A new method for the synthesis of a structural gene*

Hai-Bao Chen+, Jie-Min Weng, Kun Jiang and Jian-Shao Bao
Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Lu, Shanghai 200032, China

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ABSTRACT

A novel method of synthesizing a structural gene or gene fragment, consisting of the first synthesis of a single-stranded DNA (ssDNA), has been developed. As a preliminary test of this method, four synthetic genes or gene fragments have been synthesized. The first one with 396 base pairs (b.p.) codes for the mature rbcS from wheat, the next two with 370 and 342 b.p. respectively, for two half molecules of a gene for trichosanthin and the last one with 315 b.p. for the N-terminal 1 – 102 residues of human prourokinase. In all these syntheses, a plus-stranded DNA of the target gene was generally assembled by a stepwise or one step T_4 DNA ligase reaction of six oligonucleotides (A, *pB, *pC, *pD, *pE and *pF) of 30 – 71 nucleotides long in the presence of two terminal complementary oligonucleotides (Ab′ and eF′) and three short inter-fragment complementary oligonucleotides (bc, cd and de). After purification, the synthetic ssDNA was inserted into a cloning vector, pWR13. The resulting product was directly used to transform a host cell. The structure of the cloned synthetic gene was confirmed by DNA sequence analysis.

INTRODUCTION

The methodological investigation of gene synthesis has been developing rapidly, ever since Khorana devised the strategy of combining chemical and enzymatical method twenty years ago (1, 2). especially, great progress has been made in the organic chemical synthesis of oligonucleotide. That an oligodeoxyribonucleotide with defined sequence around 60 nucleotides long now can be conveniently synthesized on a DNA synthesizer (3) permits the development of new approach for gene synthesis.

As an alternative to Khorana's strategy, a novel method for the synthesis of a structural gene or a gene fragment of around 350 b.p. was developed in our laboratory. One of the key points of this method was to synthesize first the ssDNA of a target gene. The rationale was the same as that of Khorana's based on the premise that DNA ligase is capable of catalyzing the formation of a phosphodiester bond between a 5′-phosphate residue and 3′-hydroxyl residue in a nicked double-stranded DNA molecule. In the new method, as shown in Fig. 1a and 2, six oligonucleotides (A, *pB, *pC, *pD, *pE, and *pF) corresponding to the component fragments of the target gene's plus-stranded DNA, two terminal complementary oligonucleotides (A′b and eF′) abbreviated TerCOs and three short inter-fragment complementary oligonucleotides (bc, cd and de) abbreviated SIFCOs were designed and chemically synthesized first. Each SIFCO was designed with computer-aid to contain special nucleotide sequences enabling it to base-pair with two corresponding oligonucleotides assigned to positions conducive to ready coupling. As a result, a few local short complementary regions were formed with annealing above these fragments. Then, the 3′, 5′-phosphodiester linkages were formed by T_4 DNA ligase reactions of these locally nicked duplex DNAs. In other words, the synthetic ssDNA of a target gene was synthesized in this way by stepwise or one step T_4 DNA ligase reaction.

The molecular cloning of the synthetic ssDNA, another key point of this method, was carried out as shown in Fig. 1b: the ssDNA was first annealed with two TerCOs, which was previously designed to create two restriction endonuclease recognition sites at both 3′- and 5′-termini of the ssDNA, then ligated with a linearized plasmid formed from double digestion of a suitable plasmid by corresponding restriction endonucleases, and finally the resulting product was directly used to transform a host cell. In consequence, the synthetic ssDNA as a part of the recombinant plasmid was replicated *in vivo* to form the double-stranded DNA of the target structural gene. The clones isolated from transformants were checked with suitable restriction endonuclease digestions, and then the correct recombinant plasmid containing the synthetic gene was further confirmed by DNA sequence analysis.

As a preliminary test of the new method, a gene coding for the mature small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from wheat (abbreviated as **WrbcS**) (4) and two half molecules of a gene for trichosanthin (abbreviated as **T**) (5) have been synthesized by stepwise T_4DNA ligase reactions. This being successful, the first part of this method was further extended to one step ligase reaction as shown in Fig. 4 used in the synthesis of a gene fragment coding for the N-terminal

+ To whom correspondence should be addressed

* Zhu Ren (a postgraduate student of this group) Wen Yan (a postgraduate student from the Department of Biology, Peking University) and Xiang-Min Cui (a student also from the Department of Biology, Peking University) partook of the work. This paper is dedicated to the 80th birthday of Prof. Yu Wang.

