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Chemical synthesis of a gene for somatomedin C

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ABSTRACT

A synthetic gene for somatomedin C, a human growth factor, has been assembled by a single ligation of 23 oligodeoxyribonucleotides, which were chemically synthesized by an improved solid phase phosphotriester method.

INTRODUCTION

Somatomedin C and insulin-like growth factor I (IGF-I) are different names for a single chain basic polypeptide consisting of 70 amino acid residues, the primary sequence (1,2) of which is shown in Figure 1. It has been purified to homogeneity from human plasma and characterised by several groups (3,4) and has recently been synthesised (5). The synthetic material was shown to be identical biologically and immunologically with the natural hormone isolated from human plasma (6). Somatomedin C is a member of a family of insulin-like peptides which are present in serum and are thought to mediate certain actions of growth hormone (7,8,9). In particular it is believed to mediate most of the growth promoting actions of somatotropin (1,2,10). The biochemistry and physiology of the somatomedin family are described elsewhere (11) and a review on hereditary somatomedin deficiency, which results in Laron-type dwarfism, has been published recently (12).

Somatomedin C was required for clinical purposes by the National Health Service (NHS), however its use has been restricted by the extreme rarity of the purified product. Even using chromatographic reprocessing techniques the yield of purified peptide is less than 1 mg from 100 liters of human plasma (4). Originally Rinderknecht and Humbel (13) obtained a mere 38 mg of IGF-I from 6,000 Kg of a human plasma Cohn fraction (precipitate

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B). Moreover, the yield of purified peptide from the solid phase synthesis was only about 1% (5). Methods have been developed for simultaneous synthesis of large numbers of oligodeoxyribonucleotides (14,15), making total gene synthesis feasible. The synthesis of peptide hormones using recombinant DNA techniques has been reviewed (16), and syntheses of human growth hormone gene (17) and of a gene for human epidermal growth factor urogastrone (18) have been reported.

We planned to synthesise a whole DNA duplex coding for somatomedin C employing optimal codons for abundant proteins in E.coli (19,20,21), then clone the gene into M13 and sequence it by the standard Sanger dideoxy method (22). The correct clone would then be sent to the NHS at Porton Down and the gene would be excised and placed in a suitable expression vector containing a ribosome binding site and a strong promoter.

RESULTS AND DISCUSSION

Gene design

The desired gene sequence is shown in Figure 1 using the optimal codon principle for E.coli (19). The ends of the gene were designed with appropriate linkers including a terminal EcoRI site plus a start codon at the N-terminal end and two stop codons (both TGA) plus a terminal HindIII site at the C-terminal end. These sticky ends facilitate direct cloning into double cut (EcoRI and HindIII) M13mp8. In addition SfaNI restriction sites were incorporated at both ends of the gene such that on cleavage with this enzyme followed by trimming back of the resultant sticky ends with S_1 nuclease the entire coding region of the gene could be excised without start or stop codons. Removal of the cloned and sequenced gene permits, with the aid of appropriate linkers, the coding part to be placed in a suitable expression system. This strategy is based on the procedure used by Urdea et al. (18) in the chemical synthesis of a gene for human epidermal growth factor urogastrone.

The two strands of the gene were divided up into oligodeoxyribonucleotides of 21 or 22 bases long, such that the overlaps on opposite strands were at least 7 bases, to ensure good annealing. The sequences of the oligodeoxyribonucleotides (designated

