Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4642–4646, August 1984
Biochemistry

α-Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*

(epidermal growth factor/protein fusion/peptide processing/yeast mating type/*in vitro* mutagenesis)

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Communicated by Bruce M. Alberts, March 28, 1984

ABSTRACT ***Saccharomyces cerevisiae* cells were transformed with plasmids containing hybrid genes in which the sequence encoding mature human epidermal growth factor was joined to sequences encoding the leader region (prepro-segment) of the precursor of the yeast mating pheromone α-factor. These cells accurately process the hybrid protein and efficiently secrete authentic biologically active human epidermal growth factor into the medium.**

The secretion of proteins by the yeast *Saccharomyces cerevisiae* occurs by a mechanism very similar to that observed in mammalian cells. In studies using temperature-sensitive protein secretion (*sec*) mutants, Schekman and collaborators (1, 2) have shown that the yeast secretory pathway involves a series of membrane-bound structures that mediate the transfer of exported proteins from their site of synthesis at the endoplasmic reticulum to their site of release at the plasma membrane. As in other organisms, these secreted proteins are synthesized as larger precursors including "signal" sequences, which are cleaved by a membrane-bound protease to yield the mature gene products.

Previously published studies on the secretion of heterologous proteins from yeast have used the signal sequences of the mammalian proteins being secreted (3). When a structural gene of α-interferon was expressed in yeast to produce a peptide containing the signal sequence, only 10%–20% of the interferon synthesized was secreted into the medium. In addition, proteolytic processing seemed to be imprecise because a heterogeneous mixture of peptides was produced, apparently resulting from cleavages at several different sites within the interferon precursor (3).

Previous studies on the expression of β-lactamase–proinsulin gene fusions in *Escherichia coli* showed that the β-lactamase signal sequence is cleaved and proinsulin is secreted into the periplasm (4). We reasoned, therefore, that the leader sequences of secreted yeast proteins may allow more efficient processing and secretion of heterologous proteins by yeast.

Most studies on secretion of yeast proteins have used enzymes such as invertase and acid phosphatase (2, 5, 6), which are secreted into the periplasmic space or cell wall. We have used the peptide mating pheromone α-factor, which is efficiently secreted into the medium. α-Factor is a 13-residue peptide, secreted by cells of the α mating type, that acts on cells of the opposite *a* mating type to promote efficient conjugation between the two cell types leading to the formation of *aα* diploid cells (7). Studies on the sequence of the α-factor structural gene (8) and on the synthesis and processing of the α-factor peptide (9–11) have shown that α-factor is synthesized as a precursor of 165 amino acids containing an 83-residue leader and four α-factor coding regions, each preceded by a short spacer peptide. The leader and spacer amino acids appear to contain the signals necessary for proteolytic processing and secretion. A recent study (12) shows that fusion of the leader region of the α-factor precursor to invertase, another secreted yeast protein, directs its export.

To investigate whether the α-factor leader sequences are sufficient to allow efficient processing and secretion, we have constructed plasmids in which the genes coding for foreign secreted proteins have been fused to the yeast α-factor gene and expressed under the control of the α-factor promoter. Several different constructions were made in which the DNA sequences of the spacer region, which may encode some of the processing recognition sites, were altered to study their role in the maturation process. Yeast cells transformed with some of these plasmids efficiently synthesize, process, and secrete into the medium the mature biologically active foreign proteins. We describe below the expression, processing, and secretion of human epidermal growth factor (hEGF) from a chemically synthesized gene using the yeast α-factor expression system.

MATERIALS AND METHODS

Strains. Plasmids were propagated in *E. coli* strain HB101 grown in LB medium (13). *S. cerevisiae* strain AB103 (*Matα leu2-3,112 ura 3-52 his4-580 pep 4-3*) was constructed in this laboratory. JRY188 (*Matα sir3-8 leu2-3,112 ura 3-52 his4 rme*) was obtained from Jasper Rine. Yeast transformation was carried out as described (14).