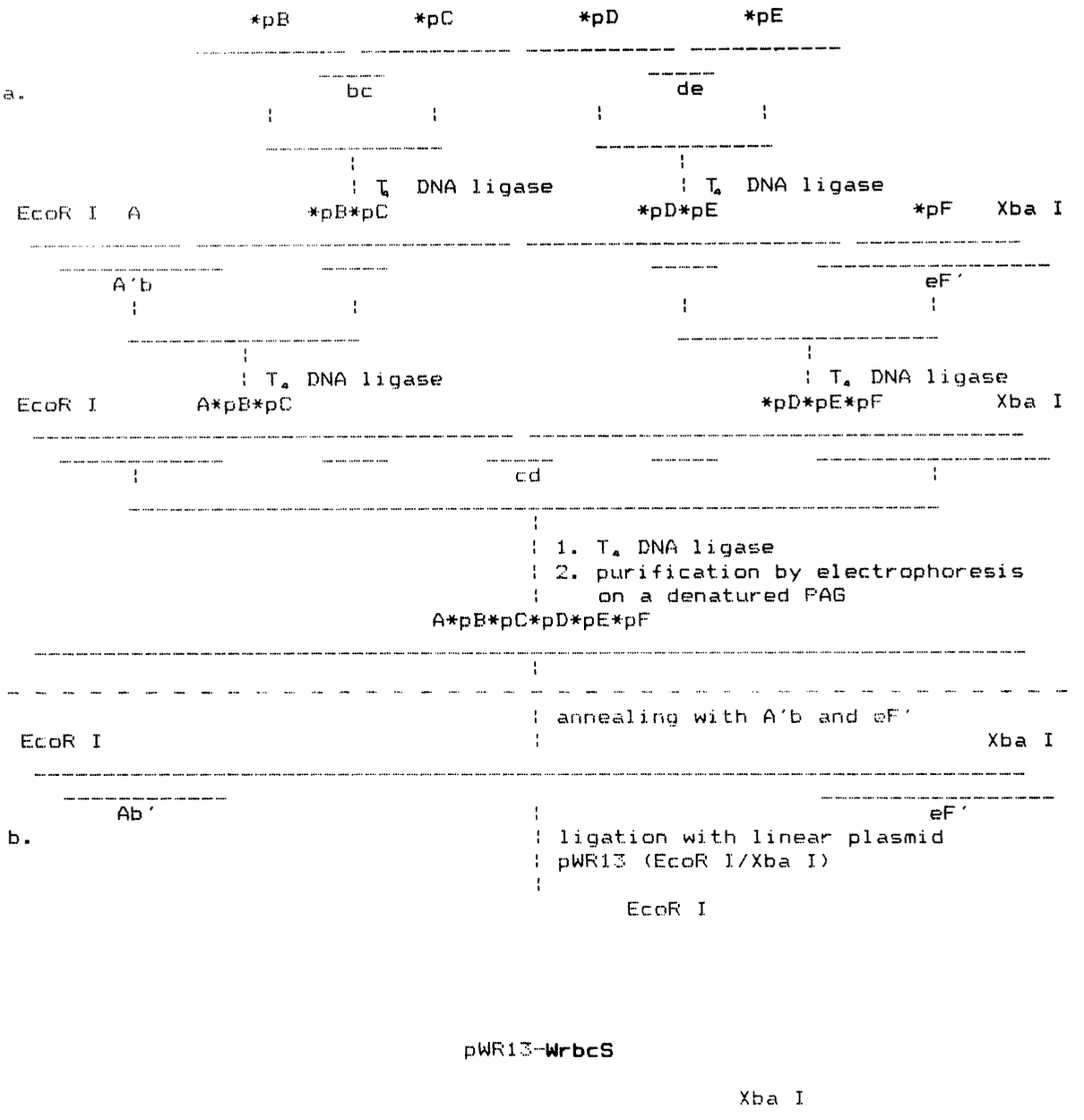

Fig. 1. Synthesis of an ssDNA corresponding to the plus-stranded DNA of a gene for **WrbcS** by stepwise T_4 DNA ligase reaction of six oligonucleotides in the presence of SIFCOs and TerCOs (a) and insertion of the synthetic ssDNA into a cloning plasmid pWR13 (b).

1−102 residues of human prourokinase (6) (**Npuk** as the abbreviation). The detailed syntheses of these genes or gene fragments will be published in separate papers. Here only the general procedure of the new method is dealt with.

MATERIALS AND METHODS

Fully protected 2′-deoxynucleoside-3′-O-methyl-N,N-diisopropylaminophosphoramidites and controlled pore glass (CPG) derivatives of 5′-O,N-protected-2′-deoxynucleosides were purchased from Applied Biosystems or prepared according to (7) and (8), respectively. [1-H]-Tetrazole was obtained from Aldrich and sublimated below 125°C under vaccum. Acetonitrile supplied by E.Merck, Darmstadt was refluxed and distilled separately from phosphorus pentoxide and calcium hydride. [δ-^{32}P]ATP was from Amersham or Institute of Isotopes, Chinese Academy of Atomic Energy, [α-^{35}S]dATP (sp act 800 Ci/mmol, 10 mCi/ml) from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Other ordinary chemical and biochemical reagents were from the local market. Restriction endonucleases, T_4 DNA ligase and T_4 polynucleotide kinase were purchased from New England Biolabs, the heat stable large fragment (9) of DNA polymerase I from the Shanghai Institute of Biochemistry.

