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Chemical synthesis of a gene for human epidermal growth factor urogastrone and its expression in yeast

(synthetic gene/yeast glyceraldehyde-3-phosphate dehydrogenase promoter/phosphoramidite)

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ABSTRACT **We have chemically synthesized and expressed in yeast a gene coding for human epidermal growth factor (urogastrone), a 53-amino-acid polypeptide that has been shown to promote epithelial cell proliferation and to inhibit gastric acid secretion. The synthetic gene, consisting of 170 base pairs, was designed with yeast-preferred codons and assembled by enzymatic ligation of synthetic fragments produced by phosphoramidite chemistry. The DNA synthesis protocol used allows for facile synthesis of oligonucleotides larger than 50 bases. Yeast cells were transformed with plasmids containing the synthetic gene under control of a yeast glyceraldehyde-3-phosphate dehydrogenase gene promoter and were shown to synthesize a biologically active human epidermal growth factor.**

Epidermal growth factor (EGF) is a single-chain polypeptide consisting of 53 amino acids of M_r of ≈6,000 (1–6). It is synthesized in the salivary glands of adult male mice (1–3). Specific, saturable membrane mouse EGF (mEGF) receptors from a variety of tissues have been characterized (7–9). This polypeptide hormone markedly stimulates the proliferation of a variety of keratinocytes derived from skin, conjuntival, or pharyngeal tissue (10, 11) and has been shown to delay the ultimate senescence of cells in culture (11).

A remarkably similar peptide, human urogastrone, has been isolated from human urine and subsequently shown to be synthesized in the duodenum and salivary glands (12). Human urogastrone is a potent inhibitor of gastric acid secretion and a promoter of epithelial cell proliferation (5, 13). Peptide sequence determination has revealed the unexpected findings that urogastrone was 70% homologous (37 of 53 common amino acids) to mEGF and that the three disulfide bonds are formed in the same relative position (4, 5). The mouse- and human-derived peptides elicit nearly identical biological responses, and all available data suggest that human urogastrone and human EGF (hEGF) are identical.

The full range of biological activity of hEGF has not yet been investigated in detail because of its low abundance in human urine. We have utilized, therefore, a combination of oligonucleotide synthesis and recombinant DNA technology to produce this polypeptide hormone in yeast. Because of its gastric antisecretory activity, it may be of therapeutic value in the treatment of duodenal ulceration (14).

Recently, Smith *et al.* have reported the synthesis of a human urogastrone gene designed for expression in *Escherichia coli* as a fusion product with a portion of the *trpE* gene (15). Twenty-three oligonucleotides ranging from 12 to 20 nucleotides in length were synthesized by phosphotriester methodology and used to construct the gene by enzymatic ligation. Two halves of the gene were independently cloned.

This communication reports the synthesis of a gene by phosphite-coupling procedures. With our procedures, we produced three derivatives of the 170-base-pair (bp) hEGF gene that were cloned directly. This was achieved with as few as 10 oligonucleotides ranging from 11 to 59 bases in length.

The codon usage bias observed in highly expressed yeast genes was used in the design of the synthetic gene. A yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was linked to the hEGF gene in an autonomously replicating plasmid. Yeast cells transformed with these vectors synthesize a biologically active hEGF.

MATERIALS AND METHODS

Materials. T4 polynucleotide kinase and terminal transferase were purchased from New England Nuclear. T4 DNA ligase was obtained from New England BioLabs. Restriction enzymes were from Bethesda Research Laboratories. Deoxyribonucleosides were purchased from Sigma. Vydac HPLC grade silica gel was purchased from The Separations Group (Hesperia, CA). The protected deoxyribonucleosides were synthesized as described (16) except that isobutyryl chloride was used to protect 2′-deoxyguanosine instead of isobutyric anhydride (17). Fully protected 2′-deoxyribonucleoside 3′-phosphoramidites were synthesized from the protected deoxyribonucleosides and chloro-*N*,*N*-(dimethylamino)methoxyphosphine (18). Silica gel supports were synthesized as described (19). The extent of coupling was quantitated by release of the dimethoxytrityl group in acid (16). Typically, the functionalized support contained 50–70 μmol of deoxynucleoside per gram.

