GCN4-Based Expression System (pGES): Translationally Regulated Yeast Expression Vectors

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ABSTRACT

The expression of foreign proteins in Saccharomyces cerevisiae is a powerful tool for basic research and the biotechnological industry. In spite of the potential of S. cerevisiae, only a few useful expression vectors have been developed for this yeast. These vectors are based on an increasing transcription rate in combination with an increase in gene dosage. Most vectors are maintained as plasmids, which forces growth of cultures on poor selective media. Expression of the yeast Gcn4 protein is regulated at the translational level and increases strongly under amino acid starvation. Because under these conditions protein synthesis in general ceases, it is conceivable that regulatory elements that control Gcn4 expression could support selective expression of foreign genes. We cloned DNA fragments residing upstream from the GCN4 coding sequence (including the 5'UTR) and ligated them to a cDNA that encodes the human serum albumin (HSA) gene. These GCN4 regulatory elements induced efficient HSA expression at the translational level under amino acid starvation. The GCN4/HSA cassette promoted efficient, inducible expression on either a multicopy or integrative plasmid. The integrated cassette induced a high level of HSA in dense cultures grown on rich media. Thus, the GCN4-based expression system (pGES) provides high protein quantities. pGES is the first expression vector to be induced at the translational level.

INTRODUCTION

The expression of proteins in microorganisms is a powerful tool for basic research and for various medical and industrial needs. A variety of advanced expression systems have been developed in prokaryotes, in particular in the bacterium *Escherichia coli* (9,20,28). In this organism, a foreign protein can be expressed efficiently, reaching a level of 10%–30% of its total protein content (7,9,20,28). However, many eukaryotic proteins expressed in bacteria are not active. For various reasons, proteins expressed in bacteria must be extensively purified and assayed in rigorous and expensive quality-control systems. Therefore, although powerful, the bacterial systems are disadvantageous in many cases (3,27).

An attractive alternative is the baker's yeast, *Saccharomyces cerevisiae*. Most foreign proteins expressed in this eukaryote are accurately processed posttranslationally and are biologically active (3,10,21,27). *S. cerevisiae* has been widely used in the food industry and is regarded as a safe organism; the risk of side effects and the odds of the presence of pathogenic agents are minimal (3,10,27). Although *S. cerevisiae* was used successfully for the production of several proteins for therapeutic and diagnostic uses (10), and in spite of its great potential as a protein factory, this organism is not widely used for the production of proteins. The major reason for this seems to be that the level of expression obtained in yeast is significantly lower than that obtained in bacteria (3,10,21,23,24).

Only a few yeast expression vectors are available. All vectors (including

bacterial and mammalian vectors) contain strong promoters (usually a promoter of a glycolytic gene; e.g., *GPD1*, *ADHI* or *PGK1*) (21,23,24). In addition, they contain the 2 μ m origin of replication or a multicopy integration system that promotes maintenance of the vector in 60–100 copies per cell (10,22). Some of the vectors contain an inducible promoter, the most widely used being the *GALI-10* promoter (21,23). The strategy of all yeast expression vectors is therefore based on a high transcription rate and the presence of many copies per cell.

Most yeast expression vectors are present in the cell as nonintegrated plasmids. This forces the growth of expressing cultures on synthetic media under selective conditions. When grown in laboratory flasks, these cultures enter the stationary phase at a low cell density, and the quantities of proteins obtained are low. When grown in industrial fermentors, high cell concentrations could be obtained but would require special treatments and long, expensive incubation time.

Here, we describe the development and use of a novel type of expression vector that is fully inducible and can be used in rich media. This vector is based on the regulatory elements of the *GCN4* gene. *GCN4* encodes a transcriptional activator with target genes that encode amino acids and nucleotides biosynthetic enzymes (15,16). Under optimal growth conditions on media supplemented with amino acids and pyrimidine and purine bases, Gcn4 is not expressed. When cells are starved for amino acids, Gcn4 expression is dramatically induced, and consequently, transcription of biosynthetic genes