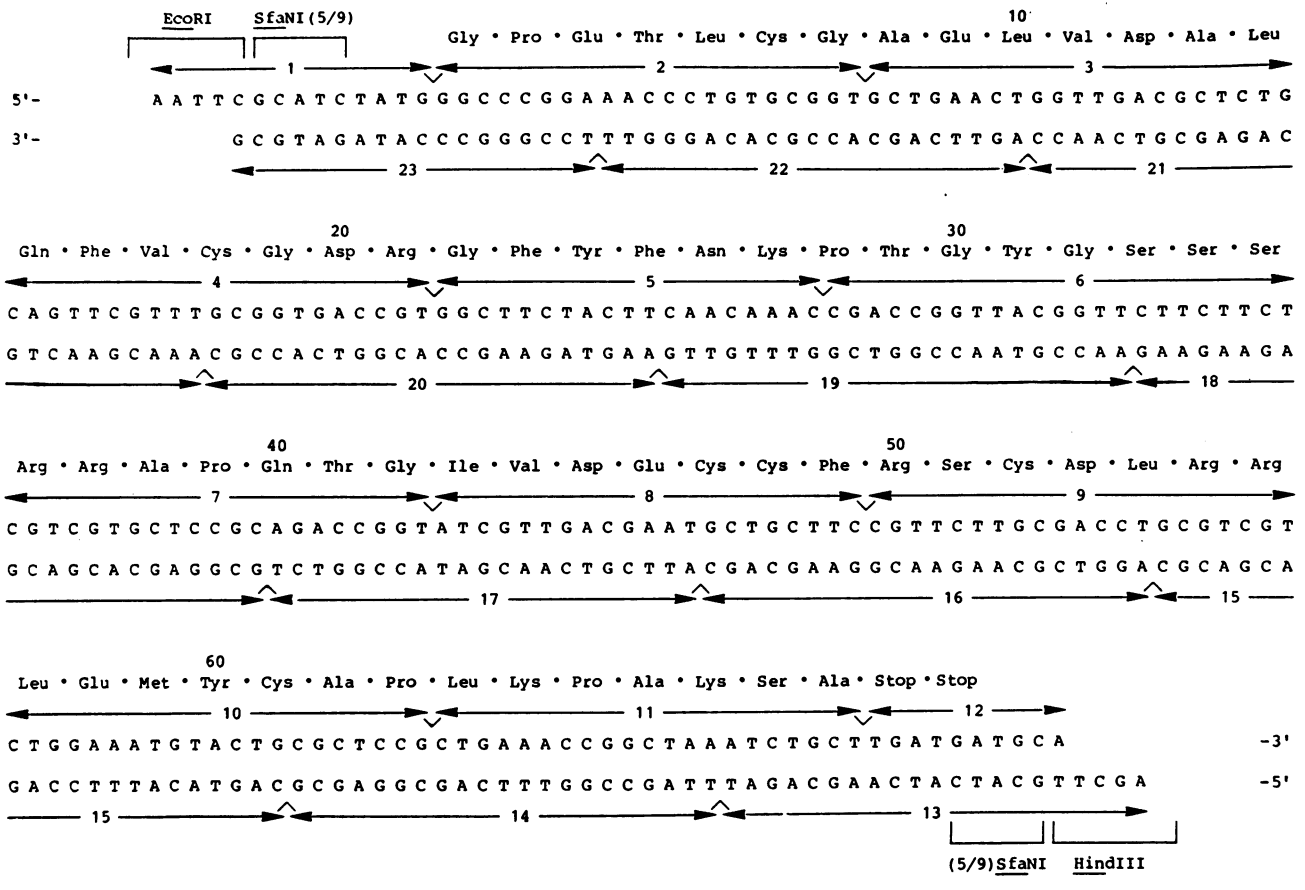

Figure 1. Amino acid sequence of somatomedin C and the construction of a synthetic gene.

S0M.C.001-S0M.C.023) required for the gene construction are indicated in Figure 1. Each oligodeoxyribonucleotide was checked for any internal self-complementarity and incorrect hybridisation (23) to ensure that only the desired hybridisations would occur. It is for this reason that the codons chosen for glycines 1 and 22 are GGC and not GGT.

Oligodeoxyribonucleotide synthesis

The 23 oligodeoxyribonucleotides, designated S0M.C.001-S0M.C.023 were assembled using the improved solid phase phosphotriester method on the new urethane linked long chain alkylamine controlled pore glass (LCAA/CPG) support (24), except for S0M.C.012, viz. d [TGATGATGCA] and S0M.C.023, viz. d[AAACGAACTGCAGAGAGTCAACC] which were synthesised on Whatman 3MM chromatography paper and on a urethane linked aminopropyl controlled pore glass respectively. The decamer, S0M.C.012, was synthesised using a slightly modified procedure to that used by Matthes (15), such that the 5'-0-protecting group was removed using 10% w/v trichloroacetic acid in 1,2-dichloroethane and the reaction was quenched by a brief wash with N,N-dimethylformamide. As Whatman paper is not a particularly good support for oligodeoxyribonucleotide synthesis by the phosphotriester method, in particular the cycle yields are at least 5% lower than those achieved with controlled pore glass supports, it was decided to prepare the remaining 22 oligodeoxyribonucleotides on controlled pore glass (CPG). Moreover, the single synthesis performed on the aminopropyl CPG support, viz. S0M.C.023 was very poor compared to any of the syntheses performed on the LCAA/CPG supports. The syntheses performed on glass were carried out 4 at a time on a 4 column Omnifit set up (25) using a reduced scale to minimise the costs, however the general assembly procedure was as previously described (24,25). The monomers which were used were protected with the 5'-0-(9-phenylxanthen-9-yl) group i.e. pixyl group (26) and the exocyclic amino group of deoxyadenosine was protected with the phthaloyl group (27,28) in order to minimize depurination during the 5'-deprotection step. These compounds were prepared as described previously (25). The urethane linked LCAA/CPG supports were prepared according to a previous publication (24). No problems were encountered working with the

