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Effective method for obtaining long nucleotide chains on partially complementary templates

Processed bovine γ-interferon gene obtained from human γ-interferon gene

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The method of obtaining the bovine γ-interferon gene by means of simultaneous multidirected mutagenesis of the human γ-interferon gene is presented. The first strand of the bovine γ-interferon gene was obtained by ligation of synthetic oligonucleotides, using the cDNA of human γ-interferon, cloned in the single-stranded phage M13mp19 as a template. The second strand was synthesized using a large fragment of *E. coli* DNA-polymerase I. The double-stranded gene was then treated by restriction nucleases and cloned in a pUC-18 derived vector. The primary structure was confirmed by sequencing.

Site-directed mutagenesis; Interferon, γ-; Sequence homology; Gene variant; (Bovine)

1. INTRODUCTION

Recent progress in chemical methods of oligonucleotide synthesis led to the possibility of using a chemical-enzymatic approach to obtain long double-stranded sequences, which created large amounts of synthetic genes, coding for different proteins. However in some cases, it is necessary to get analogs of genes, differing from existing ones by several nucleotide substitutions. When these substitutions are localized in limited areas of genes, this problem can be successfully solved using site-directed mutagenesis [2]. Each step of mutagenesis produces a new gene, related to the precursor. However, this approach is unsuitable when the gene needs to be changed at a number of points; for instance, to convert a gene, coding for a protein of one kind into another, coding for a homologous protein of the other kind.

In this paper we describe the method of simultaneous multidirected mutagenesis, which was used for transformation of the human γ-interferon gene into the processed bovine γ-interferon gene. The technique includes the following steps (fig.1): (i) Cloning of the native gene into polylinker region of single-stranded phage M13 or its analogs. (ii) Synthesis of oligonucleotides, forming the desired gene strand, complementary to the cloned one. The synthesis was planned taking into account maximum complementarity according to codon usage. (iii) Ligation of synthetic oligonucleotides using the cloned strand as a template. (iv) Recovery of the ligated strand and synthesis of the second strand with DNA-polymerase using a synthetic primer. (v) Cloning of the obtained double-stranded fragment.

A preliminary report of this work has been published [1]

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2. MATERIALS AND METHODS

Oligodeoxyribonucleotides were synthesized with semiautomatic synthesizer, designed in our laboratory, using 5′-dimethoxytrityl-*O*-nucleoside-3′-phosphoramidites as monomers [3,4].

2.1. *Preparation of the first strand*

30 pmol each of oligonucleotides IV, V, VII, X, XI, XII, XIII, 24 pmol of oligonucleotide II, 60 pmol of oligonucleotides III, VI, VIII, IX were phosphorylated with T4-polynucleotide kinase and rATP. Phosphorylation mixture contained 66 mM Tris-HCl, pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM DTT, 1 mM ATP, reaction volume was 30 μl. Oligonucleotide II (6 pmol) was labelled with [^{32}P]ATP (Amersham 5000 Ci/mmol, 12 pmol) and T4-polynucleotide kinase, phosphorylation was carried out in the same buffer. Kinase was inactivated by heating (70°C, 5 min). Phosphorylated oligonucleotides were combined with nonphosphorylated oligonucleotide I. Single-stranded phage M13, carrying cDNA of the human γ-interferon gene (4 μl, 1 pmol), was added to the mixture. To anneal oligonucleotides with the template, the reaction mixture was slowly cooled for 5 h from 70°C to 4°C. After annealing, the reaction mixture was incubated with T4 DNA ligase (1 μl, 13 U) for 12 h at 4°C, then precipitated with ethanol and run in 8% polyacrylamide gel. The gel was autoradiographed, the band containing the full-length strand was cut off and eluted by the Maxam-Gilbert procedure [5].

2.2. *Preparation of the double-stranded fragment*

The annealing mixture (0.05 pmol of full-length strand, 5 pmol of oligonucleotide XIV, used as a primer, 10 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 60 mM NaCl, the final volume 4 μl) was cooled for 2 h from 70°C to 14°C, then it was incubated (16°C, 30 min) with the DNA-polymerase I large fragment (5 U) in the presence of all dNTP solutions (final volume, 10 μl; final concentrations of dNTP, 0.1 mmol). The enzyme was inactivated by heating (70°C, 5 min). Double-stranded DNA fragment was precipitated, dried, dissolved in buffer (1 mM DTT, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl) and treated with restriction nucleases *Pst*I and *Xba*I. Enzymes were inactivated by heating (70°C, 5 min), the obtained fragment was precipitated. The vector was prepared on the base of pUC-18, hydrolysed by the same restriction nucleases and then treated with alkaline phosphatase [6]. Ligation was carried out with two-fold excess of the vector over the obtained fragment in a buffer containing 66 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM DTT, 1 mM ATP (final volume, 10 μl) and then transformed in competent *E. coli* MH-1 cells as described [7]. Transformed cells were plated on 1% LB agar (ampicillin concentration, 75 μg/ml).