commences. The expression of *Gcn4* is mainly regulated at the translational level. The mRNA levels of *GCN4* are high under most growth conditions, but the translation rate of this mRNA is extremely low (17). The low translation rate is maintained by four short open reading frames (ORFs) located at the 5' noncoding leader of the message (upstream ORFs 1–4; 4uORF) (4,15–17) (Figure 1). Ribosomes recognize uORF1 and begin translation, yet they dissociate from the mRNA at the stop codon and reinitiate translation at another short ORF. In most cases, ribosomes do not reach the fifth ORF, which is the *GCN4* coding sequence (17). The inhibition of *GCN4* translation depends on the high rate of reinitiation. Under amino acid starvation, the rate of reinitiation is reduced to a minimum, and consequently, translation activity in the cell pauses, which is an obvious protective response. Reduction in reinitiation activity is mediated by the Gcn2 kinase that phosphorylates, and thereby inhibits, the activity of initiation factor 2 α (eIF2 α) (4,17). Although translation in the cell is generally ceased under these conditions, translation of *GCN4* mRNA is specifically induced (15–17). This activity seems a logical response because the Gcn4 protein is required for the induction of amino acid biosynthesis. Following the translation of Gcn4 and the synthesis of amino acids, general translation activity restarts. In the laboratory, amino acid starvation could be easily mimicked by the treatment of yeast cells with inhibitors of biosynthetic enzymes. The most widely used compound is 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3. Cells combat the competitive inhibition effect simply by

producing more His3. The *GCN4* translation rate, which leads to *HIS3* transcription, dramatically increases in response to 3-AT treatment (12,15).

We proposed that the unusual pattern of Gcn4 expression could be advantageous for the expression of a foreign protein. We reasoned that if the mRNA of a given foreign protein is selectively translated when translation of most cellular mRNAs is halted, its concentration should be high. To test this idea, we have cloned the regulatory elements of *GCN4* (from -1067 to -1; +1 is the initiation of translation) and ligated this fragment to the coding sequence of the human serum albumin (*HSA*) gene. We show that under the control of *GCN4* regulatory elements, expression of *HSA* is rapidly and efficiently induced by 3-AT. We further show that induction occurs at the translational level and could be finely controlled by using various 3-AT concentrations. Importantly, this vector induces efficient expression as an integrated copy, which allows growth on rich media to high cell density.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

The SP1 strain (*MATa*, *his3*, *leu2*, *ura3*, *trp1*, *ade8*, *can1*⁺; obtained from M. Wigler) (18) was used as a source of genomic DNA from which the *GCN4* upstream sequences were cloned. Strain BJ2168 (*MATa*, *prc1-407*, *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*; obtained from the yeast genetic stock center at the University of California-Berkeley) (29) was used as a host for the expres-

sion plasmids. Yeast cultures were grown on either 1% yeast extract, 2% bactopectone, 2% glucose (YPD medium) or 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco, Detroit, MI, USA), 0.5% $\text{NH}_4(\text{SO}_4)_2$, 2% glucose and the required amino acids and uracil (40 mg/L) (SD medium). Strains that harbor plasmids were grown on SD(-uracil). For expression experiments, cultures were harvested at the logarithmic phase (unless mentioned otherwise) and resuspended in SD(-uracil, -histidine) [or in SD(-histidine) for integrated vectors], supplemented with 3-AT (see details of each experiment).

PCR Cloning of *GCN4* Fragments and Plasmid Constructions

PCRs were performed using genomic DNA of the yeast strain SP1 as a template and the following primers. Primer 3' (5'-GCGCCGGATCCTTTA-TTTGTATTTAATTTATTTCTTG-AGC-3') contains a *Bam*HI site and was used in all PCRs (Figure 1). The long promoter (5'-GCGCCGACTAGT-TTGCCACGTACATGACATTA-3') was used to amplify the sequence from -1067. The middle-sized promoter (5'-GCGCCGACTAGTGCCTAACACC-ACCGGCATCTTG-3') was used to amplify the sequence from -904. The short promoter (5'-GCGCCGACTAGTTCGGAAGATAAATACTCCAAC-3') was used to amplify the sequence from -706. These three primers contain *Spe*I sites. They were used at the 5' side of the *GCN4* promoter (Figure 1). PCR products were digested with *Spe*I and *Bam*HI and ligated to pBluescript® (Stratagene, La Jolla, CA, USA) to obtain pBSG-Short (pBSG-S), pBSG-Middle (pBSG-M) and pBSG-Long (pBSG-L). The cloned DNAs were sequenced and found to be identical to the sequence of this region as it appears in the *Saccharomyces* genome database.

To ligate the coding sequence of *HSA* downstream from the *GCN4* sequences, the cDNA of *HSA* was cloned as a *Bam*HI-*Eco*RI fragment to pBSG-S, pBSG-M and pBSG-L. These chimeric genes contained about 70 bp of 5' UTR sequences of *HSA*, which were found to reduce the expression level (data not shown). To remove this

Figure 1. Schematic description of the *GCN4* gene and the primers used for promoter cloning. ORFs are shown as boxes and primers as arrows.