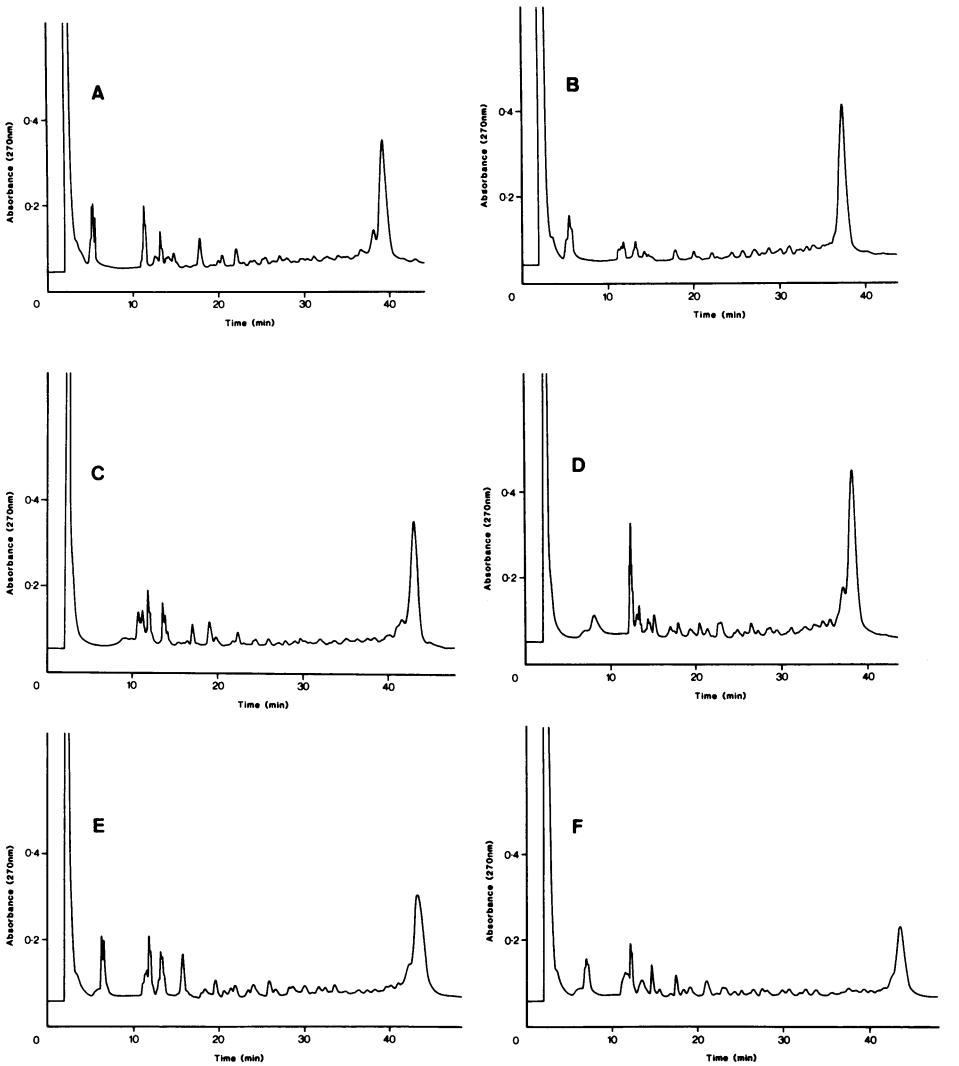

Figure 2. Ion-exchange h.p.l.c. elution profiles of 6 of the 23 crude oligodeoxyribonucleotides on Partisil 10SAX using a gradient of KH_2PO_4 (pH 6.3) in formamide/water (6:4 v/v).
A, SOM.C.003, d[GCTGAACTGGTTGACGCTCTG];
B, SOM.C.005, d[GGCTTCTACTTCAACAAACCG];
C, SOM.C.007, d[CGTCGTGCTCCGCAGACCGGT];
D, SOM.C.011, d[CTGAAACCGGCTAAATCTGCT];
E, SOM.C.016, d[AGGTCGCAAGAACGGAAGCAGC];
F, SOM.C.018, d[GCGGAGCACGACGAGAAGAAG].

Table 1

Ligation mix	Oligodeoxyribonucleotides used
A	1+2+23+22
B	1+2+23+22+3
C	1+2+23+22+3+21
D	1+2+23+22+3+21+4
E	1+2+23+22+3+21+4+20
F	1+2+23+22+3+21+4+20+5
G	1+2+23+22+3+21+4+20+5+19
H	1+2+23+22+3+21+4+20+5+19+6
I	1+2+23+22+3+21+4+20+5+19+6+18
J	13+12+11+14
K	13+12+11+14+10
L	13+12+11+14+10+15
M	13+12+11+14+10+15+9
N	13+12+11+14+10+15+9+16
O	13+12+11+14+10+15+9+16+8
P	13+12+11+14+10+15+9+16+8+17
Q	13+12+11+14+10+15+9+16+8+17+7

reduced scale which comprised 16 mg of support (0.35-0.48 μmol) and coupling mixtures consisting of only 8 μmol of monomer, 12 mg (40.5 μmol) of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole, 6 μl (75.6 μmol) of 1-methylimidazole and 80 μl of anhydrous pyridine. The activated mixtures for the couplings were prepared in dry 0.5 ml Eppendorf tubes instead of Pierce microvials.

The high quality of the products prepared by this method is easily seen from the ion-exchange h.p.l.c. traces of the crude fully deprotected oligodeoxyribonucleotides (see Figure 2). All of the oligodeoxyribonucleotides were purified by ion-exchange h.p.l.c., although normally one would purify a large number of oligodeoxyribonucleotides by polyacrylamide gel electrophoresis in order to save time. The isolated yields (narrow cuts were taken from the product peaks on h.p.l.c.) of oligodeoxyribonucleotides prepared on the LCAA/CPG supports were between 3 and 13% (it should be made clear that no attempts to obtain maximum

yields were made). The h.p.l.c. purified oligodeoxyribonucleotides were then desalted by dialysis.

Gene construction

All oligodeoxyribonucleotides except the two 5'-terminal ones, viz. S0M.C.001 and S0M.C.013 were ^{32}P-labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase, as it was intended to leave the terminal phosphate groups on the double cut M13mp8 ready for direct ligation of the double sticky ended gene. (As no reverse phase h.p.l.c. purification was carried out so some base modified materials will be present).

Initially we decided to try and assemble the gene in two halves. Trial ligations were performed using 10 pmol of each oligodeoxyribonucleotide, and the various ligation mixes tried are shown in Table 1. Small aliquots of the mixes were taken after several days and run on a thin 12% native polyacrylamide gel. The autoradiograph of the gel is shown in Figure 3. The results were very promising indeed in that the two halves of the gene, represented by the bands in lanes I and Q, had assembled very efficiently. At this point it was decided to try a preparative scale (100 pmol) ligation of all 23 oligodeoxyribonucleotides. The ligation of all 23 pieces was relatively successful as is testified by the autoradiograph (see Figure 4) of a 1.3 mm thick 6% native polyacrylamide gel. The band of the correct length (the gene migrated a little more slowly than the xylene cyanol FF dye marker) was excised and the DNA was eluted by soaking the gel slice in ammonium acetate solution. The yield of gene from the ligation and gel was 3.3%.

