A large fragment approach to DNA synthesis: total synthesis of a gene for the protease inhibitor eglin c from the leech *Hirudo medicinalis* and its expression in *E. coli*

Hans Rink, Manfred Liersch, Peter Sieber and François Meyer

Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Received 25 June 1984; Revised and Accepted 1 August 1984

ABSTRACT

A DNA containing the coding sequence for the proteinase inhibitor protein, eglin c, from the leech Hirudo medicinalis has been obtained by enzymatic assembly of chemically synthesized DNA fragments. The synthetic gene consists of a 232 base-pair fragment containing initiation and termination codon signals with restriction enzyme recognition sites conveniently placed for cloning into a plasmid vector. Only six oligonucleotides from 34 to 61 bases in length, sharing pairwise stretches of complementary regions at their 3'-termini, were prepared by phosphotriester solid-phase synthesis. The oligomers were annealed pairwise and converted into double stranded DNA fragments by DNA polymerase I mediated repair synthesis. The fragments were assembled by ligation, and the synthetic gene was expressed in high yield in E. coli under the transcriptional control of the E. coli tryptophan promoter. The expression product was purified to homogeneity and was shown to have similar physicochemical and identical biological properties as the authentic protein isolated from the leech.

INTRODUCTION

Eglin c, a proteinase inhibitor which has been isolated from the leech Hirudo medicinalis (1), is a strong and specific inhibitor of the human granulocytic proteinases elastase and cathepsin G (1) with an association rate constant of the same order of magnitude as described for the endogenous inhibitors α2-macroglobulin and α1-antitrypsin (2). Eglin c has been shown to protect hamsters from the elastase-induced pulmonary emphysema, when administered intratracheally one hour before the insult (G.L. Snider, Boston University, School of Medicine, Boston MA, U.S.A., personal communication). This polypeptide might therefore be of potential therapeutic value in both local and generalized inflammatory processes, e.g.

emphysema and septicaemia. In order to demonstrate the therapeutic usefulness of the exogenous proteinase inhibitor additional animal studies are required. Preclinical research, however, has been hampered by the fact that only 10 to 20 µg of eglin c can be purified from one leech. We have therefore considered the use of recombinant DNA for the production of eglin c. On the basis of the amino acid sequence of eglin c (3,4) we designed a 232 bp long DNA fragment which encodes the 70-amino-acid protein (MW:8100) and is amenable to total chemical synthesis.

All the genes synthesized so far have been constructed by assembling single-stranded fragments of 10 to 20 nucleotides in lenght (5,6,7,8). This process requires the ligation of a correspondingly large number of oligonucleotides, which results in a complex assembly strategy and low duplex recovery (8). Furthermore, the genetic information for both strands has to be synthesized chemically. An alternative strategy based on a combined chemical and enzymatic approach has been described recently (9). Basically, this procedure involves the chemical synthesis of large single-stranded DNA fragments (more than 30 nucleotides) which share a short stretch of complementary sequences at their 3'-termini. After annealing and in vitro repair with DNA polymerase I, a full double-stranded product is obtained.

We now report the application of this approach to the assembly of an eglin c gene from only six synthetic fragments, its expression in E. coli and the partial characterization of the gene product.

MATERIALS

Analytical-grade reagents were used for the chemical synthesis of the oligonucleotides. Pyridine was dried by refluxing with calcium hydride and then distilled. Pyridine and tetrahydrofuran were stored over Molecular sieve 4 A. Kieselgel 60 (No. 9385, 230-400 mesh) from Merck, Darmstadt, was used for the preparative purification of intermediates. Pre-coated silica gel 60 F-254 plates (Merck, Darmstadt) were used for thin-layer chromatography. HPLC chromatography of

the oligonucleotides was performed on a Spectra Physics SP8700 apparatus on PRP-1 (Hamilton) or µBondapack C18 reverse-phase columns (250x4.6 mm). Proteins were analyzed by HPLC on a Kontron Uvicon 720 LC (15 mm cell), 2 Model pump 410, programme Model 200 with a Shimadzu CR 1B integrator on Vydac 218 TP B5, 330 A pore size, columns (250x4.6 mmm).
Oligonucleotides were synthesized on a self-constructed synthesizer, which consists mainly of a microprocessor-controlled solvent-delivery system, equipped with an HPLC-pump and a device for manual injection of the coupling reaction mixture. To prevent backpressure problems with the swelling and shrinking resin during the washing procedures, a specially dimensioned cylindrical reaction vessel was designed (8 mm in length, 7 mm in diameter). Materials used were stainless steel, glass and teflon. A more complete description of the synthesizer will be published elsewhere.
Bacterial strains and media: E. coli K12 strain LM 1035 was kindly provided by Dr. N. Waller (Friedrich Miescher Institute, Basel, Switzerland). L-Broth was as described (10) and M9-medium contained $Na_2HPO_4.2H_2O$ 7 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, D(+) glucose 5 g, $CaCl_2.H_2O$ 0.02 g, $MgSO_4.H_2O$ 0.25 g, Casamino acids 2.5 g, thiamine 0.05 mg per liter of water.
Enzymes and reagents: Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs., DNA polymerase I (Klenow) and calf intestinal alkaline phosphatase were from Boehringer. T4 polynucleotide kinase and dGTP, dCTP, dATP and dTTP were obtained from P & L Biochemicals. [γ-^{32}P]ATP was obtained from Amersham International.

