NEW BIOMEDICAL TECHNOLOGIES

Expression of Human Interleukin-10 in *Escherichia coli* Cells

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The human interleukin-10 gene has been synthesized by the chemical enzymatic method. Vectors for cytoplasmic and periplasmic expression of recombinant interleukin-10 have been obtained in *E. coli* cells. A high level of protein expression was found to be characteristic of only recombinant strains producing interleukin-10 as "fused" protein (fused with the N-terminal fragment of interleukin-3).

Key Words: *interleukin-10; gene synthesis; producer strain*

Interleukin-10 (IL-10) is a cytokine produced by activated T cells, B cells, keratinocytes, and monocyte/macrophages [8]. IL-10 was first detected as a product of murine type 2 T helpers (Th2) and was at first called "cytokine synthesis inhibitory factor" (CSIF) because of its capacity to inhibit the synthesis of such cytokines as γ-interferon, interleukin-2, interleukin-3, tumor necrosis β-factor, and granulocyte and macrophage colony-stimulating factor produced by type 1 T helpers (Th1) [5,9]. The studies that ensued showed that both murine and human IL-10 exert multiple biological activities: they costimulate the proliferation and differentiation of human B cells [10], murine thymocytes, T cells [4], and mast cells [11], and regulate the expression of the class II major histocompatibility complex on murine B cells [6]. Murine IL-10 has been shown to possess a cellular receptor (IL-10R) belonging to class II (interferon-receptor-like group) of the cytokine receptor family [7]. Due to its immunoregulatory properties, IL-10 is believed to be clinically useful in various autoimmune diseases and as a potential antiinflammatory agent [8].

Investigation of the biological characteristics of IL-10 requires that methods for the preparation of recombinant proteins be developed, for this is the only route for obtaining this preparation in quantities sufficient for medicinal purposes. We synthesized the human IL-10 gene and investigated the potentialities of its expression in *E. coli* cells.

MATERIALS AND METHODS

Twenty-six oligonucleotides were synthesized using a MilliGen/Biosearch 8700 automated four-column DNA synthesizer after a modified amidophosphite synthesis protocol which involves the use of 5'-(4,4'-dimethoxytriphenylmethyl)-N-substituted-2'-deoxynucleoside-3'-O-(2-cyanethyl)-diisopropylamido-phosphites as monomers for the oligonucleotide chain build-up. Oligonucleotides were purified by high-performance liquid chromatography first on an ion-exchange Synchropak AX 300 column (250×4.6 mm, Synchrom, Inc.) in a 0.05 M po-

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tassium dihydrophosphate density gradient in 60% formamide (0.6 M ammonium sulfate and 0.05 M potassium dihydrophosphate in 60% formamide) followed by reverse-phase chromatography on a Nucleosil 300-5-C18 column (250×4 mm, Macherey-Nagel) in the system 0.1 M ammonium acetate - 0.1 M ammonium acetate in 50% acetonitrile. After purification, the oligonucleotide solutions were desalinated on a TSK gel G2000SW column (Toyo Soda). The quality of the resultant oligonucleotides was controlled by electrophoresis in denaturating polyacrylamide gel.

The gene was synthesized after the following protocol: 16 oligonucleotides forming the second sequence block were mixed in equimolar amounts (15 of them were pre-dephosphorylated using phage T4 polynucleotide kinase), the resulting mixture was annealed and ligated, and the forming second-block dimer (756 nucleotide pairs, n.p.) was isolated by electrophoresis in agarose gel. The

