It should be noted that many commonly-used strains have mutations in the galactose permease gene (*GAL2*) and are not inducible.

The large background of knowledge of galactose-regulation offers the possibility of manipulating the system in a number of ways in order to improve its characteristics for protein production. Three types of manipulations that have been carried out are: (1) the over-expression of *trans*-activator; (2) the use of mutations in the galactose-regulatory pathway or in glucose-repression; and (3) the construction of chimaeric galactose-regulated promoters.

GAL4 protein is present in one or two molecules per cell and, moreover, GAL80 repressor is in excess of this and is galactose-inducible.[201] Therefore, even with single-copy promoters, *GAL* transcription is limited by a shortage of GAL4 protein, though other factors also become limiting with multi-copy promoters.[13] With multi-copy expression vectors GAL4 limitation is exacerbated, for example β-galactosidase expression from a *GAL-lacZ* vector increases only two-fold in going from a single-copy to a 2μ vector.[413] Both with single and multi-copy vectors, two- to three-fold increases can be obtained in *gal80* strains, but expression then becomes constitutive.[413] Over-expression of *GAL4*, either by insertion of the gene into the multi-copy expression vector, or by use of a single-copy integrating *ADH1-GAL4* expression vector, increases product levels 2- to 3-fold, but again at the price of losing tight regulation.[13,158] Schultz *et al.*[334] used an integrated *GAL10-GAL4* expression vector in order to over-produce GAL4 protein in a galactose-regulated manner. Transcription from the target expression vector, encoding Epstein-Barr virus (EBV) gp350, was increased 5-fold. We have tested a similar system which used an integrated *ADH2-GAL4* expression vector in order to over-produce GAL4 protein. This system alters the regulation of *GAL* expression vectors so that they are induced by glucose-depletion. Using a constitutive *GAL4* gene, i.e. one that does not interact with GAL80 repressor, it was possible to increase levels of β-galactosidase produced from a multi-copy *GAL1* vector by 2- to 4-fold (M.A.R. and J.J.C., unpublished results).

In order to facilitate galactose-induction of glucose-grown cultures, the use of mutants defective in either global or *GAL*-specific glucose-repression has been examined. In *reg1-1* mutants efficient galactose-induction occurs with galactose to glucose

ratios of 5:1.[248] Hovland *et al.*[181] used a novel selection procedure to isolate glucose-resistant mutants, and found a *reg1* mutant (*reg1-501*) in which efficient induction occurred even with galactose/glucose ratios of 1/100. A *gal1 reg1-501* strain was made which cannot metabolize galactose so that very low levels of galactose (e.g. 0·2%) could be used, in the presence of excess glucose, for efficient induction.[181]

In an attempt to combine the high activity of glycolytic promoters with the tight regulation of *GAL* promoters, hybrid promoters have been constructed where a glycolytic UAS is replaced by a *GAL* UAS. However, the published results do not suggest that these hybrids are more efficient than *GAL* promoters. Bitter and Egan[35] have described the use of a *GAP/GAL* UAS hybrid for the expression of γ-interferon, which is toxic to yeast cells. Insertion of a 55 bp *GAL1-GAL10* UAS fragment between the *GAP* UAS and TATA element conferred galactose-regulation to the promoter. Product levels were significantly higher than with the native *GAP* promoter, due largely to a dramatically increased vector copy number, from 1 to 20–50 per cell. A similar type of hybrid promoter (*PAL*) has been constructed, by replacing the *PGK* UAS with a *GAL* UAS, and used for regulated production of α-interferon and human serum albumin.[77] However, no comparison of the efficiency of *GAL* and *PAL* promoters has been published; it would be expected that all *GAL*-UAS hybrid promoters would be severely limited by GAL4 protein.

Phosphate-regulated promoters

The promoter of the acid phosphatase gene, *PHO5*, is regulated by inorganic phosphate concentration and has been used extensively for foreign gene expression.[169] In one of the earliest studies, a 1·4 kb *PHO5* DNA fragment was used to drive production of α-interferon, which was induced 200-fold by switching to low-phosphate medium.[223] More recently, the structural features and regulation of the *PHO5* promoter have been studied in detail (reviewed in reference 394). The promoter, encompassing about 400 bp of DNA, contains two UASs which are necessary and sufficient for regulation. These contain 19 bp dyad sequences which bind the PHO4 *trans*-activator.

Since the *PHO5* promoter is not very strong, attempts have been made to use the *PHO5* UAS to confer regulation to glycolytic promoters. Hinnen *et al.*[169] constructed a series of *GAP/ PHO5* UAS hybrids and tested them in expression vectors for eglin C. Some hybrid promoters yielded up to twice the amount of product as *GAP*, but the induction ratio was 2- to 5-fold compared to 40-fold for *PHO5*. Since no copy number data were available one cannot make conclusions about relative promoter strength.

Kramer *et al.*[223] constructed a host strain temperature-sensitive in the PHO4 *trans*-activator and defective in the PHO80 repressor (*pho4*^{ts}*pho80*) to achieve phosphate-independent induction of the *PHO5* promoter regulated by temperature. On lowering the temperature from 35°C to 23°C a 50-fold induction of α-interferon was achieved, but the absolute level was only one tenth that in wild-type induced cells.

Glucose-repressible promoters

Glucose-repression is a global system regulating the expression of a number of genes, including sugar fermentation genes, by the availability of glucose. Yeasts preferentially utilize sugars such as glucose that enter the glycolytic pathway directly. Genes involved in sucrose or galactose metabolism are transcriptionally repressed by glucose. Typical examples of promoters regulated primarily by glucose-repression are those of the *ADH2, SUC2* and *CYC1* genes, encoding alcohol dehydrogenase II, invertase and iso-1-cytochrome c, respectively.

The *ADH2* promoter is both powerful and tightly regulated and has been used for foreign gene expression.[291] Since it is repressed over 100-fold by glucose, it can be used for efficient expression of toxic proteins, e.g. insulin-like growth factor I (IGF-I).[343] Deletion analysis has identified a 260 bp region 5' to the initiation site which contains two UASs sufficient for full promoter activity and regulation.[26] UAS1 is a 22 bp inverted repeat which binds the ADR1 *trans*-activator.[105] We have used the 260 bp region, which is readily assembled from synthetic oligonucleotides, in efficient expression vectors (M.A.R. and J.J.C., unpublished results).

In order to maintain repression of *ADH2*, cells must be grown in excess glucose (e.g. 8%) until induction, which is effected by changing to fresh medium containing a non-fermentable carbon source, e.g. ethanol, glycerol, raffinose, etc. Alternatively, *ADH2* can be induced by culturing initially in a lower concentration of glucose (e.g. 1%) which is gradually depleted.[291] Glucose-repressible systems have a potentially serious disadvantage in industrial fermentations: it is difficult to maintain tight glucose-repression under conditions of glucose-limitation, which are required to achieve high cell density.

Irani *et al.*[187] showed that transcriptional factors, including the *ADR1 trans*-activator, became limiting for *ADH2* transcription from multi-copy plasmids. Over-expression of *ADR1* leads to loss of regulation, but *ADH2*-regulated over-expression of *ADR1*, from a single-copy integrating vector, has been used to increase the efficiency of *ADH2* expression systems without loss of regulation.[291] In this way it has been possible to increase expression of β-galactosidase from a single-copy *ADH2-lacZ* vector by 4- to 10-fold. The system usually results in about a 3-fold increase in yield for intracellular proteins and is used for commercial production (J. Shuster, personal communication).

Hybrid glycolytic/*ADH2* promoters have been constructed by transplanting the *ADH2* UAS into *GAP* proximal promoter sequences. Cousens *et al.*[78] fused the *ADH2* UAS containing the 22 bp dyad 320 bp upstream of the *GAP* TATA element, and were able to achieve tightly regulated production of a superoxide dismutase (SOD)-proinsulin fusion to >15% t.c.p. However, no critical comparison of the strength of the *ADH2, GAP*, and hybrid promoters has been published.

Other regulated promoter systems

A few other regulated promoter systems are worthy of comment here: these are of interest because their induction is independent of culture nutrients and can therefore be controlled without otherwise perturbing the culture.

A temperature-regulated system based on mating type control has been used by a number of groups. Mutations in the *SIR* genes de-repress the silent mating-type loci, so that **a** or α-specific genes are not expressed. Repression of the *MFa1* promoter is mediated by the *MATa2* repressor which binds a 31 bp operator sequence. Brake *et al.*[42] first reported the use of a *MATa sir3*^{ts} strain for secretion of hEGF using the α-factor (*MFa1*) promoter. Switching from the non-permissive (35°C) to the permissive (25°C) temperature led to an induction in secreted hEGF from 10 ng/l to 4 mg/l. The *α2* operator has been transferred to the strong *ADH2* and *TPI* (triose phosphate isomerase) promoters and shown to confer temperature regulation in a *MAT***a** *sir3*^{ts} strain. Sledziewski *et al.*[352] inserted up to four operators between the UAS and TATA elements of the *TPI* promoter and were able to obtain tight regulation (>50-fold induction of β-galactosidase) and full activity. Inexplicably, the hybrid *ADH2* promoters were not tightly temperature-regulated. The system appears amenable to fine-tuning by use of intermediate induction temperatures. A similar system has been developed by insertion of two *α2* operators in the *PGK* promoter, achieving a >100-fold induction ratio without any reduction in activity.[398]

A novel type of promoter system uses the ability of mammalian steroid hormone receptors to function as transcriptional activators in yeast. Schena *et al.*[332] constructed a *CYC1* expression vector under the control of three tandem 26 bp glucocorticoid response elements fused upstream of the promoter. In yeast cells also expressing the glucocorticoid receptor, a CAT reporter gene could be induced 50- to 100-fold to high levels by addition of deoxycorticosterone (DOC). The degree of induction was titratable over 1 nM to 10 μM-DOC and was very rapid ($t_{1/2}$ of 7 to 9 mins). A similar system has been developed using a hybrid *PGK* promoter containing androgen response elements.[293] Addition of increasing amounts of dihydrotestosterone induced increasing amounts of β-galactosidase reporter over a several hundred-fold range, and the range could be extended to 1400-fold by varying the copy number of the receptor or reporter genes. In conclusion, steroid-regulated systems appear to be powerful and tightly-controlled, and could be used in yeasts lacking promoters with these qualities (e.g. *K.lactis* and *S.pombe*).

The promoter of the *CUP1* gene, encoding copper metallothionein, has been used in expression vectors.[113] This promoter is tightly-regulated and independent of culture parameters. The concentration of Cu^{2+} ions for induction depends on the copper-resistance of the host strain from 0·01 mM (no *CUP1* gene) to 0·5 mM (>6 copies).

Selection of novel yeast promoters

A number of selection or screening methods have been used in order to identify new promoters from a genomic library.[147,326] Selection procedures can be devised for regulated promoters, e.g. the glucose-repressible promoters of the *GUT2* and *PRB1* genes which encode glycerol-3-phosphate dehydrogenase and vacuolar endoprotease B, respectively.[354,356]

Foreign promoters systems

Foreign promoters not recognized by yeast RNA polymerase can in principle be used provided the cognate RNA polymerase is co-expressed. The bacteriophage T7 RNA polymerase is highly active and has been used in a number of prokaryotic and

eukaryotic organisms. T7 RNA polymerase, ideally with an added nuclear targetting signal, can be expressed from a *GAL1* promoter and can efficiently transcribe DNA in yeast cells.[29,63] However the T7-induced *lacZ* mRNA is not translated in yeast, due either to absence of 5' caps and/or polyadenylation, or to a stable hairpin formed in the 5' region.[29] In animal cells a similar problem has been solved using the encephalomyocarditis virus leader to promote cap-independent translation,[108] but this appears to be inactive in yeast (C.A.S., unpublished results). If the translation problem were solved a yeast T7 system would be very powerful, since *GAL1*-driven expression of the polymerase would act as an amplification step.

Yeast terminators

Yeast transcriptional terminators are usually present in expression vectors for efficient mRNA 3' end formation. Terminators of prokaryotic or higher eukaryotic genes are not normally active in yeast, though there are exceptions such as the *Drosophila ADE8* gene.[161] Efficient termination is probably required for maximal expression: deletion of 'termination' sequences 3' of the *CYC1* gene resulted in longer mRNA and a dramatic reduction in mRNA level.[415]

Transcriptional termination of yeast mRNAs is less well understood than that in bacteria and in higher eukaryotes. Bacterial transcription terminates at the mature 3' end of the mRNA. In higher eukaryotes mRNA 3' end formation involves cleavage and polyadenylation, downstream of the signal AAUAAA, of precursor mRNAs that extend several hundred nucleotides beyond the coding region. Contrary to earlier ideas, it appears that yeast mRNAs follow the same pattern of termination, processing and polyadenylation of pre-mRNA as higher eukaryotes. However in yeast these processes are tightly coupled and occur within a shorter distance, near the 3' end of the gene.[52]

A number of consensus sequences have been implicated as part of the mRNA 'terminator', especially the tripartite sequence TAG..(T-rich)..TA(T)GT..(AT-rich)..TTT[416] and TTTTTATA.[160] The commonly found tripartite motif shows poor conservation and is tolerant of large sequence alterations, suggesting that a general feature such as high AT-content may be critical.[279] However this cannot be sufficient since terminators are frequently absent in AT-rich DNA, and can be unidirectional, implying some sequence specificity. More recent evidence suggests that a variety of different signals, unidirectional and bidirectional, are used in yeast.[188]

Terminators from a number of genes have been used in expression vectors, e.g. *TRP1*,[172] *ADH1*,[385] *GAP*,[310] *MF1*,[42] etc. In order to simplify vector construction, a terminator from 2μ[371] can be used, e.g. the *FLP*[173] (pWYG7L, Figure 1) or *D* gene terminator[304] (pWYG4, Figure 1).

FACTORS AFFECTING INTRACELLULAR EXPRESSION

Initiation of transcription

Gene expression is most frequently regulated at the level of transcription, and it is generally assumed that the steady-state mRNA level is a primary determinant of the final yield of a foreign protein. The mRNA level is determined both by the rate of initiation and the rate of turnover.

In most cases the yield of a foreign protein expressed using a yeast promoter has been much lower than the yield of the homologous protein using the same promoter. Using the *PGK* promoter on a multi-copy vector several proteins accumulate to 1 to 2% t.c.p., whereas with the entire *PGK* gene phosphoglycerate kinase accumulates to over 50%.[62,254] These levels reflect to a large extent the lower amounts of the foreign *versus PGK* transcripts for as many as 20 genes,[62] although there appear to be exceptions.[154] It was suggested that the reduced levels were due to shorter $t_{1/2}$s for the foreign transcripts, but Mellor *et al.*[253] showed that α-interferon mRNA was not unstable but was initiated at a six-fold lower rate. Addition of downstream *PGK* sequences restored the mRNA level to that for *PGK* mRNA, suggesting the presence of a downstream activation sequence (DAS), localized to the first 79 codons, required for maximal transcription. Indirect evidence for a DAS has also been found with the pyruvate kinase (*PYK*) gene.[292] Attachment of the *lacZ* gene to the *PYK* promoter resulted in a 30-fold drop in mRNA molarity, whereas its $t_{1/2}$ decreased only two-fold, consistent with a 15-fold drop in the initiation rate. The putative *PYK* DAS is active in single- and multi-copy vectors, has been localized to nt 500 to 800 relative to the initiating ATG, and appears to be able to functionally replace the *PGK* DAS (A. Brown, personal communication). Evidence for DASs has also been found in the lipoamide dehydrogenase gene,[414] and in *Ty2* DNA.[232]

Table 4. Fortuitous transcriptional termination in foreign genes.

Gene	Size (kb)	AT-content %	RNA analysis[a]	Comments
K.lactis plasmid k1 ORF2	3·5	73	Truncated mRNA	Reference 305
Tetanus toxin fragment C	1·5	71	Truncated mRNA	≥6 terminators, eliminated by increasing GC-content[b,c,307]
HIV gp120	1·5	60	Truncated mRNA in *P.pastoris* not *S.cerevisiae*	Mutation of T_4ATA_3 gave several longer low-abundance mRNAs Increasing GC-content[b] gave full-length mRNA (R.G.Buckholz, personal communication and C.A.S.)
SIV gp130	1·6	62	Truncated mRNA	(C.A.S., unpublished results)
Plasmodium falciparum p195 (42K fragment)	0·88	74	Multiple short mRNAs	(C.A.S., M.A.R. and J.J.C., unpublished results)
P.falciparum pfc2 (*CDC28* homologue)	0·91	67	No data	No complementation (P.Ross-MacDonald and D.H.Williamson, personal communication)

[a]No detectable protein in any example.
[b]By chemical synthesis of the DNA.
[c]A similar problem in *P. pastoris* with two bacterial genes (63% and 67% AT) was solved by increasing GC-content (K. Sreekrishna, personal communication).

Since the evidence may now favour the existence of DASs in certain genes it is possible that they will be found in many others. There may be some circumstantial evidence for this, e.g. multi-copy *GAL7* gives levels of uridyltransferase of >15% t.c.p.,[13] whereas foreign proteins rarely reach this level. It must be emphasized that many other factors could account for these differences. If DASs are characterized, it may be possible to incorporate them into upstream promoter fragments in order to create more efficient expression vectors. Alternatively, if DASs prove to be strongly position-dependent, they could be placed within an intron which would be excised prior to translation. If neither of these options work, then maximal transcription will only be achievable using fusion proteins, for example to PGK.

An alternative to all these approaches is to use a different yeast, such as *P.pastoris* which does not appear to have the same problem.

Yeast expression vectors frequently give rise to numerous unexpected transcripts arising from fortuitous promoters in bacterial plasmid DNA.[245] It is not known whether they can affect foreign gene expression, but antisense transcripts through the foreign gene can be eliminated by using bidirectional terminators or by judicious juxtaposition with 2μ DNA (e.g. *FLP* terminator).

RNA elongation

The elongation of transcripts is not thought normally to affect the overall rate of transcription, but the yield of full-length transcripts could be affected by fortuitous sequences in foreign genes which cause pausing or termination. These could either act in the same way as natural yeast terminators or else by a different mechanism. We have found evidence of this in a remarkably high proportion of the genes we have examined, especially, but not exclusively, in those with unusually high AT-content. Though not widely recognized, this problem could be a very common reason for low yields or complete failure of foreign gene expression in yeast.

We have listed examples of lack of expression of AT-rich genes in Table 4. The presence of truncated mRNA has not been demonstrated in every case, but the clearest published example is in the expression

of the AT-rich *Clostridium tetani* DNA encoding tetanus toxin fragment C.[307] RNA was analysed from the native gene and from each of three versions of the gene containing progressively more synthetic DNA, starting from the 5' end (Figure 5). The transcript length increased through this series, with only the fully-synthetic gene giving full-length mRNA as the major species. All the short and full-length transcripts were discrete, abundant and polyadenylated. The 1·5 kb *C.tetani* DNA was shown to contain at least six fortuitous polyadenylation sites which were eliminated by increasing GC-content (from 29% to 47%). The partially-synthetic genes gave rise to low levels of run-off translation products, but efficient production of fragment C was only achieved with the fully-synthetic gene.