After hybridisation (as described in [8]), approximately 100 positive colonies were found. Twenty of them were characterised by restriction analysis. 10 clones were sequenced by the Sanger procedure [9]. Nonmutant γ-interferon gene was formed by ligation of the *Pst*I-*Eco*RV fragment from clone 1, the *Eco*RV-*Xba*I fragment from clone 2 and M13mp19 vector. *E. coli* TG-1 cells transformed by the ligation mixture and phages with the insert were chosen and sequenced.

3. RESULTS AND DISCUSSION

Mature γ-interferon and human γ-interferon are both 143 amino acid residues long but differ in 55 amino acid residues [10,11]. The ratio of homology between coding areas of these two genes is 62%. By varying the codon usage, we achieved a ratio of homology between the bovine γ-interferon gene and cDNA of the human γ-interferon gene of 81.5%. The adapted gene sequence and its division into oligonucleotides is given in fig.2, which demonstrates that the degree of complementary of the oligonucleotides varies from 65% to 100%. The following requirements were taken into consideration when the synthesis of oligonucleotides (those of which the bovine γ-interferon is composed), were planned: (i) in order to express the bovine γ-interferon gene in prokaryotic cells, the initiation codon ATG was linked to the coding region; (ii) the terminator TAA was introduced at the 3′-end; (iii) new restriction sites *Xba*I and *Pst*I, flanking the gene, were introduced to be used for cloning; (iv) additional restriction sites were introduced to facilitate analysis of recombinants as well as to combine the full-length gene from different fragments; (v) each of the oligonucleotides should be complementary to the

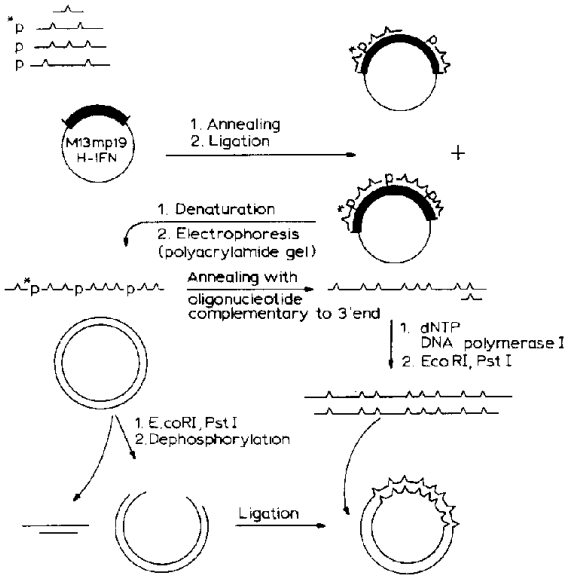

Fig.1. Scheme of the transformation of the homologous gene using synthetic oligonucleotides.