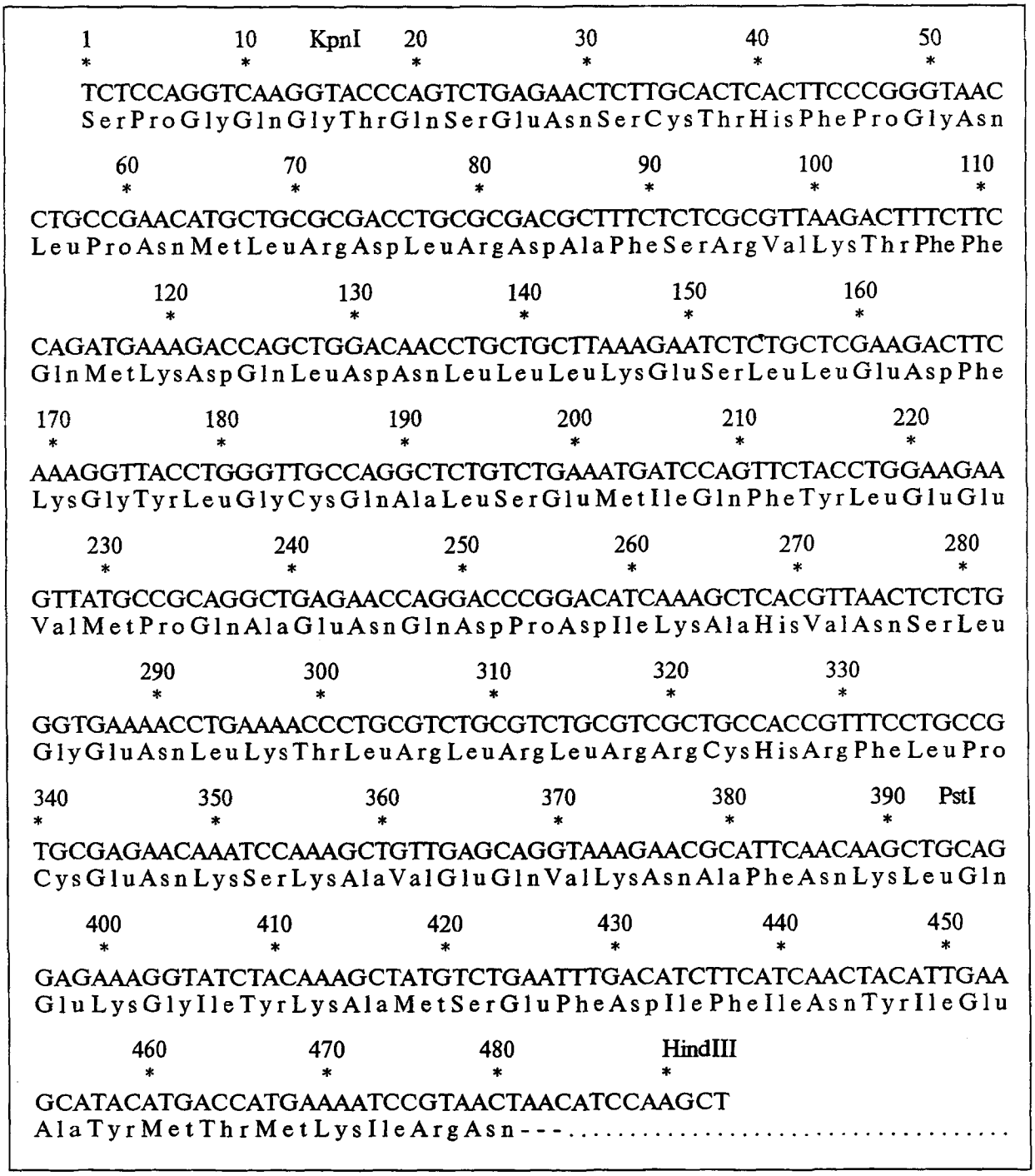

Fig. 1. Structure of the synthetic human IL-10 gene. The arrows show the sites of respective restriction endonucleases.