Low gelling temperature agarose, ATP, ribonuclease A, bovine serum albumin and lysozyme were obtained from Sigma. 2′-Deoxynucleoside-5′-triphosphates and 2′-,3′-dideoxy-nucleoside-5′-triphosphates from Pharmacia.

Part of the T_4 polynucleotide kinase we used was a gift from Chang-Qin Chen (Shanghai Inst. of Biochem.). pWR13 (10) and *E.coli* strain JM83 (11) were gifts from Li-He Guo (Shanghai Inst. of Cell Biology).

Design of the synthetic gene

The synthetic gene was designed with the aid of a computer VAX 11/780 by running the PSQ and NAQ programmes afforded by NBRF, USA.

Synthesis of oligonucleotide

The phosphoramidite triester method (12, 13) was applied to synthesize oligonucleotide on an ABI 381A DNA synthesizer by running a 0.2 umole programme with 0.5 umole of CPG derivative of protected-2′-deoxynucleosides. After deprotection, the product was purified by denatured polyacrylamide gel electrophoresis (PAGE) as usual. The purified oligonucleotide was [5′-^{32}P]-phosphorylated, isolated and charaterized as mentioned in (8, 14).

T_4 DNA ligase-catalyzed reaction of oligonucleotides

The basic mehtod was essentially the same as described in (14, 15).

(A) Stepwise reaction: The reaction mixture containing Tris-HCl (pH 8, 50 mM), $MgCl_2$ (10 mM), DTT (20 mM), oligonucleotides (10−50 μM each), SIFCOs (12−60 μM each), ATP (100 μM), BSA (50 μg/ml) and T_4 DNA ligase (2000 units/ml, the unit definitions as described in (16)) was incubated at 10°C for over 20 hrs.

Two oligonucleotides such as *pB and *pC (1 nmol each) and their respective SIFCO, bc, (1.2 nmol) were annealed in a solution of 15 μl and then subjected to ligation in a 20 μl reaction mixture at 10°C. After an aliquot was taken, the reaction mixture was mixed with a terminal duplex, for example, A/A′b formed from annealing A and A′b, and fresh ligase (1000 u/ml) was added to support further ligation in a 40 μl of reaction mixture to form the longer polynucleotide A*pB*pC. In the similar way, the polynucleotide *pD*pE*pF was formed. After the aliquots were taken, two of the afore-mentioned reaction mixtures and SIFCO cd were mixed and more ligase was added as above to complete the stepwise reaction for the synthesis of the ssDNA.

(B) One step reaction: Six oligonucleotides, two SIFCOs (bc and de), and two TerCOs (*pA′b and eF′) were combined into following four groups: A/*pA′b, *pB-*pC/bc, *pD-*pE/de and *pF/eF′, and annealed separately. Each in 15 μl of annealing solution containing Tris-HCl (pH 8, 50 mM), $MgCl_2$ (10 mM), oligonucleotides (13−65 μM each), SIFCOs (15−80 μM each) was kept at 90°C for three minutes. As the temperature of the solution was cooled slowly down to 40°C in 1 hr, two annealing solutions obtained from three oligonucleotides were mixed and to the resulting mixture was added with SIFCO cd. While the temperature was further cooled down to 25°C, other two annealing solutions were added into the above-mentioned mixture. Finally, when the temperature was lowered to 10°C, T_4 DNA ligase (2000 u/ml) and other reagents for ligation were added into the mixed solution and incubated at 10°C for 24 hrs. After this operation, another fresh portion of T_4 DNA ligase (1000 u/ml) was added into the reaction mixture and kept at the same temperature for a further 24 hrs.

Detection of thr products or intermediates

The aliquots from a series of reaction mixtures were loaded on a 12 % denatured polyacrylamide gel slab (0.03×18×36 cm), followed by running electrophoresis as usual and visualizing with autoradiography.

Purification of the synthetic ssDNA

The preparative denatured PAGE was coducted to purify the ssDNA. After autoradiography, the band containing the expected product was cut off and the radioactivity was measured. The gel band was smashed and shaken with M-G solution (17) at 35°C overnight. The purified synthetic ssDNA was isolated with centrifugal microfiltration and ethanol precipitation as usual.

Molecular cloning of the synthetic ssDNA

Before cloning, we suggest, it is better to identify the synthetic ssDNA by any means such as, for example, the nearest neighbour analysis.

The purified ssDNA(100 nM) was first annealed with two TerCOs (120 nM). Then, the partially base-paired DNA was coupled with the respective linearized plasmid in a molecular ratio of 20 to 1. The coupling reaction was carried out in 20 μl of ordinary T_4 DNA ligase reaction solution described as above except that the concentration of the linearized plasmid was 0.5 nM. The resulting product was directly used to transform the host competent cells in a routine way. The clones isolated from transformants were checked by several restriction enzyme digestions and the clones proven by restriction digestions were further confirmed by DNA sequence analysis.