The problem with AT-rich genes means that analysis of genomes with high AT-contents (e.g. *Plasmodium falciparum* or *Dictyostelium discoideum*) by complementation in *S.cerevisiae* is possibly not worth pursuing as a general strategy. Such studies might be attempted in *S.pombe*, though recent work suggests that the two yeasts have a conserved mechanism for mRNA 3' end formation.[184] More worryingly, there is evidence of the same problem with genes having an average AT-content similar to that of yeast (60%). With the HIV-1 *env* gene, truncated mRNA was found in *P.pastoris* but not *S.cerevisiae*. Mutagenesis of a sequence resembling a polyadenylation consensus inactivated a major polyadenylation site but revealed several weak sites which were removed by increasing GC-content using chemical synthesis (C.A.S. and R.G. Buckholz, in preparation).

The frequency of the problem means that it would be desirable to be able to predict its occurrence, and to solve it without resorting to gene synthesis. Unfortunately it appears to be very difficult to identify polyadenylation sites by searching for consensus sequences, especially in AT-rich DNA. In the case of the fragment C gene, one of the short mRNAs could have been due to a single TTTTTATA sequence, but a number of tripartite motifs were found in regions with no polyadenylation sites.[307]

At present the only solution is to increase the GC-content of offending sections of genes by chemical synthesis. The success of more general solutions would depend on whether the problem is due to true premature termination or to uncoupled post-transcriptional processing. In the former case a foreign RNA polymerase which uses different termination signals, e.g. T7 RNA polymerase, might produce full-length mRNA. Unfortunately, the T7 system is not fully developed in yeast (see Transcriptional promoters and terminators). If the problem is one of post-transcriptional processing then it may be necessary to use an extranuclear expression vector, e.g. one based on the linear AT-rich plasmids of *K.lactis* which can also be propagated in *S. cerevisiae*, to segregate the transcripts from nuclear processing enzymes.[365]

RNA stability

The half-lives of yeast mRNAs range from 1 to 100 min and can therefore have a profound effect on the steady-state level (reviewed in reference 47). A careful study of 15 randomly-chosen mRNAs showed two populations, one with longer $t_{1/2}$s (40 to 100 min) and one with shorter $t_{1/2}$s (6 to 20 min).[47] Within each class there was an inverse relation between mRNA length and stability. However, there is some disagreement over this division into two classes.[162] The difference between stable and unstable mRNAs may be that the latter have destabilizing elements, since sequences from the short-lived *URA3* mRNA destabilize *PYK* mRNA.[48] A number of the unstable mRNAs have recently been found to encode ribosomal proteins (A. Brown, personal communication); the foreign *lacZ* mRNA has a $t_{1/2}$ of 27 min.[292]

Insertion of premature stop codons in *URA1*, *URA3* and *PGK* mRNAs has been shown to cause their destabilization, suggesting that ribosome attachment may contribute to mRNA stability.[175,241,283] However the same result was not observed for a *PYK* and *PYK-lacZ* mRNA,[292] suggesting a complex relationship between mRNA stability and translation. Santiago *et al.*[327] found no obvious correlation between ribosome loading and stability for ten different mRNAs.

There appear to be few examples of low mRNA stability being a primary factor in poor yields of foreign proteins. Due to the lack of knowledge of yeast mRNA degradation mechanisms it is impossible to predict whether a foreign mRNA will be stable, though we might expect long mRNAs to be somewhat less stable than short ones. AU-rich sequences in the 3'-ends of some mammalian mRNAs confer instability,[339] but it is not known whether they function in yeast. Where mRNA instability is diagnosed as a problem, overall yield might be improved by (i) using a more powerful promoter, (ii) using a promoter with more rapid induction kinetics, or (iii) chemically synthesizing the gene with altered codons or deleting the 3' untranslated

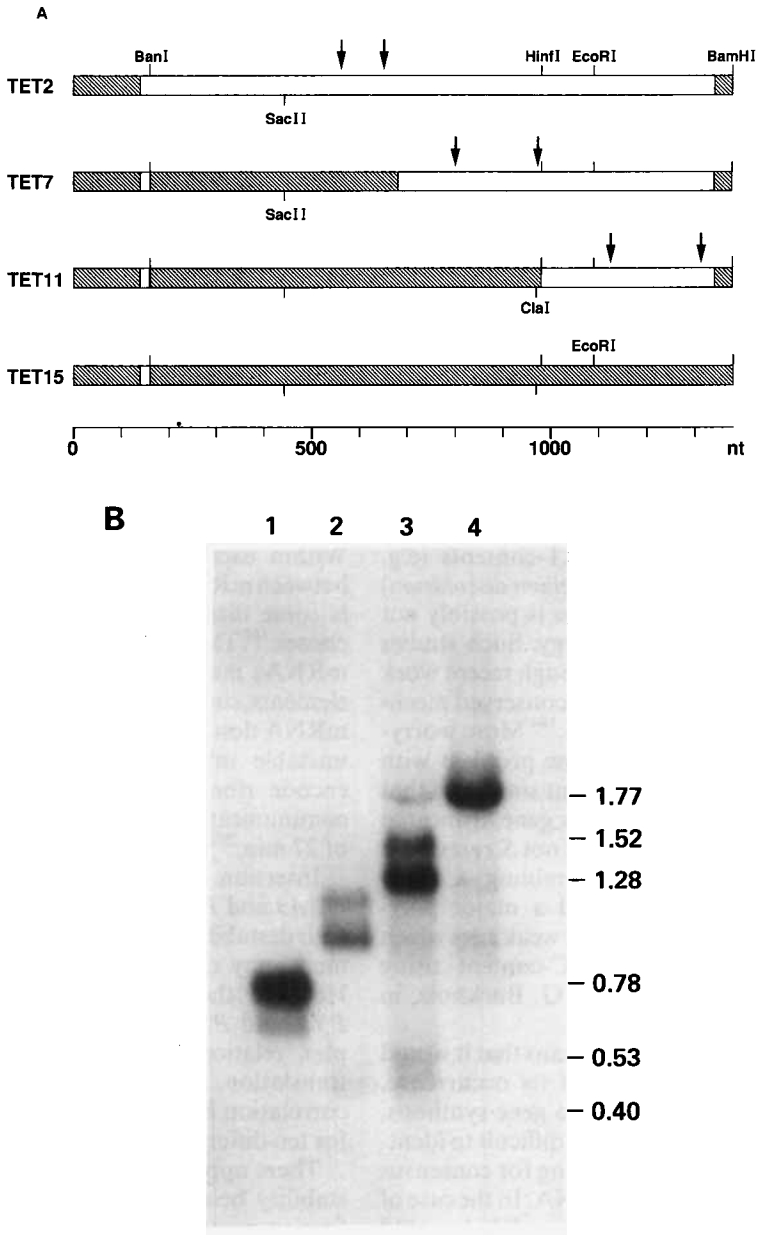

Figure 5. Fortuitous transcriptional termination in AT-rich tetanus toxin fragment C DNA. (A) Four genes encoding fragment C are shown, with synthetic DNA of increased GC-content (hatched). Approximate 3' ends of transcripts generated in yeast are indicated. (B) Northern blot showing fragment C-specific RNA from these genes: only TET15 gave abundant full- length mRNA.[307]

region[90a] in the hope that instability determinants will be removed.

Initiation of translation

Translational efficiency is thought to be controlled primarily by the rate of initiation. This is affected by the structure of the 5' untranslated leader of a mRNA, and there are examples of poor expression due to the presence of sub-optimal foreign 5' leaders. Using a multi-copy *GAP491* promoter vector to express hepatitis B core antigen (HBcAg) Kniskern *et al.*[218] found that the product accumulated to 0·05% of soluble cell protein when viral 5' and 3' sequences of about 100 nt were retained. Deletion of the 5' viral sequence raised the yield to 26%, while deletion of 5' and 3' viral sequences raised it to 41%, both without altering mRNA levels.

Initiation in eukaryotes is thought to follow a scanning mechanism whereby the 40S ribosomal subunit plus co-factors (eIF2, eIF3, eIF4C, Met-tRNA and GTP) bind the 5' cap of the mRNA then migrate down the untranslated leader scanning for the first AUG codon.[222] Any part of this process, which is affected by the structure of the leader and the AUG context, could limit the initiation rate. Yeast mRNA leaders have an average length of about 50 nt, are A-rich, have little secondary structure, and almost always use the first AUG for initiation (reviewed in reference 70). The length of the untranslated leader does not seem to be critical: although there is a 50% reduction in efficiency when the *PGK* leader is shortened from 45 to 21 nt, further shortening to 7 nt has no effect.[389] Insertion of long runs of Gs is very deleterious, however, causing a complete inhibition of translation; runs of Us have a partial effect possibly caused by interaction with the polyA tail.[390] The most significant single factor is secondary structure: hairpins of $\Delta G = -20$ kcal/mol can drastically inhibit translation of *HIS4* or *CYC1* mRNA in yeast cells,[11,71] in contrast to the much lower sensitivity of translation to leader secondary structure in mammalian cells.

The consensus sequence around the initiating AUG, A/YAA/UAAUGUCU,[70] in yeast is different from that in higher eukaryotes (CACCAUGG). It has been speculated that the mammalian context reflects an interaction with the sequence GUGG in 18S rRNA. The prevalence of UCU as the second codon may reflect the preference for stabilizing amino acids according to the N-end rule (see below). Although there is a strong bias for A at-3, especially in highly-expressed genes, alteration of the AUG context of mRNAs in yeast has at most a 2- to 3-fold effect, in contrast to the much greater effect in mammalian cells.[11,71]

As a consequence of these considerations it is preferable to avoid the inclusion of foreign non-coding sequences in yeast expression vectors. However, where this is inconvenient it should be possible to predict whether a foreign 5' leader will be deleterious by examining the sequence for secondary structure or runs of Gs or Us. For example, analysis of the sequence 5' to the HBcAg gene, found by Kniskern *et al.*[218] to inhibit translation, shows a number of predicted secondary structures of up to $\Delta G = -35$ kcal/mol (M.A.R., unpublished results).

Translational elongation

Translational elongation is not thought to affect the yield or quality of polypeptide normally, but there is now some evidence that it can become limiting with very high mRNA levels. Codon usage is known to affect the elongation rate and therefore has to be considered as a *potential* factor affecting product yield. In the past it has been discounted on the basis that some genes containing many rare codons, e.g. *lacZ* or HBcAg, are expressed at high levels; however, these gene products are unusually stable and their yield does not reflect overall translational efficiency.

Despite the degeneracy of the genetic code, a non-random codon usage is found in most organisms: whilst the codon usage of most genes reflects the nucleotide composition of the genome, highly-expressed genes show a strong bias towards a subset of codons.[28,338] This 'major codon bias', which can vary greatly between organisms, is thought to be a growth optimization strategy such that only a subset of tRNAs and aminoacyl-tRNA synthetases is needed at high concentration for efficient translation of highly-expressed genes at fast growth rates.[227] Rare codons, for which the cognate tRNA is less abundant, are translated at a slower rate in *E.coli*,[282] but this will not normally affect the level of product from an mRNA since *initiation* is usually rate-limiting. A ribosome finishing translation of one mRNA molecule is most likely to initiate translation of a different mRNA species, unless the original species comprises a large proportion of total mRNA. Thus, the overall rate of translation of an mRNA is not usually affected by a slower elongation rate unless ribosomes become limiting, which would affect all transcripts in the cell.

In contrast to the normal situation, there is evidence that codon usage may affect both the yield and quality of a protein when a gene is transcribed to very high levels. With very high levels of mRNA containing rare codons, aminoacyl-tRNAs may become limiting, increasing the probability of amino acid misincorporations, and possibly causing ribosomes to drop off. Indeed, a high misincorporation frequency has recently been observed in a foreign protein produced at very high levels in *E.coli*.[336] The presence of many very rare codons has been shown to limit the production of tetanus toxin fragment C in *E.coli*.[242] In yeast, Hoekema *et al.*[175] showed that substitution of a large proportion of preferred codons with rare codons in the 5' portion of the *PGK* gene caused a decrease in expression level. However, these substitutions were made in the region containing a putative DAS,[253] possibly contributing to the effect on expression levels. Translation of *lacZ* mRNA, which contains a high proportion of rare codons, was shown to be limiting at high levels.[292] There was a decrease in ribosome loading, possibly due to pausing and drop-off, on *lacZ* mRNA and on *TRP2* mRNA, which also has poor codon bias. Recently, expression of an immunoglobulin kappa chain in yeast was shown to increase 50-fold, without a change in mRNA levels, using a synthetic, codon-optimized gene.[220]

Thus the codon content of a foreign gene may influence the yield of protein where the mRNA is produced at very high levels. This may be more likely to occur on growth in minimal medium, when the cell produces a wide variety of biosynthetic enzymes, encoded by genes containing rare codons.[338] The effect on product quality has been difficult to measure but requires further attention since it has important implications for therapeutic proteins.[336] Proteins containing amino acid misincorporations are difficult to separate and may affect the activity and antigenicity of the product. Since small genes are now frequently synthesized chemically they may be easily and perhaps profitably engineered to contain optimal codons for high-level expression in yeast.

mRNA secondary structure, in addition to codon usage, may affect translational elongation. Baim *et al.*[10] showed that a mutation which introduced a hairpin loop ($\Delta G = -19{\cdot}6$ kcal/mol) near the 5' end of the coding region of *CYC1* mRNA reduced the amount of protein product five-fold. mRNA levels were unchanged and analysis of the distribution of *CYC1* mRNA on polysome gradients indicated that translation was affected.

Polypeptide folding

During or following translation, polypeptides must fold so as to adopt their functionally-active conformation. Since many denatured proteins can be refolded *in vitro*, it appears that the information for correct folding is contained in the primary polypeptide structure.[140] However, folding comprises rate-limiting steps during which some molecules may aggregate, particularly at high rates of synthesis and at higher temperatures.[210] There is evidence that certain heat-shock proteins act as molecular chaperones in preventing the formation and accumulation of unfolded aggregates, while accelerating the folding reactions.[140]

Due to the intrinsic nature of polypeptide folding and the low specificity of chaperones, it is very unlikely that foreign cytosolic proteins will accumulate in yeast in non-native conformations, and indeed this is generally the case. When fragments of proteins or fusion proteins are expressed, however, normal folding domains may be perturbed resulting in an insoluble product. Nevertheless, fusion proteins that are insoluble in *E.coli* may frequently be soluble when expressed in yeast, e.g. fusions to β-galactosidase (J.J.C., unpublished results), glutathione-S-transferase (GST; M.A.R., unpublished results), and HBcAg.[23] Insoluble proteins can often be renatured *in vitro* though the techniques for this can be complex and unpredictable.[246]

In contrast to intracellular proteins, naturally-secreted proteins encounter an abnormal environment in the cytoplasm: disulphide bond formation is not favoured and glycosylation cannot occur. Though many secreted proteins are insoluble when expressed intracellularly, e.g. prochymosin,[146] human serum albumin,[294] HIV gp120,[19] some are soluble and biologically-active, e.g. α-interferon,[172] α_1-antitrypsin,[310] tumour necrosis factor,[359] Factor XIIIa.[300] Factor XIIIa is interesting in that it has nine Cys residues, none of which forms disulphide bonds.

In *E.coli*, foreign proteins are frequently insoluble but low temperature has been found to increase solubility in some cases.[331] This may be due to a decreased translation rate or to the fact that hydrophobic interactions, such as occur in aggregates, become less favourable. A dramatic increase in the yield of active, soluble protein has been reported on reducing the rate of induction in *E.coli*.[219] Low temperature or reduced induction rates may increase product solubility in yeast. For example, in the intracellular expression of the bacterial membrane protein pertactin, the proportion of the product that was soluble

Table 5. Effect of penultimate amino acid in determining mature N-terminus of intracellular proteins.

	Met removed	Met retained
No N^{α}-acetylation	Pro, Val, Cys	Ile, Leu, Met, Phe, Tyr, Trp, Lys, His, Arg, Gln
N^{α}-acetylation	Gly, Ala, Ser, Thr	Asp, Glu, Asn

decreased from 100% to 10% as the expression level was increased from 0·1% to 10% t.c.p.[306]

Similar considerations apply to the assembly of foreign multimeric proteins. Examples of homopolymeric assembly include the HBcAg[218] and HBsAg.[387] The latter is inserted into intracellular yeast membranes to form immunogenic 22 nm particles resembling those found in the serum of chronic HBV carriers. In early studies only a proportion of the HBsAg was found to be immunologically-active, and later work showed that mature particles only formed during extraction, when SH residues oxidized to form inter-chain S_2 bonds.[399] More recent work in *P.pastoris* showed that a much higher proportion of HBsAg was correctly folded when produced in a strain growing slowly during induction, suggesting rate-limiting folding steps related to growth rate.[81]

There are few published examples of heteromultimeric assembly in yeast, but a notable one is the co-expression of α and β-globin cDNAs to produce haemoglobin.[396] The yield of fully-assembled, soluble haemoglobin, which incorporated yeast haem, was 3 to 5% t.c.p. Another interesting example is in the simultaneous intracellular expression of heavy and light chains of an IgG directed against ADH I: these were able to assemble in the cytoplasm and partially block ADH I activity *in vivo*, despite the fact that the inter-chain S_2 bonds would not form.[56] Similarly, intracellular expression of a catalytic antibody Fab fragment yielded functional product at 0·1% t.c.p.[39]

Post-translational processing

Amino-terminal modifications of polypeptides are the commonest processing events and occur on most cytosolic proteins (reviewed in reference 209). Two types of events normally occur: removal of the N-terminal Met residue, catalysed by Met aminopeptidase (MAP), and acetylation of the N-terminal residue, catalysed by N^{α}-acetyltransferase (NAT). Both enzymes are associated with ribosomes and act on nascent polypeptides.

The specificities of yeast MAP and NAT (Table 5) have been determined by N-terminal amino acid analysis of mutant iso-1-cytochrome *c* or thaumatin polypeptides,[183,260] and more recently by studies of purified MAP.[60] The specificity of MAP is determined by the size of the penultimate residue. When this is has a radius of gyration of $< 1{\cdot}29$ A (Gly, Ala, Ser, Cys, Thr, Pro and Val) the Met is removed, though with the larger residues Thr and Val there is only partial removal if they are followed by Pro. With other penultimate residues Met is retained. These rules appear to be reliable and highly conserved among eukaryotes, so mammalian proteins produced in yeast should have the normal N-terminal residue.

Recently it has been shown that fusions of proteins to the C-terminus of ubiquitin are rapidly processed *in vivo* when expressed in yeast, liberating the mature protein.[321,393] This approach is useful since it can be used to generate a protein with any desired N-terminus (apart from Pro), and appears to significantly increase the product yield in some cases.[101]

The factors governing N^{α}-acetylation are less clear. It appears that Met-Glu/Asp is sufficient for acetylation in eukaryotes.[260] N-terminal Gly, Ala, Ser, Thr and Met-Asn may also be acetylated, though effects of second and third residues make this less predictable.[209] However, there is evidence that the process is conserved among eukaryotes, so that proteins acetylated in mammalian cells might be expected to be acetylated in yeast cells.[156]

In most cases the structure of the N-terminus should not affect biological activity of a protein, but there may be exceptions. For example the response of haemoglobin to physiological modifiers involves the N-terminus, and correct processing of α and β-globins in yeast is therefore advantageous over expression in *E.coli*.[396] N^{α}-acetylation of melanocyte-stimulating hormone and of β-endorphin is required for full biological activity (reviewed in reference 209).