Plasmids. Plasmid DNA was isolated by alkaline Na-DodSO$_4$ lysis (15). Plasmid pAB11 was derived from pBR322 (16) as described (10). pC1/1 was derived from pJDB219 (17) by replacement of the pMB9 region by pBR322 and contains the ampicillin- and tetracycline-resistance genes and replication origin of pBR322 for selection and propagation of *E. coli* and the yeast *LEU2* gene and 2-μm plasmid sequences for selection and autonomous replication in yeast *leu2* mutants. An oligonucleotide (5′-T-T-A-G-T-A-C-A-T-T-G-G-T-T-G-G-C-C-G-G-3′) homologous to the end of the yeast α-factor structural gene (8) was used to probe a library (6) of yeast genomic DNA cloned in YEP24 (18). A plasmid (pAB101; see ref. 10) with a 7.5-kilobase-pair (kbp) insert was isolated from which was obtained a 1.76-kbp *Eco*RI fragment containing the complete yeast α-factor gene. This fragment was cloned into pAB11 to produce pAB112.

***In Vitro* Mutagenesis.** Site-directed *in vitro* mutagenesis (19) was performed using single-stranded DNA from phage M13 into which had been cloned a 421-base-pair (bp) *Pst* I/*Sal* I fragment containing the α-factor leader–hEGF gene fusion. A synthetic oligonucleotide (70 pmol) was used as a primer for the synthesis of the second strand from 1.2 pmol of the phage template using the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories; 5 units) and

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Abbreviations: hEGF, human epidermal growth factor; kbp, kilobase pair(s); bp, base pair(s).

T4 DNA ligase (New England Biolabs; 40 units). After 18 hr at 14°C, the mixture was treated with S1 nuclease (Miles; 5 units) for 15 min and was used to transfect *E. coli* JM101 cells. Bacteriophage containing the desired mutation were located by plaque-filter hybridization using the ^{32}P-labeled primer as a probe.

EGF Assays. EGF was assayed by a competitive receptor binding assay using ^{125}I-labeled mouse EGF (Amersham) and human foreskin fibroblasts (20). Standard curves were obtained by measuring the effects of increasing quantities of mouse EGF on the binding of a constant amount of ^{125}I-labeled mouse EGF.

RESULTS

Construction of α-Factor-Derived Plasmid Vectors for the Secretion of Heterologous Proteins in Yeast. For the expression and secretion of heterologous proteins in yeast, we have constructed plasmids in which the region coding for the foreign protein is fused in-frame to the α-factor leader coding region by sequences coding for the presumptive processing signals Lys-Arg-(Glu-Ala)$_3$, and variations thereof. The yeast α-factor gene used in this study corresponds to the MFα1 gene reported by Kurjan and Herskowitz (8) (see legend to Fig. 1 for details). The isolated 1720-bp *Eco*RI fragment contains ≈950 bp of 5′ flanking region, 495 bp of the region coding for the α-factor leader and α-factor protein, and 270 bp of 3′ flanking region. Fusions between the α-factor leader and heterologous proteins were obtained by digesting pAB112 with *Hin*dIII and *Sal* I and replacing the α-factor coding *Hin*dIII/*Sal* I fragment by the region coding for the foreign protein. Yeast expression vectors using this system were constructed, as shown in Fig. 1, by insertion of the α-factor gene region modified as described above in the multicopy plasmid pC1/1, which is capable of autonomous replication in yeast and *E. coli*. The heterologous gene used in this study is a synthetic DNA fragment coding for hEGF, a potent general stimulant of cell growth, and a specific promoter of epithelial cell proliferation (22). The synthesis and assembly of the hEGF gene has been described elsewhere (21).

Processing and Secretion of Proteins from a Direct α-Factor Leader–hEGF Fusion. *S. cerevisiae* strain AB103 was transformed with pYαEGF-21, and leucine prototrophs were selected. Cultures of these transformants were grown to stationary phase in leucine-selective medium. After removal of the cells by centrifugation, the culture medium of cells carrying the plasmid pYαEGF-21 was found to contain 5–10 μg of hEGF per ml in this assay. Control cells transformed with pC1/1 produced no detectable EGF (≤1 ng/ml).

Cells grown to stationary phase contained very little intracellular EGF (<5% of that secreted). However, at earlier times, a considerable fraction of the hEGF produced appeared to remain in the cells. During the exponential growth phase, as much as 67% of the hEGF produced is intracellular. The fraction of hEGF that is intracellular gradually decreases to ≈1% by 24 hr after the cells have reached saturation. Gel electrophoresis analysis of cell proteins labeled with [^{35}S]sulfate confirmed also that there is no significant intracellular accumulation of hEGF or its precursor (P. Tekamp-Olson, personal communication). The total amount of hEGF produced in these experiments represents ≈7% of the soluble cellular protein.

