MOLECULAR CLONING OF SYNTHETIC ANGIOTENSIN 1 GENE IN *ESCHERICHIA COLI*

A route to physiologically active hormone

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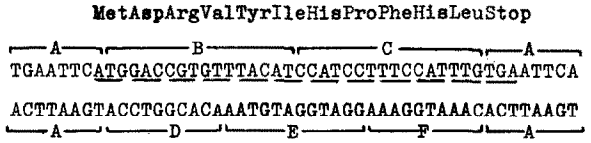
Received 14 March 1980

1. Introduction

An approach has been proposed for cloning and expression of chemically synthesized genes in *Escherichia coli* cells [1] in which the synthetic polynucleotide coding for the desired amino acid sequence was introduced into a specially designed plasmid near the end of the β-galactosidase gene. The synthetic gene contained an additional codon for methionine, which connected the β-galactosidase protein chain with the peptide coded. The peptide can be split off this chimeric protein by cyanogen bromide treatment. Potency of this method was additionally demonstrated by cloning of the synthetic genes for insulin [2] and neuropeptide leucine-encephalin [3].

We used this principle to clone and express the synthetic gene for angiotensin 1. For this purpose, a 48-membered bihelical angiotensin 1 polynucleotide was obtained by a combination of chemical and enzymatic methods:



A–F are chemically synthesized oligonuclotides. This gene was cloned in two vectors (in a plasmid and in a phage) and efficiencies of expression were compared. The specially designed pMR1 and phage λ*plac*5-1 [4] were used and expression in the latter appeared to be ~10-times more efficient than in the former. We used the phosphatase method [5] for insertion of the synthetic gene into the plasmid vector. The techniques appeared to be very convenient in our hands and provided ~100% yield of hybrid clones.

The hormone preparation obtained exhibited physiological activity in vivo.

2. Materials and methods

Restriction endonuclease *Eco*RI was isolated according to [6]; DNA-ligase and polynucleotide kinase were isolated according to [7]; alkaline phosphatase BAPF was purchased from Worthington (USA).

λ*plac*5 DNA was kindly given by Dr T. G. Maximova and DNA of vector λ*plac*5-1 [4] was isolated according to [8]. pBR322 DNA isolated from *E. coli* C600 cells transformed by this plasmid [9].

Ligation of oligonucleotide blocks was performed essentially according to [1]. The product was isolated by gel-filtration on Sephadex G-50 in 0.05 M triethylammonium bicarbonate and final purification was achieved by preparative gel-electrophoresis on 20% polyacrylamide gel.

The octanucleotide 'linkers' were added as in [10]. The product after treatment with *Eco*RI was isolated by gel-filtration as described above and analyzed by gel-electrophoresis.

2.1. *Design of the plasmid vector*

A mixture of pBR322 plasmid and λ*plac*5 DNAs was hydrolyzed sequentially by restriction endonucleases *Eco*RI and *Hin*dIII and after the removal of these enzymes was ligated by DNA-ligase. The DNA obtained was used to transform $CaCl_2$-treated *E. coli* cells BMH71-18 (Δ[*lac,pro*]*F' lac* I^qZ ΔM15 pro^+)

[11,12] and the transformants plated on 1.5% meat–peptone agar containing ampicillin (20 μg/ml), 5-bromo-4-chloroindolyl-β-D-galactoside (X-gal, 20 μg/ml) and isopropylthiogalactoside (IPTG, 5×10^{-4} M). Cells of one of the blue clones were grown and their plasmid DNA was isolated as above described.

