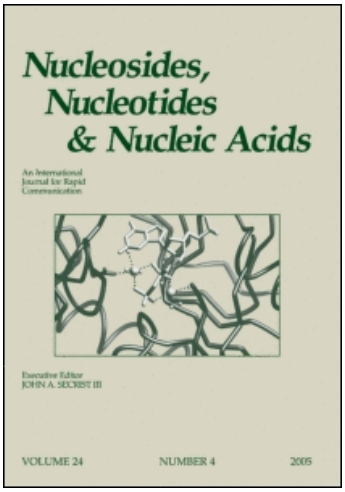

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Rational Methods of DNA Synthesis, Exemplified for the Preparation of Parts of the Human Proopiomelanocortin Gene

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RATIONAL METHODS OF DNA SYNTHESIS, EXEMPLIFIED FOR THE PREPARATION OF PARTS OF THE HUMAN PROOPIOMELANOCORTIN GENE

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Abstract. Complete genes can now be prepared in single-batch procedures a) as one long oligonucleotide strand, b) as a number of simultaneously synthesized fragments. For further increase of overall efficiency DNA fragments terminated by a 3'-ribonucleoside can be joined by solid-phase single strand ligation using RNA ligase. This paper describes the application of these techniques to the preparation of parts of the human proopiomelanocortin gene.

INTRODUCTION

New uses of synthetic DNA fragments, e.g. in medical diagnostics or protein engineering, create a continued demand for rapid and efficient preparative methods. Along this line we have pursued three developments:

A) the chemical preparation of long oligonucleotides (up to 150 b)[1] using the 5'-(4-decyloxyphenyl-)diphenylmethyl group for purification by RP-HPLC[2,3];

B) the semi-mechanized simultaneous synthesis of multiple fragments[4,5,6];

C) the single-strand ligation of 3'-ribonucleoside-terminated deoxyoligonucleotides with RNA ligase[7].

This paper will give a brief account of the application of these methods to the preparation of DNA fragments constituting parts of the human proopiomelanocortin(POMC) gene.

The protein-encoding part of this gene[8] comprises 801 bases corresponding to 267 amino acids. The primary expression product yields several well-known hypothalamic hormones by a very specific processing mechanism the study

of which may be aided by the preparation and expression of segments of the POMC gene and modifications thereof.

RESULTS

Method A: Genes for ACTH as well as for the pharmaceutically interesting ACTH(1-24) (Synacthen) were prepared as single oligonucleotides of chain length between 90 and 147 bases using synthesizers Biosearch SAM I resp. Applied Biosystems 381A. Two variants were synthesized with expression linkers coding for a) the recognition site for collagenase[9], b) the four amino acids Ile-Glu-Gly-Arg, recognized by factor X_a[10]. All genes were tailored according to maximum codon usage in E. coli[11]. The sequences A-F (CHART 1) were purified using the (4-decyloxyphenyl-)diphenylmethyl group as affinity handle[2] (average yield per cycle 97-99 %; individual yields of purified sequences: A: 2.2 $O.D._{260}$; B: 47 $O.D._{260}$; C: 3.4 $O.D._{260}$; D: 2.6 $O.D._{260}$; E: 12 $O.D._{260}$; F: 2.2 $O.D._{260}$) and cloned in E. coli K 12 using pBR 322, linearized with Eco R I and Pst I, as a vector.

Method B: For the assemblage of longer genes, conventionally done by enzymatic joining of sequentially overlapping fragments, the oligonucleotide blocks can be prepared simultaneously in a semi-automated synthesis using a combination of the commercial SAM I synthesizer (Biosearch) with the Autofix (E. Merck) pneumatic fitting system for stacked cartridges. Using this method for the construction of parts of the proopiomelanocortin gene, we have e.g. prepared genes for γ_1 + γ_3-MSH[6] and ß-Lipotropin[12] from fragments of chain length 60 bases and beyond. Details of the preparation and cloning of these genes have been described.