Oligonucleotide Synthesis. Solid-phase synthesis of oligonucleotides by sequential addition of the above phosphoramidite monomers proceeded as indicated in Table 1. Completion of steps 1–9 constitutes one cycle. All reactions were performed manually at ambient temperature in a sintered glass filtration funnel using a wrist-action shaker for agitation. After completion of the desired chain length, the protecting groups were removed as described (20). The fully deprotected sequence was purified by 15% or 20% polyacrylamide gel electrophoresis under denaturing conditions.

Assembly of the Gene. Oligonucleotides were preparatively 5′-phosphorylated as described elsewhere (21). For the ligations, the oligonucleotides were mixed and dissolved in 50 mM Tris·HCl (pH 7.8/10 mM $MgCl_2$ containing spermidine (1 mg/ml), heated to 85°C, and then cooled to 20°C at 0.1°C per min. The solutions then were made 10 mM in dithiothreitol and 3

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Abbreviations: EGF, epidermal growth factor; hEGF, human EGF; mEGF, mouse EGF; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_4furan, tetrahydrofuran; ADH, alcohol dehydrogenase; bp, base pair.

Table 1. Oligonucleotide synthesis procedure

Step	Reagent	Vol, ml	Time, min	Comments
1	$CHCl_2COOH$ (5%, wt/vol) in CH_2Cl_2	1	2	Shaken
2	Dry CH_3CN	3	0.5	Under argon
3	Amidite (60 μmol) in 0.33 M tetrazole in dry CH_3CN	0.75	2	Under argon
4	CH_3CN	3	0.5	
5	Acetic anhydride (50 μl) in 6.5% *N,N*-dimethylaminopyridine in 2,6-lutidine/H_4furan, 1:9 (vol/vol)	1	4	Shaken
6	H_4furan/2,6-lutidine/H_2O, 2:1:1 (vol/vol)	3	0.5	
7	I_2 (0.2 M) in H_4furan/2,6 lutidine/H_2O, 2:2:1 (vol/vol)	2	1	Shaken
8	CH_3CN	3	0.5	
9	CH_2Cl_2	2	0.5	

H_4furan, tetrahydrofuran.

mM in ATP, and T4 DNA ligase was added. After 2 hr at 20°C, the solutions were evaporated to dryness, and the single-stranded products of the ligation were isolated separately by 15% polyacrylamide gel electrophoresis under denaturing conditions. The desired fragments were visualized by UV shadowing and eluted as described (22). The appropriate fragments were ligated as above, digested with *Eco*RI, and purified on a 7% polyacrylamide gel under nondenaturing conditions. A 170-bp band was visualized by ethidium bromide staining, excised from the gel, electroeluted, and cloned in the *Eco*RI site of pBR328 (23). The sequence of the cloned gene was determined by the dideoxynucleotide method in bacteriophage M13 (24).

Fibroblast Receptor Binding Competition Assay for EGF. This method is based on the ability of both mEGF and EGF to compete with ^{125}I-labeled mEGF (^{125}I-mEGF) for binding sites on the plasma membrane of human foreskin fibroblasts (3). Standard curves were obtained by measuring the effects of increasing quantities of mEGF on the binding of a standard amount of ^{125}I-mEGF.

RESULTS AND DISCUSSION

Oligonucleotide Synthesis. Several advances in chemical methods used in the synthesis of DNA have been introduced recently. Letsinger and co-workers (25, 26) showed that phosphite triester intermediates could be used to synthesize oligodeoxyribonucleotides, thereby greatly increasing the rate and ease of the coupling procedure. Several groups have used solid-phase-supported nucleosides in oligonucleotide synthesis (27–29), and this has been adapted to phosphite triester chemistry (30–32). A significant problem incurred in the phosphite method has been the chemical lability of the phosphorochloridite intermediates initially investigated. The advent of relatively stable *N,N*-dialkylamino phosphites has largely circumvented this problem (18). We have utilized several modifications of the phosphite-coupling procedures (18–20, 30, 33), which increase the rate and yield of the synthetic process, permitting the synthesis of larger sequences than has been previously practical.