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sequence from each of the three plasmids, a 885 bp *Bam*HI-*Nco*I fragment that contains the entire 5' UTR and 815 bp of the *HSA* coding sequence was replaced with a 815 bp PCR product that contains only the first 815 bp of the *HSA* coding sequence. Primers used for this PCR were HSA-ATG (5'-GCG-CGCGGATCCATGAAGTGGGTAA-CCTTTATTTCCC-3') and HSA-815 (5'-GCAGATCTCCATGGCAGCATT-CC-3').

The plasmids obtained were designated pBSG-S-HSA, pBSG-M-HSA and pBSG-L-HSA. The *GCN4*-*HSA* moiety of each of these plasmids was removed as a *Spe*I-*Eco*RI fragment and inserted into pRS316 (a centromeric yeast plasmid) (25), pRS306 (an integrated yeast vector) (25) and pRS426 (a 2 μ m-based vector) (2). The terminator of *ADHI* (0.6 kb *Eco*RI fragment) was ligated to the *Eco*RI site of each of the nine plasmids obtained. The final constructs were designated as follows: (i) pGES306-S-HSA (for *GCN4*-based expression system, derived from pRS306, which contains the short *GCN4* promoter and the *HSA* cDNA; (ii) pGES306-M-HSA, which is similar to pGES306-S-HSA but contains the medium-sized *GCN4* promoter); (iii) pGES306-L-HSA, which contains the long *GCN4* promoter; (iv) pGES316-S-HSA; (v) pGES316-M-HSA; (vi) pGES316-L-HSA; (vii) pGES426-S-HSA; (viii) pGES426-M-HSA; and (ix) pGES426-L-HSA. Figure 2 depicts pGES306-L-HSA, pGES316-L-HSA and pGES426-L-HSA.

Integrated vectors were digested with *Stu*I before yeast transformation. This digestion directs the vector to the *URA3* locus of the genome. To express *HSA* under the *ADHI* promoter, a *Bam*HI-*Eco*RI fragment containing the *HSA* cDNA was rendered blunt by DNA *Po*II (Klenow fragment) and inserted into the *Sma*I site of pAD4 Δ (1).

Western Blot Analysis

Protein extracts were prepared as previously described (8). From each extract, 30 μ g were used for Western blot analysis. The SDS-PAGE gels contained 10% acrylamide. Protein blotting was performed with a semi-dry blotter (LKB, Sweden) for 2 h under

constant current (mA = 0.8 $\times$ gel area in cm²). Polyclonal anti-HSA antibodies (RAHu/albumin) were purchased from Nordic Immunology (Tilburg, The Netherlands) and used in a dilution of

1:50 000. Peroxidase-conjugated goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and used at the dilution of 1:25 000. Puri-

Figure 2. The basic pGES family containing the *HSA* cDNA. Note that pGES306-HSA is an integrative vector, pGES316-HSA is a centromeric vector and pGES426-HSA is a multicopy vector. All vectors shown contain the long *GCN4* promoter (see text for details).

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fied HSA (Sigma, St. Louis, MO, USA) was used as a standard positive control. RNA preparations and primer extension analysis were performed as previously described (6) using the following primers: HSA-extension (5'-A-CCTCACTCTTGT-GTGCATCTC-3') and GCN4-extension (5'-ATAATTC-GCTAGTGAACTGATGGGC-3').

Primer extension products were separated on 6% acrylamide 7 M urea gel.

RESULTS AND DISCUSSION

A 1067 bp Fragment of the *GCN4* Upstream Sequences is Required for Efficient Expression of a Heterologous Gene

To test whether regulatory elements of *GCN4* could be used to regulate the expression of a heterologous gene, we cloned a DNA fragment that resides upstream from the *GCN4* coding sequence. Initially, we cloned (from a genomic DNA prepared from the SP1 strain) a fragment (-706 to -1, designated short promoter; see Materials and Methods and Figure 1) previously shown to be sufficient for regulated expression of Gcn4 and of a *GCN4*/β-galactosidase (β-gal) chimeric gene (containing 53 amino acids of Gcn4 fused to β-gal) (13,14). The capability of this 706 bp fragment alone (containing no *GCN4* coding sequences) to control the transcription and translation

of another gene has not been reported. We constructed a *GCN4*-S-HSA (S stands for short promoter; see Materials and Methods) cassette that was introduced into a multicopy yeast plasmid as well as into centromeric and integrated plasmids. The terminator of the *ADH1* gene was inserted downstream to *GCN4*-S-HSA. The resulting plasmids were introduced into yeast cells that were subsequently grown under various conditions, including amino acid starvation and 3-AT treatment.