The nature of the secreted products was investigated by gel electrophoresis and by amino acid sequence analysis. NaDodSO$_4$/polyacrylamide gel electrophoresis (see Fig. 3, lane 3) shows that the material concentrated from culture medium of cells transformed with pYαEGF-21 contains a single protein species with the properties of hEGF. This protein is absent in the culture medium from control cells and

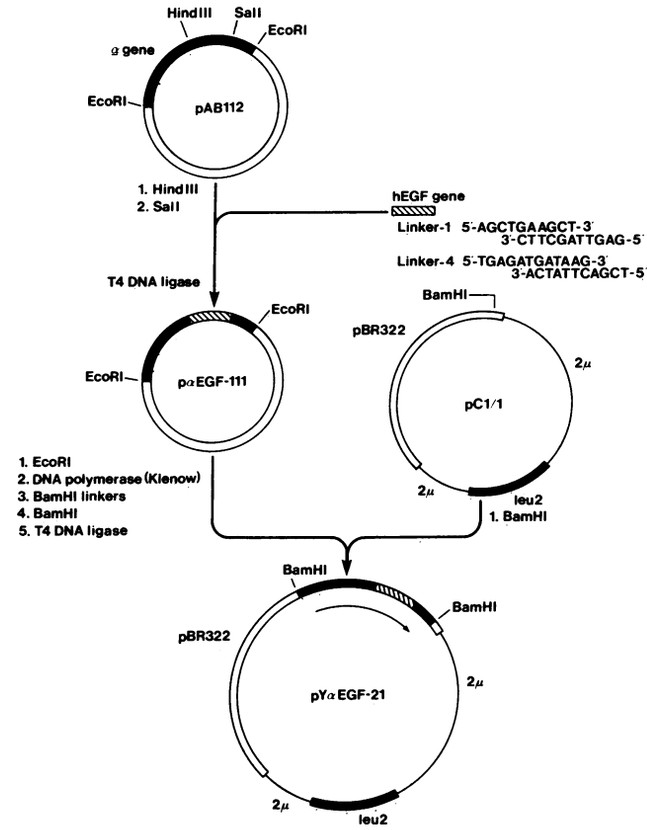

FIG. 1. Construction of α-factor-based vectors for the synthesis and secretion of heterologous proteins in yeast. Fusions were made by digestion of pAB112 with *Hin*dIII and *Sal* I and ligation to synthetic oligonucleotide linkers and to a 159-bp *Hga* I fragment containing a chemically synthesized region coding for hEGF (21). Linker I restored the α-factor processing signals interrupted by the *Hin*dIII digestion and joins the *Hga* I end at the 5′ end of the hEGF gene to the *Hin*dIII end of pAB112. Linker 4 contained two stop codons and joins the *Hga* I end at the 3′ end of the hEGF gene to the *Sal* I end of pAB112. The resulting plasmid is named pYαEGF-111. Plasmid vectors for the synthesis and secretion of hEGF in yeast (pYαEGF-21) were constructed by digestion of pYαEGF-111 with *Eco*RI, ligation to *Bam*HI linkers, digestion with *Bam*HI, and ligation to *Bam*HI-digested pC1/1.

migrates in NaDodSO$_4$ gel electrophoresis as a molecule of M_r ≈6000. hEGF has a great tendency to polymerize through disulfide exchange, as shown by the fact the oligomers (dimers and trimers) are also seen even after treatment with iodoacetamide. NH$_2$-terminal amino acid sequence analysis (Table 1) indicates that this protein corresponds to an incompletely processed hEGF derivative containing six additional amino acids (Glu-Ala)$_3$ at the NH$_2$ terminus of the molecule. The (Glu-Ala)$_3$-hEGF derivative has the same receptor binding and other biological activities as authentic hEGF (see below).