Use of $ZnBr_2$ for the detritylation of the dimethoxytrityl-blocked 5′ hydroxyl of the nucleoside bound to the support and for the subsequent deblocking of the growing oligonucleotide chain has been advocated because there is little associated depurination (20, 33, 34). We have found that, under the con-

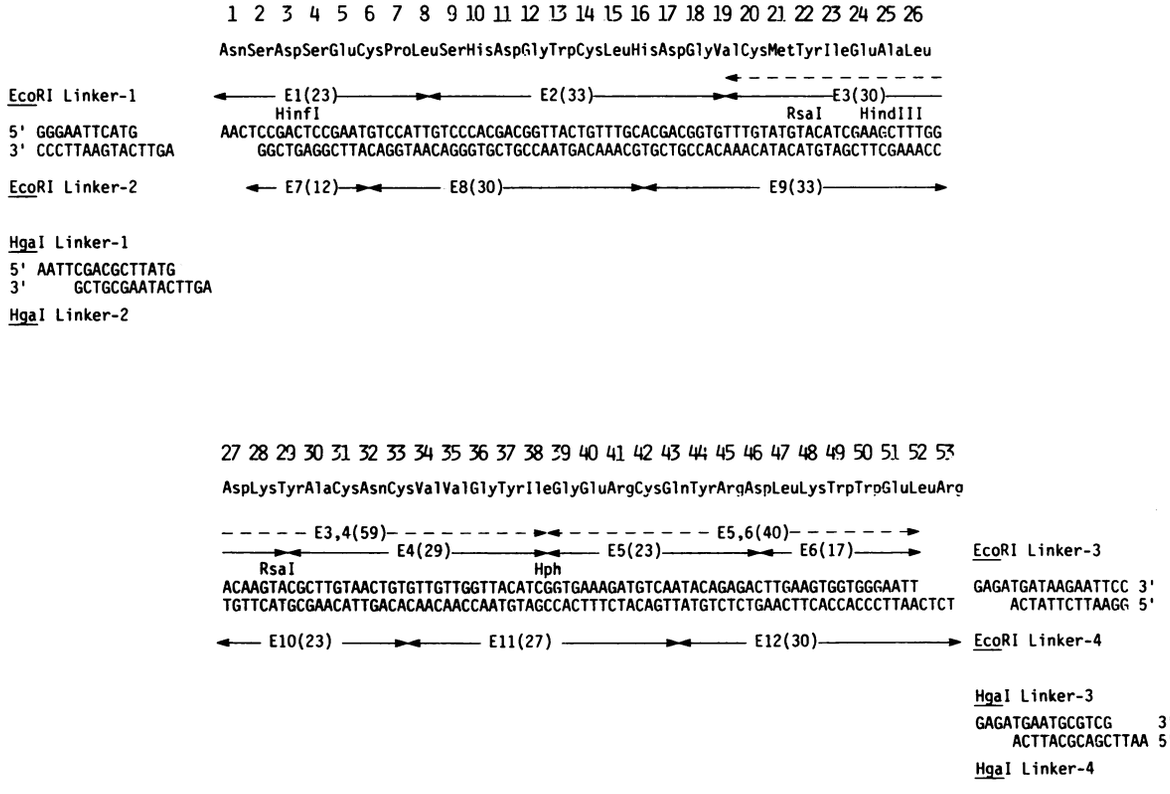

FIG. 1. The amino acid and nucleotide sequences of hEGF and the synthetic gene. Fragments E3,4 and E5,6 (---) were synthesized as single pieces directly as well as subfragments E3, E4, E5, and E6. Two different sets of linkers (*Eco*RI and *Hga* I linkers) were synthesized and used in two different gene constructions. Numbers in parenthesis indicate the length of oligomers in nucleotides.