2.2. *Insertion of the synthetic polynucleotide into plasmid DNA*

Hybrid plasmid DNA (2 μg) was treated by *Eco*RI and dephosphorylated by *E. coli* phosphatase according to [5]. Phosphatase was thoroughly removed by 3 phenol extractions, the DNA precipitated and washed with ethanol. The precipitate was dissolved in 20 μl ligase buffer [10] and divided into two equal parts. To one part (experiment) 5 pmol 'linkered' synthetic polynucleotide was added and both parts diluted with water to 20 μl. The mixtures were cooled to 4°C, and 1 activity unit of DNA-ligase was added to each of them. These preparations were used for transformation of $CaCl_2$-treated *E. coli* BMH71-18 in 12 h incubation, and the transformants were plated on meat–peptone agar containing ampicillin (20 μg/ml). Cells of 10 experimental clones were grown in ampicillin-supplemented L-broth, collected, treated with cyanogen bromide in formic acid [1] and the lysates tested by radioimmunoanalysis for angiotensin 1, using a Clinical Assay (USA) kit according to the protocol enclosed.

2.3. *Insertion of synthetic polynucleotide into DNA of λplac5-1*

λp*lac*5-1 DNA (1 μg) treated by *Eco*RI was mixed with 1 pmol synthetic polynucleotide and 0.5 activity units of DNA-ligase in 10 μl ligase buffer [10] and this mixture was kept for 12 h at 4°C. The DNA obtained was used for transfection of *E. coli* BMH71-18 as in [13]. The cells were plated on meat–peptone agar containing X-gal and IPTG. Recombinant clones giving colourless plaques were grown and used to infect *E. coli* BMH71-18. The cells were collected 30 min after infection and assayed for angiotensin 1 as above.

3. Results and discussion

3.1. *Synthesis and ligation of oligonucleotide blocks*

The synthesis of oligonucleotides A–F was performed using a modification of the triester method [14] from the corresponding dinucleotide blocks. The primary structures of all the oligonucleotides were confirmed by Sanger techniques [15].

The oligonucleotides B–F were phosphorylated by polynucleotide kinase, purified by high-performance liquid chromatography, and joined into a bihelical polynucleotide by means of T4 DNA-ligase. The fidelity of joining was checked by sequencing using the Maxam-Gilbert procedure [16]. The polynucleotide duplex B–F was purified by preparative gel-electrophoresis, and flanked by the linker oligonucleotide A using DNA-ligase. Treatment of the product of this reaction by *Eco*RI afforded the bihelical polynucleotide with protruding *Eco*RI sticky ends. Details of the chemical synthesis wil be published elsewhere.

3.2. *Design of the plasmid vector*

To clone and to express the synthetic gene of angiotensin 1 we constructed the plasmid pMR1 incorporating the main part of the gene of *E. coli* β-galactosidase; pMR1 was made by a single-stage procedure from DNAs of the plasmid pBR322 and of λp*lac*5 phage. It is known [17], that combined cleavage of λp*lac*5 DNA by *Eco*RI and *Hin*dIII affords a mixture of fragments one of which contains the operator zone and 3015 base residues of the structural part of the β-galactosidase gene. DNA of pBR322 plasmid contains a single site for each of *Eco*RI and *Hin*dIII; the latter site resides in the promotor region of the tetracycline operon [9].

Hence, ligation of the mixture of *Eco*RI + *Hin*dIII fragments of λp*lac*5 and pBR322 afforded recombinant molecules. Recombinant clones were identified by their ability to form blue colonies when grown on a medium with X-gal and IPTG. Plasmid DNA of one such clone was isolated and characterized by restriction analysis (fig.1). Hydrolysis by either *Eco*RI, or *Hin*dIII afforded a single product of ~6 Mdaltons, whereas combined hydrolysis gives two products of 2.6 and 3.8 Mdaltons. The first fragment corresponds to the plasmid part of the vector, the second one to the gene of β-galactosidase. *E. coli* BMH71-18 cells transformed by this plasmid are resistant to ampicillin and produce active β-galactosidase.