Analysis of α-Factor Leader Processing by Site-Directed Mutagenesis. To further understand the specificity of the processing of the α-factor leader, we have studied the efficiency of secretion and the nature of the products exported from plasmids containing direct α-factor leader–hEGF fusions and from plasmids containing modified forms of α-factor leader–hEGF gene fusions. In the latter fusions, the sequence of the spacer region, which encodes the processing signals, has been specifically altered by use of synthetic oligonucleotide linkers or by site-directed mutagenesis (Fig. 2). In pYαEGF-21, the α-factor leader has been fused to the hEGF gene by sequences coding for Lys-Arg-(Glu-Ala)$_3$ as in the natural yeast α-factor gene. In pYαEGF-22, a se-

Table 1. NH_2-terminal amino acid sequence of products secreted by yeast strains containing α-factor leader–EGF gene fusions

Protein product	NH_2-terminal sequence
Authentic EGF (18)	*NH_2-Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly*
Secretion from pYαEGF-21	NH_2-Glu-Ala-Glu-Ala-Glu-Ala-*Asn-Ser-Asp-Ser-Glu-Cys* (100%)
Secretion from pYαEGF-22	NH_2-Glu-Ala-Glu-Ala-Ser-Leu-Asp-Lys-Arg-*Asn-Ser-Asp* (20%)
	NH_2-Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly (80%)
Secretion from pYαEGF-23	*NH_2-Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly* (100%)

Proteins were concentrated from the culture medium by absorption on Bio-Rex 70 resin and elution with 0.01 HCl in 80% ethanol and were purified by high-performance liquid chromatography (HPLC) on a reversed-phase C18 column. Sequence at the NH_2-terminal end was obtained by the Edman degradation method (23) using a gas-phase (24) protein sequencer. Sequences corresponding to hEGF are italicized.

quence encoding an additional cathepsin B-like maturation site Ser-Leu-Asp-Lys-Arg has been inserted, replacing the last Glu-Ala pair immediately preceding the first amino acid of mature hEGF. In a third construction, pYαEGF-23, the sequences encoding the dipeptidyl aminopeptidase maturation sites $(Glu\text{-}Ala)_3$ have been removed. In the construction pYαEGF-24, the triplet TCT coding for serine-81 of the α-factor leader has been changed to CCT, coding now for proline. This change was made to generate a *Kpn* I site useful in the construction of fusions of the α-factor leader and other heterologous proteins without the $(Glu\text{-}Ala)_3$ residues. An example is pYαEGF-25, in which the new *Kpn* I site has been used together with synthetic oligonucleotide linkers to make such a fusion with hEGF. In this case, the serine at position 81 has been changed to a glutamine.

Yeast strain AB103 was transformed with DNA from pYαEGF-22, pYαEGF-23, pYαEGF-24, and pYαEGF-25 and assayed for hEGF as indicated above for pYαEGF-21. Cultures from cells carrying pYαEGF-22 secrete 5–8 μg of hEGF per ml, measured either as protein or in the receptor binding assay. Analysis by $NaDodSO_4$ gel electrophoresis indicates that two protein species are secreted, a major component migrating with an apparent M_r of 5500 and a minor component with an apparent M_r of 6000 (Fig. 3, lane 2). Dimers and trimers of both proteins are also seen. NH_2-terminal amino acid sequence analysis (Table 1) indicates that the proteins secreted correspond to a mixture of 80% authentic hEGF and 20% of a hEGF derivative containing nine additional amino acids at the NH_2 terminus. This result indicates that the hybrid protein can be cleaved at either Lys-Arg maturation site.