Cloning and sequencing

The double sticky ended M13 vector was prepared by HindIII digestion of the replicating form of M13mp8 DNA (linearisation of the DNA was checked on a 0.8% HGT agarose mini-gel), followed by a further digestion with EcoRI. The vector was then purified on a preparative 0.6% HGT agarose gel using ethidium staining to locate the DNA. The DNA was then isolated from the gel slice by binding it to powdered glass (29). The purified gene and vector were then ligated overnight and the recombinant was used to

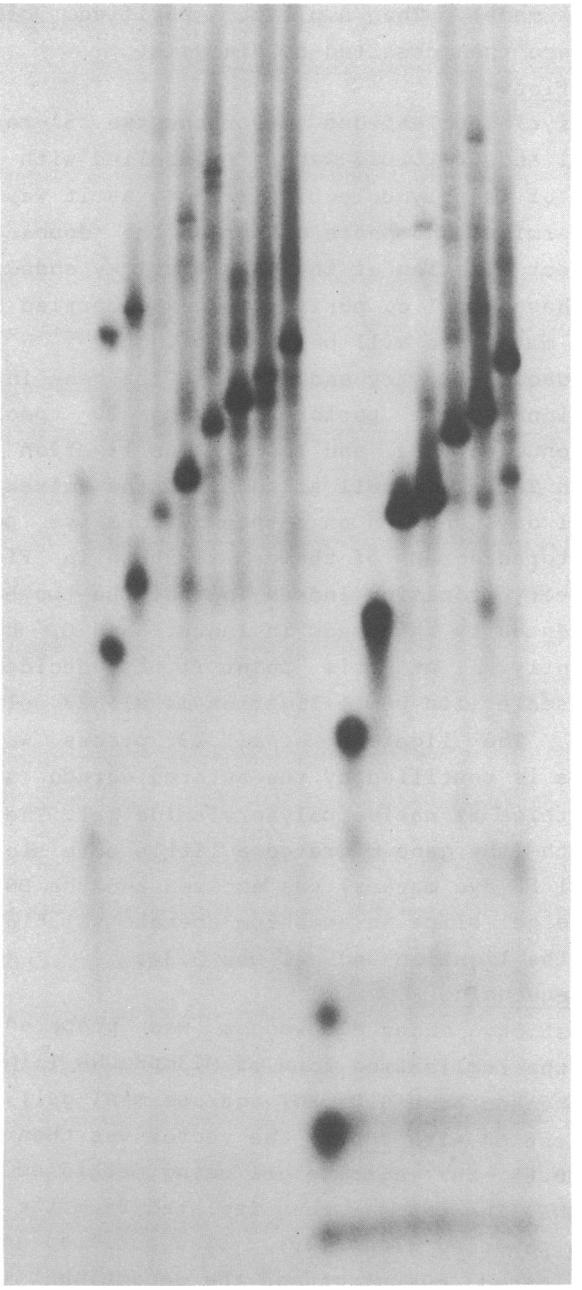

Figure 3. Autoradiograph of a 12% native polyacrylamide gel of the products of the various oligodeoxyribonucleotide ligation experiments (see Table 1), reading A to Q from left to right.

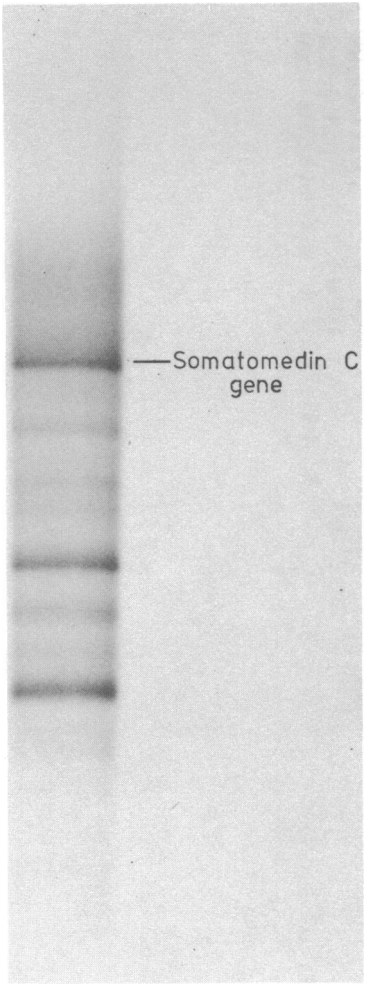

Figure 4. Autoradiograph of a 6% native polyacrylamide gel of the product of the ligation of all 23 oligodeoxyribonucleotides required to produce the somatomedin C gene.

transform an r_K-derivative of E.coli JM101 (constructed by Toby Gibson in Cambridge) made competent by the DMSO method (30). (This method was preferred to the calcium ion method (31) as it generally gives a higher transformation efficiency). Various amounts of the transformation mixes were plated out on agar with isopropyl-β-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIG) present. Blue plaques are produced when β-galactosidase is produced. Plaques due to M13 carrying inserted gene were expected to be white.