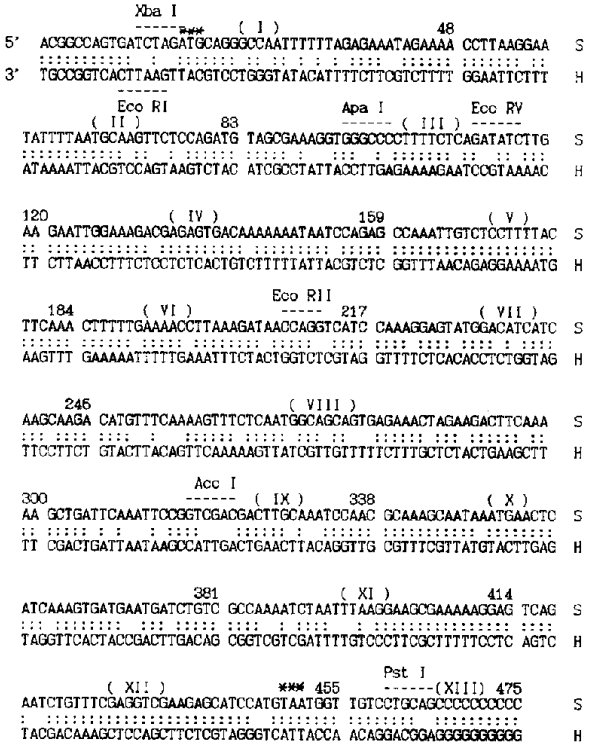

Fig.2. Comparison of nucleotide sequences of the synthetic bovine γ-interferon gene (S) and cDNA of the human γ-interferon gene (H). The distribution of oligonucleotides is shown (numbers in brackets); ATG initiating and TAA terminating codons are marked with stars.

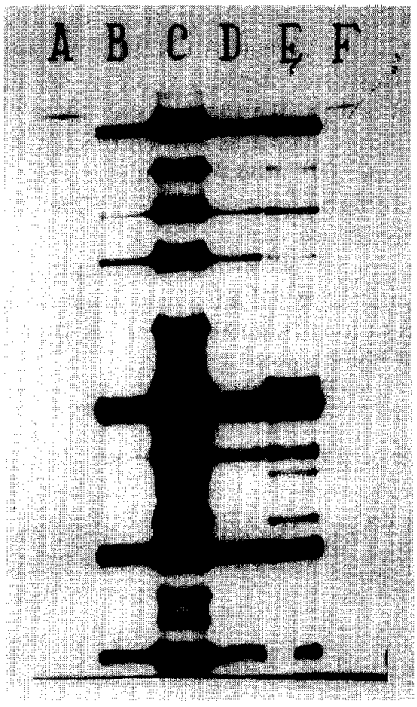

Fig.3. Recovery of the full-length chain by electrophoresis: B,D,E, 30-fold excess of every oligonucleotide over template; C, 150-fold excess of every oligonucleotide over template; B, ligation during 2 days; C–E, ligation during 3.5 days; B,E, 5-fold excess of DNA ligase VS C,D; A,F, the human γ-interferon gene chain.

template at both ends so that the number of complementary residues is not less than 4.

Though the nucleotide sequence of the synthetic gene differs from the native one, the amino acid sequence, coded by the synthetic gene, completely corresponds to the published data.

The degree of complementary is of essential importance to double strand yield: the smaller the complementary, the lower the yield of fully ligated strand (fig.3). The increase of ligation time or increase in concentrations of all oligonucleotides does not raise the yield of the full-length chain. The recovery of the full-length strand is a very important stage, for a great number of clones with chimerical genes and short inserts would make screening more difficult. After ligation the complete strand was recovered electophoretically in denaturating polyacrylamide gel.

Synthesis of the second strand was performed with DNA polymerase I (Klenow fragment) using as primer the synthetic oligonucleotide XIV, d(GGGGGGGCTGCAGGACAACCA), which is complementary to the 452–472 area of the synthetic gene and includes the *Pst*I restriction site.

The double-stranded chimera was treated with *Xba*I and *Pst*I restriction nucleases and cloned in the pUC-18-derived vector. Recombinants were analyzed by hybridisation in situ with a ^{32}P-labelled synthetic oligonucleotide, complementary to the 5′-end region of the gene. 10 clones with the required restriction map were sequenced. Point mutations and deletions were found in all clones: in the insert from clone 1 there is a C⟶T (98) substitution; from clone 2, T⟶G (264); from clone 3, C⟶T (232), T⟶C (234), G⟶C (278), A⟶C (309); from clone 4, C⟶T (197); from clone 5, A⟶CA (113), C⟶T (197), C⟶T (232), A⟶C (240); from clone 6, A⟶T (372); from clone 7, C⟶T (181), G⟶T (280); from clone 8, A⟶C (90), G⟶A (93); from clone 9,

G⟶T (259), C⟶T (263); from clone 10, C⟶T (75), T (261) is absent. A (242) in the insert from clone 5 is absent, A (300) is absent in all inserts from clones 3–6. We suppose that these nonplanned mutations appear as a result of contamination of the monomers used for synthesis. The nonmutant γ-interferon gene was easily formed from two fragments of two clones.

These results show the advantages of simultaneous multidirected mutagenesis in semichemical synthesis of long fragments of genes, transforming available homologous structures.

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