This result was confirmed by using the pYαEGF-23 con-

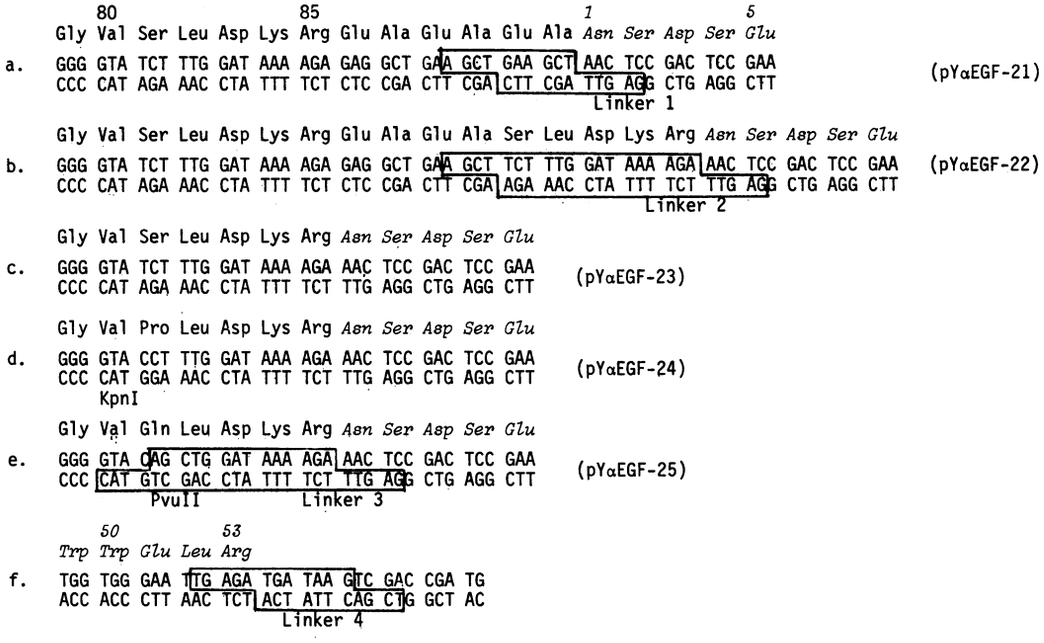

FIG. 2. Amino acid and nucleotide sequences of modified α-factor leader–hEGF gene fusions. (*a–e*) Sequences of fusions at the NH_2-terminal region of hEGF, which differ among several constructions; (*f*) sequences at the COOH-terminal region of hEGF, which is the same for all constructions. The amino acid sequences corresponding to hEGF are shown in italics. Synthetic oligonucleotide linkers used in these constructions are boxed. Fusions were made as follows. Construction *a* was made as shown in Fig. 1 from a synthetic hEGF gene flanked by *Hga* I sites (21). Construction *b* was made in a similar way except that linker 2 was used instead of linker 1. The resulting yeast plasmid is named pYαEGF-22. Construction *c* was obtained by *in vitro* mutagenesis of construction *a*. A *Pst* I/*Sal* I fragment containing the α-factor leader–hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A 31-mer oligonucleotide of sequence 5′-T-C-T-T-T-G-G-A-T-A-A-A-A-G-A-A-A-C-T-C-C-G-A-C-T-C-C-G-A-A-T-3′ was used as a primer for *in vitro* mutagenesis of the above template. RF DNA from the mutant phage was isolated, digested with *Pst* I/*Sal* I, and the resulting fragment was inserted into pAB112 that had previously been digested to completion with *Sal* I and partially digested with *Pst* I. The resulting yeast plasmid is named pYαEGF-23. Construction *d*, in which a new *Kpn* I site was generated, was made as described for construction *c* except that the 36-mer oligonucleotide primer of sequence 5′-G-G-G-T-A-C-C-T-T-T-G-G-A-T-A-A-A-A-G-A-A-A-C-T-C-C-G-A-C-T-C-C-G-A-A-T-3′ was used. The resulting yeast plasmid is named pYαEGF-24. Construction *e* was derived by digestion of the plasmid containing construction *d* with *Kpn* I and *Sal* I and by insertion of the *Hga* I-derived EGF gene using linkers 3 and 4 instead of linker 1 or 2. The resulting yeast plasmid is named pYαEGF-25.

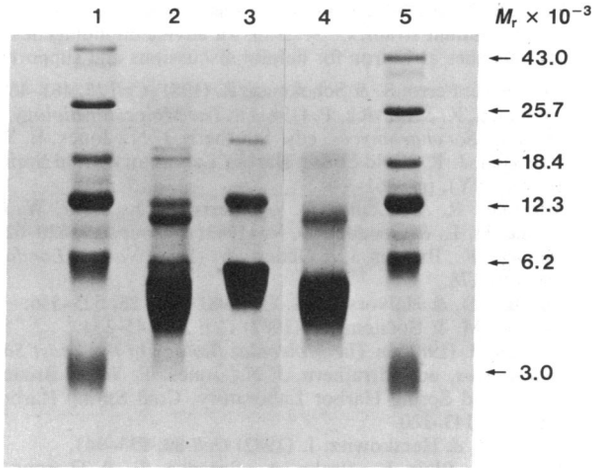

FIG. 3. $NaDodSO_4$ gel electrophoresis of hEGF and derivatives secreted by transformed yeast cells. Proteins were concentrated by absorption on Bio-Rex 70 resin, carboxymethylated with iodoacetamide, and subjected to gel electrophoresis in 12.5% polyacrylamide gels as described by Laemmli (25). Lanes 1 and 5 are M_r standards: ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), cytochrome *c* (12,300), bovine trypsin inhibitor (6200), and insulin A and B chains (3000). Lanes 2, 3, and 4 are proteins secreted by yeast cells transformed with pYαEGF-22, pYαEGF-21, and pYαEGF-23, respectively.