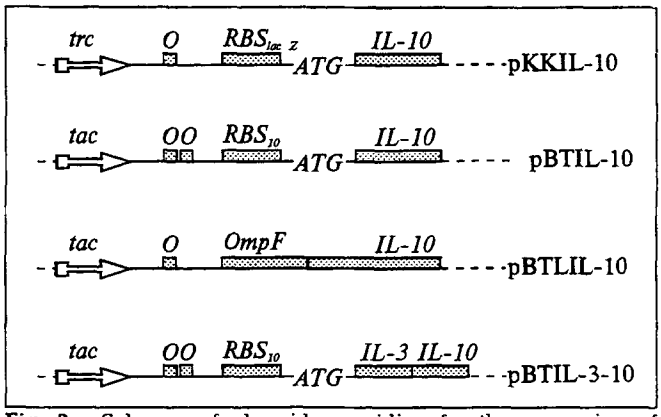

Fig. 2. Schemes of plasmids providing for the expression of the human IL-10 gene (pKKIL-10, pBTIL-10, pBTLIL-10, pBTIL-3-10). *tac* and *trc*: promoters; *O*: operator site of lac-repressor binding; RBS_{lacZ} and RBS_{10}: binding sites for ribosomes of lacZ genes and phage T7 protein 10; *ATG*: start codon; *OmpF*: fragment of OmpF gene containing 5'-untranslated sequence and the sequence of the leader peptide of the *E. coli* OmpF gene; *IL-3*: fragment (204 n.p.) of IL-3 gene; *S*: spacer DNA site coding for the amino acid -Asn-Ser-Gln-Ile- sequence; *IL-10*: human IL-10 gene. Broken lines show the sequence of pKK223-3 plasmid.

dimer was then cleaved by the respective restriction enzyme and the resultant fragment (378 n.p.) was cloned in pUC18/KpnI-PstI vector. The third block of the IL-10 gene (94 n.p.) was synthesized similarly using 4 oligonucleotides and then cloned in vector pUC18/PstI-HindIII. Plasmids pUC18-blockII and pUC18-blockIII were isolated from the resultant clones and analyzed by the sequence of corresponding fragments after Sanger.

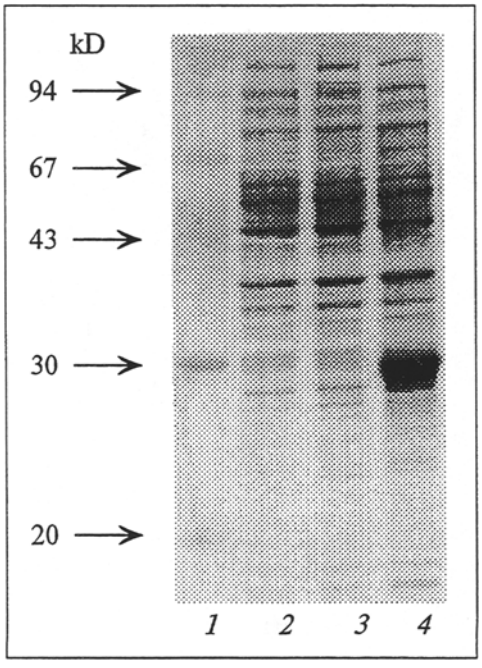

Fig. 3. Electrophoregram of the total cellular protein of strain TG1(pBTIL-3-10). *1*) molecular weight markers (Pharmacia); *2*) TG1(pKK223-3) strain used as control preparation; *3*) TG1(pBTIL-3-10) strain grown in the presence of inductor IPTG; *4*) TG1(pBTIL-3-10) strain grown without inductor.

Then a DNA fragment (94 n.p.) from plasmid pUC18-blockIII was cloned by PstI and HindIII sites of pUC18-blockII plasmid, as a result of which plasmid pUCnc was obtained containing the IL-10 gene sequence (18-489 n.p.). This gene fragment and oligonucleotides containing the 17 n.p. sequence of the 5'-terminal site of the IL-10 gene and the "sticky ends" of the corresponding restriction enzymes (three variants of sequences) were cloned in expression plasmids. The structures of all the resultant variants of the IL-10 gene in expression vectors were confirmed by sequencing the respective sequences after Sanger.

All the genetic engineering manipulations, polyacrylamide gel electrophoresis of oligonucleotides, DNA, and protein, culturing of recombinant strains, and cell lysis were carried out using standard methods [2]. Plasmid pKK233-2 (Pharmacia) and plasmids from the laboratory collection derived from pKK223-3 plasmid (Pharmacia) were used for the construction of expression vectors. *E. coli* TG1 strain was used in the study [2].