Method C: A further approach to rational gene synthesis is the single-strand ligation of gene fragments using RNA ligase. The constituent DNA fragments are constructed to have a 3'-riboadenosine acceptor terminus[7]. This approach was further developed to a solid-phase single strand ligation[13] using the assemblage of a part of the ß-endorphin gene as a test system. The two fragments A and B, both pre-

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I :                  (NH2-) Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val Gly Lys Lys Arg Arg Pro Val Lys Val Tyr Pro .....
II :                 (NH2-) Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val Gly Lys Lys Arg Arg Pro Val Lys Val Tyr Pro (COOH)

III :             Ala Gly Pro ...

A :                5' GGT CCG AGC TAC AGC ATG GAA CAC TTC CGT TGG GGT AAA CCG GTG GGC AAA AAA CGT CGT CCA GTT AAA GTT TAC CCG TAG CTG CAG
B :              A CGT CCA GGC TCG ATG TCG TAC CTT GTG AAG GCA ACC CCA TTT GGC CAC CCG TTT TTT GCA GCA GGT CAA TTT CAA ATG GGC ATC GAC GTC TTA A  5

IV :   Ala Asp Ile Glu Gly Arg ...

C :    5' GAT ATC GAG GGT AGG AGC TAC AGC ATG GAA CAC TTC CGT TGG GGT AAA CCG GTG GGC AAA AAA CGT CGT CCA GTT AAA GTT TAC CCC ....
D :  A CGT CTA TAG CTC CCA TCC TCG ATG TCG TAC CTT GTG AAG GCA ACC CCA TTT GGC CAC CCG TTT TTT GCA GCA GGT CAA TTT CAA ATG GGG ....

E :    5' GAT ATC GAG GGT AGG AGC TAC AGC ATG GAA CAC TTC CGT TGG GGT AAA CCG GTG GGC AAA AAA CGT CGT CCA GTT AAA GTT TAC CCC TAA TAG AAT TC
F :  A CGT CTA TAG CTC CCA TCC TCG ATG TCG TAC CTT GTG AAG GCA ACC CCA TTT GGC CAC CCG TTT TTT GCA GCA GGT CAA TTT CAA ATG GGG ATT ATC TTA AGA  5'

I (cont.) :             ..... Asn Gly Ala Glu Asp Glu Ser Ala Gln Ala Phe Pro Leu Glu Phe (COOH)

C (cont.) :             ..... AAC GGC GCC GAG GAC GAG TCT GCG GAG GCC TTT CCG CTG GAG TTC TAA TAG
D (cont.) :             ..... TTG CCG CGG CTC CTG CTC AGA CGC CTC CGG AAA GGC GAC CTC AAG ATT ATC TTA A   5'
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CHART 1:

Synthetic gene sequences specific for human ACTH and ACTH(1-24) = Synacthen.

I, II = amino acid sequence of ACTH and Synacthen; III, IV = additional amino acids expressed as linker sequences for fusion peptide cleavage by collagenase (III) (9) resp. factor X_a (IV) (10).

A/B = DNA fragments constituting a gene for Synacthen with codons for collagenase recognition site; C/D resp. E/F = DNA fragments constituting genes for ACTH resp. Synacthen with codons for factor X_a recognition site.