METHODS

General Methods

DNA sequencing was performed by the method of Maxam and Gilbert (11). In situ colony hybridisations were performed by the Grunstein-Hogness procedure (12). Transformation and preparation of competent cells were as described (13). Competent cells were stored in 50 mM $CaCl_2$, 20% glycerol at -80°C. Transformations were performed by adding 1 - 10 µl

ligation mixture to a transfection mixture containing 50 µl 10 mM $CaCl_2$, 10 mM $MgCl_2$, 10mM Tris-HCl (pH 7.5) and 150 µl of a suspension of competent E. coli. The transformants were plated on McConkey agar plates containing 50 µg/ml ampicillin (Sigma) or 10 µg/ml tetracycline (Sigma) for selection. Plasmid DNA was prepared according to the method of J. Ecsödi and C. Weissmann (personal communication), a modification of the procedure of Currier and Nester (14). DNA fragments were isolated from polyacrylamide gels by electroelution (15) and from agarose gels by electrophoresis on 1% low-melting agarose gels (low-melt agarose, Sigma). Aliquots of the gel slices were melted at 65°C and used directly for the ligation reactions (16). Ligations were performed (except when stated otherwise) in 20 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM ATP at 15°C for 2-16 hours, using a concentration of 5-10 pmole/ml for Vector DNA fragments. Protein concentrations were determined according to Lowry (17). Restriction enzymes were used as suggested by the suppliers except that bovine serum albumin was replaced by gelatin.

Synthesis of oligonucleotides

The 5'-monomethoxytrityl (MMT) derivatives of N-benzoyl-2'-deoxy-adenosine, N-benzoyl-2'-deoxycytidine, N-iso-butyryl-2'-deoxyguanosine and thymidine were prepared as described (18). The protected trimers were synthesized in gram amounts using 2-chlorophenyl-di-(1-benzo-triazolyl)-phosphate as phosphorylating reagent and the cyanoethyl residue for full protection of the terminal 3'-phosphate group (19). The trimers and intermediates were purified on silica-columns with dichloromethane-methanol gradients and their purity was checked by thin layer chromatography on silica. Immediately prior to solid phase synthesis all trimers were simultaneously decyanoethylated as follows. About 10 µmole protected trimers were treated with 60 µl pyridine-acetonitrile-triethylamine (1:1:1) at room temperature for 1 hr in Eppendorf tubes and the salts precipitated with 0.7 ml ether each. After centrifugation the salts were dissolved in 50 µl dry pyridine, reprecipitated and dried

in vacuo in a centrifuge. Aminomethyl-polystyrene (1% cross-linked) was substituted with the appropriate base protected 5'-O-MMT-2'deoxriboynucleoside derivative via a 3'-O-succinamido linkage (50-80 µmole/g) according to described methods (20). The excess of amino groups was acetylated. In a typical synthesis, 20 mg resin (about 1 µmole functionality) in a 300 µl reaction vessel were washed sequentially (flow rate: 2ml/min) with dichloromethane (5 min), dichloromethane-isopropanol (85:15) (2 min), 1 M $ZnBr_2$, 0.02 M 1,2,4-triazole in dichloromethane-isopropanol (85:15) (1.5-3 min) to remove the MMT-groups, dichloromethane-isopropanol (85:15), 0.5 M triethylammonium-acetate in dimethylformamide (10 min), dry pyridine (5 min), dry tetrahydrofurane (peroxid free) (5 min), dry nitrogen (10 min). 10 equivalents of dry trimer salt were dissolved in a solution of 30 equivalents mesitylenesulfonyl-3-nitro-1,2,4-triazolide (21) in 160 µl pyridine under dry argon and manually injected into the reaction cell and coupled for 30 min at 40°C, followed by washings with pyridine (5 min), 5% acetanhydride, 2.5% 4-dimethylaminopyridine in pyridine (5 min) for capping, pyridine (5 min) and pyridine-isopropanol (1:1) (3 min). This cycle was repeated with the appropriate trimers until the desired sequence was completed. The oligonucleotide was cleaved from the resin and the protecting groups (except MMT) removed by sequentially reacting with 1 M tetramethylguanidinium 2-nitrobenzaldoximate (22) in 300 µl 95% pyridine (3 h, 50°C and 12 hr, room temperature) and 1.6 ml 33% ammonia (24 hr, 50°C). After filtration through Biogel P6, aliquots were purified by reverse phase HPLC. Columns were eluted at a flow rate of 1 ml/min with an acetonitrile gradient (15%-30%) in .05 M triethylammonium acetate pH 7, over a period of 20 min at 50°C. The most hydrophilic peak of the MMt containing set of oligomers was collected (Fig. 2) (23). The MMT group was removed by treating with 80% aqueous acetic acid for 45 min at room temperature. After lyophilisation, samples were purified by electrophoresis on a 8% denaturing polyacrylamide gel (7 M urea) (11), electroeluted (15) and the DNA fragments were concentrated by ethanol precipitation (0.1-1 OD).