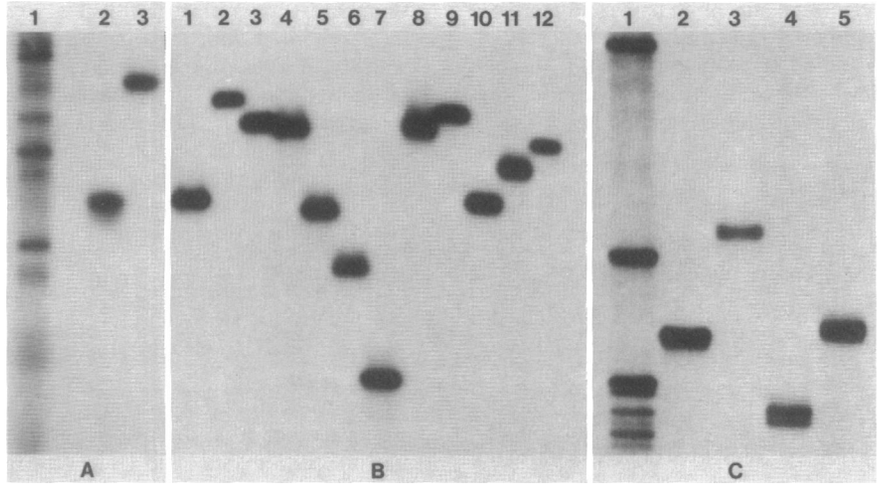

FIG. 2. Gel electrophoresis of purified synthetic oligonucleotides and ligation products. Unless otherwise indicated, ≈1–10 pmol of the fragments were 5′-phosphorylated with T4 polynucleotide kinase, [γ-^{32}P]ATP (10 μCi; 1 Ci = 37 GBq) and analyzed in 20% polyacrylamide gels under denaturing conditions. Autoradiography was performed at room temperature for 0.5–12 hr. (*A*) ^{32}P-Labeled *Sau*3A fragments of pBR322 (lane 1), E5,6 (40 mer, lane 2), and E3,4 (59 mer, lane 3). The most prominent *Sau*3A size markers (lane 1) correspond to 75, 46, and 36 bases in length. (*B*) Fragments E1–E12 (lanes 1–12, respectively; see Fig. 1 for sizes). (*C*) ^{32}P-Labeled *Hae* II-digested pBR322 (lane 1) and ligation products A (67 mer, lane 2), B (90 mer, lane 3). C (57 mer, lane 4), and F (70 mer, lane 5). Ligation products were labeled with [α-^{32}P]cordycepin triphosphate and terminal transferase. The lengths of the most prominent *Hae* II size markers (lane 1) correspond to 181, 83, and 60 bases.

ditions described by previous workers (20, 34), the detritylation step is incomplete. This was evident from the finding that, upon treating the washed $ZnBr_2$-detritylated support with 5% trichloroacetic acid in dichloromethane, 5–10% further detritylation took place. Use of dichloroacetic acid has alleviated these problems (35). For example, by using a support-bound protected tetramer of sequence 5′ A-T-C-T 3′, the release of *N*-6-benzoyladenine, monitored at 292 nm for several days, showed a first-order rate constant of $3.80 \pm 0.04 \times 10^{-4}$ min^{-1} (data not shown). This corresponds to a $t_{1/2}$ of 30.4 ± 0.3 hr.

We found that capping of failure sequences is important for synthesis of oligomers larger than ≈10 nucleotides. Despite high yields at each step, the population of N-1 oligomers generated from incomplete coupling becomes significant. At approximately the 20-mer stage, without capping the N-1 population can be equally represented along with the desired product, even with >95% efficiency per coupling step. If the detritylation step is quantitative throughout the synthesis, then efficient capping will eliminate all N-1 sequences except the one generated by incomplete coupling on the last step. This greatly simplifies purification. The acetylation of the 5′ hydroxyl is efficiently catalyzed by 4,4-dimethylaminopyridine, but upon standing, a solution containing both compounds discolors (19) and loses its reactivity. As a result we have stored and added these components separately (Table 1).

Our procedures (Table 1) have permitted us to synthesize sequences with a cycle time of 12 min (for one synthesis) to 20 min (for four simultaneous syntheses). Typically 0.1–1.0 mg of purified material is obtained after work-up from 50 mg of solid support (2.5–3.5 μmol of bound nucleoside).

Fig. 1 shows the amino acid sequence of hEGF and the nucleotide sequences synthesized. Oligonucleotides ranging from 11 to 59 bases were synthesized by phosphoramidite chemistry and purified by gel electrophoresis. In order to demonstrate