Protein extracts were prepared from the cultures, and the expression of HSA was monitored by Western blot analysis. Unexpectedly, in all cases, the expression of HSA was not detectable (data not shown). Therefore, we further cloned two longer fragments of *GCN4* upstream sequences, one containing the

sequence from -904 to -1 and another containing the sequence from -1067 to -1 (designated middle-sized and long promoters, respectively; see Figure 1 and Materials and Methods). When the *HSA* gene was ligated to these fragments (plasmids pGES-M-HSA and pGES-L-HSA) and introduced into yeast cells, a high expression level of HSA was detected (Figure 3). Note that an additional band of 45 kDa cross-reacted with anti-HSA antibodies. This band, which is a result of proteolytic activity of the Yap3 protease (19), was observed also by others that expressed HSA in yeast (19,26).

In all constructs, HSA expression was barely detectable before induction with 3-AT, which suggests that expression from pGES plasmids is tightly controlled. Expression from constructs

Figure 3. Induction and expression of HSA by pGES vectors containing either the long *GCN4* promoter (Long) or the middle-sized promoter (Mid.). Western blot analysis using anti-HSA antibodies was performed on protein extracts prepared from cultures (BJ2168 cells) harboring either pGES306-L-HSA (Long) or p306-M-HSA (Mid.) 8 and 10 h after addition of 3-AT.

Figure 4. Time course and dose response analysis of induction of HSA expression. (A) A culture of BJ2168 cells harboring pGES426-L-HSA was grown to logarithmic phase and then supplemented with 20 mM of 3-AT. Samples were removed at indicated time points and subjected to Western blot analysis. (B) A similar culture was grown to logarithmic phase and then divided into 6 fractions. Each fraction was supplemented with the indicated concentration of 3-AT. Cultures were further grown for 7 h, collected and analyzed by Western blotting. Purified HSA was loaded on the right lanes of each gel.

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containing the long promoter was usually higher than that obtained with the middle-sized promoter (note, for example, the level of expression after 10 h induction; see Figure 3). All further experiments were carried out with the long promoter.

Expression of HSA Under *GCN4* Regulatory Elements Is Induced by 3-AT

We tested whether the cloned *GCN4* elements regulate HSA expression in a pattern similar to that of *Gcn4* regulation. Yeast cells (strain BJ2168) that harbor pGES426-L-HSA were grown to logarithmic phase on SD(-uracil). Then, cells were collected and resuspended in SD(-uracil, -histidine) medium supplemented with 20 mM 3-AT. The samples were removed from the culture at indicated time points, and the expression of HSA was measured. Two hours after the addition of 3-AT, the expression of HSA was easily detected. The expression reached its maximum level between 6 and 8 h after induction (Figure 4A). These results (Figure 3 and Figure 4A) show that a vector based on *GCN4* upstream elements is useful for the inducible expression of foreign genes.

To test whether the level of expression could be finely controlled, we grew the BJ2168/pGES426-L-HSA cells to logarithmic phase, collected the cells, divided them into fractions and resuspended each fraction in medium supplemented with a different 3-AT concentration. The level of HSA expressed was proportional to the concentration of 3-AT provided up to 40 mM. Higher concentrations of 3-AT reduced the expression level of HSA (Figure 4B).

Expression of HSA from pGES Vectors Is Regulated at the Translational Level

To verify that the induction of HSA expression occurred at the translational level, we measured the levels of HSA mRNA and protein before and after induction with 3-AT (Figure 5). Protein extracts were tested by Western blot analysis using anti-HSA antibodies (Figure 5A), while RNA preparations were subjected to primer extension

analysis. The extended primers were separated on sequencing gels, which allowed accurate detection of each mRNA initiation site (Figure 5, B and C). As determined by Hinnebusch (14), the transcription of *GCN4* initiates at three major sites (+1, +18 and +30) and at several minor sites, which gives rise to several mRNA species. Figure 5C

shows that, as in the case of *GCN4* mRNA, the *HSA* mRNA is expressed at a similar level before and after 3-AT is provided. Most initiation sites used by RNA polymerase to transcribe the *GCN4*/*HSA* mRNA are identical to those found in *GCN4* mRNAs (compare Figure 5, B and C). Curiously, the level of *GCN4* mRNA in cells that ex-