Fragment assembly

100 pmole of each of the fragments 2,3,5 and 6 were phosphorylated with T4 polynucleotide kinase and [γ-^{32}P] ATP (1 µCi/100 pmole) and concentrated by ethanol precipitation. The duplexes I, II and B were constructed by incubating 50 pmole of each oligomer pair in 24 µl of water at 90°C for 3 min, cooling to 12°C within 5 min and adjusting the solution to 10 mM Tris-HCl pH 7.5, 6.6 mM $MgCl_2$, 6.6 mM β-mercaptoethanol, 60 mM NaCl, 0.5 mM of each of the four deoxyribonucleoside triphosphates in 40 µl. 20 units DNA polymerase I (Klenow) were added and the samples were incubated for 30 min at 12°C. The reactions were terminated by heat inactivation (90°C, 3 min) and aliquots were examined on 8% polyacrylamide gels in the presence or the absence of 7 M urea (11). The reaction mixture containing fragment B was phenol extracted ethanol precipitated and digested with BamHI. The mixtures containing fragments I and II were incubated with 6 units of T4 DNA ligase (Boehringer) in 60 µl volume, adjusted to 66 mM Tris-HCl pH 7.5, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM ATP and incubated for 21 hr at 20°C. After heat inactivation (5 min, 70°C), phenol extraction and ethanol precipitation the ligation mixture wasfractionated on an 8% polyacrylamide gel and the full-size DNA fragment A was excised.

Cloning and expression

Subcloning of fragment A and B into pBR322: pBR 322 DNA (3 µg) was digested with EcoRI and BalI and the fragments fractionated through a 1 % agarose gel in 40 mM Tris-acetate pH 7.8, 1 mM EDTA. The larger fragment (2.9 kb) was isolated and 0.3 pmole were ligated to the EcoRI-cleaved fragment A (127bp) (0.84 pmol). The reaction mixture was used to transform E. coli HB 101, and colonies were screened by colony hybridization using ^{32}P-labelled fragment 4 as a hybridization probe. Plasmid DNA was isolated from positive colonies and subjected to restriction analysis with EcoRI and HpaII. One of the recombinant plasmids, which contained the desired 127 bp EcoRI-HpaII restriction fragment, pML 87 was cleaved with EcoRI and HpaII, the digest was fractionated on

an 8 % polyacrylamide gel (24) and the 127 bp fragment (fragment C) was isolated by electroelution. Fragment D was obtained similarly. The cloning vector was the large fragment (3.7 kb) from NruIand BamHI-cleaved pBR322. The colonies were screened by using ^{32}P-labelled fragment 6 as a hybridization probe. The 103-bp fragment was excised from pML 136 with BamHI and HpaII.

Construction of plasmid pHR 148 carrying the trp promoter: As a source of the E. coli trp promoter we used the plasmid pGM 91, which was obtained from Dr. G. Miozzari. pGM 91 is derived from pGM 1 (25) (G. Miozzari, personal communication). pGM 91 was digested with EcoRI and BglII. The digest was fractionated on a 1 % low-melting agarose gel in 40 mM Tris-HCl pH 7.8, 1 mM EDTA. The 0.3-kb EcoRI-BglII fragment containing the trp POL region was isolated and subjected to partial TaqI digestion. The resulting 283-bp fragment, which comprised the promotor-operator region and the SD sequence of the trp leader, but lacked the ATG initiation codon, was cloned into the EcoRI and the ClaI sites of pBR322 to form plasmid pFM 159. pFM 159 was cleaved with EcoRI and the EcoRI site was filled in with E. coli polymerase I (Klenow) in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 mM NaCl and 0.1 mM dTTP, dATP at 12°C for 15 min. After heat inactivation (85°C, 5 min), the plasmid DNA was recircularized with T4 DNA ligase and transformed into HB 101 to generate plasmid pHR 145. The EcoRI-resistant plasmid pHR 145 was cleaved with ClaI. The ends were filled in with E. coli DNA polymerase I (Klenow), and after phosphatase treatment a multirestriction site-linker (GAATTCCATGGTACCATGGAATTC) was inserted by ligation. After heat inactivation and KpnI digestion, the mixture was fractionated on a 1% low-melting agarose gel. The linear 4.7-kb DNA fragment was isolated, and after religation the DNA was transfected into HB101 to yield plasmid pHR 148.