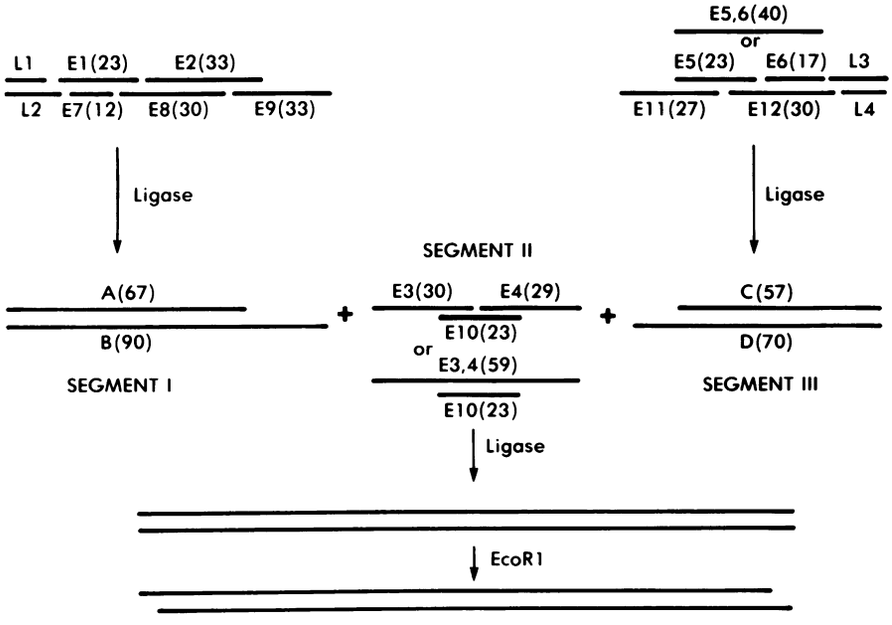

FIG. 3. Scheme for the ligation of the synthetic of hEGF gene. L-1, L-2, L-3, and L-4 refer to the *Eco*RI linkers as shown in Fig. 1.

the capability of producing fragments of 40 bases and more by these methods, we also synthesized and purified fragments E3,4 and E5,6 (Fig. 1) of 59 and 40 nucleotides in length, respectively. As described below, both were successfully used in the gene assembly in place of the smaller fragments, E3, E4, E5, and E6. Gel electrophoresis analysis of the purified fragments is shown in Fig. 2 *A* and *B*.

Gene Design. The gene was designed to maximize the overlaps among all the complementary fragments in order to facilitate faithful enzymatic ligation. At the ends of the gene were incorporated 4-bp overhangs to permit the attachment of specific linkers. The first set of linkers, designated *Eco*RI linkers, are shown in Fig. 1. These linkers include *Eco*RI restriction sites to facilitate direct cloning, an ATG start codon preceding the first asparagine codon, and two stop codons (TGA and TAA) after the terminal arginine codon. The second set of linkers, designated *Hga* I linkers (Fig. 1), similarly contains a start and stop codon and terminal *Eco*RI sites. *Hga* I restriction sites were incorporated at both ends of the gene so that upon cleavage with this enzyme, the entire coding region of the gene could be excised without start or termination codons. This can be achieved because *Hga* I cleaves at positions 5 and 10 bases away from the recognition site (36). Removal of the gene from a cloning vector in this manner permits, with the aid of specific linkers, the precise transposition of only the coding portion of the gene into other restriction sites or specific expression systems.

Because our intention was to obtain expression in yeast, the EGF gene was constructed with the set of preferred yeast codons inferred from codon usage in the highly expressed yeast genes for GAPDH, alcohol dehydrogenase (ADH) (37), and pyruvate kinase (38).

Gene Assembly. The gene, equipped with the *EcoRI* linkers, was assembled from three segments (Fig. 3). With the exception of *EcoRI* linkers 1 and 4, all of the fragments were preparatively 5′-phosphorylated with T4 polynucleotide kinase. The anticipated sizes of the single-stranded intermediates were verified by gel analysis (Fig. 2*C*). C(57) was constructed by combining *EcoRI* linker 3 with E5 and E6 or with E5,6. The latter derivative is presented in Fig. 2*C* and was used to produce the complete *EcoRI* linker-equipped gene. There are two bands present in lane 4 of Fig. 2*C* representing C(57) because the ligation by-product of E11 and E12 in segment III was most likely copurified.

Segments I and III were mixed in approximately equimolar quantities with the 5′-phosphorylated oligomers E3, E4, and E10 and ligated. After *EcoRI* digestion and gel electrophoresis, a 170-bp band was isolated.

In a similar manner, the *EcoRI* linker-equipped gene containing the 59-mer (E3,4) was constructed and cloned. Another construct containing the *Hga* I linkers (Fig. 1) also was assembled and cloned in bacteria as described below.