Sequence analysis of the synthetic gene

The dideoxy method (19) was performed on a denatured double-stranded plasmid. A denatured plasmid (1 pmole) formed from alkaline treatment (20, 21) was mixed well with primer (5 pmol of 3′- or 5′-universal primer) in 10 μl of buffer containing Tris-HCl (10 mM, pH 7.5) and $MgCl_2$ (5 mM). Into the mixture of template/primer, [α-^{35}S]-dATP (20 μCi) and the heat stable large fragment of DNA polymerase I (0.5 u) in 3 μl of buffer as described above were added. The sample was immediately dispensed into four tubes of A-, G-, C- and T- specific dideoxy-deoxynucleotide mixture (2μl each) as usual.

The reactions were carried out at 65°C for 10 min. and chased for another 10 min. the following procedure was routine.

Determination of radioactivity

A scintillation prob FJ-367 and an automatic counter FH408 manufactured by the 261 Factory in Beijing were used for the measurement.

RESULTS

Design of the synthetic gene

With the aid of a computer, VAX 11/780, which was running the PSQ and NAQ programmes, the design of the synthetic gene in a preliminary nucleotide sequence was finished after inputing the corresponding amino acid sequence and selecting the suitable nucleotides based on the assignment of codon usage and the arrangement of restriction sites.

To ensure correct ligation, which consists of the assembling of several oligonucleotides into an ssDNA, the preliminary nucleotide sequence of the ssDNA needs to be refined besides the design of a few TerCOs and SIFCOs. This is because, in an ssDNA, there are many segments of oligonucleotide, which are able to base-pair with each other inter- or intra-molecularly; among them those showing the potential tending to form base-pairing structure greater than 7 b.p. need to be avoided. With the computer-aid, this sort of oligonuceotide segments were found out and deleted by changing one base, or, in another words,

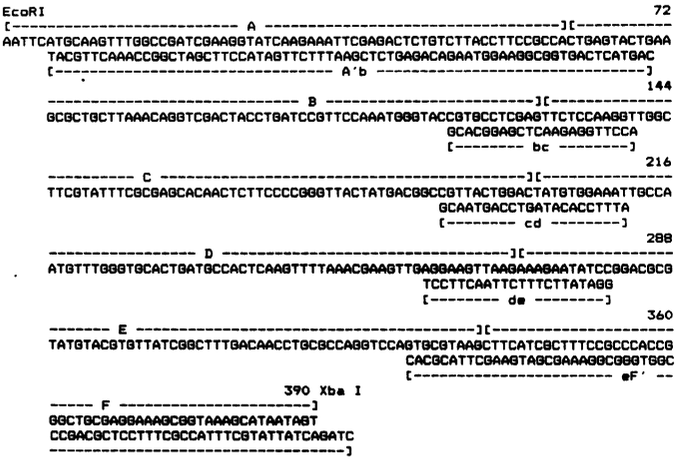

Fig. 2. The oligonucleotide fragments devided by the computer-aid-design for the synthesis of an ssDNA, which corresponds to the plus-stranded DNA of a gene for the mature **WrbcS**.

replacing one original codon by its synonym. As an example, the nucleotide sequence of a gene determined for the mature **WrbcS** is shown in Fig. 2.

Synthesis of the ssDNA

(A) Stepwise reaction: The synthesis of the plus-stranded DNA of a gene for the mature **WrbcS** is described here as an example. Six oligonucleotide fragments (A, B, C, D, E and F as shown in Fig. 2), three SIFCOs (bc, cd and de) and two TerCOs (A'b and eF') were designed, chemically synthesized and purified. Except for fragment A, all the fragments corresponding to the components of the plus-stranded DNA were [5'-^{32}P]-phosphorylated. The fragment *pB and *pC were joined by T_4 DNA ligase reaction in the presence of SIFCO bc , then the reaction mixture was mixed with annealed A/A'b and addition of fresh T_4 DNA ligase to complete the ligation of A, *pB and *pC (Fig. 1a). Similarly, the ligation of *pD, *pE and *pF was finished in the same way. In the end, the synthesis of the plus-stranded DNA was completed by further DNA ligase reaction of the mixed solution formed from the above two reaction mixtures, SIFCO cd and fresh T_4 DNA ligase.

The results of ligation shown in Fig. 3 is another example for the synthesis of the plus-stranded DNA of the 5'-half molecule of a gene for **T**, in this case by a slightly different procedure from the above in the ligation of *pD, *pE and *pF(see legend in Fig. 3). After purification, The purified synthetic ssDNA (20 pmoles) was isolated in a total yield of 2%.

In the similar way, the 3'-half molecule of the gene for **T** was synthesized.