struction. Cultures from cells carrying pYαEGF-23 produced ≈5 μg of hEGF per ml, measured either as protein or in the receptor binding assay. Analysis by $NaDodSO_4$ gel electrophoresis indicates that only one protein species is secreted. This protein migrates with an apparent M_r of 5500 (Fig. 3, lane 4). Dimers and trimers of this species are also seen. NH_2-terminal amino acid sequence analysis (Table 1) indicates that the secreted protein corresponds to authentic hEGF.

Cells carrying pYαEGF-24 and cells carrying pYαEGF-25 also synthesize and secrete hEGF in yields similar to those of cells transformed with pYαEGF-23. This indicates that the secretion and maturation process occurs with similar efficiency regardless of whether the amino acid at position 81 in the α-factor leader is serine, proline, or glutamine.

Biological Activity of hEGF Produced Yeast Cells. Biosynthetic hEGF was found to have the same biological activities previously reported for hEGF isolated from human urine (20, 26), such as early eyelid opening and incisor eruption in newborn rats and inhibition of acid gastric secretion in adult rats (A. Gonzalez, personal communication).

Regulated Production of hEGF in *sir3* Mutants. Although yeast cells transformed with pYαEGF-21 grow only slightly more slowly than control cells transformed with the vector pC1/1, expression of fusion proteins between α-factor and other heterologous genes appears to be somewhat deleterious, as manifested by difficulty in obtaining transformants and by the slow growth of such transformants (unpublished results). Therefore, it was desirable to devise a method to regulate the expression of such fusion proteins easily. This was accomplished by using a temperature-sensitive *sir3* mutant. Mutations in any of the *SIR* genes result in a non-mating phenotype because the normally silent *MATa* and *Matα* sequences present in the *HML* and *HMR* loci are expressed (27). Such mutants behave like *a*/α diploid cells and do not express either α or *a* specific gene products.

A *MATα sir3* strain (JRY188) was transformed with pYαEGF-21, and leucine prototrophs were selected at 37°C. Cells were grown at the restrictive temperature in selective medium, then shifted to either permissive or non-permissive temperature, and the culture fluid was assayed at various times for the presence of hEGF. Supernatant medium from cells grown at 36°C contained no detectable hEGF (≤10 ng/ml) (Table 2). However, after a lag of several hours, cells grown at 24°C secreted up to 4 mg of hEGF per liter.

Table 2. Regulated synthesis and secretion of hEGF in transformed yeast temperature-sensitive *sir3* mutants

Temperature	Transformant	OD_{650}	hEGF (ng/ml)
36°C	3a	3.5	0.010
		5.4	0.026
	3b	3.6	0.020
		6.4	0.024
24°C	3a	0.4	34
		1.3	145
		2.1	1075
		4.0	3250
	3b	0.4	32
		1.4	210
		2.2	1935
		4.2	4600

Yeast strain JRY188 (*Matα, sir3-8, leu2-3, leu2-112, trp1, ura3, his4, rme*) was transformed with pYαEGF-21 and leucine prototrophs selected at 37°C. Cells were then grown in selective medium (without leucine) at 37°C. Saturated cultures were then diluted 1:100 in fresh medium and grown at 24°C or 36°C. Culture medium was assayed for hEGF.

DISCUSSION

We have shown that fusions of the α-factor leader to heterologous proteins such as hEGF are efficiently processed by yeast cells, resulting in the secretion of mature hEGF into the medium. Nearly all of the hEGF synthesized by the yeast transformants appears to be processed and secreted, with very little (<5%) found in an intracellular form. These results indicate that the leader region of the α-factor precursor can provide the amino acid sequence information necessary for recognition by the yeast secretory and processing apparatus. Analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis and by reversed-phase HPLC shows that hEGF makes up >90% of the peptide material present in the culture supernatant of yeast cells carrying these recombinant plasmids.