Plates from 20 μl of transformation mix obtained from a ligation

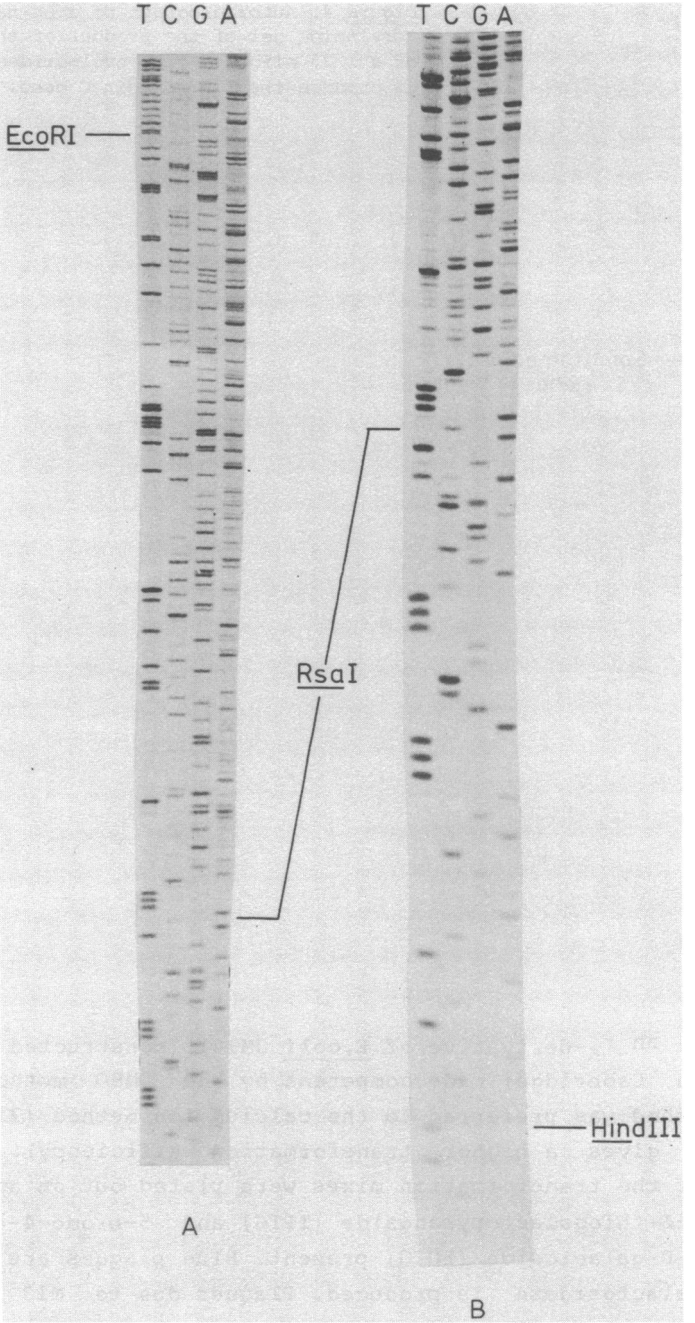

Figure 5. Autoradiographs of A, 6% and B, 10% denaturing polyacrylamide sequencing gels of an M13 clone containing the somatomedin C gene.

mix of 0.024 pmol of vector with 0.13 pmol of insert, and from 2 µl of transformation mix obtained from a ligation mix of 0.024 pmol of vector with 0.26 pmol of insert gave 9 blue + 139 white plaques and 1 blue + 22 white plaques respectively. Phage stocks were grown from 20 small white plaques from the first plate and from 20 of the 22 white plaques on the second plate. In order to reduce the amount of sequencing to be done the 40 clones were sized on preparative 0.6% HGT agarose gels containing ethidium bromide. Eighteen of the 40 clones appeared to be of identical size and were somewhat larger than an M13mp8 reference, and so contained insert DNA.

The DNA from 4 of the 18 clones containing insert DNA was then cleaned up and sequenced by the Sanger dideoxy method (22) using [α-^{35}S]ATP and a gradient gel (32). Two of the four clones contained the correct gene sequence (see Figure 5) whereas the other two clones had gene sequences containing one or two mutations. In one such clone there was a G→T transversion in the ala 27 codon, and in the other clone there was a C→T transition in the ala 38 codon and a G→A transition in the met 59 codon. The reason for these mutations is not clear although presumably they arose because of some base modification in the oligodeoxyribonucleotides. It would be interesting to see if the frequency of occurrence of mutations was less if all the oligodeoxyribonucleotides had been subject to a further purification step using reversed phase h.p.l.c.

In conclusion, a gene for somatomedin C was synthesised and cloned without difficulty, and moreover this type of method opens up many possibilities for protein engineering by recombinant DNA techniques utilising total gene synthesis.

EXPERIMENTAL PROCEDURES

Oligodeoxyribonucleotide synthesis and purification

Pyridine was purified and dried by an initial refluxing with and distillation from ninhydrin followed by refluxing with and distillation from barium oxide. 1,2-Dichloroethane was purified by passage through a column of basic alumina followed by distillation from phosphorus pentoxide. 1-Methylimidazole was purified by vacuum distillation under reduced pressure of dry

nitrogen. The urethane linked LCAA/CPG supports (24) and nucleotide monomers (25) were prepared as described previously. The oligodeoxyribonucleotides (except for S0M.C.012 which was prepared on Whatman 3MM paper in a separate experiment) were assembled 4 at a time on a 4-column Omnifit system (25) using the procedure described, except that the scale was reduced. Each assembly was carried out using 16 mg (0.35-0.48 μmol) of support, and the coupling mixture per cycle consisted of 8 μmol of 5'-O-pixyl protected monomer (as the triethylammonium salt of the 3'-O- [2-chlorophenyl phosphate]), 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (12 mg, 40.5 μmol), 1-methylimidazole (6 μl, 75.6 μmol) and anhydrous pyridine (80 μl). The coupling mixtures were prepared in dry 0.5 ml Eppendorf tubes, which were discarded after use, in place of Pierce reacti-vials. The normal wash cycle (24) was used for the columns except that the overall flow rate was increased slightly to give a flow of about 1 ml min^{-1} through each column.

The glass supports carrying the fully protected oligodeoxyribonucleotides were deprotected in 2 ml screw top vials initially with 350 μl of a solution of pyridine-2-carbaldoxime (0.42 M) and 1,1,3,3-tetramethylguanidine (0.4 M) in dioxan/water (1:1 v/v) for 38 h at room temperature. The samples were then lyophilised in the microvials. Concentrated aqueous ammonia (1 ml, 35%) was added to each vial and the tightly sealed vials were kept at 56°C for 42 h in order to remove the base protecting groups and complete the cleavage from the support. The vials were cooled, the beads were filtered off, washed with a little water and the filtrates were evaporated *in vacuo*. The residual glasses were dissolved in water (1 ml) and the solutions were transferred into 10 ml conical centrifuge tubes and then lyophilised. The residues were dissolved in 80% acetic acid (2 ml) and the solutions were kept at room temperature for 30 min to remove the 5'-terminal pixyl protecting groups. Water (2 ml) was added to each tube and the solutions were washed with diethyl ether (3x4 ml). The aqueous phases were then evaporated to dryness *in vacuo*. Finally, water (3 ml) was added to each residue and the solutions were evaporated once more *in vacuo* to remove residual acetic acid.