A variety of other post-translational modifications which are often critical for biological activity appear to be conserved between yeast and higher eukaryotes. The phosphoproteins fos and c-myc are correctly phosphorylated in yeast.[258,325] Myristylation is a co-translational modification of N-terminal Gly important for the membrane targetting of certain proteins, e.g. G proteins, src tyrosine kinases, and retroviral gag proteins, and this also occurs in yeast.[195] Isoprenylation affects an important class of membrane proteins including G proteins and ras proteins:[330] the Cys of C-terminal Cys-X-X-X-CO_2H is isoprenylated, following which the three C-terminal residues are removed and the Cys-CO_2H is methylesterified. Mammalian proteins such as H-ras p21 are isoprenylated in yeast.

Stability of intracellular proteins

So far, processes affecting the rate of synthesis of proteins have been considered, but the ultimate yield is equally affected by the rate of degradation. In fact the few examples of very high level expression (>25% t.c.p.) in *S.cerevisiae* are of unusually stable proteins, e.g. SOD,[156] HBcAg,[218] and schistosomal GST.[237] This reflects a difficulty in achieving a very high rate of synthesis of foreign proteins in *S.cerevisiae*, probably at the level of transcription.

Very low yields are obtained with proteins which are naturally short-lived, such as myc,[258] or with some polypeptides which are naturally secreted, such as insulin[366] and hEGF.[385] In some cases fusion to a stable protein has given high-level accumulation in yeast, for example with a hybrid TAT-Ty particle,[40] a SOD-proinsulin fusion protein,[78] fusions to GST (M.A.R., unpublished results), and a variety of peptides fused to HBcAg.[23] Alternatively, secretion has been used to segregate the product from intracellular proteases. In the case of HBsAg particles containing the pre-S2 peptide, proteolytic cleavage occurred at a specific site and could be reduced by using a protease-deficient (*pep4*) strain or by mutation to remove the susceptible region.[190] Where these approaches fail, yields might logically be improved by the following measures: (i) using a more rapidly-induced promoter, (ii) using additional protease inhibitors to minimize degradation during extraction, (iii) inducing at lower temperature, (iv) harvesting cells in the exponential growth phase.

A number of different pathways of protein degradation exist and therefore there are multiple molecular determinants that confer instability (reviewed in reference 96). Unfortunately, we do not know all the determinants nor the relative importance of different pathways. Vacuolar degradation is responsible for non-selective bulk turnover of long-lived proteins (average $t_{1/2}$ approx. 160 h), whereas short-lived proteins ($t_{1/2}$ <2·5 h) are degraded in the cytosol by an ATP-dependent pathway involving ubiquitin.

A number of proteases, activated by the *PEP4* and *PRB1* gene products, are responsible for yeast vacuolar degradation.[170] Mutations in both genes dramatically reduce cellular proteolysis and should also reduce the risk of proteolysis during extraction. *pep4* mutants are widely used, but do not normally appear to offer an advantage in product yield. Indeed a general reduction in protein turnover rate would not be expected to increase the *relative* accumulation of a foreign protein, though it might increase the total protein yield. Vacuolar proteolysis is affected by culture conditions and increases several-fold during N- or C-starvation or in stationary phase.[170]

In the ubiquitin pathway proteins are marked by covalent attachment of ubiquitin, a 76-residue polypeptide, and become substrates for rapid degradation by a cytosolic ATP-dependent proteasome.[96] In addition to short-lived proteins, damaged or denatured proteins conjugate ubiquitin more efficiently and are targetted for degradation.

Varshavsky and co-workers have identified one component of ubiquitin recognition as the N-terminal amino acid (the N-end rule pathway; reviewed in reference 393). The N-end rule was uncovered by the production of β-galactosidase variants containing different N-terminal residues, which were generated by the expression and spontaneous processing of ubiquitin-β-galactosidase fusions; the $t_{1/2}$s of these variants ranged from 2 min to >20 h. The N-end rule degradation signal actually comprises two determinants: a destabilizing N-terminal amino acid and a proximal internal Lys. Destabilizing residues are classified as Type I (positively charged: Arg, Lys, His), Type II (bulky hydrophobic: Phe, Tyr, Trp, Leu, and Ile in yeast), and Type III (small uncharged: Ala, Ser, Thr), the latter are not destabilizing in yeast. Additionally, the residues Asp and Glu are secondary destabilizing residues since they are substrates for post-translational addition of Arg; Asn and Gln are tertiary destabilizing residues since they can be deamidated *in vivo*. Recognition of a destabilizing N-terminus results in multiple ubiquitination of the internal Lys residue which is the prelude to degradation.

In agreement with the N-end rule, almost all cytosolic proteins with known N-termini have stabilizing amino acids. This might be expected from the

striking inverse correlation between the N-end rule and N-termini that are generated by MAP. (In contrast most secreted proteins have destabilizing N-termini.) However, recent data suggest that the N-end rule is only one component, possibly a minor one, of the ubiquitin pathway. Mutations in the yeast gene (*UBR1*) for the Type I and II recognition component dramatically stabilized Arg-galactosidase but did not affect either bulk proteolysis or ubiquitin-dependent degradation.[20] A similar result was obtained using inhibitors of Type I (Arg-Ala) or Type II (Leu-OMe) recognition *in vivo*, resulting in a ten-fold stabilization of Arg- or Leu-galactosidase, respectively, and > 50-fold increases in accumulated protein.[12] Therefore other, undefined, signals are more important in the ubiquitin pathway.

Another proposed signal for rapid degradation is a variable sequence rich in Pro, Glu, Ser, and Thr (PEST) found in the majority of short-lived proteins.[301] Recently, addition of PEST sequences has been shown to destabilize DHFR.[235] It is not clear whether degradation of proteins containing PEST sequences involves the ubiquitin pathway.

SECRETION OF FOREIGN PROTEINS

Introduction

A wide variety of heterologous proteins have been secreted from yeast and this approach offers certain advantages over intracellular production. Although *S.cerevisiae* secretes only 0·5% of its own proteins, this level can be increased several-fold so that a secreted foreign protein can be almost pure in the medium. Many pharmacologically-important proteins are naturally secreted and can often only adopt their correct conformation by folding within the secretory pathway. As a consequence, intracellular expression is often unsuitable since the product may be insoluble and may also have an incorrect N-terminus, although this can be overcome by fusion to ubiquitin (see 'Factors affecting intracellular expression'). Thus secretion is used mainly for the production of correctly-folded, naturally-secreted proteins, but there are, in addition, other instances when secretion may be preferable. For example, some proteins are unstable or toxic when cytoplasmically-expressed and these problems may be circumvented by secretion.

As in higher eukaryotes, protein secretion in yeast is directed by an amino-terminal signal sequence which mediates co-translational translocation into the endoplasmic reticulum (ER). The signal peptide is removed by a signal peptidase. In the lumen of the ER

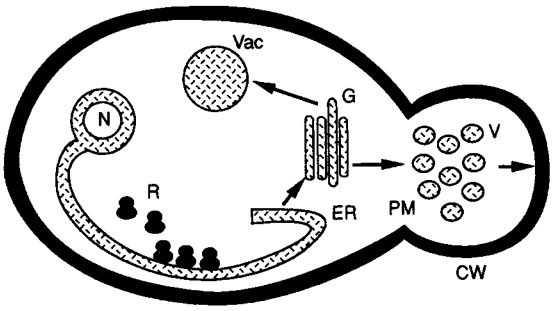

Figure 6. The yeast secretory pathway. Arrows indicate the route taken by proteins through the secretory pathway to either the vacuole or the plasma membrane. N, nucleus; R, ribosomes; ER, endoplasmic reticulum; G, Golgi; Vac, vacuole; V, secretory vesicles; PM, plasma membrane; CW, cell wall.

asparagine-linked glycosyl structures may be added. The signal for the addition of these N-linked sugars is the same for yeast and mammalian glycoproteins (Asn-X-Ser/Thr). O-linked oligosaccharides may also be added. Proteins are then transported in vesicles to the Golgi where modifications to these glycosyl structures take place. These modifications differ from those made by higher eukaryotic cells and, as a result, glycosylation is increasingly regarded as a major drawback to the secretion of therapeutic glycoproteins from yeast. From the Golgi, proteins are packaged into secretory vesicles and are delivered to the cell surface (Figure 6).

Once in the ER, it is probable that a default pathway directs a protein to the plasma membrane unless it contains specific signals to cause retention in the ER or Golgi, or to target it to the vacuole. Therefore it might be thought that if a foreign protein could be targetted to the lumen of the ER, it should be successfully secreted. However, there are a number of stages in the secretory process at which problems may occur. The yeast proteins which assist in folding and disulphide bond formation differ from their counterparts in higher eukaryotes and this may affect folding of foreign proteins. Malfolding can result in retention in the ER and degradation. There are also reports of proteins being retained in the Golgi, again possibly due to malfolding. Retention in the cell wall has also been a problem, especially with larger proteins, although factors other than molecular mass are known to be important. In addition to problems of transport, other undesirable events such as aberrant processing or hyperglycosylation may take place during the secretory process. It should be noted, however, that proteins which are poorly secreted from *S.cerevisiae* may be efficiently secreted from other yeasts, for

example prochymosin secretion is much more efficient from *K.lactis* and *Yarrowia lipolytica* than from *S.cerevisiae* (see 'Expression in non-*Saccharomyces* yeasts').

The secretion of heterologous proteins from yeast has been most successful with peptides, many of which are commercially important, e.g. epidermal growth factor (EGF) and insulin. In high-density cultures hundreds of milligrams of these proteins may be secreted per litre. The secretion of large proteins has proved less predictable, nevertheless there have been some notable successes.[186,284,388] The successful secretion of proteins which are not naturally secreted, such as HIV-1 protease has also been reported,[288] but the presence of fortuitous glycosylation sites can cause problems.[307]

Vectors and signal sequences

Promoters and selectable markers Most commonly-used secretion vectors are based on high-copy, 2μ plasmids. Integrated vectors have been reported to give higher yields of secreted product than episomal vectors; four integrated copies of a prochymosin expression unit resulted in similar overall expression levels but higher secretion yields than were achieved with a multi-copy vector.[357] The reason for this difference is unclear. However, yields of EGF, which in contrast to prochymosin is efficiently secreted, were not higher from integrated gene copies.[74] Sakai *et al.*[322] compared secretion of human nerve growth factor from a 20-copy *Ty* δ-integrant with that from a 2μ vector. Levels were three- to four-fold higher (3–4 mg/l) from the δ-integrant, but the use of a constitutive promoter (*PGK*) may have led to copy number reduction with the 2μ vector.

A number of the promoters described above (see 'Transcriptional promoters and terminators') have been used in secretion vectors, but frequently a promoter and signal sequence from the same gene are chosen e.g. *MFa1*, *PHO5* or *SUC2*. Ernst[110] reported that up to a two-fold increase in somatomedin-C secretion could be obtained by using the weak *CYC1* promoter rather than the moderate actin promoter. Product toxicity may be more acute with powerful promoters and this may work to reduce plasmid copy number; the use of weak or moderate constitutive promoters, or regulated promoters which are repressed during early growth phases, may minimize this effect.

The choice of selection marker on a secretion vector may be particularly important since culture conditions may dramatically affect the final yield in the medium. Selection of plasmid-containing cells in a defined medium may result in lower cell density and, in some cases, lower levels of secreted product per cell. Wheat α-amylase secreted from yeast has been shown to reach much higher levels in rich medium than in selective, defined medium.[314] At laboratory scale, it may be preferable therefore to use a dominant marker such as that for G418 resistance.

The nature of signal sequences A classical signal sequence comprises a charged N-terminus, a central hydrophobic core and a consensus sequence for cleavage in the ER by signal peptidase. Some secreted proteins, such as the yeast mating pheromone, α-factor, have additional pro sequences which may aid secretion.

As described below, heterologous proteins may be secreted from yeast using either a foreign signal, often derived from the protein being secreted, or a yeast signal. Since signal sequences are recognized with low specificity in yeast,[205] it could be assumed that foreign signals would work as efficiently as those from yeast, but this is often not the case.

A number of studies aimed at identifying the features of yeast signal peptides have been performed. Ngsee *et al.*[267] analysed mutated signal sequences from the yeast invertase gene, *SUC2*. They concluded that the essential feature of a signal peptide is a hydrophobic core of 6–15 amino acids, which may be interrupted by non-hydrophobic residues. Additionally, many signal peptides were found to contain one or more basic amino acids preceding the hydrophobic core. Small neutral and α-helix-disrupting amino acids were often present in the vicinity of the cleavage site.

Secretion of α-factor was shown to be drastically reduced if the hydrophobic core of the signal was interrupted by a hydrophilic residue, or a proline which disrupts-helical secondary structure.[4] Ngsee and Smith[268] noted that yeast invertase signal sequence is predicted to have an α-helical conformation, whereas that of bovine prolactin has a tendency to form an extended coil. Substitutions in the prolactin signal which increased the probability of it having an α-helical conformation improved its functioning in yeast. Thus there may be more stringent requirements for an α-helical secondary structure in yeast signal sequences compared to those of higher eukaryotes.

Surprisingly, even if the signal peptide is deleted, some proteins, e.g. carboxypeptidase Y[36] and acid phosphatase,[349] may still inefficiently enter the secretory pathway, indicating that the mature protein sequence may also contain features which can be recognized by elements of the secretory pathway.

Use of heterologous signal sequences Early attempts to secrete foreign proteins from *S.cerevisiae* utilized the protein's own signal sequence, e.g. *E.coli* β-lactamase,[303] human α- and γ-interferon (IFN),[173] mouse immunoglobulin heavy and light chains[406] and influenza virus haemagglutinin.[191] However the expression levels were often very low, with only a proportion of the protein being secreted. Processing of the polypeptide was not always correct and, in the case of IFN, degradation of the preprotein took place.[173] Nevertheless, some foreign proteins have been successfully secreted from yeast using their own signal, e.g. *Aspergillus awamori* glucoamylase,[186] barley α-amylases 1 and 2[358] and human serum albumin (HSA).[355] De Baetselier *et al.*[89] have reported the production of up to 3 g/L of active, secreted *Aspergillus niger* glucose oxidase.

The secretion from yeast of *Bacillus amyloliquefaciens* α-amylase[316] using its own signal demonstrated that a prokaryotic signal sequence may also efficiently direct secretion. The signal peptide was correctly cleaved, but the α-amylase was core glycosylated in contrast to the native product. However, the glycosylated enzyme retained its activity.

Foreign signals may also be used to drive secretion of other heterologous proteins—the human gastrin signal sequence has been shown to drive secretion of human α-amylase in *S.cerevisiae*.[328] In general, the heterologous signal sequences which function best in *S.cerevisiae* tend to be those from plant or fungal proteins (e.g. barley α-amylase,[358] *Trichoderma reesei* endoglucanase,[388] *Mucor pusillus* aspartic proteinase[410]).

The leader sequence from the killer toxin *ORF2* gene of *K.lactis*, a yeast closely related to *S.cerevisiae*, has been employed to direct the secretion of human interleukin-1β[15] and diphtheria toxin-hormone fusion proteins.[285] HSA has also been secreted from *S.cerevisiae* using this leader sequence, however it was shown to be less efficient than the native HSA leader or an *S.cerevisiae* signal sequence.[355]

Although there have been some notable successes, the use of foreign leaders often results in intracellular accumulation (e.g. α-IFN,[173] α-1-antitrypsin[53]), and it must be concluded that the yeast secretory pathway has slightly different requirements from higher eukaryotic secretion systems. Therefore, for most cases of heterologous protein secretion from yeast, it is preferable to use a yeast signal sequence and it may be simpler to do this as a matter of course with a new gene.

Use of yeast signal sequences Owing to the difficulty of predicting whether a particular foreign signal sequence will function in yeast, much work has been carried out using homologous *S.cerevisiae* signal sequences. The three most widely studied are those from acid phosphatase, invertase and α-factor.

Acid phosphatase (*PHO5*) has a classical signal sequence[7] yet there are few examples of its use for heterologous protein secretion. When used to secrete tissue-type plasminogen activator (tPA), less than 5% of the total tPA activity was found in the medium,[169] however the secretion of this protein has been problematic.[231] Human salivary α-amylase has been secreted using the *PHO5* signal[328] which in this instance gave similar results to the heterologous α-amylase and human gastrin signals and to the yeast α-factor leader.

The yeast invertase (*SUC2*) signal[286] has been used more widely for foreign protein secretion. Human α2-IFN has been secreted using this signal, which was correctly cleaved from all secreted molecules[59] unlike the native IFN signal peptide.[173] The invertase signal has also been used to secrete human α-1-antitrypsin (α-AT), however approximately 80% of the protein remained inside the cell.[262] The passage of the α-AT from the ER appeared to be the rate-limiting step in this case. Production of mouse–human chimaeric antibody has been achieved using the invertase signal to secrete immunoglobulin heavy and light chains.[180] Melnick *et al.*[255] reported the efficient secretion of human single-chain urinary plasminogen activator (scuPA) using the invertase signal.

Attachment of a heterologous protein to a yeast signal sequence will usually result in a change in the amino acid immediately C-terminal to the signal peptidase cleavage site. However, there appears to be flexibility in recognition of cleavage junctions. Fusions of the invertase signal with proteins which alter the amino acids at the junction, such as HSA or two forms of insulin, are still cleaved.[171]

The most extensively used signal sequence for heterologous protein secretion from *S.cerevisiae* is the prepro region from α-factor (*MFa1*), frequently used with the *MFa1* promoter. *MFa1* encodes a 165 amino acid protein, prepro-α-factor, which comprises a signal sequence of 19 amino acids (the pre region) and a pro region, followed by four tandem repeats of the mature 13 amino acid α-factor sequence (Figure 7). Each repeat is preceded by a short 'spacer peptide' with the structure Lys-Arg-$(Glu/Asp\text{-}Ala)_{2\text{-}3}$. Processing of prepro-α-factor

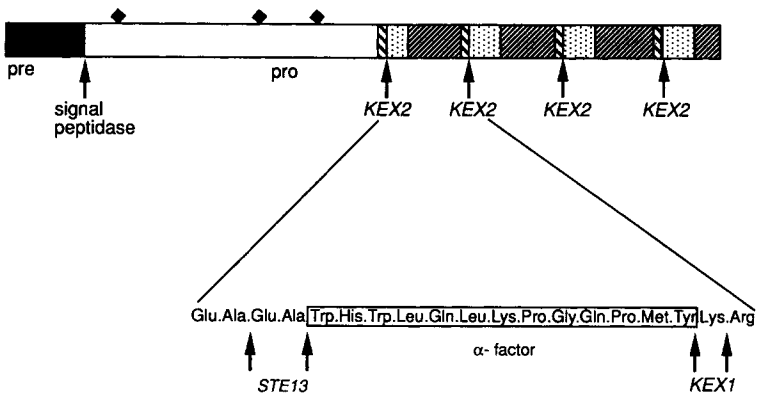

Figure 7. The structure and processing of prepro-α-factor. The product of the *MFα1* gene is shown schematically. The three N-linked glycosylation sites in the pro region are marked (◆). The peptide product from the cleavage of prepro-α-factor by KEX2 is also indicated and sites for further processing by STE13 and KEX1 are shown. See text for a description of the activities of the processing enzymes.

involves four proteolytic enzymes: (i) the pre region is cleaved by signal peptidase, (ii) the KEX2 protease cleaves on the carboxyl terminal side of the Lys-Arg sequence in the pro region and at the junction of each repeat, (iii) STE13, a dipeptidyl aminopeptidase, removes the spacer residues at the amino terminus of each repeat and (iv) the KEX1 carboxypeptidase removes the Lys and Arg residues at the carboxyl terminus of the first three repeats (see Figure 7; reviewed in reference 41).