Although the hEGF synthesized in strains carrying pYαEGF-21 is biologically active, amino acid sequence analysis indicated that the major hEGF species secreted contained the spacer sequence $(Glu\text{-}Ala)_3$ at the NH_2 terminus. This indicates that the amount of dipeptidyl aminopeptidase present in these yeast strains is insufficient to process the large amounts of the fusion proteins being made. Similar results were observed in strains overproducing α-factor itself, because of the presence of the α-factor gene on a multicopy plasmid (9).

Since it had not been determined whether all of the spacer sequence is required for proper processing of α-factor or α-factor fusion proteins, we decided to determine whether alteration of some of these residues still allows processing at an intact Lys-Arg cleavage site. Oligonucleotide-directed mutagenesis of M13 phage DNA containing the α-factor–hEGF gene fusion allowed us to delete the $(Glu\text{-}Ala)_3$ sequence of the spacer peptide. When such gene fusions were introduced into yeast, they resulted in the synthesis and secretion of mature hEGF, as judged by NH_2-terminal sequence analysis. Using a similar *in vitro* mutagenesis procedure, we were also able to generate new restriction sites, which are useful in the construction of fusion between α-factor and other heterologous proteins. Although these changes alter a residue in the α-factor leader (from serine to either proline or glutamine), processing of these proteins at

the nearby Lys-Arg site appears to occur normally. Only authentic hEGF, rather than partially processed forms, are found in the culture medium of these strains. Thus, the $(Glu\text{-}Ala)_3$ sequence of the spacer region in the α-factor precursor appears not to be required for proper processing of the hEGF fusions. It is conceivable that these residues are necessary for α-factor maturation. Alternatively, these amino acids may prevent accumulation of intracellular active α-factor, because the presence of the Glu-Ala residues greatly decreases the biological activity of α-factor (9). Similar spacer sequences in honey bee promellitin probably prevent the toxicity of the mature venom protein (28). It is also possible that $(Glu\text{-}Ala)_3$ sequences are required for the proper folding of the α-factor precursor but may not be necessary in the case of the α-factor–hEGF fusions. Correct folding of other fusions between the α-factor leader and heterologous proteins may require the $(Glu\text{-}Ala)_3$ site.

Although yeast strains transformed with α-factor–hEGF gene fusions appear to tolerate the production of large amounts of product, expression of high levels of some other heterologous proteins may be toxic to yeast cells. To regulate the expression of the desired gene product, we have taken advantage of a conditional mutation in a gene involved in regulating the expression of mating-type specific genes (27). At restrictive temperatures, the *sir3* mutant used here does not express α-specific genes including the plasmid-born α-factor–hEGF fusion. As shown in Table 2, a temperature shift from 36°C to 24°C results in a $>2 \times 10^5$-fold increase in expression and secretion of hEGF after ≈6 generations of growth. Growth for shorter times at the permissive temperature (≈3 generations) did not result in full induction of expression of secreted hEGF (data not shown), probably because this did not allow sufficient time for expression of the α-mating phenotype due to the inactivation or dilution of the *MAT1* gene product or, more likely, synthesis or activation of sufficient *SIR3* gene product.

The experiments described here suggest that the α-factor expression–secretion system may be generally useful for the expression of heterologous proteins. Yeast cells can be cultivated readily in large-scale fermentations and have the advantage of releasing relatively little extraneous protein material into the medium. This situation greatly facilitates the purification of proteins secreted by strains carrying the appropriate recombinant plasmids. An additional advantage is that some may be degraded or may be toxic if expressed as intracellular proteins. For example, when the synthetic hEGF gene used here was linked directly to a constitutive yeast promoter (from the glyceraldehyde-3-phosphate dehydrogenase gene), only small amounts of hEGF could be recovered from cell lysates (21). This is in striking contrast to the large amounts of the same protein that were produced when using the α-factor expression and secretion system reported here. We have recently (refs. 29 and 30; unpublished results) achieved secretion of a number of other human proteins using the α-factor system, including growth hormone-releasing factor, insulin-like growth factors, and interleukin 2. Thus, we have demonstrated the general utility of the α-factor expression system.

We thank R. Najarian for DNA sequencing, J. Rine for providing the yeast *sir3* mutant strain, C. S. Craik for advice on mutagenesis, and our colleagues at Chiron for helpful discussions and support.

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