The residues were dissolved in water (1 ml) and a 100 µl aliquot of each solution was purified by ion-exchange h.p.l.c. on Partisil 10SAX as described previously (25). The centre part of each product peak was collected and the 23 oligodeoxyribonucleotides were desalted by dialysis against water (3x5 l) at 4°C. The amounts of each oligodeoxyribonucleotide were then estimated spectrophotometrically.

All the deoxyribonucleotides except S0M.C.001 and S0M.C.013 were phosphorylated on a 500 pmol scale with [γ-^{32}P]ATP (1 pmol, 3 µCi) diluted with cold ATP (1.5 nmol) in the presence of T4 polynucleotide kinase. Small samples of the kinased oligodeoxyribonucleotides were then checked on a thin 20% denaturing polyacrylamide gel. The 21 ^{32}P-labelled oligodeoxyribonucleotides and the two unlabelled ones were then diluted with water to give a concentration of 10 pmol µl^{-1}, and the solutions were stored at 4°C.

Gene construction

Trial ligations of the oligodeoxyribonucleotides were performed on a 10 pmol scale. Ligation mixes A to Q were prepared (see Table 1) in small Eppendorfs. Each mix was prepared from 1 µl of each oligodeoxyribonucleotide required plus 1.5 µl of 10x ligase salts (100 mM Tris-HCl, 100 mM NaCl, and 100 mM $MgCl_2$, pH 7.4) plus water to give a final volume of 13.5 µl. The tubes were heated at 80°C for 2 min, cooled to room temperature over 20 min, and then cooled in ice for 10 min. Added 1.5 µl of 5 mM ATP solution and 1 µl of 0.1 M DTT solution to each tube followed by 1 µl of DNA ligase solution (40 U). The mixtures were then kept at 4°C for 5 days. The ligations were terminated by addition of EDTA (2 µl, 0.5 M) and SDS (2 µl, 10% w/v solution). Small aliquots of the various ligation mixes were then run on a thin (0.35 mm) 12% native polyacrylamide gel. The autoradiograph of this gel is shown in Figure 3.

The preparative scale ligation of all 23 oligodeoxyribonucleotides was carried out as follows: 10 µl of each oligodeoxyribonucleotide solution (100 pmol) and 30 µl of 10x ligase buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 100 mM $MgCl_2$) were placed in an Eppendorf tube and the solution was heated at 80°C for 2 min, and then allowed to cool to room

temperature over 30 min. The solution was cooled on ice for 10 min then 5 mM ATP (30 µl), 0.1 M DTT (20 µl) and T4 DNA ligase (2 µl, 400 U μl^{-1}) were added. The ligation mix was kept at 4°C and the course of the reaction was monitored by electrophoresis on a thin 6% native polyacrylamide gel. The gene fragment ran a little more slowly than the xylene cyanol FF marker. After 5 days it was judged that the reaction was progressing no father so EDTA solution (30 µl, 0.5 M) and SDS (33 µl, 10% w/v) were added. Added 40 µl of 3 M NaOAc solution (pH 5) and transferred 200 µl of the resultant solution into each of 2 siliconised 1.5 ml Eppendorf tubes. Cold 95% EtOH (600 µl) was added to each tube, the contents were mixed and the tubes were kept at -20°C overnight to precipitate the DNA. The DNA was pelleted by centrifugation at 4°C and the two pellets were redissolved in 0.3 M sodium acetate (250 µl). Cold 96% EtOH (500 µl) was added to each tube, the caps were closed and the contents were mixed by inversion. The tubes were kept at -20°C for 3 h and the DNA was then pelleted. The pellets were washed with 70% EtOH and then dried *in vacuo* for 10 min.

Added 10 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8) and 10 µl of 8% Ficoll-dye solution (consists of 0.8 g Ficoll 400, 100 µl of 0.5 M EDTA, 2 mg of bromphenol blue, 5 mg of xylene cyanol FF and water to 10 ml) to each tube, and vortexed to solubilise the DNA. The combined samples (total volume 40 µl) were loaded ito a 2.5 cm slot in a 1.3 mm thick 6% native polyacrylamide gel (20x40 cm). which was electrophoresed for 6 h at 300 V and 30 mA in TBE buffer (0.1 M Tris-borate, 2.5 mM EDTA, pH 8.3) until the slow blue dye had migrated about half way down the gel. The position of the gene was located by autoradiography (see Figure 4) and the gel piece containing the gene was excised and cut into small pieces. The gel slices were then soaked overnight in 300 µl of 0.5 M ammonium acetate, 1 mM EDTA, pH 8.4 in a 1.5 ml siliconised Eppendorf tube mounted in a wrist action shaker. The supernatant was removed carefully with a fine drawn out capillary, and the gel slices were washed with a further 200 µl of ammonium acetate solution. The DNA in the combined supernatants was then precipitated with 2 volumes of cold 95% ethanol and the mixture was kept at -20°C for 90 min. The DNA

was then pelleted, washed, dried, and then redissolved in 25 µl of TE buffer. The amount of DNA was then determined by scintillation counting of a small sample of this solution adsorbed on a glass fibre filter. 3.3 pmol of somatomedin C gene were obtained.

