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Cloning and expression of the hEGF gene in *Saccharomyces cerevisiae*

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Yeast has been successfully used as a host for the synthesis and secretion of the epidermal growth factors (EGF)[1] from different species. EGF is a small single-chain polypeptide consisting of 53 amino acids with molecular mass about 6000 daltons. Its structure is stabilized by three intramolecular disulfide bonds [1]. We have investigated a novel secretion vector designed to synthesize and direct efficient secretion of human EGF from *S. cerevisiae* cells.

The gene coding for human EGF was designed with yeast-preferred codons and assembled by enzymatic ligation of 10 synthetic fragments. The ligation products were separated by polyacrylamide gel electrophoresis. A 170-bp band was isolated and cloned into bacteriophage M13mp19. The nucleotide sequence of the cloned gene was confirmed by the dideoxynucleotide method [2].

A number of signal sequences have been used successfully to secrete EGF protein in yeast [3]. In our studies we constructed a hybrid plasmid in which the region coding for the foreign protein was fused in-frame to that coding for the leader peptide of the killer toxin of *Kluyvermyces lactis* [4]. Construction of the expression vector based on the pEMBL18 plasmid is shown in Fig. 1. The synthetic oligonucleotides coding for the 16 amino acid long leader peptide of the killer toxin and a synthetic gene for hEGF were inserted into appropriate restriction sites of the polylinker between the inducible promoter *CTA1* and the transcription termination signals (3′ end of FLP from 2 μ DNA) The resulting pYET-EGF plasmid (Fig. 2) is capable of autonomous replication both in yeast (the *Hin*dIII restriction fragment of plasmid pJDB219 contains the genes: *STB* of 2 μ plasmid and the 3′ end *FLP* of 2 μ plasmid, *leu* 2-d gene) and in *Escherichia coli* (*ori* Col E1). *Leu* 2-d gene is a poorly expressed allele of *Leu* 2 which appears to increase the stability and the copy number of 2 μ plasmid derivatives. Additional features of pYET involve the presence of a selectable marker *URA3* and the f1 phage origin of replication (IG); the latter makes possible the existence of this plasmid in a single-stranded form. The junction between the toxin leader peptide and the 5′ end of the hEGF sequence is formed by four amino acids (Thr-Arg-Gly-Ser), specified by the polylinker sequences preceding the *Bam*HI cloning site. The sequence Thr-Arg-Gly is recognized in yeast as an endopeptidase cleavage site.

The hybrid plasmid was then used for transformation of different strains *S. cerevisiae*. The stability of the Ura^+ phenotype in transformed cells was analyzed by growing the cells in a non-selective medium for 8 generations and plating the same number of cells onto selective and non-selective media. The same number of colonies arose on both media, indicating a stable phenotype.

The synthesis and secretion of hEGF were assayed by SDS-polyacrylamide gel electrophoresis of cell supernatants prepared from cultures grown in synthetic medium, lacking leucine, and containing ethanol as a carbon source. Electrophoretic analysis showed the presence of a protein of molecular mass about 6000 daltons in the supernatants of the pYET-EGF strain GC1-8b and pYET-EGF strain 4A

[1]Abbreviations: EGF, epidermal growth factor; hEGF, human EGF; h.p.l.c., high performance liquid chromatography