Cloning of the Synthetic EGF Gene in Bacteria. The 170-bp DNA band was ligated to pBR328 (23), which had been linearized with *EcoRI* and treated with calf intestine alkaline phosphatase. The ligation mix was used to transform competent *E. coli* HB101 cells and recombinant clones selected by their resistance to ampicillin and sensitivity to chloramphenicol. A 170-bp *EcoRI* fragment from one plasmid (p328EGF-1) was isolated by gel electrophoresis; its sequence was identical to the sequence designed for the synthetic EGF gene.

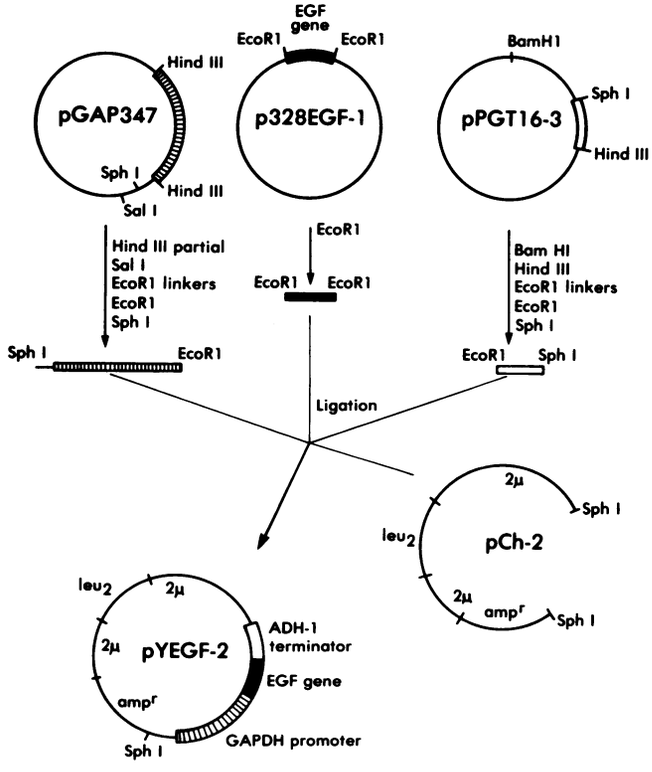

FIG. 4. Construction of plasmids for the expression of hEGF in yeast. pGAP347 [20 μg; a plasmid containing a promoter fragment of the gene for GAPDH, from base −1063 to base −3 upstream of the methionine initiator codon (39)] was digested to completion with *Sal* I and partially with *Hin*dIII. A 1,800-bp *Hin*dIII–*Sal* I fragment was isolated, filled in with deoxynucleotides by using the Klenow fragment of DNA polymerase I, ligated to an excess of *Eco*RI linkers, and digested with *Sph* I and *Eco*RI. A 1,700-bp *Sph*I–*Eco*RI fragment was isolated by gel electrophoresis. pBR328 EGF-1 was digested with *Eco*RI, and the expected 170-bp *Eco*RI fragment (1 μg) was isolated by gel electrophoresis. pPGT16-3 (20 μg; a plasmid containing the terminator region of the yeast gene for ADH-1 cloned in pBR322) was digested with *Bam*HI and *Hin*dIII. A 570-bp fragment was isolated by gel electrophoresis, filled in as described above, ligated to *Eco*RI linkers, and digested with *Sph* I and *Eco*RI. An *Eco*RI–*Sph* I fragment of 380-bp was isolated by gel electrophoresis; 25 ng of the synthetic EGF gene *Eco*RI fragment, 200 ng of the *Sph* I yeast GAPDH promoter fragment, and 55 ng of the *Eco*RI-*Sph* I yeast ADH-1 terminator fragment were ligated together in the presence of T4 DNA ligase, digested with *Sph* I, and ligated to 50 ng of Sph I-digested pCh-2 [a derivative of pJDB219 (40)]. The mixture was used to transform *E. coli* HB101 cells. DNA from a selected clone (pYEGF-2), in which the promoter, gene, and terminator were in the correct relative orientation, was prepared and used to transform yeast GM3C2 cells. Transformants were selected by their leu phenotype.