Vector preparation

To RF M13mp8 DNA solution (5 µl, 1 mg ml^{-1}) added 5 µl of 250 mM NaCl, 5 µl of 50 mM Tris-HCl pH 7.4, 5 µl of 50 mM $MgCl_2$, 2.5 µl of 10 mM DTT and 2.5 µl of HindIII solution (50 U). The solution was kept overnight at 37°C and then a small sample was analysed on a 0.8% HGT agarose min-gel, run at about 50 mA and 64 V for 45 min, to check that all the DNA had been linearised. Added 1.4 µl of 1 M NaCl, 1.1 µl of 1 M Tris-HCl and 2 µl of EcoRI solution (40 U). After overnight incubation at 37°C the reaction was terminated by addition of TE buffer (114 µl) and Ficoll-dye mix (57 µl). The solution was heated at 68°C for 10 min and 40 µl aliquots of solution were placed in each of five 2 cm wide wells in a preparative 0.6% HGT agarose gel (20 cm x 20 cm). The gel was run overnight at 30 V and about 25 mM in TAE buffer (40 mM Tris-acetate, 20 mM Na acetate, 1 mM EDTA, pH 8.2) containing 0.5 mg l^{-1} of ethidium bromide. The gel was washed with water and the DNA was visualised by long wavelength u.v. radiation. The fluorescent bands were excised and the gel slices (1.27 g) were cut into small pieces and placed in a 10 ml centrifuge tube. Ice-cold saturated sodium iodide solution (2.6 ml) was added and the mixture was incubated at 37°C for 30 min to dissolve the agarose. 50 µl of well vortexed glass powder (29) was then added and the mixture was incubated for 2 h at 4°C with gentle shaking. The tube was then spun for 3 min at 2000 rpm and the supernatant was discarded. The beads were suspended in 1 ml of cold saturated sodium iodide solution and the mixture was transferred into an Eppendorf tube. The tube was spun for 10 s in a microfuge and the supernatant was discarded. The glass powder was then suspended in 75% ethanol (0.5 ml), the tube was spun and the supernatant discarded. This wash procedure was repeated twice. Finally the glass powder was suspended in 75 µl of TE buffer and incubated at 37°C for 30 min. The tube was spun for 10 s and the supernatant was collected in a fresh Eppendorf tube. The TE addition and

incubation were then repeated. After spinning the tube for 10 s the two supernatants were combined, the tube was spun briefly, and the supernatant containing the double cut M13mp8 DNA was transferred into a fresh Eppendorf tube and stored at 4°C. The DNA concentration was estimated to be about 17 ng μl^{-1} (i.e. about 0.0042 pmol μl^{-1}) by mini agarose gel and comparison of band fluorescence with the fluorescence (intercalated ethidium) of known concentrations of HindIII restriction fragments of bacteriophage λ.

Ligation of vector and insert

The vector concentration in the stock solution was 0.0042 pmol μl^{-1} and the insert concentration was 0.132 pmol μl^{-1} in the stock solution. The following 6 ligation mixes were then prepared:

	1	2	3	4	5	6
insert (5x diluted)	-	-	1 µl	2 µl	5 µl	2 µl
vector	6 µl	6 µl	6 µl	6 µl	6 µl	6 µ
10x ligase buffer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
DTT (0.1 M)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
ATP (5 mM)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
H_2O	8 µl	7 µl	6 µl	5 µl	2 µl	5 µl
ligase (1 µl of 10x diluted)	-	+	+	+	+	+

Placed the vector, insert, 10x ligase buffer and the water in 0.5 ml siliconised Eppendorf tubes and mixed the contents by vortexing. Spun the tubes for a few seconds and then cooled them on ice for 10 min. Next, added the DTT, ATP and the T4 DNA ligase (4 U) and kept the mixes overnight at 4°C.

Transformation of E.coli

Streaked cells from a frozen stock of an r_K-derivative of E.coli JM101 onto an H plate and incubated overnight at 37°C. Inoculated two sterile tubes of SOB medium (15 ml in each; 2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$) each with a 2.5 mm diameter colony taken from the H plate. The cells were dispersed by gentle vortexing and the tubes were incubated at 37°C for 3 h in an orbital shaker. The contents were then poured into 2 x 50 ml Falcon tubes, kept on ice for 10-15 min and then spun for 12 min at 2500

rpm at 4°C. The supernatant was decanted off and the cells were resuspended in 5 ml of TFB (10 mM K-MES pH 6.2, 100 mM RbCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM $HACoCl_3$) by gentle vortexing. Kept the tubes on ice for 10-15 min and then spun them for 10 min at 2500 rpm at 4°C. Supernatant was decanted off and each batch of cells was resuspended in 1.2 ml TFB. Distilled DMSO (42 µl) was added to each tube, the contents were swirled and the tubes left for 5 min on ice. DTT (42 µl of 2.25 M stock solution in 40 mM KOAc, pH 6) was added to each tube, the contents were swirled and left for 10 min on ice. Added DMSO (42 µl) and kept the tubes on ice for a further 5 min, then aliquoted 210 µl of the solution into each of 7 Eppendorf tubes cooled on ice. Next, added 10 µl of the 6 recombinant DNA samples to separate tubes (control 7 was just 0.4 µg of RF M13mp8 DNA) and swirled the contents gently. The tubes were kept on ice for 30 min, heat pulsed at 42°C for 90 s and then placed on ice for 1-2 min.

Prepared 15 tubes containing 2.8 ml of agar overlay mixture and added 30 µl of BCIG solution (20 mg ml^{-1} in DMF) and 20 µl of IPTG solution (20 mg ml^{-1} in H_2O). Added all of the transformation mixes [1] and [2] to overlay mixes and poured the solution onto fresh H plates. Used only 10 µl of control [7] + overlay mix for plating out. For experiments [3]-[6] used 2 µl, 20 µl, and the remaining transformation mix for plating out. The fifteen H plates were then incubated overnight at 37°C and then the blue and white plaques were counted. Control [1] gave 7 blues and 35 whites, control [2] gave 123 blues and about 800 whites, control [7] was covered with blues. On all other plates the white:blue ratio was about 20:1.