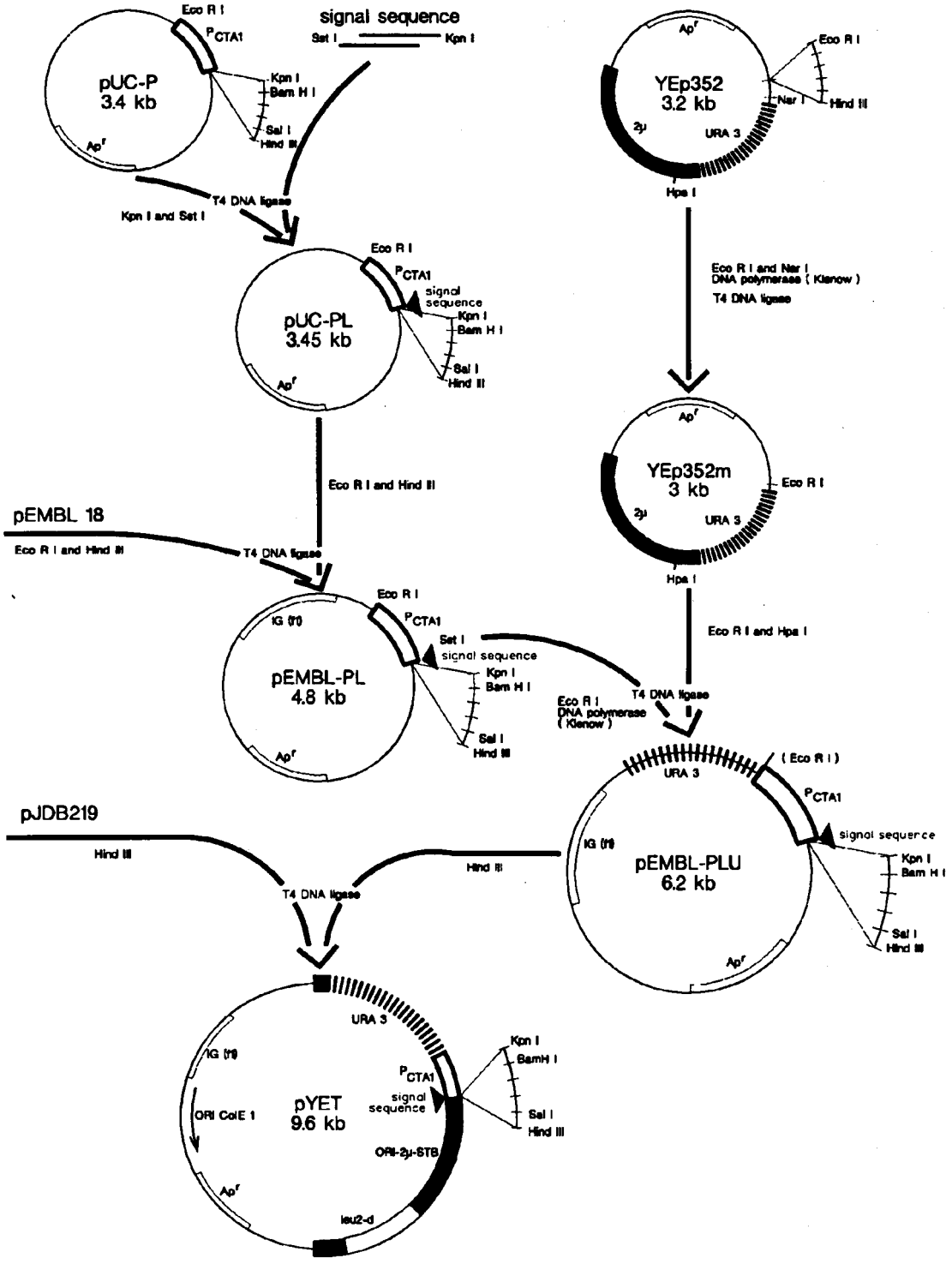

Fig. 1. Construction of vector for the synthesis and secretion of heterologous protein in yeast.
Plasmid pUC-PL was constructed by inserting the synthetic sequence coding for the leader peptide of *K. lactis* killer toxin downstream from the promoter *CTA1*, in the polylinker *Sst*I *Kpn*I sites. The *Eco*RI - *Hin*dIII restriction fragment of this plasmid was isolated and inserted in the *Eco*RI - *Hin*dIII polylinker of pEMBL18, giving the plasmid pEMBL-PL. The YEp352 plasmid was modified by digestion with *Eco*RI and *Nar*I rendered blunt-end with DNA polymerase I (Klenow fragment) and self-ligation. The *Eco*RI restriction site was re-created and the *Eco*RI - *Hpa*I fragment then excised and inserted into the filled *Eco*RI site of pEMBL 18, forming plasmid pEMBL-PLU. The plasmid vector for synthesis and secretion of heterologous protein was constructed by digestion of pEMBL-PLU with *Hin*dIII and ligation with *Hin*dIII fragments of pJDB219 plasmid, forming the vector pYET