Table 2. Receptor binding activity in hEGF in yeast extracts

Nature of competitor	Amount	^{125}I-mEGF bound to receptor, cpm
No competitor		4,200
Lysis buffer	10 μl	3,500
mEGF	0.5 ng	2,500
	2 ng	1,350
	4 ng	900
	8 ng	500
Extract from GM3C2	10 μl	2,500
cells (control)	5 μl	2,550
Extract from GM3C2	10 μl	507
cells transformed	5 μl	870
with pYEGF-2		

Yeast cells were pelleted, washed with lysis buffer (10 mM phosphate, pH 7.5/0.1% Triton X-100), suspended in 1 vol of the same buffer, and broken by mixing with 1 vol of glass beads. The receptor binding competition assay was carried out as reported by Savage *et al.* (3) with ^{125}I-mEGF tracer from Amersham and standard mEGF from Collaborative Research (Waltham, MA). Each cpm value is the average of three independent assays. Consistently similar results have been obtained in repeated experiments.

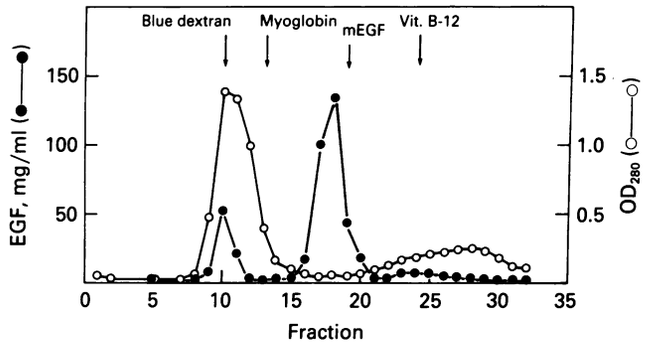

FIG. 5. Gel filtration of yeast extracts containing EGF activity. Cells from cultures of yeast GM3C2 transformed with pYEGF-2 were pelleted, washed with 10 mM phosphate buffer (pH 7.5)/0.1% Triton X-100, suspended in the same buffer, and broken by mixing with glass beads. After centrifugation, a 0.1-ml aliquot was loaded on a 1 × 14 cm column of Bio-Gel P-30. The column was equilibrated and eluted with phosphate/saline buffer, pH 7.3. Fractions of 0.5 ml were collected and assayed for protein and EGF receptor binding activity.

Expression of hEGF in Yeast. We constructed a plasmid containing the yeast GAPDH promoter, hEGF gene, and yeast ADH-1 terminator in tandem. This genetic unit was inserted into a yeast plasmid vector that contains the yeast 2-μm sequences, a DNA fragment containing the yeast *leu* coding sequences, and a fragment of pBR322 containing the origin of replication and the ampicillin resistance gene (pYEGF-2, Fig. 4). The vector gene system used in this experiment should direct the synthesis of a 54-amino-acid hEGF derivative containing an extra NH_2-terminal methionine residue ($M_r \approx 6{,}000$). The portions of the genes for GAPDH and ADH-1 used in this construction do not contain their amino acid coding regions and, therefore, this construction does not result in a fusion product with any portion of either protein. Extracts from mid-logarithmic-phase cultures of cells transformed with pYEGF-2 were assayed by the receptor competition binding assay (Table 2). Preliminary expression experiments indicated that extracts prepared from 1-liter cultures of yeast ($OD_{650} \cong 2$) contained $\approx$30 μg of EGF. Fig. 5 shows that most of the hEGF activity was retained on a Bio-Gel P-30 column migrating between myoglobin ($M_r = 16{,}800$) and vitamin B-12 ($M_r = 1{,}160$), slightly ahead of a mEGF standard, consistent with the expected M_r of the biosynthetic product. A more detailed account of the structure, including amino acid sequence data of the hEGF synthesized from the synthetic gene in yeast will be published elsewhere.

Conclusions. Considering the difficulty in working with human salivary or duodenal tissue, we chose to synthesize the hEGF gene chemically as opposed to obtaining it by cDNA cloning techniques. With our methods for DNA synthesis and purification, we have shown that large fragments can be synthesized rapidly and used to produce biologically active genes. By placing the synthetic hEGF gene under control of a yeast promoter, we were able to express a product of the predicted size that possesses EGF receptor binding activity. Biosynthetic hEGF produced from this synthetic gene also elicits the biological activities characteristic of EGF from human origins—e.g., precocious eyelid opening and incisor eruption in newborn mice and inhibition of gastric acid secretion (unpublished data).

Approaches demonstrated here have provided an alternative means for obtaining significant amounts of hEGF, thus permitting detailed studies of the pharmacological properties of this scarce hormone. The potential therapeutic value of this material in the treatment of duodenal ulceration and the promotion of wound healing can now be assessed more easily.

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