Construction of pML 147: Plasmid pHR 148 was digested with EcoRI and BamHI. The digest was treated with calf intestinal alkaline phosphatase, subjected to electrophoresis on a 1 % agarose gel (low-melting) and the large (4.3-kb) fragment

vector DNA was isolated. 10 ng each of fragments C and D and were incubated with T4 DNA ligase, and after heat inactivation (5 min, 85°C) the ligation mixture was digested with EcoRI and BamHI. After heat inactivation, the mixture was ligated to 50 ng of vector DNA and transfected into E. coli LM 1035. Plasmid DNAs were isolated from transformants and digested with EcoRI, BamHI and HpaI. Plasmid pML 147 was selected.

Purification of recombinant eglin c

150 ml cultures of transformed E. coli pML 147 were grown in M9 medium containing 100 µg/ml ampicillin to OD 650 of about 1. The cells were centrifuged, washed and resuspended in 1/10 of the original volume of 50 mM Tris-HCl pH 8.0, 30 mM NaCl. Lysozyme was added to 1 mg/ml, and after 30 min at 0°C the cell suspensions were frozen and thawed four times and centrifuged at 16000 r.p.m. for 30 min at 4°C. Solid ammonium sulfate was added to 65 % saturation of the supernatants, which contained about 1 mg/ml protein. The pellets were collected by centrifugation, dissolved in 50 mM Tris-HCl pH 8.0 and dialyzed against the same buffer. The crude extract (16 mg protein in 1.2 ml) was applied at 7 ml/h on an immunoadsorbent column (4.5 x 0.5 cm, 19 mg of purified monoclonal antibody F-299-20-10 was coupled to Affi-Gel 10) equilibrated with the same buffer. Similarly, an affinity column (4.5 x 0.5 cm) containing anhydrochymotrypsin coupled to Affi-gel as described (26) was used. The columns were washed with 10 volumes of 50 mM Tris-HCl pH 8.0. About 95 % of the proteins applied to the column were collected in the flow-through fractions. The recombinant eglin c was eluted with a gradient from 0-5 M NaSCN, the fractions containing pure material (.15 mg) were pooled and dialyzed against 0.5 mM Tris-HCl pH 7.5, lyophilized and redissolved in water at a concentration of 0.2 mg/ml. Aliquots from the crude extract and from the purified fractions were analyzed on a 15% polyacrylamide gel in SDS (Fig. 5). HPLC analysis of crude bacterial lysates and purified proteins (Fig. 6) were performed on Vydac 218 TP B5 (330 A) using the following gradient at a flow rate of 1.2 ml/min. A: .1 % TFA

(trifluoroacetic acid), B: acetonitrile-water (8:2) + .07% TFA. Gradient: 1 min 30 % B, 14 min 30 % - 70 % B.

HLE Assay

The enzyme activity of human leucocyte elastase (HLE) (0.05 µM) and the elastase - inhibiting activity were measured at 37°C with methoxy-succinyl-L-Ala-L-Ala-L-Pro-L-Val-p-nitroanilide (0.5 mM) (Bachem AG) as a substrate according to (1). The substrate hydrolysis was determined at 405 nm in a final volume of 1 ml of 0.2 Tris-HCl pH 7.5 containing 100 µg/ml BSA.

RESULTS AND DISCUSSIONS

Design of the gene

The structure of the synthetic gene is outlined in Fig. 1. The coding sequence corresponding to the most abundant tRNAs from E. coli (27) was used, except for the codons for amino acids 3,11,42 and 58, in which potential EcoRI- and HpaII-sites were eliminated (Fig. 1). The sequence was provided with an ATG at the 5'-end and a TAG at the 3'-end. An HpaII site at position 38 facilitates the assembly of parts A and B

Figure 1: Nucleotide and amino acid sequence of the synthetic eglin c gene. Chemically synthesized oligonucleotides (1-6) are arrowed. Numbers refer to amino acid position.

(Fig. 4), and the flanking EcoRI and BamHI restriction sites allow the subsequent insertion of the fragments in an appropriate cloning vector. For the gene synthesis the two strands were subdivided into 6 oligomers (34 to 61 nucleotides long) with pairwise overlaps of 10 to 13 bases (Fig. 1). A computer analysis showed that with such large overlaps, there is little probability of the appearance of complementarities which could interfere with correct annealing.

Chemical synthesis of the oligomers

It has been demonstrated that both, the phosphoramidite method (28,29) and the phosphotriester assembly of presynthesized oligonucleotide blocks (30) are applicable to the solid-phase synthesis of relatively large DNA fragments. However, the former method has the drawback of accumulating 4.5 times more chemical steps to assemble a fragment than the trimer triester approach. This can strongly affect the overall yield of long fragments and prevent the isolation of the desired product in sufficient amounts. We therefore opted for the triester approach and prepared a library of trimers by means of the effective triester hydroxybenzotriazolide method described by van Boom (19) and assembled the blocks on polystyrene (20). The purity of the blocks is most important for successful solid phase assembly, and the presence of free 5'-hydroxy contaminants especially must be avoided. We therefore replaced the widely used dimethoxytrityl group by the more stable monomethoxytrityl (MMT) group for 5'-hydroxy protection. This simplified the chromatographic purification of the intermediates on silica columns and also allowed the trimers to be stored for at least two years at -20°C. In the course of the solid-phase synthesis, the increased stability of the MMT residue against acidolysis is not a serious disadvantage, since we found that the zinc bromide cleavage conditions described earlier (20,31,32) can be improved significantly by adding the trityl scavenger 1,2,4-triazole. Using a continuous flow mode, this process is fast at room temperature (1.5-3 min) and does not result in significant debenzoylation or depurination of the growing chain.