(B) One step reaction: Similarly, six oligonucleotides, three SIFCOs and two TerCOs were designed (Fig. 4) for the synthesis of a gene fragment of **Npuk**. After synthesis and purification, the oligonucleotides B, C, D, E, F, and A'b were [5'-^{32}P]-phosphorylated. The preparation of *pA'b was convenient for the detection of the results of annealing stated below. The annealing and ligation of A/*pA'b, *pB-*pC/bc, *pD-*pE/de and *pF/eF' were carried out as described in METHODS. To detect the annealing results, a series of aliquots from annealing mixtures were analyzed in a nondenatured PAGE (12 %). As the annealing results were shown to be in a good

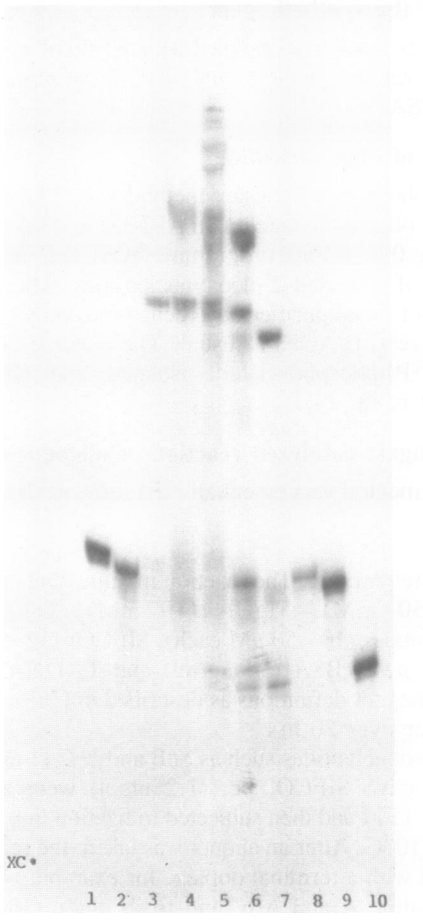

Fig. 3. A stepwise T_4 DNA ligase reaction of chemically synthesized oligonucleotides, A, *pB, *pC, *pD, *pE and *pF to assemble an ssDNA corresponding to the plus-stranded DNA of the 5'-half molecule of a gene for trichosanthin: 1) *pB, 2) *pC, 3) the ligation of *pB and *pC in the presence of SIFCO bc, 4) the ligation of reaction mixture '3' with A/A'b duplex, 5) the ligation of mixture '4' with mixture '6' in the presence of SIFCO cd, 6) the ligation of mixture '7' with *pD in the presence of SIFCO de, 7) the ligation of *pE and *pF in the presence of TerCO eF', 8) *pD, 9) *pE, 10) *pF. The intermediates and the final product in the reaction mixture were not purified, the aliquots from each step were taken and directly separated by electrophoresis in a denatured 12% PAG and visualized by autoradiography.

state, the ligation of the annealed mixture was started by addition of T_4 DNA ligase and other reagents for ligation. After separation with a denatured 12 % PAGE, the ligation mixture resulted in not only production of the expected product but formation of all likely fourteen intermediates as shown in Table 1 and Fig. 5. Bands No. 2 and No. 3 were not separated in a denatured 12 % PAGE, as the difference in base number was too small for discrimination.

After purification, from band No. 1 twenty-one picomoles of synthetic ssDNA with full length proven by nearest neighbour were obtained.

Cloning of the synthetic ssDNA

The synthetic ssDNA, which had been passed by nearest neighbour analysis, was annealed with two TerCOs, the product of annealing was ligated with a corresponding linearized plasmid and the resulting product was directly used to transform the competent cell of *E. coli* JM83 as decribed in METHODS. When plasmid pWR13 was used as the cloning vector, white colonies