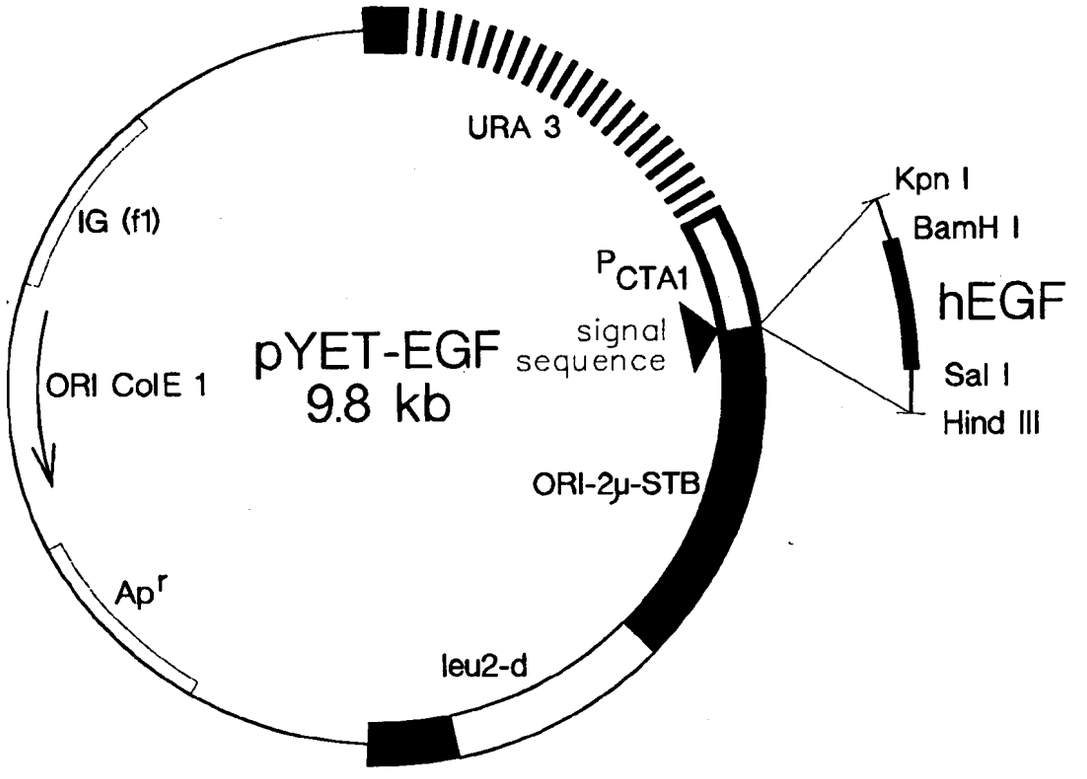

Fig. 2. Construction of the plasmid for expression and secretion of hEGF in S. cerevisiae.
The *Bam*HI-*Sal*I restriction fragment containing a chemically synthesized region coding for hEGF was inserted into the appropriate polylinker sites of the pYET vector

Table 1

Stimulation of DNA synthesis in human skin fibroblasts by the tested fractions f5, f6, f9, murine EGF (mEGF), recombinant human EGF (rEGF, SIGMA), and 10% serum.

As the control the same cells without the growth factor were used. Cells were incubated at 37°C for 24 h, then the growth factor and 8 h later [^{3}H]thymidine were added. Incubation was continued for another 16 h. Acid precipitable radioactivity was determined. Measurements were repeated 4 times and S.D. are given

Probe			c.p.m.	S.D.
Control			357.8	71.9
Serum			7634.5	461.1
rEGF	5	ng	513.7	60.1
mEGF	5	ng	787.0	29.0
f5	0.1	ng	593.5	130.4
f5	1.0	ng	1094.7	88.2
f5	10	ng	1438.7	80.4
f6	5	ng	1016.5	100.5
f6	15	ng	1085.5	92.5
f9	0.1	ng	1653.0	282.1
f9	1	ng	1575.0	91.6
f9	10	ng	1263.2	85.6

only when the cells were grown on ethanol. The protein was partially purified, using the procedure of Savage & Harper [5] with fractionation on reversed-phase h.p.l.c. as the final step. The activity of chosen fractions was confirmed in the biological assay. Aliquots of the eluted fractions were added to human skin fibroblast cultures and [^{3}H]thymidine incorporation into proliferating cells was measured. Three tested fractions, with murine EGF as a control, specifically stimulated synthesis of DNA in human fibroblasts (cf. Table 1). This result shows that the 6 kDa protein secreted by recombinant *S. cerevisiae* reveals certain properties similar to those of natural hEGF.

In conclusion, the plasmid vector constructed by us, with the synthetic hEGF gene transformed to *S. cerevisiae* cells, efficiently secretes the 6 kDa protein into the culture medium. The purified protein has the same proliferation activity as the hEGF isolated from human urine [6].

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