Fusions of the *MFα1* prepro to genes encoding mature human α1-IFN,[350] a consensus α-IFN,[34] β-endorphin[34] or human EGF (hEGF)[42] resulted in efficient secretion of the heterologous protein. Processing of the prepro region took place, but the Glu-Ala spacer at the N-terminus of the secreted protein was not always removed. 5% of the hEGF and 50% of the α1-IFN was estimated to be in an intracellular form. Many foreign proteins have now been secreted from yeast using the α-factor leader, and this system has been demonstrated to be generally applicable. Changes can be been made to the *MFα1* prepro to facilitate cloning, such as the introduction of a *Xho* I site towards the end of the pro region.[74] Despite a resultant amino acid change (Asp to Glu), this was shown to be fully functional.

The requirement for the pro region of the α-factor signal sequence seems to vary. Glycosylation of this region appears to be important for efficient transport of α-factor.[55] Ernst[111] reported that the pro region was not important for the secretion and processing of aminoglycoside phosphotransferase or human granulocyte-macrophage colony stimulating factor (GM-CSF). Furthermore, prepro fusions to interleukin-1β were not cleaved whereas pre fusions were correctly processed.[111] However, secretion of human insulin-like growth factor I (IGF-I) from yeast is dependent on the entire α-factor leader; the pre region is not sufficient. It may be that the pro region aids movement of the IGF-I into the ER or assists folding of the protein in the ER (A. Hinnen, personal communication). Additionally, both the pre and pro regions were required to bring about translocation of a reporter protein, α-globin.[312] Reppe *et al.*[296] compared secretion of human parathyroid hormone (hPTH) directed either by the α-factor prepro region or by the pre region alone. When the pro region was not present, there was a considerable reduction in hPTH mRNA levels, with a concomitant reduction in levels of the protein product both in the growth medium and inside the cells. This suggests that the pro region may additionally play a role in stabilizing the mRNA or may affect transcription of the gene.

Glycosylation

Many potential therapeutic proteins are glycosylated, including monoclonal antibodies, blood clotting factors, and many IFNs, hormones, growth factors and viral antigens. The carbohydrate side chains appear to be involved in diverse processes including cell–cell recognition, hormone–receptor binding, protein targetting, host-microorganism interactions, solubility and stability.[295] Glycosylation is both organism and cell-type specific and

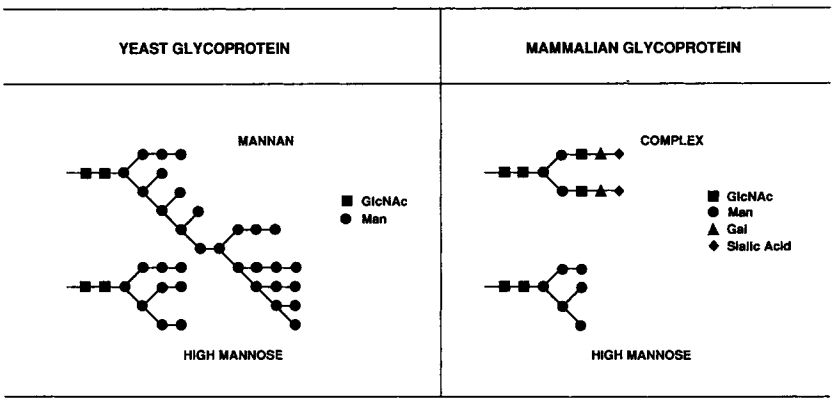

Figure 8. Comparison of yeast and mammalian glycosylation. Schematic diagram to compare the structures of oligosaccharide side chains of glycoproteins produced in yeast and mammalian cells.

therefore expression of a protein in a heterologous system will almost certainly result in a product with different modifications from the native material. This may affect the function or immunogenicity of the protein. It is interesting to note that differences in glycosylation of tPA produced in either Chinese hamster ovary or murine cell lines led to differences in the kinetics of its fibrin-dependent activation of plasminogen.[280]

Oligosaccharides may be either N-linked to asparagine or O-linked to serine or threonine residues. O-linked oligosaccharides synthesized by yeast are very different from those of higher eukaryotes, being composed only of mannose residues. Many higher eukaryotic proteins have sialylated O-linked chains of the mucin-type. N-linked glycosylation in yeast and higher eukaryotes is more conserved, and entails the addition in the ER of a core oligosaccharide unit comprising two N-acetylglucosamine (GlcNAc), nine mannose (Man) and three glucose residues. The glucose residues are subsequently trimmed from the side chain and one mannose is also removed. These steps are common to yeast, plants and higher eukaryotes.

Processing of the core oligosaccharide unit takes place in the Golgi and at this stage there is divergence between yeast and other eukaryotes (Figure 8). In animal cells further mannose residues are removed and additional sugars may be added. The resultant oligosaccharide side chains are of three types: (i) high-mannose ($GlcNAc_2Man_{2-6}$), (ii) complex, which comprises a core of $GlcNAc_2Man_3$ plus additional residues including GlcNAc, galactose (Gal), fucose and sialic acid, and (iii) hybrid, containing features of both high-mannose and complex-type oligosaccharides. (N-linked glycosylation is reviewed in reference 221). In contrast, *S.cerevisiae* does not trim back the mannose residues as it lacks the Golgi mannosidases present in higher eukaryotes. Instead, elongation of the chain may take place through a stepwise addition of further mannose residues. These additional mannose units comprise the outer chain which may be up to 75 residues long, with many branch chains. (For a review of protein glycosylation in yeast see reference 225).

Many heterologous glycoproteins have been secreted from yeast, including *Aspergillus* glucoamylase,[186] somatostatin,[148] human α-1-antitrypsin,[262] porcine urokinase,[417] human erythropoetin[106] and *Trichoderma reesei* endoglucanase 1.[388] The addition of both N- and O-linked oligosaccharides has been reported (e.g. *Aspergillus* glucoamylase[186]). Analysis has revealed that not all *S. cerevisiae* N-linked oligosaccharides contain the outer chain of mannose residues. Addition of the outer chain to heterologous proteins is regarded as 'hyperglycosylation' because it results in more extensive glycosylation than is found in higher eukaryotic glycoproteins. Additionally, it should be noted that glycosylation can be very heterogeneous, giving rise to a mixed product with a varying degree of oligosaccharide attached.

Some foreign proteins are fortuitously glycosylated when secreted from yeast. This problem is more likely to occur with proteins from bacteria (for example see references 307 and 316), in which there is no glycosylation, or with proteins which are not normally secreted. Interleukin (IL)-1α and 1β are naturally-secreted proteins, but do not pass through the normal secretory pathway in mammalian cells,

and are therefore not glycosylated. However, when secreted from yeast using a signal sequence, both proteins are glycosylated. Although this has no significant effect on the biological activity of IL-1α,[233] the activity of IL-1β is reduced five-fold.[234]

Although many glycoproteins produced in yeast are active, yeast glycosylation, especially hyperglycosylation, may give rise to problems, depending on the intended use for the protein. In some instances, extensive glycosylation may inhibit reactivity with antibodies, as was the case for EBV gp350 secreted from *S.cerevisiae*.[335] This is obviously an undesirable effect for potential vaccine antigens. Furthermore, α1,3-linked mannose units, which occur in large numbers in the outer chain, appear to be immunogenic[16] and thus yeast-derived glycoproteins may be unsuitable as therapeutics.

Problems with yeast glycosylation of foreign proteins may be circumvented by mutating the glycosylation site(s), as was done in the case of scuPA[255] to yield a fully-active, non-immunogenic product. Alternatively, the use of glycosylation mutants allows the production of more homogeneous proteins with limited glycosylation. Mutants in mannan biosynthesis (*mnn*) may be useful for the production of heterologous proteins; *mnn9* mutants do not add the extensive outer chain of mannose units,[225] however immunogenic terminal α1,3 mannose linkages are still present. Use of the *mnn1 mnn9* strain, which additionally lacks these residues, may enable the production of non-immunogenic, non-hyperglycosylated proteins. However some *mnn* mutants, e.g. *mnn9*, exhibit slower growth and greater osmotic sensitivity than wild-type cells. Sledziewski *et al.*[353] created a strain in which *MNN9* expression was temperature-regulated, using the α2 repressor system in a *MAT***a** *sir3*^{ts} strain (see 'Transcriptional promoters and terminators'). Cultures were grown to high density at the permissive temperature for *MNN9* expression (25°C), then switched to the non-permissive temperature (35°C). Glycoproteins produced at the higher temperature were shown to have the shorter, homogeneous mannose side chains characteristic of *mnn9* mutants.

Some 'super-secreting' mutants are also hyperglycosylation-deficient and may be suitable host strains for glycoprotein production. *pmr1* mutants (previously called *ssc1*; see below) possess a defective Ca^{2+} ATPase ion pump and their growth is calcium-dependent.[315] The mutation is thought to cause secreted proteins to by-pass the Golgi. Proteins secreted from this strain are core-glycosylated only.

Protein folding and transport

Folding of secreted proteins occurs in the ER and involves accessory proteins such as BiP (heavy chain binding protein, the product of the *KAR2* or *GRP78* gene[272,309]), and protein disulphide isomerase.[126] Nascent proteins bind to BiP co-translocationally and are released upon folding, assembly and glycosylation; malfolded proteins bind permanently and are retained in the ER. The assembly of an oligomeric bacterial enterotoxoid in *S.cerevisiae* has recently been shown to be dependent on BiP, which acts as a molecular chaperone.[333] During the secretion of foreign proteins, problems might arise either from saturation of these accessory proteins or from their inability to aid the folding of heterologous proteins.

Glycosylation of a protein can aid both generation of the correct conformation and passage of the molecule through the secretory pathway. Inhibition of glycosylation with tunicamycin results in the intracellular accumulation of inactive invertase.[136] At lower temperatures, unglycosylated protein may be secreted: at 25°C, 50% of unglycosylated invertase is secreted.[116] Similarly, active, unglycosylated acid phosphatase can be secreted from tunicamycin-treated cells at 20°C or 25°C, but not at 30°C.[259] Unglycosylated glycoproteins are transported only if they achieve the correct conformation and the lower temperature allows this to happen. Progressive elimination, by mutation, of each of the 12 glycosylation sites of acid phosphatase resulted in increasing irreversible retention of the malfolded protein in the ER as more sites were removed.[299] At lower temperatures under-glycosylated or unglycosylated protein could be secreted.

Sato *et al.*[328] studied the expression of a series of human salivary α-amylase mutants which either lacked regions encoding cysteine residues or contained mutations at cysteine codons. The prevention of the possible formation of a disulphide bond abolished secretion. Elimination of either of the two glycosylation sites did not affect secretion and indeed α-amylase is secreted from tunicamycin-treated cells in a fully active form. Thus, at least for α-amylase, the unique conformation of the protein appears to be much more important than glycosylation for secretion in yeast. This phenomenon indicates that attempts to secrete protein fragments or fusions could lead to intracellular accumulation if normal folding and disulphide bond formation cannot take place.

In addition to requiring successful targetting and folding in the ER, a foreign protein also has to pass

through the secretory organelles in order to be released into the culture medium. Transport from the ER to the Golgi has often been shown to be rate-limiting. Hepatitis B virus large surface protein is retained in the ER, provoking the enlargement of this organelle.[31] This retention may be due to mal-folding or to a specific retention signal. α-1-anti-trypsin[262] and erythropoetin[106] are also retained in the ER. Soybean proglycinin, although expressed to high levels and correctly processed, was mostly insoluble and accumulated in Golgi-like structure.[386] Insolubility was due to interaction of the acidic region of the polypeptide with cellular components.[386] A Golgi or post-Golgi bottleneck was also postulated to represent a major obstacle in the secretion of IGF-I.[367] Retained material may have been malfolded and this is likely to be a common reason for intracellular accumulation of proteins within the secretory pathway.

A blockage or bottleneck in the secretory pathway caused by foreign protein secretion may also interfere with the secretion of host proteins. This was observed for acid phosphatase during the secretion of tPA[169] and may result in toxicity.

Correct folding, assembly and transport are especially important in the production of multimeric proteins. The first multimeric protein to be secreted from yeast was a mouse-human chimaeric antibody. Co-expression of immunoglobulin heavy and light chains resulted in the secretion of properly folded and assembled antibody.[180] Both whole antibody and Fab fragments were functional. The pentameric *Torpedo californica* nicotinic acetylcholine receptor with a stoichiometry $\alpha_2\beta\gamma\delta$ has also been produced in yeast by co-expression of the four subunits.[199,412] These integral membrane proteins entered the secretory pathway and were processed and glycosylated. However, no functional receptor was detected, possibly due to improper folding or assembly.

Membrane proteins often cause problems when secreted due to non-specific insertion into intracellular membranes (see 'Physiology of foreign gene expression'). EBV membrane glycoprotein gp350 was highly toxic, but could be secreted in a membrane anchor-minus form.[335] Nevertheless, some membrane proteins have been successfully produced in yeast. The human β_2-adrenergic receptor was co-expressed with a mammalian G protein.[214] Coupling of these components to each other and to downstream effectors of the yeast mating signal transduction pathway was demonstrated, indicating that correct folding and targetting took place. This *in vivo* reconstruction system provides a useful new approach for the study of signal transduction pathways.

The presence of the cell wall complicates the secretion process in yeast. Permeability may be a limiting factor and it is notable that most success has been with very small proteins. Some proteins have been reported to be localized mainly in the cell wall when secreted from *S.cerevisiae*, e.g. α-IFN[420] (166 amino acids, 20 kDa). Although it has been reported that only molecules with a molecular mass below 760 are able to diffuse freely through the cell wall, a number of very large proteins such as EBV membrane glycoprotein[335] (842 amino acids, approximately 400 kDa) and cellobiohydrolase[284] (up to 200 kDa) have been shown to be capable of passing through the cell wall and therefore this property is not related simply to the size of the molecule. It may be that there are a few large holes in the cell wall with the average pore size being small. It is difficult to draw conclusions from observations of permeability since many other factors, including strain, growth phase and composition of the medium, may have an effect on cell wall porosity (reviewed in reference 91). Additionally, glycosylation and the charge on a protein are thought to affect its passage through the cell wall. *mnn9* mutants have been reported to have increased porosity and release invertase octamers, which are normally retained.[373] *kre1* mutants, which have altered (1,6)-β-D-glucan in the cell wall, over-secrete secreted yeast proteins, possibly due to the cell wall being more leaky.[51]

Proteolytic processing

There are two ways in which proteolytic processing is relevant to heterologous protein secretion in yeast. Firstly, correct processing of the signal peptide or prepro region must take place so that the mature product is secreted. Secondly, fortuitous, undesirable processing events may occur as a result of cleavage by processing proteases.

Yeast signal peptidase (SPase) is a polypeptide complex which includes the *SEC11* gene product and a glycoprotein.[407a] Although eukaryotic SPases and the *E.coli* SPase I are disparate, it is interesting to note that homologous overproduction of the *E.coli* protein resulted in increased efficiencies of export and maturation of two poorly-processed hybrid secretory proteins.[392] This indicates that, at least in *E.coli*, the availability of the SPase can be a limiting factor. Indeed, *S.cerevisiae* strains carrying multiple copies of the wild-type *PHO5* gene

accumulate unprocessed precursor, suggesting that there is saturation of some component of the secretory pathway, possibly SPase.[155]

Deletion of the SPase cleavage site in yeast acid phosphatase leads to unprocessed, core glycosylated protein which accumulates inside the cell.[155] However, even where processing of the signal does take place, cleavage may be aberrant, giving rise to heterogeneous product. 64% of human IFN secreted from yeast using its own signal was properly processed, but 36% contained an additional three amino acids of the pre sequence.[173] Furthermore, 90% of the total IFN produced was not secreted and this intracellular material also included a third form which retained eight amino acids of pre sequence. It is unlikely that molecules which retain part or all of the pre sequence will be secreted since the hydrophobic core may be retained in the membrane.

The processing of the prepro sequence from α-factor has been described above and is a more complex process involving the *KEX2* and *STE13* gene products in addition to signal peptidase (reviewed in reference 49). The KEX2 protease cleaves on the carboxyl side of the dibasic residues, Lys-Arg and Arg-Arg. In a fusion of the α-factor prepro and a heterologous protein, these residues are at the junction, and cleavage by KEX2 liberates the heterologous protein from the leader region. The Glu/Asp-Ala spacer residues, which provide a hydrophilic environment at the KEX2 cleavage site can be dispensed with in certain cases, circumventing the problem of incomplete STE13 processing, e.g. for hEGF[42] and α1-IFN.[350] However, removal of the spacer peptide does sometimes lead to a failure in KEX2 processing, as observed for interleukin-6 (IL-6)[152] where the presence of a proline residue on the carboxyl side of the cleavage site inhibited cleavage. However, accurate recognition and cleavage occurred when the construct was modified to include alanine N-terminal to the proline. Expression of the modified gene fusion had then to be carried out in a *ste13* mutant, to avoid trimming at the N-terminus of IL-6 by the STE13 protease.

Another solution to the problem of inefficient processing is to over-express the processing enzyme genes. This approach was successfully employed in the expression of transforming growth factor α (TGFα).[19] Inclusion of the *KEX2* gene on the same multi-copy plasmid as the TGFα gene eliminated the presence of unprocessed forms of the α-factor leader-TGFα fusion protein and resulted in increased levels of secreted TGFα. Additionally, a novel *S.cerevisiae* aspartyl protease (*YAP3*) has been identified which allows KEX2-independent processing of the α-factor precursor.[102] This could be over-expressed when KEX2 processing was inefficient and limiting. Over-expression of *STE13* could also improve inefficient processing; wild-type cells carrying multiple copies of the *MFα1* gene produce mainly incompletely processed α-factor, indicating that the dipeptidyl aminopeptidase is rate-limiting.[204]

In addition to inefficient proteolytic processing, problems may also be caused by aberrant processing at internal sites in the protein. In the extreme case of β-endorphin, no complete mature protein was secreted into the medium;[34] two trypsin-like cleavage sites were observed after internal lysine residues. Use of the vacuolar protease mutant *pep4-3* did not reduce the degradation, suggesting that proteolysis was taking place during passage through the normal secretory pathway.[34] Internal processing was also a problem in the expression of hPTH in which cleavage, probably by the *KEX2* gene product, was observed after two basic residues.[131] A mutant form of hPTH which lacked one of the basic residues was no longer subject to internal proteolytic processing.[297] The mutant form retained full biological activity and was produced in significantly higher yield. Differences in proteolysis between strains and/or growth conditions may exist since two reports of secretion of GM-CSF identified different aberrant processing events. Miyajima *et al.*[257] reported cleavage after the arginine residue at position 4 of the mature protein. Price *et al.*[290] however observed both the full-length mature species and the product of cleavage after the proline at position 2 of the mature protein. Differences were also apparent in the efficiency of α-factor leader processing. This indicates that it may be possible to eliminate or to minimize internal cleavage of heterologous proteins by optimizing growth conditions and selecting the best strain.