3.3. *Integration of the synthetic gene into the plasmid vector*

The synthetic gene of angiotensin 1 was inserted

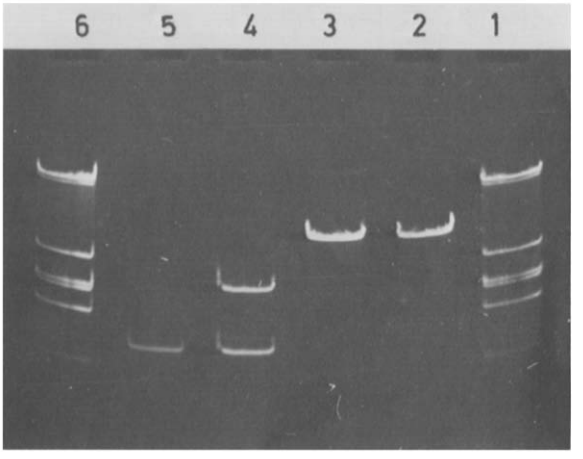


Fig.1. Gel-electrophoresis of pMR1 plasmid DNA digested by *Eco*RI and *Hin*dIII restriction endonucleases. Lanes (1,6) *Eco*RI hydrolysate of λCI857 DNA(markers). Lanes (2–4) hydrolysates of pMR1 DNA: (2) *Eco*RI; (3) *Hin*dIII; (4) *Eco*RI + *Hin*dIII. Lane (5) *Eco*RI hydrolysate of pBR322 DNA. Electrophoresis was run in 1% agarose gel for 4 h at 60 V.

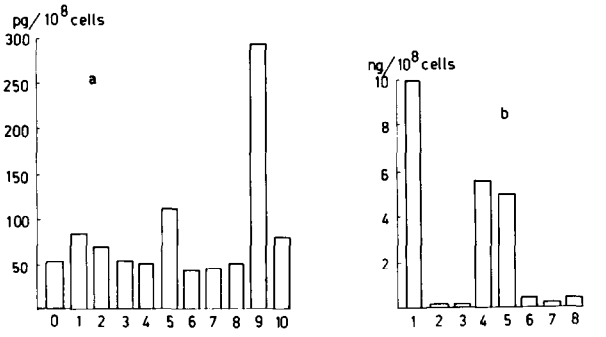


Fig.2. Radioimmunoanalysis of cell lysates for angiotensin 1 content. (a) Clones with the recombinant plasmid; (○) control, clone with the starting plasmid pMR1. (b) Cells infected by clones of the recombinant phage.

into plasmid pMR1 at the site of hydrolysis by restriction endonuclease *Eco*RI. Transformants having the hybrid and the starting plasmids were expected to be phenotypically identical. Therefore it was necessary to increase the yield of hybrid clones to a maximum in order to select them directly on the basis of their ability to produce angiotensin 1. Hence, we employed the method of molecular selection [5]. DNA of pMR1 plasmid was treated by restriction nuclease *Eco*RI and subsequently by alkaline phosphatase, and introduced into DNA ligase-catalyzed reaction with the linkered synthetic gene. This procedure must give circular DNA only with hybrid molecules containing the synthetic sequence. It is known that plasmid DNA in a linear form has a much smaller transforming activity than in the circular form and thus hybrid plasmids obtain a great advantage. Hybrid DNA obtained by the above procedure was used to transform *E. coli* BMH71-18, and the cells plated on meat–peptone agar with ampicillin. The experiment gave 205 clones, controls which omitted the synthetic gene gave no transformants. Cells of 10 clones were grown on a medium containing IPTG, treated with cyanogen bromide in formic acid [1], and assayed for angiotensin 1 by radioimmunoanalysis (fig.2a). It is seen, that the content of immunoreactive materials in the products of clones 1, 2, 5, 9 and 10 is significantly greater than in the control. It is noteworthy that the relative amount of the labelled hormone displaced by the product of clone 9 strongly depends on the amount of this product added, whereas no such dependence was found with the control. Content of angiotensin 1 in the product of clone 9 is ~300 pg/10^8 cells.

3.4. *Integration of the synthetic gene into phage vector*

The level of expression of inserted fragments is in some cases greater with phage vectors as compared to plasmids. Therefore we attempted also to introduce the synthetic angiotensin 1 gene into DNA of vector phage λ*plac*5-1 [4]. This DNA has a single *Eco*RI site within the gene of β-galactosidase. Insertions at this site are easily identified because hybrid phages form colourless plaques on a medium containing X-gal and IPTG.