Figure 5. Expression of HSA from pGES vectors is induced at the translational level. A culture harboring an integrated copy of pGES306-L-HSA and a culture harboring an integrated copy of the control plasmid pRS306-S (a similar vector but with no *HSA* cDNA) were grown to logarithmic phase when 3-AT was added. At the indicated time points, the samples were removed. Protein extracts and RNAs were prepared from each sample and analyzed by Western blot and primer extension analysis, respectively. (A) Western blot analysis. (B) Primer extension analysis using *GCN4* primer. (C) Primer extension analysis using *HSA* primer.

press HSA is high (Figure 4B). The reason for this phenomenon is unknown to us. In contrast to the constitutive and uniform expression of the *HSA* mRNA, the HSA protein was barely detectable before induction with 3-AT, and its expression increased in time following induction. We conclude that 3-AT-dependent induction of HSA expression occurs at the translational level.

***GCN4*-Based Vector Induces Efficient Expression as an Integrated Copy**

The transcription rate of *GCN4* seems to be high under most growth conditions in various strains and mutants (5,14). Accordingly, mRNA levels of *HSA* expressed from pGES plasmids do not change significantly under different growth conditions (Figure 5 and data not shown). We assume therefore that the efficient expression from the pGES426-L-HSA stems mainly from the efficient translation rate and less from the high copy number of the plasmid. If this is the case, we should be able to use the *GCN4/HSA* cassettes as integrated copies. Cells carrying an integrated vector could be grown on YPD medium to high cell densities and produce high yields of HSA.

To test this possibility, we have inserted the *GCN4-L-HSA* fragment into an integrative vector and thereby obtained the pGES306-L-HSA plasmid (see Materials and Methods). This construct was integrated into the yeast genome, and the resulting clones were

grown on rich nonselective media to a high cell concentration (5×10^8 cells/mL) before the addition of 3-AT. Under these conditions, the expression of HSA was still efficiently induced (Figure 6). The level of HSA obtained per cell was just slightly less than that obtained using the multicopy pGES426-L-HSA vector. However, as the culture grown on YPD reached higher cell concentration, the quantity of intracellular HSA protein obtained (about 0.5 mg/L) was about two times higher of that obtained with the multicopy plasmid pGES426-L-HSA. Notably, all experiments described in this study were performed on cultures grown in laboratory flasks, conditions that do not support maximal yields.

To compare the pGES vector to commonly used vectors, we have ligated the cDNA that encodes *HSA* downstream from the *ADHI* promoter in the vector pAD4 Δ (1). Cultures that harbor either pADH-HSA or pGES426-L-HSA were grown in parallel, and the expression of HSA was compared. In the case of pGES426-L-HSA, expression was compared after induction. The levels of HSA expression obtained from both plasmids were similar. Using commercial purified HSA as a standard, we calculated that about 250 μ g/L of intracellular HSA were obtained in this experiment, about half of the amount obtained using the integrative version (pGES306-L-HSA) on rich medium. We conclude that the pGES system could be more efficient than currently available yeast expression vectors.

Figure 6. pGES system is inducible in highly concentrated cultures. A culture harboring an integrated copy of pGES306-L-HSA was grown on YPD medium to $A_{600} = 3.3$ when the medium was replaced with SD(-histidine) supplemented with 40 mM 3-AT. Samples were removed and analyzed by Western blot analysis at the indicated time points after induction. Purified HSA was loaded on the right lane of the gel.

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Although pGES plasmids already seem quite efficient, they could be regarded as archetypes that could be further manipulated and improved. For example, it might be possible to increase the mRNA level of *HSA* through a combination of a strong constitutive promoter (of another gene) with the 5' UTR of *GCN4*. Such a chimera might be more efficient than pGES and still be translationally regulated. The regulation of *GCN4* translation is well studied, and mutants are available in which the *GCN4* translation rate is constitutively high and not regulated (*gcd* mutants) (11,14,17). Also, *GCN4* translation is induced by the yeast Ras/cAMP cascade and is high in *RAS^{val19}* and *bcy1* strains (5). The pGES plasmids could be used in *gcd* strains and in *RAS^{2val19}* and *bcy1* strains as constitutive vectors. These ideas are only a few of the many ways in which the pGES vectors could be used to obtain large quantities of proteins for research and industry.

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