An analysis of secreted hirudin (hir) revealed full-length hir65, but also two C-terminally degraded products, hir64 and hir63.[372] Use of the protease mutants *prc1* and *kex1* showed that this degradation was due both to yscY and yscα activities. Similarly, secreted EGF was found to be C-terminally trimmed.[74,138]

Mutants in the gene for the extracellular protease SKI5 were shown to give higher yields of secreted yeast proteins due to decreased degradation in the medium.[51]

An interesting development in protein production has been the exploitation of yeast processing

enzymes to cleave heterologous precursors. Proinsulin is converted to insulin as the result of cleavage at two dibasic sequences separated by a spacer peptide. These processing reactions can be carried out by the KEX2 protease, and various forms of proinsulin/insulin have been secreted from yeast.[376] Human proalbumin has also been shown to be processed after the Arg-Arg sequence by KEX2 to yield mature albumin.[21] *Aspergillus* glucoamylase secreted from yeast using its own signal sequence was shown to be cleaved both by SPase and by KEX2, which removed an additional six amino acids from the N-terminus.[186] This yielded a product with the same N-terminus as that of the *Aspergillus* enzyme and showed yeast to be capable of this additional processing step.

Strategies to improve secretion

Several *S.cerevisiae* strains with a 'super-secreting' phenotype have been isolated by screening for mutants with increased secretion of a particular product. Such methods have yielded strains which show a general increase in heterologous protein secretion. On finding that less than 1% of the prochymosin made in yeast was secreted, Smith *et al.*[357] employed a mutagenesis approach coupled with a rapid screening assay to isolate super-secreting colonies. The secreted material, which was activated by the low pH of the medium, was assayed by overlaying the surface of the plate with a mixture of milk and molten agarose. The chymosin clots the milk and the speed of appearance of the opaque clotted regions and their size and intensity indicate the level of prochymosin secretion. Two super-secreting strains in particular were identified and designated *ssc1* and *ssc2*.[357] The effects appeared to be additive in the double *ssc1 ssc2* mutant. The *SSC1* gene was later found to be identical to *PMR1*, which encodes a Ca^{2+} ATPase[315] (see above). The *pmr1* mutation significantly increased the secreted levels of prochymosin, bovine growth hormone and scuPA by five- to 50-fold.[357,384] However secretion of α-1-antitrypsin, which was efficiently exported in the wild-type strain, was not further improved, indicating that the *pmr1* mutation may be most effective at improving the secretion efficiency of proteins that are secreted poorly by wild-type cells.

In addition to using colony assay screens to find the most efficient secretors, Moir and Davidow[261] described a screening procedure which involved *in vitro* mutagenesis of scuPA genes on plasmids. This enabled identification of mutant forms of scuPA with either decreased or increased activity compared to the parental form. The assay uses fibrin-agar and colonies are scored by measuring zones of clearing.

Other workers have employed a similar approach to identify super-secreting strains. A plasminogen-casein assay for secreted tPA was used to isolate secretor strains;[141] previous attempts to secrete tPA from yeast had not been successful.[169,231] A general screen for secreted proteins based on a visual antibody precipitation assay has been described.[354] Repeated rounds of mutation and selection enhanced the secretion of HSA six-fold and the resultant strains were also able to produce higher levels of internally-expressed α-1-antitrypsin and human plasminogen activator inhibitor 2. This screen is suitable for any protein for which antibodies are abundantly available. Multiple rounds of mutagenesis and selection were also used to isolate a strain which secreted 70-fold more endoglucanase I than the original wild-type parent strain.[6] Again, the mutant strain was found to secrete elevated levels of other proteins.

By expressing a gene fusion of HSA and hygromycin B phosphotransferase, Chisholm *et al.*[68] selected for increased expression on the basis of the level of resistance to hygromycin B. This method was initially used to isolate strains with increased intracellular expression of the HSA fusion protein. However, when cured of the intracellular expression vector and retransformed with a HSA secretion vector, these strains also showed significant increases in secretion. Genetic analyses suggested that multiple mutations were responsible for the observed effects.

Selection may be used to identify mutants which are resistant to the toxic effects of a foreign protein. The slow growth rate of IGF-I-expressing cells was exploited by Shuster *et al.*[347] who isolated fast-growing IGF-I-resistant mutants which gradually accumulated in a population of IGF-I-expressing cells under selection. Mutations at a single locus, designated *HPX1* (for *H*eterologous *P*rotein e*X*pression) were found to confer both resistance to the toxic effects of IGF-I and its increased production.

In summary, the screening approach has frequently proved successful in the isolation of super-secreting strains, even in cases where the initial yield was high. Therefore, for industrial applications when a high yield is required, this approach deserves consideration.

Table 6. Comparison of the expression of two genes in *E.coli*, Baculovirus, *S.cerevisiae* and *P.pastoris*.

Expression system	Expression level (%) Fragment C	Pertactin
E.coli	24[241a]	30[242a]
Baculovirus	10♯,[60a]	>40*
S.cerevisiae GAL7	2–3[307]	0·1[306]
P.pastoris	28§,[73]	10¶,[306]

♯Estimate; yield variable and greatly reduced on scale-up.
*Estimate; yield variable and greatly reduced on scale-up; I.G. Charles, personal communication.
§Scale-up without loss of yield to > 12 g/l.
¶Scale-up without loss of yield to >4 g/l.

EXPRESSION IN NON-*SACCHAROMYCES* YEASTS

Introduction

In some ways *S.cerevisiae* could be regarded as a non-optimal host for the large-scale production of foreign proteins due to drawbacks such as the lack of very strong, tightly-regulated promoters, the need for fed-batch fermentation to attain high-cell densities, and hyperglycosylation. Although these have been addressed by exploiting its sophisticated molecular genetics, another important approach has been to develop expression systems in other yeasts. Most of these alternative systems are based on commercially-important yeasts that have been selected for their favourable growth characteristics at industrial scale, or on yeasts which have other favourable intrinsic properties (e.g. high-level secretion).

The most extensively developed system is based on *Pichia pastoris*. An efficient and tightly-regulated promoter coupled with very straightforward techniques for high-biomass cultivation make this a powerful expression system. It is currently the simplest of eukaryotic systems to scale up, and there are now several comparative studies suggesting it can be used to avoid limitations on transcription which are sometimes encountered with *S.cerevisiae* (see Table 6). *Hansenula polymorpha* has similar properties and there are some promising examples of its use. A potential problem however, especially with toxic products, may be that under the conditions normally used for high-density growth, expression is significantly derepressed. *K.lactis* and *Y.lipolytica* are both industrial yeasts which have been examined largely because of their capacity for high-level secretion. *K.lactis* is similar to *S.cerevisiae* and has well-developed molecular genetics. Unlike the other systems discussed here it has the advantage of highly stable episomal vectors. Finally the distantly-related fission yeast, *S.pombe*, has very advanced genetics but its use in foreign gene expression has mainly been in isolating and studying homologous mammalian genes, rather than protein production. The following sections will briefly review these non-*Saccharomyces* heterologous expression systems. A summary of the various proteins that have been expressed in each system is given in Table 7.

Pichia pastoris

The methylotroph *P.pastoris* has now been used to express high levels of many different intracellular and secreted proteins. Strains were originally developed for the large-scale, high-yield production of single-cell protein using defined medium containing methanol.[401] This fermentation technology subsequently provided the basis for an efficient expression system which uses the promoter from the tightly-regulated *AOX1* gene.[81] *AOX1* encodes alcohol oxidase, which catalyses the first step in the assimilation of methanol, and can be induced to give levels of up to 30% of t.c.p. by the addition of methanol.[76]

Since *P.pastoris* has no native plasmids (other than linear DNAs, K. Sreekrishna, personal communication) expression vectors designed for chromosomal integration have been developed. These vectors have used the *HIS4* gene for selection, but a limited number of alternative selection markers are now available, e.g. *ARG4* from *S.cerevisiae*[80] or *P.pastoris* (J. Cregg, personal communication), the *Tn903* G418-resistance gene (K. Sreekrishna personal communication), and the *S.cerevisiae SUC2* gene[361] which can be used as a dominant marker since *P.pastoris* cannot use sucrose as the sole carbon source. Multi-copy episomal vectors containing the *P.pastoris* ARS sequences, PARS1 and PARS2, have also been constructed.[79] However, for maximum stability, particularly in large-scale inductions, the expression cassette has been transplaced into *AOX1*, or integrated by single cross-over into *AOX1* or *HIS4* (see Figure 9). It is worth noting that, using *HIS4* selection, *HIS4* integrants are the least stable type of transformant since recombination can result in excision of the expression cassette leaving behind a wild-type marker. Although transplacement into

AOX1 results in gene disruption, transformants are still capable of slow growth on methanol due to the presence of a second alcohol oxidase gene, *AOX2*, which is less well expressed.[80]

The unusually high level of alcohol oxidase together with evidence for transcriptional regulation[107] suggested a very powerful and efficiently regulated promoter suitable for foreign gene expression. This was first tested by isolating the *AOX1* promoter, and also that from the co-regulated dihydroxyacetone synthase gene (*DHAS*), and fusing them to the *lacZ* gene.[380] In single-copy the *AOX1-lacZ* hybrid gene was tightly repressed by glucose or glycerol and was efficiently induced by methanol; with multi-copy *PARS* vectors some expression in glycerol occurred. The *AOX1* promoter has subsequently been used to produce a variety of foreign proteins. Promoters from several other *P.pastoris* genes have been isolated, e.g. from the constitutive *GAP* and *PGK* genes, and from the *AOX2* gene,[80] but there are no published examples of their use.

A number of factors may potentially affect expression levels using *P.pastoris* integration vectors. In particular, disruption of *AOX1* by transplacement might be expected to have important physiological consequences which may influence foreign gene expression. During induction *aox1* transformants do not simultaneously produce high levels of alcohol oxidase and heterologous protein, and they also grow more slowly ('*m*ethanol-*u*tilization *s*low', Mut^s), and have a much lower O_2 demand than wild-type (Mut$^+$) strains. Indeed, in the first report of foreign gene expression in *P.pastoris*, a higher proportion of immunogenic HBsAg particles were produced in a Mut^s host compared to Mut$^+$, although expression levels were similar.[81] This was attributed to one or more events during assembly being rate-limiting in fast-growing cells, though there is evidence that particle maturation in *S.cerevisiae* occurs during protein extraction.[399] The parameters affecting foreign gene expression in *P.pastoris* were investigated in a study using tetanus toxin fragment C.[73] In a direct comparison, similar amounts were produced in Mut^S and Mut$^+$ hosts, even at levels approaching 28% t.c.p. Expression levels were also independent of the site (*HIS4 versus AOX1*) and type (single *versus* double cross-over) of integration. However, in another study, a Mut^S integrant expressed *Bordetella pertussis* pertactin at 2% t.c.p. in shake flasks and 10% t.c.p. in fermenters, while a Mut$^+$ strain gave 5–6% t.c.p. in shake-flasks or fermenters.[306] Thus, the significant physiological differences between Mut^S and Mut$^+$ strains during induction may in some instances influence the expression of foreign genes.

The abundance of alcohol oxidase led to the assumption that single-copy integrants would yield sufficient levels of foreign proteins using the *AOX1* promoter. However, with single-copy vectors it has not been possible to obtain yields of heterologous proteins that are as high as alcohol oxidase.[73,74,306] This is not due to the presence of DAS elements since, unlike the *S.cerevisiae PGK* gene[253], replacement of the *AOX1* coding region with foreign sequences does not affect mRNA levels[73] (R. Buckholz personal communication). Rather, higher levels of alcohol oxidase are probably due to its exceptional proteolytic stability which may be enhanced as a result of sequestration to peroxisomes. Multi-copy integrant strains are now used for increased yields of foreign proteins. These can be obtained using vectors containing multiple expression cassettes[375] or, for very high copy number, by screening for multiple integration events.[73,74,306] Surprisingly, very high copy number integrants can be isolated from transformations using DNA fragments designed for single-copy transplacement. Sreekrishna *et al.*[359] observed a high degree of variation (from 1–30% t.c.p) in the level of tumour necrosis factor (TNF) expressed in different Mut^S transformants and found this was due to differences in gene copy number.

The mechanism of this 'multi-copy transplacement' was initially unclear and it was not apparent whether this was a general phenomenon, nor whether high-level expression would invariably occur as a result. However, multi-copy transplacement has now been observed in several other instances.[73,74,306] The use of such strains has frequently resulted in remarkably high yields which compare very favourably with other expression systems (see Table 6). These studies also show that multi-copy transplacement strains are stable during high-density growth and induction. The clonal variation in vector copy number and protein yield among transplacement transformants expressing pertactin is illustrated in Figure 10.

Multi-copy transplacement occurs at a variable frequency, and is observed in about 1–10% of Mut^S transformants.[73,74,306] To understand the mechanism involved and the factors controlling this, Clare *et al.*[73] carried out a detailed DNA analysis of fragment C-vector transformants. An interesting finding was that 75–95% of transformants had not undergone a transplacement event (i.e. were Mut$^+$) but were single or multiple integrations at *AOX1* or

Table 7. Production of foreign proteins in non-*Saccharomyces* yeasts.

Yeast	Protein[a]	Location[b]	Promoter[c]	Reference
Pichia pastoris	β-galactosidase	I	AOX1, DHAS	380
	HBsAg	I	AOX1	81
	Tetanus toxin fragment C	I	AOX1	73
	Pertactin	I	AOX1	306
	TNF	I	AOX1	359
	Streptokinase	I	AOX1	154a
	SOD	I	AOX1	43, 375
	HIVgp120	I, S	AOX1	C.A.S., R. Buckholz, unpublished results
	S.c. invertase	S	AOX1	381
	Bovine lysozyme	S	AOX1	97
	Human EGF	S	AOX1	43
	Murine EGF	S	AOX1	74
	Aprotinin	S	AOX1	375
	HSA	S	AOX1	K. Sreekrishna, personal communication
Hansenula polymorpha	β-lactamase	I, S	MOX, FMD, DAS	198
	HBsAg	PERI	MOX, FMD	340, 197
	PreS1-S2-HBsAg	PERI	MOX	197
	α-galactosidase	S	MOX	115
	Glucoamylase	S	FMD	137
	HSA	S	MOX	174
	S.c. invertase	S	MOX	198
Kluyveromyces lactis	Prochymosin	S	LAC4	391
	IL-1β	S	*S.c.*PHO5,*S.c.*PGK	119
	HSA	S	LAC4,*S.c.*PHO5,*S.c.*PGK	120
	HSA-CD4	S	*S.c.*PGK	R. Fleer, personal communication

	Schw.o. α-amylase	S	Homologous	368
	tPA	S	?	411
	TIMP	S	?	411
Yarrowia lipolytica	β-galactosidase	I	LEU2	134
	S.c. invertase	S	XPR2	270
	Bovine prochymosin	S	XPR2, LEU2	124
	Porcine IFN	S	XPR2	164, 271
Schizosaccharomyces pombe	Polyoma middle-T Ag	I	*S.c.*PGK	27
	β-galactosidase	I	54/1, fbp, adh, GRE, CaMV35S*	224, 176, 287
	CAT	I	nmt1, HCGα, CMV*, SV40*,GRE	249, 379, 287
	Human epoxide hydrolase	I	adh	192
	Factor XIIIa	I	adh	45
	IBD virus VP3	I	adh,*S.c.*ADH1	194
	E.coli β-glucuronidase	I	CaMV35S*	289
	Single-chain Ab	I	adh	88
	Bacterio-opsin	PLM	adh	166
	STP1 glucose transporter	PLM	adh	329
	S.c. invertase	PERI	Homologous	263
	S.dia. glucoamylase	PERI?	Homologous	112
	S.c. α-mannosidase	CWALL?	Homologous	226
	S.c. exoglucanase	CWALL?	Homologous	226
	S.c. endochitinase	CWALL?	Homologous	226
	Antithrombin III	S	*S.c.*ADH1,*S.c.*CYC1	46
	Schw.o. α-amylase	S	Homologous	368[a]

[a]*S.c.*, *Saccharomyces cerevisiae*; *Schw.o.*, *Schwanniomyces occidentalis*; *S.dia.*, *Saccharomyces diastaticus*.
[b]Location of expressed protein: I, intracellular; PERI, periplasmic; PLM, plasmamembrane; CWALL, cell wall; S, secreted.
[c]Promoters given are native to the organism except: *S.c.*, *Saccharomyces cerevisiae*; *, viral; homologous, homologous to the gene expressed; GRE, glucocorticoid response elements; HCGα, human chorionic gonadotrophin α.

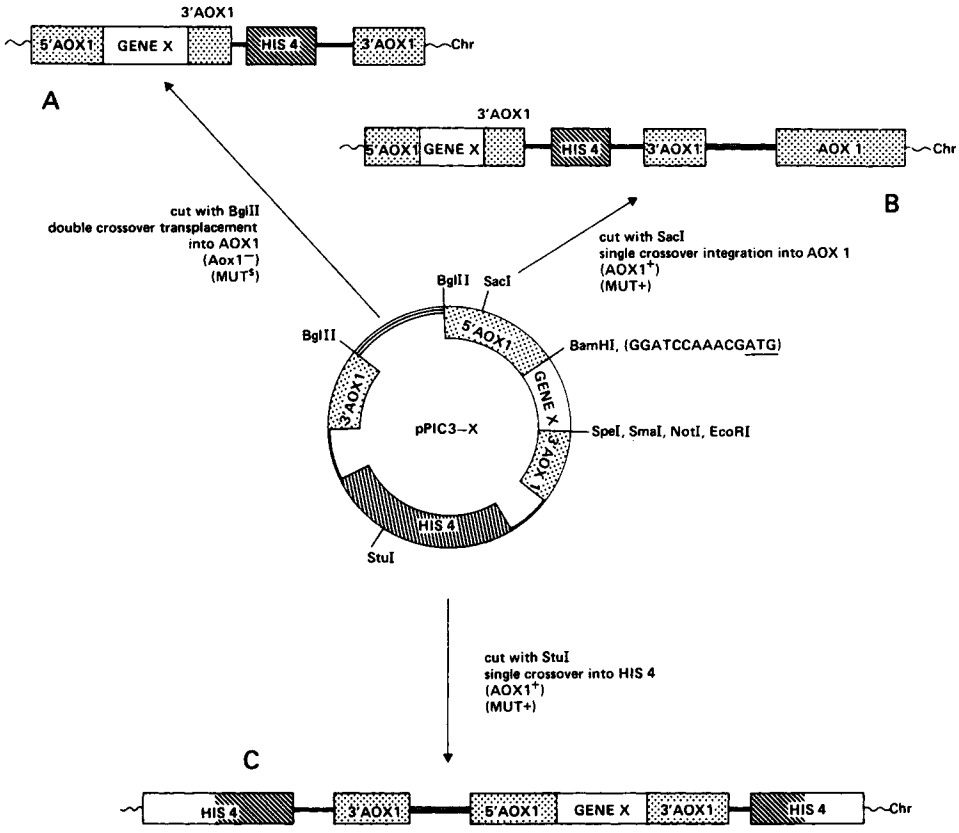

Figure 9. Plasmid map of the *P.pastoris* expression vector pPIC3 showing the three different modes of chromosomal integration. (A) Digestion with *Bgl*II yields a fragment with ends that are homologous to the 5' and 3' regions of *AOX1* that is targetted to transplace into *AOX1*. (B) Linearizing with *Sac*I targets integration by single cross-over into *AOX1*. (C) Linearizing with *Stu*I targets single cross-over integration into *HIS4*.

HIS4, or lacked vector sequences and were presumably *HIS4* gene convertants. The multi-copy transformants predominantly contained head-to-tail tandem arrays of the transplacing fragment located at *AOX1*. Both these and the other structures observed could be explained by intramolecular ligation of transplacement cassettes *in vivo*, prior to repeated single cross-over integration into *AOX1*, *HIS4* or into previously transplaced vector. Intermolecular ligation also occurred at low frequency since integrants containing the entire vector or head-to-head repeats were also found.