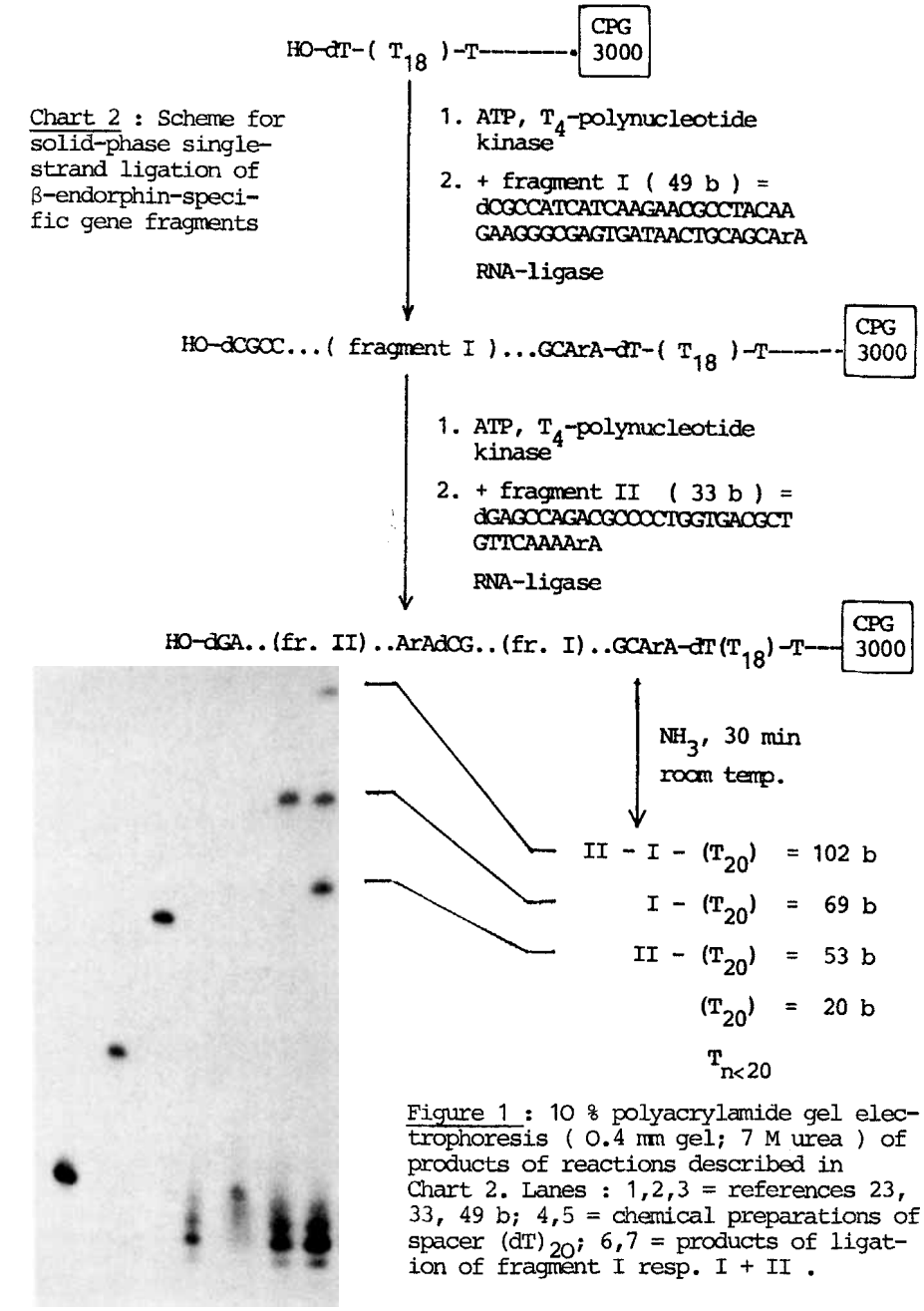

Chart 2 : Scheme for solid-phase single-strand ligation of β-endorphin-specific gene fragments

Figure 1 : 10 % polyacrylamide gel electrophoresis (0.4 mm gel; 7 M urea) of products of reactions described in Chart 2. Lanes : 1,2,3 = references 23, 33, 49 b; 4,5 = chemical preparations of spacer $(dT)_{20}$; 6,7 = products of ligation of fragment I resp. I + II .

pared by automated DNA synthesis on rA-supports[7], were joined to CPG 3000-icosathymidylate in 46 and 19 % yield, as outlined in CHART 2. The ligation product was characterized by ammoniacal cleavage of the single strand from the support and gel electrophoretic separation (FIG. 1). Replication of the immobilized template was effected by DNA polymerase (Klenow fragment) using oligo-dA as a primer. The full-length double strand was cleaved from the support by Pst I and the correct sequence established according to the method of Maxam and Gilbert.

CONCLUSION

Using one of the above described methods or a combination of these methods, the preparation of long eukaryotic genes is now within the reach of synthetic chemistry. Further work along the lines described here should simplify not only the preparative work, but also the still tedious purification of gene fragments. Experiments aiming at the improvement of yields of RNA ligase joining reactions are under way.

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