Immediately before synthesis, the appropriate trimers were

simultaneously decyanoethylated and the acrylonitrile removed by ether precipitation using a fast parallel work-up procedure instead of the pyridine coevaporation processes described earlier (6,20).

The washing procedures were performed by a modification of the protocol reported by Ito et al. (20). The processing times, however, were not optimized.

The application of the semiautomatic synthesizer generally gave more reproducible yields and fragments of higher quality than manual devices such as those described (20). It also allowed the scaling down of the synthesis to 0.5 - 2 µmole, which resulted in an economical use of the trimers, which are laborious to prepare. Usually 10 equivalents of these, providing a concentration of 0.05 - 0.1 M, were used for coupling in the presence of 30 equivalents of mesitylenesulfonyl-3-nitro-1,2,4-triazolide (21). Although coupling yields, derived from spectrophotometric monitoring of the cleaved MMT cation, are rather unreliable since the guanines can be phosphorylated to some extent at position 6 (33), we made an attempt to quantitate the reaction. At the end of syntheses of the 61-mers, about 50% of the initial MMT amount could still be recovered (an average of >95% yield per coupling) indicating that the approach can be applied to the synthesis of even longer fragments (oligonucleotides up to 67 bases have been prepared: H. Rink, unpublished results). However, we observed a tendency for yields to decrease with increasing size of the growing chain, which might reflect a less efficient MMT cleavage from large fragments (20).

HPLC purification of the partially deprotected fragments using the MMT group as a lipophilic handle as described by Dembek (23) was first performed on µBondapack C18. Later we preferred the more stable polystyrene (PRP-1, Hamilton) columns (recommended by K. Itakura, personal communication). An example, illustrating the HPLC profile of crude fragment 6 is shown in Fig. 2. The fragments were then further purified by polyacrylamide gel electrophoresis. The overall yields based on the initial polymer loading ranged from 0.3%

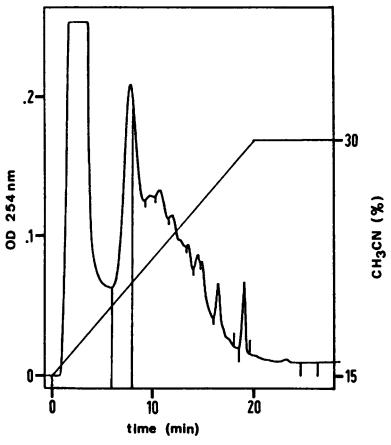

Figure 2: HPLC profile of crude fragment 6 on PRP-1. The indicated fraction containing the MMT-61-mer were collected.

(fragment 6) to 3% (fragment 3). An autoradiogram of the purified fragments, labelled by T4 polynucleotide kinase at their 5'-ends, is shown in Fig. 3. The correctness of their structures was confirmed by sequencing the gene after assembling and cloning.

Assembly of the gene fragments

The assembly was performed according to the scheme in Fig. 4. In order to prevent the formation of multimers upon ligation when constructing part A, only fragments 2 and 3 were phosphorylated. After annealing their 3' ends, the partial hybrids were converted into complete duplexes by the fill-in reaction of E. coli polymerase I (Klenow). The product was analysed on a denaturing 8 % polyacrylamide gel; approximately 5% of filled-in fragments were found to be full-size DNA as estimated by visual inspection of the autoradiogram.

Segments I and II were ligated to part A and the resulting duplex of the appropriate size (127 bp) was isolated (yield 2%) from a polyacrylamide gel by electroelution and amplified by subcloning in E. coli. Part B (109 bp) was prepared from fragments 5 and 6 and the mixture was subcloned without selection of the full-size DNA.

Fragments A and B were excised from the isolated recombinant plasmids, gel-purified and the nucleotide sequence of both fragments was determined by sequencing both strands; it was

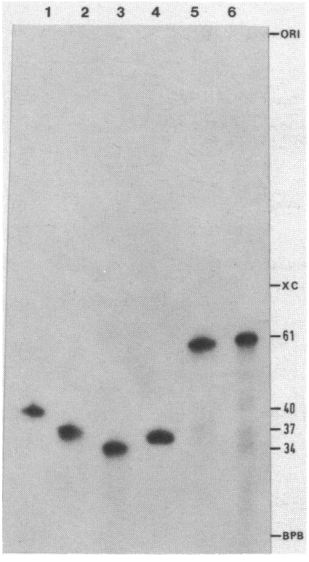

Figure 3: Autoradiogram of the purified fragments (lanes 1-6) on a 8 % denaturing polyacrylamid gel. Oligomer sizes are indicated.

found to be in complete agreement with the expected coding sequence.