This mechanism of multi-copy formation (i.e. repeated single cross-overs of circularized transplacement cassettes) suggests that DNA fragments designed for targetted single cross-over integration rather than transplacement could be used to generate multi-copy transformants more readily, since higher transformation frequencies can be achieved.

For the routine isolation of high-copy integrants, mass screening methods based on colony hybridization have been used[74,306] (Figure 10). More recently, vectors containing the G418-resistance marker have been used to identify high-copy integrants by resistance to increasing concentrations of the drug (K. Sreekrishna, C.A.S., unpublished results). Thus, the most efficient procedure for isolating high-copy integrants is probably to use these vectors in single-cross-over integrations and to select clones, from amongst primary His$^+$ transformants, that are resistant to high concentrations of G418 (e.g. 2 mg/ml). Multi-copy Mut^S clones can be obtained by using a strain carrying a disrupted copy of *AOX1* (e.g. KM71[80]).

With some foreign proteins there is a direct correlation between gene dosage and expression level, although the yield per expression unit is usually reduced at very high copy number. This can be clearly

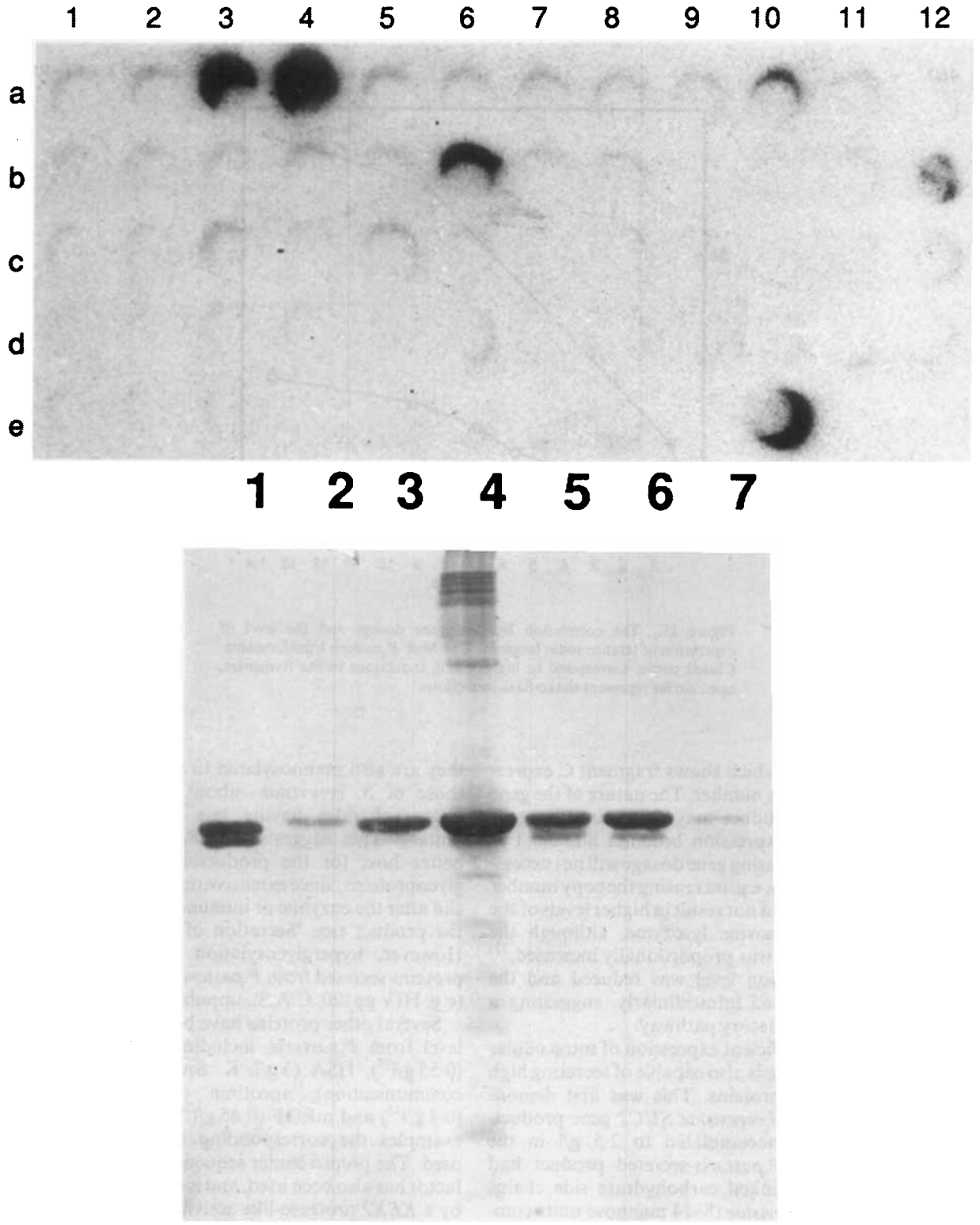

Figure 10. Screening for high copy number *Pichia* integrants. Mut^s transformants of a pPIC3 vector expressing pertactin were isolated prior to screening for high copy number.[306] (A) DNA dot blot of intact whole cells which were grown in microtitre wells then lysed on nitrocellulose. Most dots correspond to single-copy transformants (e.g. a1) while some are multi-copy (e.g. a4). (B) Western blot showing variation of pertactin expression levels among transformants showing a correlation with vector copy number. Track (1) native pertactin, (2) single-copy *Pichia* transformant, (3) 13-copy, (4) 30-copy, (5) 12-copy, (6) 21-copy, (7) *S.cerevisiae* pWYG7-based vector.

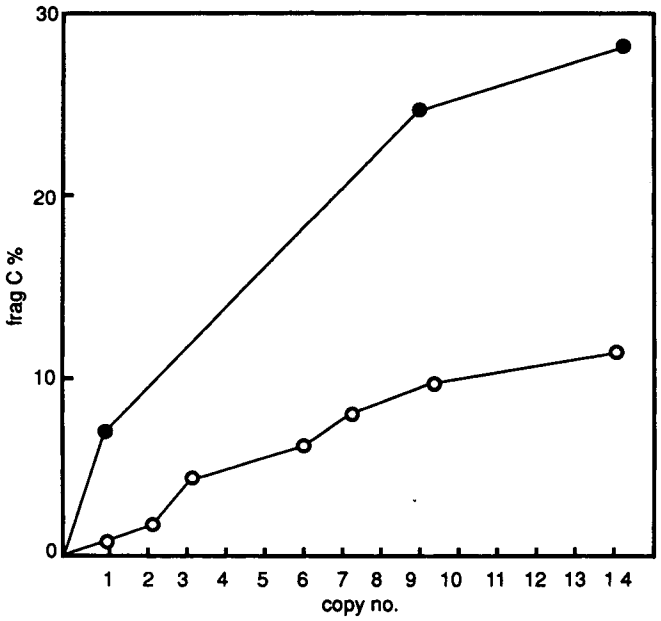

Figure 11. The correlation between gene dosage and the level of expression of tetanus toxin fragment C in Mut^s *P.pastoris* transformants. Closed circles correspond to high density inductions in the fermenter, open circles represent shake-flask inductions.

seen in Figure 11, which shows fragment C expression at varying copy number. The nature of the gene and its protein product may determine the gene dosage at which expression becomes limited. For toxic proteins increasing gene dosage will not necessarily improve yields, e.g. increasing the copy number from one to three did not result in higher levels of the secreted enzyme bovine lysozyme, although the amount of mRNA was proportionally increased.[375] Indeed, the secretion level was reduced and the enzyme accumulated intracellularly, suggesting a blockage of the secretory pathway.

In addition to efficient expression of intracellular products, *P.pastoris* is also capable of secreting high levels of foreign proteins. This was first demonstrated using the *S.cerevisiae SUC2* gene product, invertase, which accumulated to 2·5 g/l in the medium.[381] The *P.pastoris*-secreted product had much shorter N-linked carbohydrate side chains than that of *S.cerevisiae* (8–14 mannose units compared to >50). In addition, these did not contain the terminal α1,3-mannose linkages[379a] which are present in *S.cerevisiae* glycoproteins and have been shown to be immunogenic.[16] Studies of bulk endogenous glycoproteins from *P.pastoris* revealed that they are also mannosylated to a lesser extent than those of *S. cerevisiae*—about 35% of N-linked oligosaccharides having less than 14 mannose units.[149] This suggests that *P.pastoris* may be a better host for the production of heterologous glycoproteins, since extensive outer chain structures can alter the enzymic or immunogenic properties of the product (see 'Secretion of foreign proteins'). However, hyperglycosylation of certain foreign proteins secreted from *P.pastoris* has been observed (e.g. HIV gp120, C.A.S., unpublished results).

Several other proteins have been secreted at high level from *P.pastoris*, including bovine lysozyme (0·55 g/l[97]), HSA (3 g/l, K. Sreekrishna, personal communication), aprotinin (0·93 g/l[375]), hEGF (0·4 g/l[43]) and mEGF (0·45 g/l[74]). In some of these examples the corresponding signal peptide was used. The prepro leader sequence of *S.cerevisiae* α-factor has also been used, and is efficiently processed by a *KEX2* protease-like activity in *P.pastoris*.[74,375] Interestingly, Thill *et al.*[375] have reported that *S. cerevisiae* invertase is secreted by *Pichia* with much faster kinetics using the α-factor prepro sequence than with its own secretion signal. This may be an important factor in determining the gene dosage at

which secretion of a particular protein becomes limited and may also influence the glycosylation pattern of the final product (J. Tschopp, personal communication).

A problem that has occurred with several proteins secreted by *P.pastoris* is proteolytic instability in the culture medium, e.g. mEGF,[74] hEGF,[43] HSA (K. Sreekrishna, personal communication), HIV gp120 (C.A.S., unpublished results). This can be minimized by altering the pH of the culture medium upon induction (e.g. raising to pH 6·0,[74] or lowering to pH 3·0, G. Thill, personal communication), or by using a strain deleted in the *PEP4* homologue (M. Gleeson, personal communication). In the long term it would be desirable to identify the specific extracellular proteases responsible in order to construct strains lacking these activities.

An advantage of the *Pichia* system is the ease of high-density growth and scale-up without any reduction of specific productivity.[81] This feature is especially advantageous for secreted products since the concentration in the medium increases with cell density. In fact shake-flask inductions are normally sub-optimal, and there is often a large improvement in productivity using controlled fermenters[73,74,306] probably due to the high O_2 demand of the organism.

Hansenula polymorpha

The expression system developed in the methylotroph, *H.polymorpha*, is similar to that of *P.pastoris*. The gene encoding the peroxisomally-located enzyme, methanol oxidase (*MOX*), has been isolated[229] and the promoter used to express foreign genes. As with *P.pastoris AOX1*, the *MOX* gene is highly-expressed, giving methanol oxidase levels up to 37% t.c.p.,[142] and its transcription is tightly regulated. One important difference, however, is that expression of the *H.polymorpha* gene is significantly derepressed during glucose limitation or in the absence glucose, e.g. using substrates such as glycerol, sorbitol or ribose.[103,104] Thus, tight regulation of the promoter is lost in the conditions normally used for high-biomass fermentations.[137]

Transformation systems were developed using the *LEU2* and *URA3* genes from *S.cerevisiae* as selectable markers. The *LEU2* vector, YEp13, gave a low frequency of transformants which maintained the plasmid autonomously, but with low mitotic stability.[143] No integration was observed, even with linearized vector DNA, suggesting a limited homology between *H.polymorpha* and *S.cerevisiae* *LEU2* genes, although integration has subsequently been shown to occur.[348] The bacterial plasmid sequences may promote autonomous replication since the 2μ replication origin probably does not function in *H.polymorpha*.[302] The *URA3* vector, YIp5, gave a low frequency of very unstable transformants that occasionally gave rise to stable derivatives in which the plasmid had randomly integrated.[302] The transformation frequency was significantly increased by the presence of two independently isolated chromosomal DNA sequences, *HARS1* and *HARS2*, which also conferred unstable autonomous replication. Following prolonged propagation of such transformants in non-selective medium, stable *Ura+* segregants could be isolated which contained multiple (up to 75), tandemly integrated copies of the vector. Although the mechanism of multi-copy integration is unclear, this procedure can be used to generate stable, high-copy strains for the expression of foreign proteins.[197] The use of the dominant G418-resistance marker in *H.polymorpha* has also been described.[144,197]

The *H.polymorpha* system has been used for the efficient expression and assembly of HBsAg particles containing preS2 sequences.[340] More than 95% of the protein produced could be recovered as particles, over half of which were secreted to the periplasm and could be released into the medium by inducing cells in the presence of β-glucanase. The overall yield in these permeabilized cells was several fold higher than in untreated cells, suggesting that the intracellular accumulation of particles inhibited further synthesis. Janowicz *et al.*[197] produced mixed particles containing preS1-S2-HBsAg and HBsAg by co-expression with two promoters (from the methanol-regulated formate dehydrogenase gene, *FMD*, and from *MOX*) and by selection with *URA3* and G418. Multiple integration of both genes was achieved using HARS vectors by the method described above. By obtaining strains with different copy numbers of each gene, composite particles containing various ratios of preS1-S2 and S antigen could be produced, with a total yield of 2–8% t.c.p.

The secreted plant enzyme, α-galactosidase has been produced in *H.polymorpha*.[115] The *S.cerevisiae* invertase signal sequence was used, which gave efficient secretion and was correctly processed. Attempts to isolate high-copy integrants with the YEp13-derived expression vector, by passage of transformants in non-selective medium, resulted in a strain containing two copies integrated at *MOX* and one at *LEU2*. This low integration frequency

and copy number may be because 2μ sequences prevent integration or are unstable when integrated,[348] or because the plasmid is less stable than HARS vectors and is lost before multiple integration can occur. The three-copy strain secreted 42 mg/l, which was equivalent to about 5% t.c.p. The *H. polymorpha*-derived material was over-glycosylated compared to the native enzyme and had a lower specific activity. Full activity could be restored on treatment with endoglycosidase H.

As with *P.pastoris*, *H.polymorpha* can be grown to high density (100–130 g/l), resulting in very high volumetric yields of secreted proteins. Using an integrant containing four copies of a *Schwanniomyces occidentalis* glucoamylase gene, up to 1·4 g/l of secreted enzyme was obtained.[137] The yield from an eight-copy integrant was much lower suggesting a blockage of the secretory pathway at the higher gene dosage. The secretion of several other foreign proteins in *H.polymorpha* has been reported including HSA[174], invertase and β-lactamase.[198]

Kluyveromyces lactis

K.lactis has been used in the food industry for many years in the production of β-galactosidase (lactase). Thus, its large scale cultivation has been extensively studied, and it is well accepted for the production of proteins for human use. The ability to grow on cheap substrates, such as lactose and whey, further increases its potential as a host for the production of heterologous proteins, especially for low-value products.

Transformation systems were initially developed by isolating *K.lactis* ARS sequences, since neither the *S.cerevisiae ARS1* nor 2μ replicates in *K.lactis*.[84,362] However, as with *S.cerevisiae*, *K.lactis* ARS vectors are highly unstable and are of limited use in expression systems. A number of selection markers are available for *K.lactis*, e.g. *S.cerevisiae TRP1*[84] and *URA3*,[90] *K.lactis TRP1*,[365] *URA3*[346] and *LAC4*,[84] and the G418-resistance gene,[84,362]

The two cytoplasmic linear plasmids, k1 (8·9 kb) and k2 (13·4 kb), present in killer strains of *K.lactis* have been considered as a potential vector system. They are stably maintained at 100–200 copies per cell and the regions of k1 that encode killer toxin can be deleted without affecting maintenance (reviewed in reference 364). However, their 5'-termini are covalently linked to protein, hindering manipulation *in vitro* and amplification in *E.coli*. Additionally, these plasmids encode their own cytoplasmic transcription system which does not recognize nuclear promoters. The difficulties in manipulation can be overcome by targetted integration of foreign DNA into native k1. If conventional nuclear selection markers are used, e.g. *LEU2*, this results in linear nuclear plasmids containing telomeres.[206] However, by fusing such markers to k1 promoters, recombinant linear plasmids which are cytoplasmic and stable can be generated.[207,374] It should be possible to use this system for foreign gene expression, although the k1/k2 promoters appear to be rather weak and further development, e.g. the use of the bacteriophage T7 transcription system, may be necessary.

Stable high-copy *K.lactis* expression vectors have been constructed based on the *Kluyveromyces drosophilarum* plasmid, pKD1. Although there is little sequence similarity, pKD1 is organizationally very similar to the 2μ plasmid of *S.cerevisiae*. It encodes analogous replication, amplification and segregation functions,[65] which are also active in *K.lactis*.[30] Several different types of vector based on pKD1 have been constructed which behave similarly to the analogous 2μ vectors.[30] Vectors carrying just the *cis*-acting replication element, located near one of the inverted repeats, can be maintained in host strains which have resident pKD1. However, vectors containing the entire pKD1 plasmid are significantly more stable and can be maintained in any *K.lactis* host strain.[64] The unique *Eco*R1 site adjacent to one of the inverted repeats can be used to insert foreign DNA without interruption of plasmid functions.[64] Such vectors are highly stable in pKD1° strains, even in the absence of selection, although stability is somewhat reduced in pKD1$^+$ hosts, perhaps due to incompatibility or competition. These vectors are currently used for optimal foreign gene expression in *K.lactis*.[119,120] Another reported method of producing highly stable pKD1-based vectors is to transform pKD1$^+$ hosts with plasmids containing one of the inverted repeats, in addition to the desired foreign DNA. Once introduced, these can recombine with resident pKD1 at relatively high frequency, due to the pKD1-encoded FLP recombinase, giving stable recombinant vectors.[402]

A small number of promoters have been used in *K.lactis* expression vectors. The best-characterized *K.lactis* promoter is that of the *LAC4* gene, encoding β-galactosidase, which is induced up to 100-fold by lactose or galactose. Its regulation parallels the *S.cerevisiae GAL* system, though there are significant differences: the *K.lactis GAL* genes, including *LAC4*, show no glucose repression, and the *GAL1,7* and *10* genes are only induced five- to ten-fold.[96a] Some of these differences can be ascribed

to the fact that *LAC9*, the *GAL4* homologue of *K.lactis*, is not involved in glucose repression in many strains. Several *S.cerevisiae* promoters are active in *K.lactis* and have been used for foreign gene expression, e.g. *PGK*[120] and *PHO5*.[119] In future, promoters from the recently isolated *K.lactis* genes encoding GAP[344] and alcohol dehydrogenase[324,344] may be used.

A number of studies show that *K.lactis* can efficiently secrete foreign proteins, including prochymosin which is only poorly secreted by *S.cerevisiae*. Signal peptides derived from HSA, the *K.lactis* killer toxin α-subunit, and the prepro peptides of α-factor from both *K.lactis* and *S.cerevisiae* have been used. In marked contrast to *S.cerevisiae*, prochymosin was efficiently secreted by *K.lactis* in a fully soluble, acid-activatable form, using a single-copy integration vector carrying the *LAC4* promoter.[391] Remarkably, about 80% of the prochymosin produced was secreted even when expressed without a signal peptide, though the overall level was reduced. The highest levels were obtained using the native leader peptide or the α-factor prepro sequences from either *K.lactis* or *S.cerevisiae*. The stability of the integrated expression cassette was sufficient to allow scale-up to 41,000 litres, and the product is used commercially in the manufacture of milk products.