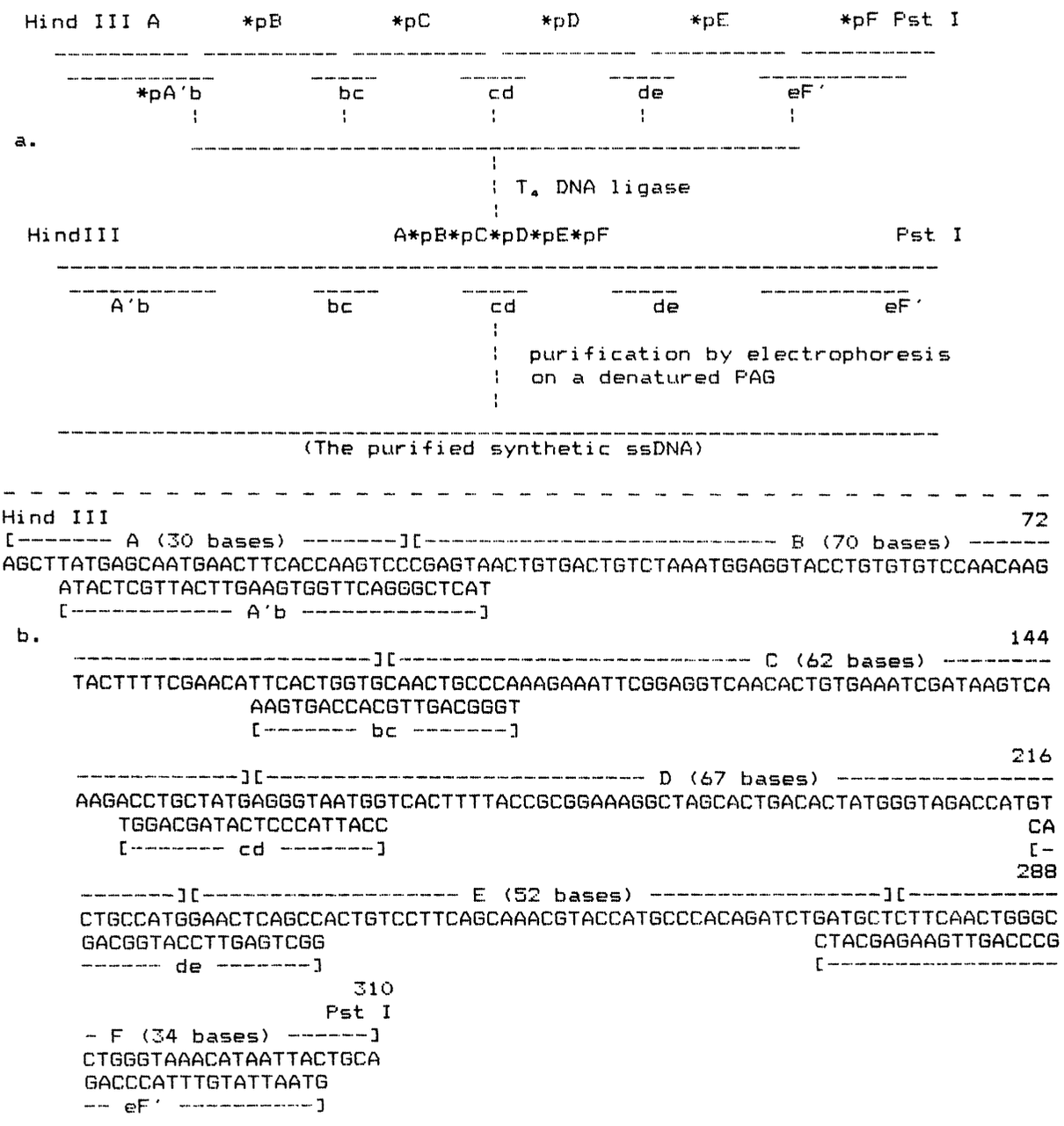

Fig. 4. a). One step T_4 DNA ligase reaction of six oligonucleotides (A, *pB, *pC, *pD, *pE and *pF) in the presence of SIFCOs (bc, cd and de) and TerCOs (*pA'b and eF') to assemble a synthetic ssDNA of the gene fragment coding for the N-terminal 1 – 102 residues of human prourokinase. b). Oligonucleotide fragments, devided by the computer-aid-design, for the synthesis of the synthetic ssDNA.

were chosen because there were several stop codons in the frame of the β-galactosidase gene. The recombinant plasmid isolated from each chosen colony was characterized by several restriction enzyme digestions. Usually five colonies were found to contain the synthetic gene among ten chosen white colonies.

As an example shown in Fig. 6, the plasmid pWR13-**WrbcS**, a recombinant plasmid formed from cloning the synthetic ssDNA for **WrbcS** into plasmid pWR13, and the pWR13, were subjected to digestions with restriction enzymes Sty I, Mlu I and Bgl I, and to double digestion with EcoR I/Xba I separately. The results showing that only pWR13-**WrbcS** was cleaved by enzymes Sty I and Mlu I clearly proved that the synthetic ssDNA has been inserted into plasmid pWR13, because these two unique restriction sites, which did not exist in pWR13, were placed into the synthetic gene by design.

These two plasmids were cleaved by Bgl I into three (e) and two (f) fragments respectively, because of existance of two Bgl I sites in the original plasmid, one of which was located up to about 150 b.p. from the EcoR I site of the polylinker in pWR13, whereas the unique Bgl I in the synthetic gene was designed close to the 3'-end, so the largest fragment resulted from pWR13-**WrbcS** was shorter than that from pWR13, i.e. the former moved faster than the later slightly. EcoR I/Xba I double digestion of pWR13-**WrbcS** resulted in one more band as compared with that from pWR13. Actually, this new band shown in the lane g of Fig. 6 was exactly the one of synthetic gene.