Our results clearly demonstrate the usefulness of the triester methodology for the rapid and reliable synthesis of long DNA fragments and their applicability in the assembly of genes. Once a trimer library has been established, this approach dramatically reduces the number of oligonucleotides required in the chemical synthesis of a gene and the time involved, e.g. a 120-bp duplex can be obtained within 2 weeks.

Expression of recombinant eglin c in E. coli

The expression vector used contained the E. coli trp promoter, operator and trp leader ribosome binding site followed by the initiation codon ATG (34). A number of unique restriction sites, which can be used for insertion of heterologous genes have been located between the Shine-Dalgarno sequence and the initiator triplet (Fig. 4). The EcoRI and NcoI sites can be used to insert genes containing the translation initiation codons. Genes lacking the ATG can be inserted as well by cleaving the vector DNA with either NcoI or KpnI followed by conversion into blunt-ends with DNA polymerase I. In addition, double-stranded cDNA can be inserted upon cleavage with KpnI using

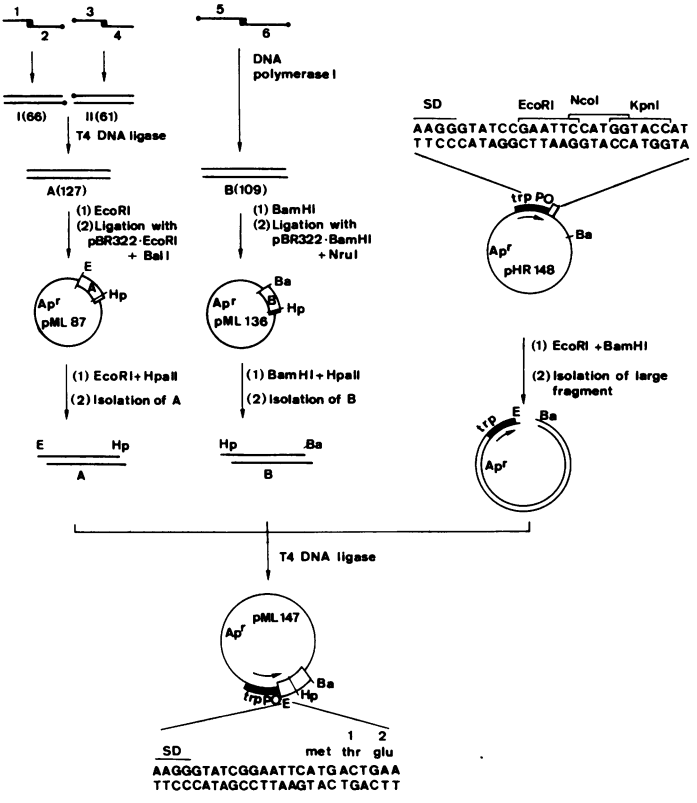

Figure 4: Construction of the synthetic eglin c gene and of recombinant plasmid containing the gene under trp promoter control.
5'-end phosphorylated oligonucleotides are marked with heavy dots (top left). The nucleotides between the Shine-Dalgarno (SD) sequence and the initiation triplet ATG are shown enlarged.
E: EcoRI. Ba: BamHI. HP: HpaII.

the standard homopolymeric tailing technique. If the translational reading frame coincides with the ATG initiation codon, the gene will be expressed in E. coli. An expression vector analogous to this has been recently described (35). The eglin c fragments A and B were ligated in a triple ligation reaction into EcoRI- and BamHI-cleaved vector DNA and the gene thereby positioned in the proper orientation for expression (Fig. 4). Plasmid DNA prepared from individual transformants were analyzed by restriction analysis and gave

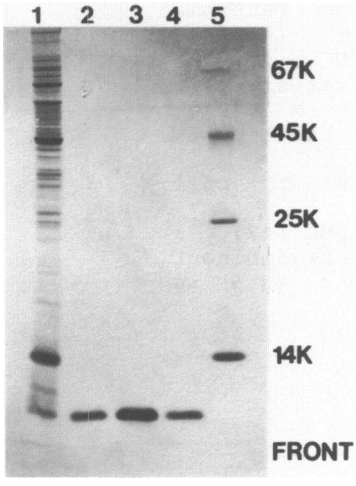

Figure 5: Analysis of recombinant and authentic eglin c on a 15 % SDS polyacrylamide gel.
1: E. coli (pML 147) lysate (60 µg crude extract).
2: recombinant eglin purified on a monoclonal antibody column (3.5 µg).
3: authentic eglin c (leech) (4.0 µg).
4: recombinant eglin c purified on an anhydrochymotrypsin column (4.0 µg).
5: MW:markers. The proteins were stained with coomassie blue.

the expected pattern. One of them (pML 147) was further analyzed, and the proper orientation of the inserted DNA was confirmed by determining the nucleotide sequence of the gene including the ribosome binding site.