Other heterologous proteins have been secreted using pKD1-derived vectors. Fleer *et al.*[120] described the secretion of HSA using its own secretion signal, or that of the *K.lactis* killer toxin α-subunit. The level of HSA produced was highly strain-dependent and this was largely due to differences in stability of the vector. Using the *S.cerevisiae PGK* promoter, the highest-expressing strain produced about 300 mg/l HSA in shake flasks. In high density (80–90 g/l dry weight of cells), fed-batch fermentations several g/l HSA were produced from cultures of up to 1000 litres. This system has also been used for the production of a HSA-CD4 fusion protein designed as a potential therapeutic agent for use in HIV infection (R. Fleer, personal communication).

The secretion of interleukin 1β (IL-1β) by *K.lactis* has also been reported, using the toxin α-subunit signal, either with or without the pro region derived from HSA.[119] These signal peptides were accurately and efficiently processed, but only about 20% of the product was secreted (80 mg/l in shake flasks). As in *S.cerevisiae*, the *K.lactis*-derived IL-1β was fortuitously glycosylated and largely inactive, though full activity was restored by digestion with endoglycosidase H. A mutant form of IL-1β which lacked the unique N-linked glycosylation site was also fully active. Plasmid stability was significantly reduced in IL-1β expressing cells, but this could be improved by replacing the constitutive promoter (*S.cerevisiae PGK*) with a regulated one (*S.cerevisiae PHO5*).

The glycosylation pattern of two other heterologous proteins secreted in *K.lactis* was examined by Yeh *et al.*[411] Variants of tPA and the tissue specific inhibitor of metalloproteinases (TIMP) were secreted using their own secretion signal or that of the killer toxin. In each case the secreted material was reported to be over-glycosylated and was not immunoreactive unless treated with endo H.

Yarrowia lipolytica

Y.lipolytica has been investigated for use in a number of industrial processes, including the production of a various metabolites (e.g. citric acid,[337] 2-keto glutarate,[243] erythritol,[93] mannitol,[94] isopropyl malate[92]), the bioconversion of alkanes and fatty acids into alcohol, and the production of single cell protein from *n*-paraffins.[133,163] In addition, this yeast secretes a variety of high molecular weight proteins including acid proteases,[408] lipases,[2] a ribonuclease,[66] and an alkaline extracellular protease (AEP).[377] Under optimal conditions AEP can be induced to levels of 1–2 g/l and is the major component of the culture supernatant.[377] This inherent capacity for high-level secretion, plus the ability to grow to high cell density at industrial scale, have prompted the investigation of *Y.lipolytica* as a host for heterologous gene expression.

Early studies of *Y.lipolytica* genetics were hampered by its low sporulation frequency and low spore viability. Indeed, these characteristics led to its original classification as *Candida lipolytica*, since no sexual cycle was demonstrated until 1970.[403] However, improved techniques for mating, sporulation, and ascospore recovery, combined with improved strains derived from inbreeding programmes have led to the development of a genetic map, comprising at least five linkage groups.[273] *Y.lipolytica* is a dimorphic yeast, being unicellular in minimal medium containing glucose or *n*-hexadecane, forming mycelia in minimal medium containing olive oil or casein, and giving a mixture of both forms in complex medium. Mutants which form smooth colonies containing only yeast-phase cells have been isolated[123,274] but the molecular events involved in the regulation of growth morphology are uncharacterized.

Transformation of *Y.lipolytica* was first achieved using two different selectable markers. Davidow

et al.[85] used the homologous *LEU2* gene to transform cells permeabilized by lithium acetate. Transformation occurred by integration into the chromosomal *LEU2* locus via homologous recombination. Linearization of the vector within the *LEU2* gene resulted in a 1000-fold increase in transformation frequency. Gaillardin *et al.*[135] used vectors containing random *Y.lipolytica* genomic fragments inserted into the upstream region of the *S.cerevisiae LYS2* gene for the selection of transformed sphaeroplasts. Transformants commonly contained several tandemly integrated copies of the vector suggesting that the *LYS2* gene was poorly expressed. Unexpectedly, no sequences capable of supporting autonomous replication were isolated. Wing and Ogrydziak[405] were also unable to isolate ARS sequences using a similar strategy. This was subsequently found to be because ARSs occur relatively infrequently in *Y.lipolytica*, and because plasmids containing these sequences are unusually stable.[123] Thus, such transformants were probably erroneously scored as integrants. Using mutant host strains in which replicating vectors were fortuitously less stable, Fournier *et al.*[123] isolated the *Y.lipolytica* ARS elements, *ARS18* and *ARS68*. Vectors containing these sequences were present in only 1–3 copies per cell and were very stable, being lost at a rate of 0·5–5% per generation. Since only two different ARSs were isolated, a maximum frequency of only one element per 1000 kb of genomic DNA can be calculated. These characteristics suggested that the *ARS18* and *ARS68* elements could also contain centromere function, and this has been demonstrated genetically.[133] Expression vectors based on the *ARS18* element have now been developed.[271]

In addition to *LEU2*, several other *Y.lipolytica* genes have now been cloned and used as selection markers for transformation, e.g. *LYS1*, *LYS5* and *ADE1*,[163,407] *HIS1* and *URA3*.[86] *Y.lipolytica* is resistant to many of the antibiotics commonly used for *S.cerevisiae*, including G418 and chloramphenicol, but alternative dominant selection markers have been developed. The *LEU2* promoter was fused to the phleomycin-resistance gene from Tn5 and used for the direct selection of transformants.[134] A high proportion of resistant colonies were found to be untransformed mutants, but this could be reduced by introducing an expression phase prior to plating. An alternative dominant transformation marker is the *SUC2* gene of *S.cerevisiae*, which was fused to the promoter and secretion signal sequence from the AEP gene, *XPR2*.[270] Direct selection on sucrose medium was possible, probably since most of the secreted invertase remains in the periplasm, preventing crossfeeding of untransformed cells.

The *XPR2* promoter, which is tightly regulated by pH and by carbon and nitrogen sources,[275,276] and the *XPR2* secretion signal sequence have been used to produce other foreign proteins in addition to invertase. The *XPR2* gene was independently cloned by three groups and found to encode a prepro-enzyme.[87,247,270] The mature protease is produced by a series of processing events which sequentially remove the presequence and two or three pro-regions.[247] The presequence is cleaved on translocation into the endoplasmic reticulum and, after trimming of N-terminal X-Pro and X-Ala dipeptides, the pro sequences are then cleaved by a KEX2-like activity, most likely encoded by the *XPR6* gene. Deletion analysis suggests that the proregion is required for efficient secretion of AEP.[133]

The AEP prepro sequences have been used to direct the secretion of two commercially-important proteins, bovine prochymosin[124] and porcine α-interferon.[164,271] Franke *et al.*[124] fused prochymosin cDNA to five positions within the AEP coding sequence, resulting in the expression of prochymosin fusion proteins containing the presequence alone, the preproI region, the preproIproII region, and the preproIproII region plus either 14 or 90 amino acid residues of the mature AEP. Each of these fusion proteins was efficiently secreted and released active chymosin on treatment with acid. Thus, the AEP proregion was not necessary for the secretion of prochymosin. In the case of the preproIproII fusion, the AEP secretion and processing signal sequences were shown to be accurately recognized and cleaved. Porcine α-interferon was efficiently secreted when fused to either the preproI or the preproIproII regions of AEP[164,271] although some incorrect processing was reported[133]. The final level of α-interferon secreted was increased about two- to three-fold using an *ARS18* rather than an integrating vector.[271] However, this improvement was less than the six-fold increase found with AEP since α-interferon expression resulted in reduced vector stability. Similar results were obtained for prochymosin which gave only 1·3-fold higher levels using the ARS vector. Regulation of the *XPR2* promoter was also impaired using this vector, since basal AEP levels were found to be 50-fold higher than normal under repressing conditions.

Schizosaccharomyces pombe

Aside from *S.cerevisiae*, the fission yeast, *S.pombe*, is the most intensely studied and well

characterized of the yeast species. This is largely because, like budding yeast, its life cycle and growth characteristics are particularly suited to genetic and biochemical analysis. The early development of a transformation system[22] has led to the cloning and characterization of a large number of genes, and to the development of an array of genetic manipulation techniques comparable to those used in *S. cerevisiae* (for review, see reference 319). Such studies have highlighted how distantly related these two yeasts are. In fact, sequence comparisons show that for many *S.pombe* genes, the mammalian homologues are only marginally more divergent than corresponding *S.cerevisiae* genes,[319] and some are actually less divergent (e.g. *ras*[127]). The utility of *S.pombe* in isolating mammalian genes by complementation of corresponding mutant homologues has been demonstrated,[230] and this remains the most important use of this expression system. In contrast, the use of *S.pombe* for protein production has been limited since little fermentation technology has been developed, and because relatively few inducible promoters were available.

Transformation of *S.pombe* has been described using sphaeroplasts,[22] lithium salts,[165] and electroporation.[179] A highly efficient method uses lipofectin to enhance uptake of DNA by sphaeroplasts.[5] A number of selectable markers have been described (see reference 409) but the *LEU2* and *URA3* genes from *S.cerevisiae*, which complement the *S.pombe leu1* and *ura4* mutations, are most commonly used. The corresponding *S.pombe* genes have also been used,[9,212] and dominant selection using the G418 and bleomycin resistance genes has been demonstrated.[224,323]

S.pombe expression vectors normally contain sequences derived either from the 2μ plasmid of *S. cerevisiae* or from the *S.pombe ars1*. The 2μ sequences that have ARS activity in *S.pombe* do not include the complete 2μ *ORI* and do not depend on any 2μ-encoded function.[132] These vectors have relatively low copy number (5–10 per cell) and mitotic stability (30–45% loss per generation without selection[165]). The *ars1* vectors behave similarly but have higher copy number (about 30/cell[165]). Stability and copy number of *ars1* vectors is greatly enhanced by the presence of an *S.pombe*-derived sequence called *stb*, which appears to provide a partitioning function.[165] These *ars1/stb* vectors, e.g. pFL20,[240] have a copy number of about 80 and are lost at a rate of 13% per generation.[165]

A number of promoters have been used for the expression of foreign genes in *S.pombe*. Promoters from *S.cerevisiae* genes generally function poorly, giving inefficient and aberrant initiation of transcription.[317,318] Nevertheless, the *S.cerevisiae PGK* promoter has been used to express polyoma middle T antigen,[27] and the *ADH1* and *CYC1* promoters used to produce biologically active, secreted antithrombin III.[46] In addition, the *S.cerevisiae* genes for three glycosidases (α-mannosidase, exoglucanase and endochitinase[226]) and the *Saccharomyces diastaticus* glucoamylase gene[112] have all been expressed in *S.pombe* using their respective promoters. However, the SV40 early promoter and the promoter from the *S.pombe* alcohol dehydrogenase (*adh*) gene have been most commonly used. The *adh* gene is constitutive and gives alcohol dehydrogenase levels of about 0·5–2% t.c.p.[320] With the SV40 early promoter transcripts initiate at the same position in *S.pombe* as in mammalian cells[203] although it is weaker than the *adh* promoter. Toyama and Okayama[379] used the chloramphenicol acetyltransferase gene to test a number of other mammalian promoters for activity in *S.pombe*. The human chorionic gonadotrophin α and human cytomegalovirus promoters were about ten-fold stronger than the SV40 promoter and several others, although weaker, were also functional. Other constitutive promoters that have been used are the cauliflower mosaic virus (CaMV) 35S promoter,[145,289] which gave expression levels similar to the *adh* promoter, and a promoter isolated from random *S.pombe* genomic fragments, called 54/1,[224] which gave β-galactosidase levels of 5% t.c.p.

Regulatable expression systems for *S.pombe* have also been described. One uses the promoter from the *S.pombe* fructose bisphosphatase (*fbp*) gene which is expressed at very low levels in cells grown on 8% glucose and is derepressed 100-fold in media containing non-repressing carbon sources.[176] However it should be noted that, even in 8% glucose, the *fbp* promoter is partially induced on entry into stationary phase. A second regulated system uses mammalian glucocorticoid response elements to drive expression. These are induced up to 70-fold by glucocorticoid hormones when the hormone receptor is co-expressed.[287] The fully-induced expression level was about 20% of that using the *adh* promoter. A promising regulated system uses the promoter from a highly-transcribed *nmt1* gene which is strongly repressed by thiamine.[249] This was used to regulate a multi-copy chloramphenicol acetyltransferase (CAT) reporter gene, and gave a 200-fold thiamine-dependent repression of CAT expression.

In addition to the examples already described above, a diverse selection of other heterologous

proteins have been expressed in *S.pombe*. The human liver microsomal enzyme, epoxide hydrolase, was expressed in an active form that could be isolated from *S.pombe* microsomal fractions.[192] Active factor XIIIa was produced at 2 mg/l using a high copy number, *adh* expression vector derived from pFL20.[45] The expression of functional single-chain antibody molecules capable of binding their cognate hapten, the aromatic dye fluorescein, has been described.[88] Jagadish *et al.*[194] expressed the large polyprotein of infectious bursal disease virus, which was processed to give stable VP3 protein. Strasser *et al.*[368] expressed the α-amylase gene from *Schwanniomyces occidentalis*. The bacterial proteins, β-glucuronidase,[289] xylose isomerase[58] and bacterio-opsin[166] have also been produced in *S.pombe*. The latter is correctly processed by cleavage at the N-terminus, is inserted into membranes, and forms photoactive bacteriorhodopsin pigment when cells are grown in the presence of its chromophore, retinal.[166] Another integral membrane protein expressed in active form by *S.pombe* is the glucose transporter from *Arabidopsis thaliana* encoded by the *STP1* gene.[329]

The secretion of foreign proteins is a relatively unexplored area of potential interest since, like higher eukaryotes, *S.pombe* is known to galactosylate glycoproteins (e.g. invertase,[263] acid phosphatase[95]). However, *S.cerevisiae* invertase was not galactosylated when expressed in *S.pombe*,[263] and both this protein and homologous ones are highly glycosylated. In addition, antithrombin III was hyperglycosylated when secreted by *S.pombe*.[46]

PHYSIOLOGY OF FOREIGN GENE EXPRESSION

High-level expression of a foreign gene can place a significant metabolic burden on the host cell, reducing its growth rate and affecting the efficiency of gene expression. Expression of some genes causes a more acute effect, either through a severe effect on metabolism or by direct toxicity. In constitutive systems where growth and expression are linked there is then strong selection for cells with reduced foreign gene expression, so that low-expressing variants arise. Variants may also be selected without growth, for example through effects of the product on cell viability during freezing or long-term storage on agar. Therefore it is highly desirable to use tightly-regulated promoters where the growth and induction phases can be largely separated. This is particularly important with large-scale fermentations where growth is over many generations. With regulated systems only highly toxic products should be problematic, because of their ability to rapidly affect host cell metabolism.

Mechanisms of toxicity

Toxicity is diagnosed by a difficulty in obtaining transformants with constitutive expression vectors or a reduction in growth rate when regulated vectors are induced. Which metabolic process is predominantly affected depends on the foreign gene and vector system. The maintenance of ultra-high-copy 2μ/*LEU2*-d plasmids alone is sufficient to reduce the host cell growth rate,[397,169] whereas no effect would be expected with integrated vectors. Multi-copy promoters may compete for transcription factors and inhibit expression of co-regulated genes: thus multi-copy *GAL7* vectors inhibit *GAL10* gene transcription, and could affect galactose metabolism.[14] High levels of mRNAs containing rare codons could deplete cognate tRNAs and inhibit the translation of host mRNAs containing rare codons. This appears to be the effect of *lacZ* mRNA in yeast, which at high levels is toxic, especially during growth on minimal medium.[292]

Toxicity is a relatively common problem with secreted proteins, especially complex ones. This may be due to the complexity of the secretory pathway, offering a number of possible bottlenecks, or else to the possibility that foreign secreted proteins may be frequently malfolded and cause a blockage in the pathway. Examples of highly toxic secreted proteins are tPA,[169] IGF-1,[343] EBV gp350.[335] In the case of tPA, acid phosphatase secretion is reduced, suggesting a general block in secretion. Membrane proteins, e.g. *E.coli ompA*,[196] influenza virus haemagglutinin,[191] polyoma virus middle T antigen,[27] are particularly likely to be toxic, possibly due to non-specific insertion and disruption of yeast intracellular membranes. This may be the mechanism of toxicity of HBsAg, since constitutively-expressing strains have reduced viability on freezing and thawing (M.A.R., unpublished results).

Certain proteins have specific, acutely toxic effects. As an extreme example, induction of intracellularly expressed A-chain of ricin instantaneously kills yeast cells, and this has been used as a selection for non-toxic A-chain mutants.[125] Over-expression of proteases, such as HIV-1 protease, may be toxic. Mammalian transcriptional *trans*-activators are frequently toxic, presumably through their ability to sequester components of the yeast transcriptional apparatus.

A number of approaches can be used in an attempt to overcome product toxicity. Secretion may be used to segregate the product from the site of toxicity even if it is not normally secreted, e.g. HIV-1 protease.[288] Alternatively, fusion to other proteins may inhibit biological activity and toxicity. The approach of selecting for mutants resistant to toxicity has been used successfully with IGF-1.[347] Toxin-resistant mutants have been used to express diphtheria toxin fragment A, which normally kills yeast cells, and chimaeric diphtheria toxins.[285]

Generation of low-expressing variants

To a certain extent it is possible to limit the generation of variants having reduced expression levels, which can arise with constitutive systems. The most common response to toxicity with 2μ vectors is reduced copy number, which can reach a level as low as 1 per cell even with selection.[35] However, when *LEU2*-d selection is used the drop in copy number that can occur without affecting growth is limited, and other mechanisms such as structural rearrangement predominate. Where the selection marker is flanked by 2μ DNA, recombination can result in its transfer to native 2μ with loss of the expression cassette.[99] One way of preventing this is by using plasmids such as pJDB219 in 2μ-free strains.

Another mechanism for plasmid loss involves homologous recombination between the selection marker and chromosomal allele to generate a prototroph. This can be prevented by using a host strain with a deletion in homologous chromosomal sequences or by use of recombination-deficient mutants (e.g. *rad52*[244]).

An unusual plasmid mutation was observed during the constitutive expression of polyomavirus middle T antigen.[27] Transformants grew very slowly, and there was either plasmid loss or spontaneous generation of plasmids expressing a truncated antigen, which lacked the membrane-spanning domain and was non-toxic. Mutant plasmids arose by deletion of one G.C base pair in a run of nine, causing a frame-shift 5' of the DNA encoding the hydrophobic region.

We have observed an example where a mutation in a host cell gene inactivated the promoter used in the expression vector. The system was a *PHO5* vector expressing HBsAg in a constitutive *pho80* background (M.A.R., unpublished results). Mutant non-expressing cells were enriched during freezing and thawing of stock cultures, and out-grew the expressing cells in fermenters, resulting in greatly reduced product yields. Mutant cells were detected using a chromogenic plate assay for secreted acid phosphatase expressed from the chromosomal *PHO5* gene (Figure 12). The problem was eliminated by a combination of measures: (i) using a freezing protocol which gave higher viability to reduce enrichment of mutant cells and (ii) plating out stock cultures and using the plate assay to monitor for non-expressing colonies. The mutants did not arise at detectable frequencies without the enrichment that occurred during low-viability freezing. This example illustrates the type of problem that can occur on scale-up of a constitutive system expressing a mildly toxic product.