RESULTS

We determined the structure of the IL-10 gene proceeding from the published amino acid sequence of IL-10 protein [12] with consideration for codon incidence in *E. coli* cells [3] and for the insertion of endonuclease sites needed for the subsequent chemicoenzymatic synthesis of the gene and its cloning in the DNA sequence. Introduction of endonuclease NcoI, KpnI, PstI, and HindIII sites in the gene sequence made it possible to synthesize the gene by consecutive assembly of three blocks: the first (1-17 n.p.) limited by the NcoI* (the site not restored after gene cloning in expression vector) and KpnI (coding for the N-terminal protein site) endonuclease sites; the second block (18-395 n.p.), limited by sites KpnI and PstI, codes for the central protein fragment; the third (396-489 n.p.) is restricted by the PstI and HindIII sites and codes for the C-terminal fragment of the protein. Plasmid pUCnc constructed at the first stage of our research contained the main part of the IL-10 gene sequence (18-489 n.p.). Using oligonucleotides of the corresponding structure, we obtained the following expression vectors containing the complete sequence of mature IL-10 protein gene (Fig. 1):

For cytoplasmic expression of IL-10:

1. pKKIL-10 plasmid, constructed on the basis of pKK233-2 vector (Pharmacia) containing trc

promoter, lacZ gene RBS, and the IL-10 gene sequence with an extra start codon ATG.

2. pBTIL-10 plasmid constructed on the basis of pBTIL-3 vector (from the laboratory collection) contains tac promoter, two operator binding sites for lac-repressor, and RBS of phage T7 protein 10 gene.

For periplasmic expression of IL-10:

3. pBTLIL-10 based on pBT-hEGF vector from the laboratory collection [1] contains tac promoter, the sequence of *E. coli* protein OmpF leader peptide, and the mature part of the IL-10 gene.

For expression of IL-10 as "fused" protein:

4. pBTIL-3-10 based on pBTIL-3 vector contains tac promoter, two operator sites for lac-repressor binding, RBS of phage T7 protein 10 gene, the N-terminal fragment of the IL-3 gene (204 n.p.) "fused" with the IL-10 gene via a spacer site coding for the amino acid sequence: -Asn-Ser-Gln-Ile-. The schemes of the resultant plasmids are presented in Fig. 2.

Plasmids pKKIL-10, pBTIL-10, pBTLIL-10, and pBTIL-3-10 were inserted in *E. coli* strain TG1 by Ca^{2+}-dependent transformation. Bacterial cell cultures were grown in LB medium or minimal M9 medium in the presence of casamine acids (0.5%) and ampicillin (20 μg/ml) at 37°C. The production of recombinant protein was induced by the addition of 0.5 mM isopropylthiogalactoside (IPTG). Aliquots of noninduced and induced cultures were collected and total preparations of cellular protein derived from these cultures were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. It is noteworthy that this method permits detection of recombinant protein only if its share is at least 2-3% of the total *E. coli* cell protein. Attempts at the detection of IL-10 protein in the analyzed clones of strains TG1(pKKIL-10), TG1(pBTIL-10), and TG1(pBTLIL-10) by the above method failed (no electrophoregrams presented). Hence, these strains do not produce IL-10 in amounts surpassing 2 to 3% of the total cellular protein under the aforesaid conditions of cell culturing. A high level of recombinant IL-10 production as "fused" protein was observed when culturing strain TG1(pBTIL-3-10) in the absence of an inductor (Fig. 3). The growth of this strain in the presence of IPTG was associated with accelerated death of plasmid-containing cells, this leading to a marked drop of recombinant protein production.

Hence, attempts at obtaining a high level of production of IL-10 authentic to mature human IL-10 in *E. coli* cells failed despite the use of two variants of ribosome binding sites during cytoplasmic expression of the gene and a sufficiently effective leader peptide in periplasmic expression. This effect may be due to both proteolytic cleavage of the synthesized protein and to specific features of the mRNA structure of IL-10. Strain TG1(pBTIL-3-10) ensured a sufficiently high level of IL-10 production as "fused" polypeptide (20 to 30% total cellular protein). Further modification of this expression vector is possible, for example, insertion of the corresponding DNA fragments coding for the peptides - cleavage sites for such proteases as collagenase or Xα factor. Specific proteolytic cleavage of hybrid protein and renaturation of the resultant polypeptide will make it possible to obtain active human IL-10 in sufficient amounts.

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