Table 1. Separation of the one step ligation mixture by denatured PAGE from the synthesis of an ssDNA of a gene fragment for human **Npuk**

Band No. on PAGE	Product or intermediates	chain length in bases	fragments linked
1	A*pB*pC*pD*pE*pF	315	6
2	*pB*pC*pD*pE*pF	285	5
3	A*pB*pC*pD*pE	281	5
4	*pB*pC*pD*pE	251	4
5	A*pB*pC*pD	229	4
6	*pC*pD*pE*pF	215	4
7	*pB*pC*pD	199	3
8	*pC*pD*pE	181	3
9	A*pB*pC	162	3
10	*pD*pE*pF	153	3
11	*pB*pC	132	2
12	*pC*pD	129	2
13	*pD*pE	119	2
14	A*pB	100	2
15	*pE*pF	86	2

Sequencing of the cloned gene or gene fragment

Clones confirmed by restriction enzyme digestions were sequenced by the 'dideoxy method' using a denatured double-stranded plasmid as the template from two directions. Here, as an example, the sequence analysis results of a gene for **WrbcS** is shown in Fig. 7. The autoradiogram of the sequencing gel clearly pointed out that the synthetic ssDNA as a whole has been inserted into plasmid pWR13 and replicated *in vivo* to become a double-stranded DNA.

DICUSSION

Actually, nearly twenty years ago, Khorana first reported on a coupling reaction (2) in a three-component joining system:

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      5'  C-C-G-G-T-T-C-G-A-T-T   3'
          | | | | | | | | | | | |
3'  A-G-A-G-G-C-C-A-A-G C-T-A-A-G-G-C-C  5'
                        | |
                     32 p OH
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This is the earliest ligation of two oligonucleotides in the presence of a SIFCO to form a larger oligonucleotide. In 1982, Wu and coworkers adopted this kind of joining system to form a partial duplex followed by repair synthesis to complete the complementary strands used in the cloning of a synthetic human insulin A-chain gene (22).

The idea that the ssDNA can be synthesized by T_4 DNA ligase reaction in the presence of a SIFCO was learned from Khorana's laboratory, M.I.T. by one of the authors, who was a visiting scholar in that laboratory. Doctor Michael Nassal, a postdoctor from the Federal Republic of Germany, has tried to couple two oligonucleotides each of chain length around 60 bases long in the presence of a SIFCO or 'cramp' as called by M. Nassal. Now this idea was extended to assemble the ssDNA of

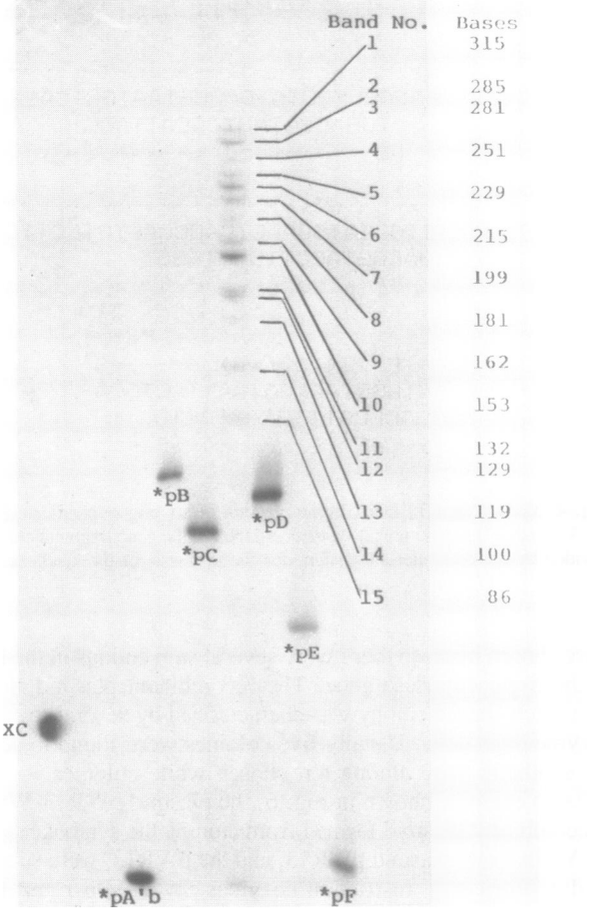

Fig. 5. Autoradiograph of a PAGE showing the results from the one step ligation of six oligonucleotides to assemble an ssDNA corresponding to the plus-stranded DNA of a gene fragment, which was coding for the human **Npuk**. After identification by nearest neighbour analysis, Band No. 1 was the expected product.

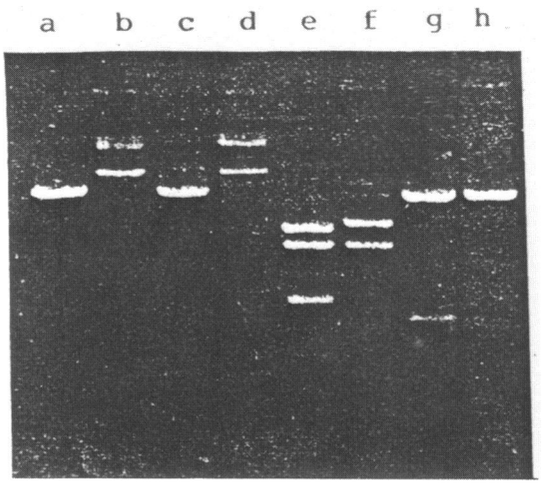

Fig. 6. Agarose gel electrophoretic analysis of a recombinant plasmid pWR13-**WrbcS**, which was subjected to several restriction endonuclease digestions: digestions with a) Sty I, c) Mlu I, e) Bgl I and g) double digestion with EcoR I and Xba I. The respective digestions of pWR13: b), d), f) and h) were as the control.

a gene or gene fragment of around 350 bases, and further developed into a new method for the synthesis of a structural gene after realizing the insertion of an ssDNA into a plasmid.