Large-scale fermentation and optimization

In the development of a large-scale industrial fermentation process, the first step is transfer to small fermenters. These offer the possibility of controlling a number of culture parameters, resulting in much greater reproducibility and the ability to reach very high cell densities. However, a significant effort is frequently required in order to maximize product yield, though in theory it should be possible to control conditions so as to give higher yields than in shake-flasks. High-density fermentation is generally desirable in order to minimize capital expenditure on fermentation equipment; it is particularly valuable with secretion, where the concentration of secreted protein in the medium can increase almost proportionately to cell density.[213]

In the case of recombinant *S.cerevisiae*, glucose is almost always used as the carbon-source. The two extremes of glucose-utilization are fermentation, which yields ethanol as a by-product, and respiration, which is much more efficient for conversion to biomass. However, growth to high density on glucose is problematic since *S.cerevisiae* has a limited capacity to metabolize glucose oxidatively, accumulating ethanol, which eventually inhibits growth, above a specific growth rate of 0·2 to 0·25 h^{-1}.[117] Secondly, glucose concentrations above approx. 0·1% repress respiration.[298] In aerobic batch cultures, such as shake-flasks, growth on glucose is diauxic: the glucose is rapidly fermented to ethanol and CO_2, and the ethanol is metabolized in a second growth phase once the glucose is exhausted.

In order to prevent the conversion of glucose to ethanol and maximize biomass, bakers' yeast is grown using a fed-batch process: molasses and nutrients are slowly fed to the fermenter, the feed rate increasing as the cell density increases, so as to obtain

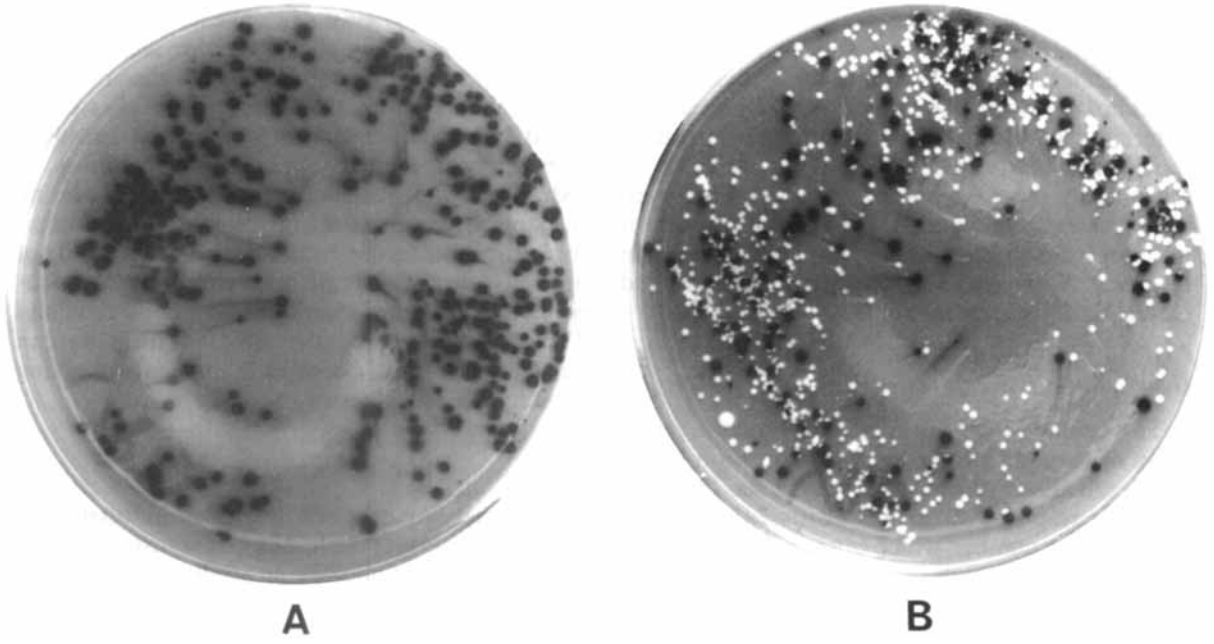

Figure 12. Accumulation of non-expressing cells during fermentation of an *S.cerevisiae pho80* strain containing a *PHO5* vector for HBsAg expression. Samples were taken at the end of 10 litre fermenter runs of the constitutively-expressing strain, plated on non-selective agar and assayed for secreted acid phosphatase (Pho phenotype) by overlaying with agar containing a chromogenic substrate. (A) High-yield fermenter run contained Pho^+ cells only. (B) Low-yield run contained a high proportion of Pho^- cells in which the *PHO5* promoter is inactive. Low-yield runs resulted from enrichment of mutant non-expressers during low-viability freezing. Improved freezing protocols resulted in high-yield runs.

an almost constant specific growth rate. Similarly, fed-batch using calculated glucose feed rates has been used for high-density culture of recombinant *S.cerevisiae*.[151,182] More refined methods have also been applied: computer control has been used to couple glucose feed rate to respiratory quotient (RQ = CO_2 evolved/O_2 consumed; maintained at 1·0 to 1·2) using on-line gas analysis,[118,213,384] or to ethanol concentration using a biosensor,[3] resulting in cell densities of 80 to 200 g/l dry mass. These results have been achieved with semi-synthetic media containing protein hydrolysate (casamino acids) or yeast extract.

With an ideal tightly-regulated system, biomass accumulation would be separated from foreign protein induction, and the two could be optimized separately. In constitutive systems this is clearly not possible, but even with regulated systems a combined growth/induction process must usually be developed. We will illustrate some principles of process development by referring to *S.cerevisiae* systems using three types of promoters: constitutive glycolytic, glucose-repressible, and galactose-inducible.

As described above, product toxicity is a serious problem with constitutive systems: Fieschko *et al.*[118] found severe plasmid-loss using a *PGK* vector expressing γ-interferon, and were unable to achieve high cell densities. However, an optimized process has been developed for secretion of pro-urokinase from a super-secreting *pmr1* strain using the *PGK* or *TPI* promoters.[384] Accumulation of secreted product was growth-associated but was not as high in an RQ-controlled fed-batch culture (77 g/l dry mass) as in a perturbed batch fermentation (45 g/l), where glucose was pulsed into a batch culture. This difference may be due to activation of glycolytic promoters by high concentrations of glucose. In batch fermentations the secretion of human lysozyme, using the enolase promoter, was increased four-fold by increasing starting glucose concentration from 2 to 10%, even though growth was unaffected.[185] In view of these observations another approach would be to use glycolytic promoter systems in two-stage fermentations, an initial glucose-limited stage for biomass accumulation followed by an increased glucose feed for induction.

Glucose-repressible expression systems present problems because of the conflicting requirement to maintain high glucose in order to repress the promoter, and to limit glucose in order to maximize

biomass. However, a fed-batch process for the production of a SOD-proinsulin fusion protein using a *GAP/ADH2* promoter has been developed, though the cell density achieved was only 30 g/l.[378] Initial experiments indicated that a glucose excess was important to repress the promoter and maintain plasmid stability. Quite high yields of product (>0·5 g/l) were obtained in simple batch cultures with glucose concentration starting at 5%, but biomass was low. A fed-batch procedure was developed using a constant, empirically-determined glucose feed which led to glucose excess during most of the growth phase but to very low glucose at later stages as the cell density increased. Sub-optimal feed-rates would have resulted either in plasmid loss or in incomplete induction. Apparently the residual glucose (20 mg/l) was partially repressing, since changing from glucose to a final ethanol feed yielded a two-fold improvement to 1·2 g/l of product (increasing to 1·6 g/l at 26°C).

The first published study of galactose induction in a high-density fermentation involved the expression of γ-interferon using the *GAP/GAL* hybrid promoter.[118] Although biomass yields of 200 g/l were possible using RQ-controlled glucose-limited fermentations, biomass was restricted to <100 g/l because of O_2-transfer limitation. Three induction methods were compared: (i) 10 g/l pulses of galactose, (ii) replacement of the glucose feed by galactose, (iii) (i) followed by (ii). The third method gave the highest yields and this was rationalized as being due to the partial induction of galactose-metabolizing enzymes during galactose pulsing, preventing temporary carbon-source starvation on switching the feeds. However, in another study galactose was rapidly metabolized in glucose-limited cultures.[3] A further improvement (to 2 g/l of product) was made using a diploid host strain, however the yield of γ-interferon was only 3 to 5% t.c.p. compared to 10% in shake-flasks.

More recently, the production of β-galactosidase was examined using a galactose-inducible, hybrid *CYC1/GAL* promoter.[3] It was found that galactose added to glucose-limited cultures was rapidly metabolized and depleted, since the cells were derepressed, and it was preferable to feed a glucose/galactose mixture so that glucose was preferentially metabolized. In this way β-galactosidase was induced to 7% t.c.p. at a cell density of 100 g/l; addition of a final 50% galactose feed increased the yield to 8%, considerably higher than the level in shake-flasks.

Expression systems based on several yeasts other than *S.cerevisiae* have been scaled-up. A recombinant *K.lactis* strain expressing secreted HSA under the control of the *S.cerevisiae PGK* promoter has been grown to high density (80 to 90 g/l) using glucose-limited fed-batch at the 1000 l scale, yielding HSA at several g/l.[120] A commercial process for the secretion of prochymosin has been scaled-up to 41,000 litres.[391]

P.pastoris is particularly suited to high-density culture and can be grown to >100 g/l on simple defined media containing glycerol without the need for complex fed-batch procedures.[80] The tight regulation of the *AOX1* expression vectors means that the growth phase is identical for different protein products, and scale-up to large fermenters does not affect biomass or product yield.[80] Additionally, contrary to the experience with *S.cerevisiae*, an immediate improvement in percentage yield of foreign protein is generally seen on going from shake-flasks to fermenters.[73,74,306] This appears to be due, at least in part, to the high O_2-demand of the organism, which is not satisfied in shake-flasks.

Figure 13 shows the results of a high-density fermentation using a *Pichia* Mut^s recombinant containing 14 integrated copies of a tetanus toxin fragment C expression vector.[73] The culture was started with an initial batch growth in glycerol; when this was used up a slow glycerol feed was initiated, during which growth was glycerol-limited and the *AOX1* promoter derepressed, allowing rapid induction during the subsequent methanol feed. Most of the growth occurred during the glycerol feed; product accumulated for about 30 h into the methanol feed, during which time there was no cell doubling, reaching a level of 27% t.c.p. (*cf.* 10% in shake-flasks) or approx. 12 g/l. In contrast, Mut$^+$ strains continue to grow rapidly during the methanol feed and are fed methanol at a higher rate. Using similar protocols high levels of several commercially-important proteins have been achieved: e.g. SOD (1·3 g/l[375]), HBsAg (0·4 g/l[81]), tumour necrosis factor (10 g/l[360]), *Bordetella pertussis* pertactin (3 g/l[306]), secreted HSA (3 g/l; K. Sreekrishna, personal communication), secreted EGF (0·45 g/l;[74] G. Thill, personal communication). Mut$^+$ strains can also be grown in continuous culture to increase fermenter productivity.[97]

Apart from the growth and induction protocols, a number of other variables have been found to affect yields and should be looked at systematically. Lower temperatures may increase yield by reducing proteolysis.[378] Added phosphate in the medium has increased heterologous secretion in at least two

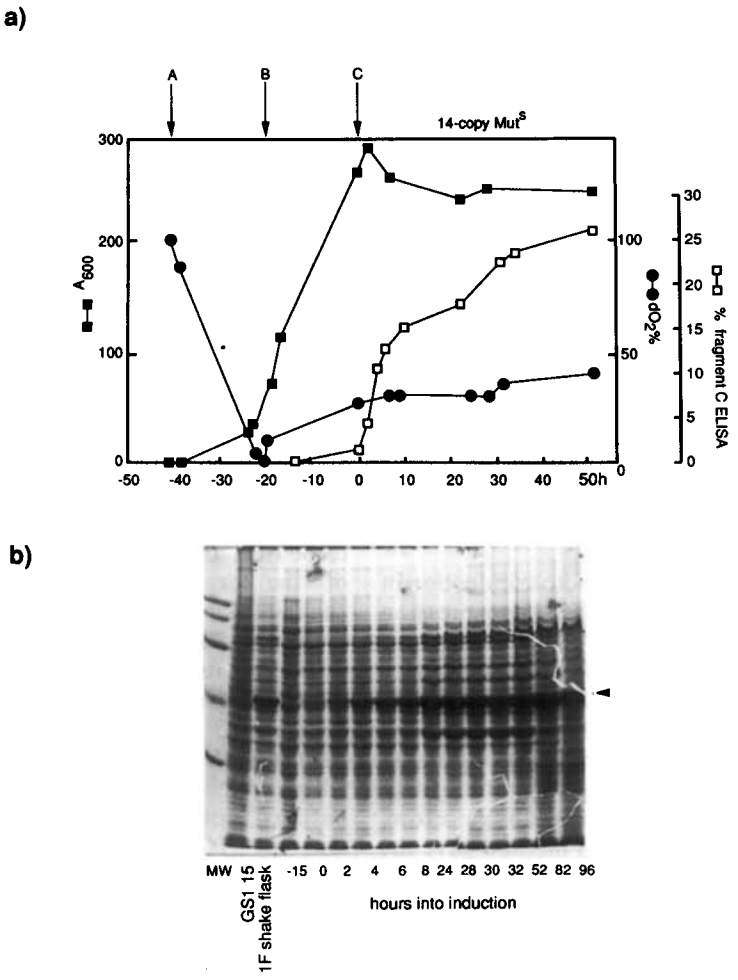

Figure 13. Fermentation of a 14-copy *Pichia* Mut^s transformant expressing tetanus toxin fragment C. (a) Cell density (A_{600}), dissolved oxygen (dO_2), and fragment C as per cent of cell protein are plotted against time (0 h = start of induction). Fermentation conditions were as described elsewhere.[73] Timepoint A, addition of shake-flask inoculum, B, glycerol-limited feed, C, methanol feed. (b) Coomassie blue-stained SDS polyacrylamide gel showing protein extracts from cells taken at different times during induction. The arrow indicates the position of fragment C. For comparison, a sample from the same transformant induced for 48 h in a shake flask is shown in lane 3, and extract from an untransformed control strain is shown in lane 2.

cases.[114,384] Buffering to high pH with *S.cerevisiae*[384] and high or low pH with *P.pastoris*[74] (K. Sreekrishna, personal communication) has reduced proteolysis of secreted proteins. Increased growth rate increases plasmid stability and can result in greater product yield.[82] Finally, different strains of *S.cerevisiae* may have dramatically different growth and induction properties.

CONCLUDING REMARKS

In its early stages the use of yeast for heterologous expression was highly favoured due to the obvious advantages of a microbial eukaryotic system. However, interest waned with the discovery of unforseen problems and with the advent of powerful alternative systems such as baculovirus. Yeast expression

systems are now in a period of resurgence, for a number of reasons. One of the major disadvantages compared to *E.coli* and baculovirus has been the generally lower yield of product, frequently due to the difficulty in obtaining high-level transcription of foreign genes. This problem has now been addressed in *S.cerevisiae* in a number of ways: for example, by over-expressing transcriptional *trans*-activators (e.g. with *ADH2*[291]), by constructing glycolytic promoters with superimposed regulation,[398] and by random screening for super-expressing mutant strains.[354] The use of yeasts such as *Pichia pastoris*, which naturally have powerful, tightly-regulated promoters, has provided an alternative solution. Table 6 (see 'Expression in non-*Saccharomyces* yeasts) shows data for two proteins produced in four expression systems, *S.cerevisiae, P.pastoris, E.coli* and baculovirus, illustrating the comparative strength of the *P.pastoris* multi-copy *AOX1* vector system. Another factor has been the accumulation of experience in the high-density growth and induction of recombinant yeasts, comparing favourably with the difficulties encountered in scaling up other eukaryotic systems. *Pichia* is particularly suited to scale-up and should continue to become more widely used for industrial production.

A number of other areas have shown significant advances, for example the development of more stable 2μ vectors, of rDNA and *Ty* multi-copy integrating vectors, and the use of autoselection markers. The development of episomal vectors for *Kluyveromyces lactis* is also worthy of mention since this yeast appears to be particularly efficient in secretion. However, there are areas where improvements are needed. In *S.cerevisiae* it appears that foreign genes are frequently poorly transcribed using certain promoters, possibly due to a requirement for intragenic yeast sequences (DASs) for maximal transcription. Unfortunately, it is not clear how general the problem is, nor is there yet direct evidence for DASs. In *P.pastoris* there appears to be no such problem using the *AOX1* promoter since very high levels of foreign transcripts can be obtained with multi-copy integrants.[73]

Despite several important successes in secreting proteins, especially unglycosylated polypeptides, this is an area which can present problems. It is still not clear why some foreign proteins are not correctly folded and transported. The current rapid progress in our understanding of protein folding and chaperones may eventually provide explanations and possibly solutions. Recently, however, the most progress in increasing yields of secreted proteins has been made using the powerful empirical approach of random mutagenesis and screening. The secretion of pharmaceutical glycoproteins remains problematic due to the differences in glycosylation between yeast and mammals. Even glycoproteins which are not hyperglycosylated contain antigenic mannose linkages: an engineered strain which could overcome this problem has been reported,[353] though no results on product antigenicity are available yet. An alternative possibility is *P.pastoris* which does not appear to add the antigenic α1,3-linked terminal mannose.[379a] The production of glycoproteins for non-pharmaceutical use, e.g. glucoamylases and xylanases, has been highly successful and presents no such problems.

In the last few years there have been many successes in the production of therapeutic proteins from yeast, for example the recombinant subunit vaccine against hepatitis B virus, human proinsulin, EGF, HSA, etc. There have also been developments in the food industry, such as the experimental use of recombinant yeast secreting glucoamylase in brewing,[157] and the production of chymosin from *K.lactis*.[391] However, in future the requirements of an expression system may be increasingly to provide reagents for research and drug discovery rather than in the production of therapeutic agents. For rapid isolation of such products it may be advantageous to make use of fusion proteins or 'tags' which allow simple affinity purification: a variety of systems are now available.[341,351] Since many pharmacological target proteins may be toxic when expressed to high levels, or may have short half-lives, it may be necessary to accept low yields and concentrate products using an affinity tag.

A highly significant development has come from the realization that many pharmacological target proteins can function in yeast cells *in vivo*. It has been reported that adrenergic receptors can be expressed in yeast and coupled to the signal transduction pathway for α-factor; using a *lacZ* reporter fused to the α-factor regulated *FUS1* promoter, a colorimetric assay that could be used for drug screening was developed.[214] There is intense commercial interest in such systems because of their advantages over mammalian cells in setting up robust drug screens, but there is scope for improvement at present. Other examples of systems that have been or could be used for *in vivo* pharmacological screening are: HIV translational frameshifting,[404] HIV TAT *trans*-activation,[100] steroid receptor binding and *trans*-activation.[293,332]

Finally, yeast expression technology is now important in the genetic analysis of organisms whose genetics are less well developed. A number of mammalian genes have been isolated by their ability to complement mutant yeast homologues, e.g. the human homologue of the *S.pombe cdc2* gene.[230] *S.pombe* may frequently be more suitable than *S.cerevisiae* for screening human cDNA expression libraries because of its closer phylogenetic relationship, and because mammalian expression vectors using the SV40 promoter are active in *S.pombe*.

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