Phage stock preparation and sequencing

Picked 20 small white plaques from plate [5] + 20 µl transformation mix and 20 out of the 22 white plaques from plate [6] + 2 µl transformation mix and grew up single stranded phage stocks in 1.5 ml of E.coli JM101 cell stock in 2XTY medium (16 g Tryptone, 10 g yeast extract, 5 g NaCl to 1 l with water, pH adjusted to 7.4 with NaOH) for 5 h at 37°C. Cells were then removed by centrifugation. Took 20 µl aliquots of the supernatants + 8 µl of 3% SDS and bromophenol blue and ran the 40 samples on a 0.6% preparative HGT agarose gel run in 1 x TAE

buffer (40 mM Tris-acetate, 20 mM Na-acetate, 1 mM EDTA, pH 8.2) containing ethidium bromide (0.5 mg l^{-1}) for 6 h at 170 mA and 70 V. An M13mp8 standard was also run on the gel. On examination of the gel under u.v. light it was observed that 18 of the 40 clones tested carried insert DNA. Four of the 18 positive clones were then sequenced.

The phage was precipitated with polyethylene glycol and the coat protein removed by phenol extraction. The single stranded DNA was then cleaned up by ethanol precipitation prior to sequencing by the Sanger dideoxy method using [α-^{35}S]ATP and a gradient gel (32).

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REFERENCES

1. Rinderknecht, E. and Humbel, R.E. (1978) J. Biol. Chem. 253, 2769-2776.
2. Klapper, D.G., Svoboda, M.E. and Van Wyk, J.J. (1983) Endocrinology 112, 2215-2217.
3. Liberti, J.P. and Miller, M.S. (1980) J. Biol. Chem. 255, 1023-1029.
4. Svoboda, M.E., Van Wyk, J.J., Klapper, D.G., Fellows, R.E., Grisson, F.E. and Schlueter, R.J. (1980) Biochemistry 19, 790-797.
5. Li, C.H., Yamashiro, D., Gospodarowicz, D., Kaplan, S.L. and Van Vliet, G. (1983) Proc. Natl. Acad. Sci. USA 80, 2216-2220.
6. Van Wyk, J.J., Russell, W.E. and Li, C.H. (1984) Proc. Natl. Acad. Sci. USA 81, 740-742.
7. Van Wyk, J.J. and Underwood, L.E. (1978) in Biochemical Actions of Hormones, Litwack, G. ed. pp. 101-148, Academic Press, New York.
8. Van Wyk, J.J., Underwood, L.E., Hintz, R.L., Clemmons, D.R., Voina, S.J. and Weaver, R.P. (1974) Recent Progr. Horm. Res. 30, 259-318.
9. Froesch, E.R., Zapf, J., Meuli, C., Mader, M., Waldvogel, M., Kaufman, M. and Morell, B. (1975) Adv. Metab. Disord. 8, 211-235.

10. Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D., Van den Brande, J.L. and Van Wyk, J.J. (1971) Nature (London) 235, 107.
11. Herington, A.C., Cornell, H.J. and Kuffer, A.D. (1983) Int. J. Biochem. 15, 1201-1210.
12. Laron, Z. (1984) Ergeb. Inn. Med. Kinderheilkd. 51, 117-150.
13. Rinderknecht, E. and Humbel, R.E. (1976) Proc. Natl. Acad. Sci. USA 73, 2365-2369.
14. Frank, R., Heikens, W., Heisterberg-Moutsis, G. and Blöcker, H. (1983) Nucleic Acids Res. 11, 4365-4377.
15. Matthes, M.W.D., Zenke, W.M., Grundström, T., Staub, A., Wintzerith, M. and Chambon, P. (1984) EMBO J. 3, 801-805.
16. Wengenmayer, F. (1983) Angew. Chem. Int. Ed. Engl. 22, 842-858.
17. Ohtsuka, E., Taniyama, Y., Iwai, S., Kitano, K., Miyamoto, S., Ohgi, T., Sakuragawa, Y., Fujiyama, K., Ikari, T., Kobayashi, M., Miyake, T., Shibahara, S., Tokunaga, T., Ikehara, M., Ono, A., Ueda, T., Tanaka, T., Baba, H., Miki, T., Sakurai, A. and Oishi, T. (1983) Nucleic Acid Res. Symposium Series no. 12, 79-82.
18. Urdea, M.S., Merryweather, J.P., Mullenbach, G.T., Coit, D., Heberlein, U., Valenzuela, P. and Barr, P.J. (1983) Proc. Natl. Acad. Sci. USA 80, 7461-7465.
19. Gouy, M. and Gautier, C. (1982) Nucleic Acids Res. 10, 7055-7074.
20. Ikemura, T. (1981) J. Mol. Biol. 146, 1-21.
21. Ikemura, T. (1981) J. Mol. Biol. 151, 389-409.
22. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
23. Gait, M.J. (1984) in Oligonucleotide Synthesis: A Practical Approach, Gait, M.J. Ed., Chapter 1, IRL Press, Oxford, UK.
24. Sproat, B.S. and Brown, D.M. (1985) Nucleic Acids Res. 13, 2979-2987.
25. Sproat, B.S. and Gait, M.J. (1984) in Oligonucleotide Synthesis: A Practical Approach, Gait, M.J. Ed., Chapter 4, IRL Press, Oxford, UK.
26. Chattopadhyaya, J.B. and Reese, C.B. (1978) J. Chem. Soc. Chem. Commun., 639-640.
27. Kume, A., Sekine, M. and Hata, T. (1982) Tetrahedron Lett. 23, 4365-4368.
28. Kume, A., Sekine, M. and Hata, T. (1983) Chem. Lett., 1597-1600.
29. Vogelstein, B. and Gillespie, D. (1979) Proc. Natl. Acad. Sci. USA 76, 615-619.
30. Hanahan, D. (1983) J. Mol. Biol. 166, 557-580.
31. Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53, 159-162
32. Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) Proc. Natl. Acad. Sci. USA 80, 3963-3965.