Our original purpose in developing the new method was to save the material used in the chemically synthesized oligonucleotides. As our results showed, not only was one fourth of the material saved by using this method instead of the routine one, but a considerable percentage of the synthetic work was saved as well. The SIFCO also can be used as a primer in DNA sequencing, if neccessary. Furthermore, the new method is convenient indeed, when the one step ligation of several oligonucleotides to assemble an ssDNA is adopted. Although a few aspects need further improvement. To delineate possible improvements, the following questions may be asked: [1] Why is the yield of ligation so low? no matter which procedure was applied, one step or stepwise. [2] Is it possible by any means to improve the yield to a significant percentage? Whether our method will turn out to be very useful depends to a large extent upon the answers to these questions.

Though the total yield of the synthetic ssDNA produced by the new method was very low (2 %), when used for the synthesis of a gene it was still practicable, because a few picomoles of

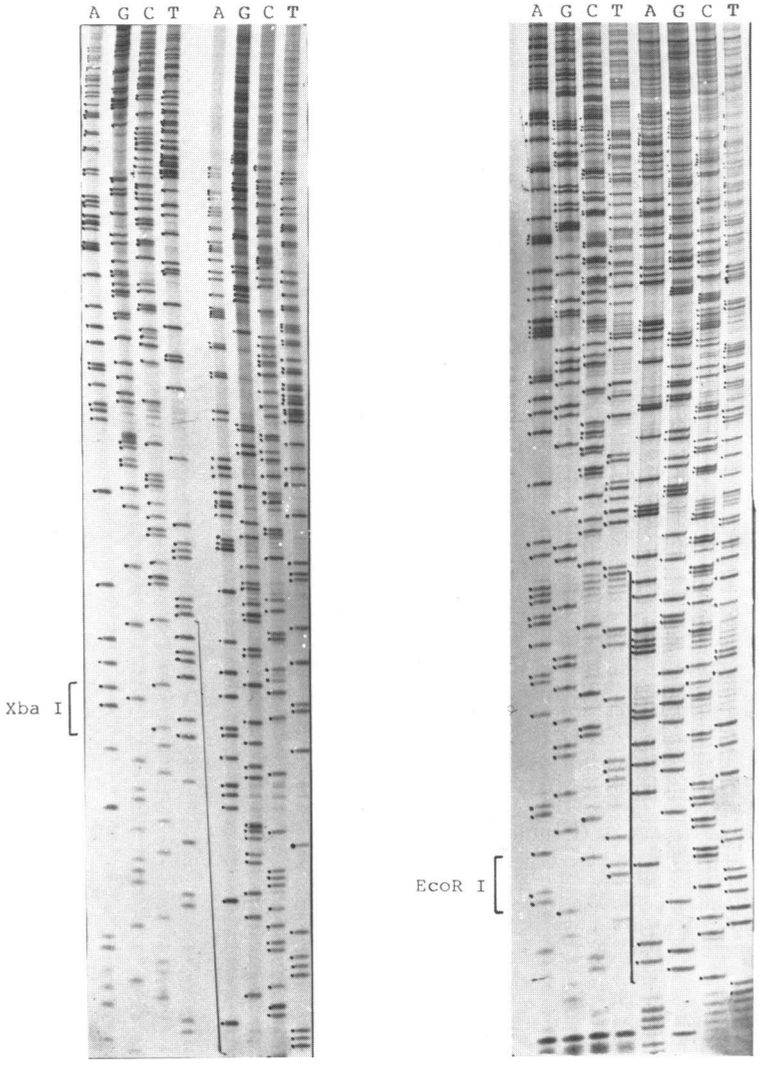

Fig. 7. The nucleotide sequence analysis of a synthetic gene, which was coding for the mature small subunit of RuBisCO from wheat, was achieved by practicing the 'dideoxy method' with [α-^{35}S]-dATP and the heat stable large fragment of DNA polymerase I at 65°C from both directions: in the left, a 3'-universal primer was used and in the right, the result was from a 5'-universal primer.

synthetic ssDNA were enough to finish the cloning of a synthetic gene fragment.

One base being changed in the gene fragment for **Npuk** was detected from the DNA sequencing, the same phenomenon has been observed (23, 24) by other authors, who adopted the general method to synthesize a stuctural gene. Most probably, it has nothing to do with the new method, whereas it came from other causes such as a side chemical reaction taking place during the capping procedure in the synthesis of oligonucleotide (24). This sort of mutation actually can be reduced nearly 50 % by this new method in comparison with the general one.

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