12 transformants carrying the recombinant plasmids were assayed for eglin c activity using the human leucocyte elastase (HLE) inhibition assay (1). Since the majority of the transformants were positive and gave similar activities, only one was selected for further study (pML 147). The amounts of recombinant eglin c synthesized ($3x10^5$ molecules/cell) were considerably higher than we have obtained earlier for human mature lymphoblastoid interferon (α-8) (2.5×10^4 molecules/cell) using similar constructions in E. coli (F. Meyer, unpublished results). This remarkable difference might be due to the strong resistance of eglin c to denaturation and proteolytic degradation (36) rather than a higher efficiency in transcription and/or translation. The strains produce 200-300 mg/lt of fermentor culture (J.A.L. Auden, unpublished results).

Purification and partial characterization of recombinant eglin c

The recombinant eglin c was purified in a one-step procedure by immuno-affinity chromatography using monoclonal antibodies directed against authentic eglin c. Similarly, the

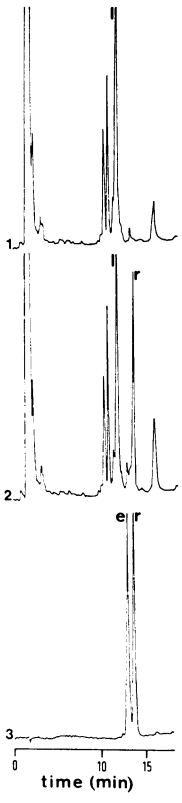

Figure 6: HPLC profile of recombinant and authentic eglin c (absorbance unit full scale, 220 nm: 0.2). 50 µl bacterial lysates were mixed with 50 µl 0.18 M acetic acid, centrifuged and the supernatants injected.
1: control lysate from E. coli (pHR 148).
2: lysate from E. coli (pML 147).
3: mixture of purified recombinant and authentic eglin c (2.5 µg each).
l :lysozyme
r :recombinant eglin c
e :authentic eglin c

polypeptide could be purified to homogeneity in a one-step procedure by affinity chromatography using anhydrochymotrypsin coupled to Affi-Gel. The recombinant and the authentic eglin c comigrated on SDS-polyacrylamide gels (Fig. 5). However, purified recombinant eglin c, when analyzed by reverse phase HPLC, showed a slightly higher retention time than authentic eglin c (Fig. 6). Since the E. coli gene product had an amino acid composition indistinguishable from authentic eglin c (data not shown), the possibility that the observed higher lipophilicity might be due to an additional methionine residue on the recombinant molecule was excluded. In fact, detailed physicochemical structural analysis of the purified recombinant polypeptide indicated that the amino group of the N-terminal threonine is blocked, suggesting that the gene product in E. coli is post-translationally modified

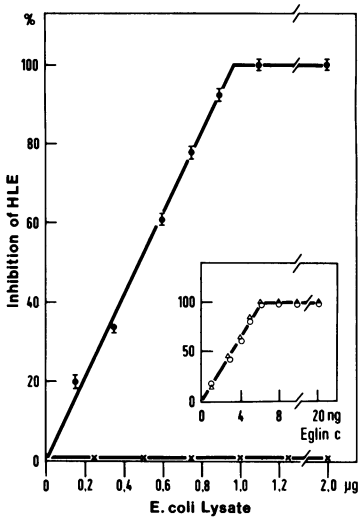

Figure 7: Inhibition of human leucocyte elastase by eglin c.
The bacterial lysates were adjusted with 0.2 M Tris-HCl pH 7.5, 100 µg/ml BSA to the indicated concentrations and assayed by using a chromogenic assay as described.
•: lysate from E. coli (pML 147)
x: control lysate from E. coli (pHR 148)
o: authentic eglin c
Δ: recombinant eglin c

(manuscript in preparation).
The biological activity in vitro, e.g. the inhibition of HLE (Fig. 7), cathepsin G and chymotrypsin (data not shown) by the recombinant and the authentic eglin c was identical.
The bacterially synthesized eglin c thus exhibits immunological and biological characteristics indistinguishable from the authentic eglin c isolated from the leech. The pharmacokinetics and the toxic effects of recombinant eglin c in animals are currently under investigation. Studies are under way to determine whether eglin c could be of therapeutic value.

ACKNOWLEDGEMENTS

We would like to thank E. Spieser, E. Manz, P. Leber, J. Bietenhader, B. Horvath, H. Richener and D. Zaninetta for skillful technical assistance, Dr. S. Alkan for preparation of the hybridomas, Dr. J.Y. Chang for amino acid analyses, Dr. W. Märki for analyzing the proteins by HPLC, Dr. H.P. Schnebli for providing us with HLE, Dr. H. Fritz (University of Munich) and the Plantorgan Werk KG (Bad Zwischenahn, FRG) for the generous gift of eglin c from the leech Hirudo medicinalis.