The DNA of phage λ*plac*5-1 was cleaved by *Eco*RI, ligated with the synthetic angiotensin 1 gene, and the resulting material used to transfect *E. coli* BMH71-18. About 15% of the phages produced colourless plaques on meat–peptone agar containing X-gal and IPTG. We studied the ability of 8 such clones to produce angiotensin 1. To this purpose, phages of the clones were grown and the resulting progeny used to infect *E. coli* BMH71-18 at a multiplicity of 10. The infected cells were collected 30 min after infection, treated with cyanogen bromide in formic acid, and the presence of angiotensin 1 in the lysates tested for by radioimmunoanalysis (fig.2b). It is seen that the content of immunoreactive materials in clones 1, 4 and 5 is much greater than in the other clones.

The average production of angiotensin 1 in such infected cells was some 10-times greater than in cells bearing the above hybrid plasmid. Presumably, this is due to the higher dose of angiotensin 1 gene in cells infected by the hybrid phage. It is known, that *E. coli* cells in the middle of the log-phase contain some 20–30 copies of the plasmid ColE1 and of related plasmid pBR-series [9], whereas phage-infected cell contains some 100 copies of phage DNA molecules.

Noteworthy is also the fact that about a half of the recombinants obtained are producers of angiotensin 1. Presumably, this is due to the two possible orientations of the inserted synthetic gene having identical sticky ends on both sides. Only one orientation of these two results in correct addition of the codogenic strand of the synthetic gene to the codogenic chain of β-galactosidase.

The identical sticky ends of the synthetic gene promote formation of oligomers, and the orientation of monomeric units in such oligomers may be random. However, translation of chimeric mRNA in recombinant clones containing oligomeric gene must stop within the first monomer unit because there are nonsense-codons at the ends of both the codogenic and the noncodogenic strands. Therefore, recombinant clones containing oligomeric synthetic gene may also produce angiotensin 1 at a probability of 50%.

3.5. *Physiological activity of angiotensin 1 produced by recombinant strains*

Of the communications describing design of bacterial strains which produce peptide hormones only one demonstrated the physiological activity of the preparation in vitro [1]. It was therefore of interest to find out whether angiotensin 1 produced by our strains exhibits biological activity in vivo. To this end the cells of clone 9 (fig.2a) containing the recombinant plasmid were grown, subjected to lysis by the method in [1], and the peptides extracted with ethanol. The extract was evaporated to dryness, dissolved in sterile physiological solution and tested for vasopressor activity on rats as in [18] by measuring the arterial pressure. The results are shown in fig.3. It is seen that the preparation (arrows 4–6) gives a strong vasopressor effect.

This work was performed according to the 'Temporary rules of safety for experiments with recombinant DNA', class K-3, with physical protection F-3 and biological B-1.

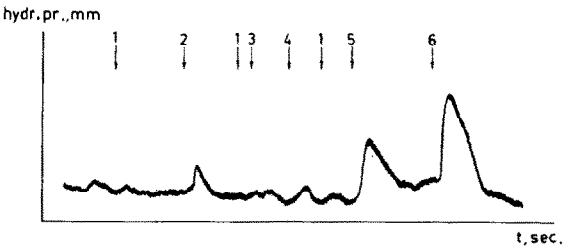


Fig.3. Effect of angiotensin 1 produced by clone 9 (fig.1) on the arterial pressure of rat. The times of injection of the tested preparations are indicated by the arrows. (1) Physiological solution; (2) 2.5 ng authentic angiotensin 1; (3) control, 20 μl lysate of cells having the starting plasmid pMR1; (4–6) 5, 10 and 20 μl, respectively, of lysate of clone 9.

Acknowledgements

The authors are thankful to Professor D. K. Belyaev for his interest and encouragement, and to Dr M. A. Grachev for helpful discussion.

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