REFERENCES

1) Seemüller,U., Meier,M., Ohlsson,K., Müller,H.-P. & Fritz,H. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1105-1117.
2) Schnebli,H.P., Liersch,M., Virca,G.D., Bodmer,J.L., Snider,G.L., Lucey,E.C. and Stone,P.G. (1984) Europ. J. Resp. Dis. (in press).
3) Seemüller,U., Eulitz,M., Fritz,H. & Strobl,A. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1841-1846.
4) Knecht,R., Seemüller,U., Liersch,M., Fritz,H., Brown,D. & Chang, J.Y. (1983) J. Analyt. Biochem. 130, 65-71.
5) Khorana,H.G. (1979) Science 203, 614-625.
6) Edge,M.D., Greene,A.R., Heathcliffe,G.R., Meacock,P.A., Schuch,W., Scanlon, D.B., Atkinson,T.C., Newton,C.R., Markham,A.F. (1981) Nature 292, 756-762.
7) Smith,J., Cook,E., Fotheringham,I., Pheby,S., Derbyshire,R., Eaton,M.A.W., Doel,M., Lilley,D.M.J., Pardon,J.F., Patel,T., Lewis,H. and Bell,L.D. (1982) Nucl. Acids Res. 10, 4467-4482.
8) Edge,M.D., Greene,A.R., Heathcliffe,G.R., Moore,V.E., Faulkner,N.J., Camble,R., Petter,N.N., Trueman,P., Schuch,W., Hennam,J., Atkinson,T.C., Newton,C.R., Markham,A.F., (1983) Nucl. Acids Res. 11, 6419-6435.
9) Rossi,J.J., Kierzek,R., Huang,T., Walker,P.A. and Itakura K. (1982) The Journal of Biological Chemistry 257, 9226-9229.
10) Miller,J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, New York.
11) Maxam,A.M. & Gilbert,W. (1978) Proc. Natl.Acad.Sci. U.S.A. 74, 560-564.
12) Grunstein,M. and Hogness,D.S. (1975) Proc. Natl.Acad. Sci. U.S.A. 72, 3961-3965.
13) Cohen,S.N., Chang,A.C.Y. and Hsu,L. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2110-2114.
14) Currier,T.C. and Nester,E.W. (1979) Analyt. Biochem. 98, 305-309.
15) Smith,H.O. (1980) Meth. Enzymol. 65, 371-380.
16) Frischauf,A.M., Larroff,H. and Letrack,H. (1980) Nucl. Acids Res. 8, 5541-5549.
17) Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275.
18) Büchi,H. and Khorana,H.G. (1972) J. Mol. Biol. 72, 251-288.
19) Van Boom,J.H., Van der Marel,G.A., Van Boeckel,C.A.A., Wille,G. and Hoyng,C.F., Chemical and Enzymatic Synthesis of Gene Fragments, A Laboratory Manual, Edited by H.G. Gassen and Anne Lang, Verlag Chemie Weinheim/ Deerfield Beach, Florida/ Basel 1982.
20) Ito,H., Ike,Y., Ikuta,S. and Itakura,K. (1982) Nucl. Acids Res. 10, 1755-1769.
21) Jones,S.S., Rayner,B., Reese,C.B., Ubasawa,A., Ubasawa,M. (1980) Tetrahedron Lett. 36, 3075-3085.
22) Reese,C.B. and Zard,L. (1981) Nucl. Acids Res. 9, 4611-4626.
23) Dembek,P., Miyoshi,K., and Itakura,K. (1981) J. Am. Chem. Soc. 103, 706-708.

24) Peacock,A.C. and Dingman,C.W. (1967) Biochem. 6, 1818-1827.
25) Miozzari,G.F. and Yanofski,C. (1978) J. Bact. 133, 1457-1466.
26) Ako,H., Ryan,C.A. and Foster,R.J. (1972) Biochem. Biophys. Res. Comm. 46, 1639-1645.
27) Ikemura,T. (1981) J. of Mol. Biol. 151, 389-409.
28) Beaucage,S.L., Caruthers,M.H. (1981) Tetrahedron Lett. 22, 1859-1862.
29) Adams,S.P., Kavka,K.S., Wykes,E.J., Holder,S.B. and Galluppi,R.R. (1983) J. Am. Chem. Soc. 105, 661-663.
30) Miyoshi,K., Arentzen,R., Huang,T. and Itakura,K. (1980) Nucl. Acids Res. 8, 5507-5517.
31) Kohli,V., Blöcker,H. and Köster,H. (1980) Tetrahedron Lett. 21, 2683-2686.
32) Matteucchi,M.D. and Caruthers,M.H. (1980) Tetrahedron Lett.21, 3243-3246.
33) Reese,C.B. (1978) Tetrahedron 34, 3143-3179.
34) Goeddel,D.V., Shepard,H.M., Yelverton,E., Leung,D. and Crea,R. (1980) Nucl. Acids. Res. 8, 4057-4074.
35) Nishi,T., Sato,M., Saito,A., Itoh,S., Takaoka,C. and Taniguchi,T. (1983) DNA 2, 265-273.
36) Seemüller,U., Fritz,H., & Eulitz,M. in Methods in Enzymology, Vo. 80 Part C (Lorand, ed.), 804-816, Academic Press New York (1980).