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(56) Documents cited by ISA
US 4582800 A
Zhurnal Vsesojuznogo khimicheskogo obschestva im. D.I.Mendel ceva, tom XXIX Nr. 2, 1984 see pages 86-89
"Biotekhnologia" 1984, see pages 204-205

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(54) Recombinant plasmid DNA pPR–IFNβ1–13, coding for synthesis of human fibroblast β–1 interferon, method of its construction and strain of bacteria Escherichia coli VNIIGENETIKA VL903 (pPR–IFNβ1–13) as producer of human β1–interferon containing it

(57) A recombinant plasmid DNA pPR-IFNβ1–13, coding for synthesis of human β1–interferon has a size of 3.9 k.b.p.and consists of the following elements: BamHI–Bgl II, a fragment of a size of 3.4 k.b.p. of vector plasmid obtained on the basis of pPR40 and pML24; EcoRI–Sau 3A, a fragment of plasmid plNβ–trp7 of a size of 510 b.p. deposited on 12.01.87 under No. 1825 at the culture collection of the USSR Research Institute for Antibiotics. A method of construction of the above plasmid DNA comprises the preliminary construction of vector plasmid pPR124B, then the construction of intermediary plasmid pPR–IFNβ1–123 with subsequent construction of plasmid pPR–IFNβ1–13. A strain of bacteria Escherichia coli VNIIGENETIKA VL903 (pPR–IFNβ1–13) as producer of human β1-interferon deposited on 12.01.87 under No. 1825 at the culture collection of the USSR Research Institute for Antibiotics.

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RECOMBINAT PLASMID DNA pHR-IFNβ1-13 CODING SYNTHESIS OF FIBROBLASTIC HUMAN β 1 - INTERFERON, METHOD OF ENGINEERING THEREOF AND STRAIN OF BACTERIA Escherichia Coli VNIIGENETIKA VL 903 (pHR-IFNβ1-13) PRODUCING HUMAN β1-INTERFERON CONTAINING SAME

Field of the Invention

The present invention relates to the art of genetic engineering and biotechnology and, more particularly, to a novel recombinant plasmid DNA pPR-IFN.1-13 coding the synthesis of a fibroblastic human ! 1-interferon, to a method for preparing thereof and to a strain of bacteria Escherichia Coli VNIIGenetika VL 903 (pPR-IFN! 1-13), which is a producer of human /1-interferon, incorporating the same.

Prior Art

Interferons are proteins synthesized by specialized human cells in response to a viral infection or an effect of various inductors. The experimental data available at the present time point to antiviral, antiproliferative and immunomodulating effects of interferon upon treatment, therewith, of individual cells, tissues and the organism as a whole. As regards its versatile character and significance for the organism, the system of interferon is comparable with the system of immunity. In accordance with antigene, biological and chemical properties, as well as depending on the type of cells producing them, human interferons are divided into three groups: α - leukocytic, β - fibroblastic, γ - immunic which are produced mainly by cells of leukocytes, fibroblasts and T-lymphocytes respectively.

By the present time the ranges of application of interferons in medicine are determined in general. These applications are keratitises and dermatoses, treatment of respiratory infections induced by various viruses (influenza, adenoviruses) Hepatitises B and the like.

However, the mechanism of action of interferons, their structural and functional features and the clinical potential have not been studied adequately enough. This is to a great extent associated with the difficulties of obtaining

preparative amounts of pure interferons by traditional methods based on the use of induced cultures of human cells as a source of this protein. Furthermore, the interferon synthesized in this case comprises, as a rule, a mixture of its different subtypes and forms differing in their structural-and-functional characteristics. In this respect, the preparation of individual interferons by a microbiological synthesis acquires an ever-growing scale.

Known in the art is a number of methods for the preparation of human interferons α, β and γ based on the use of bacteria as producers, in particular various strains of Escherichia coli, Bacillus subtilis, Pseudomonas sp. containing recombinant plasmid DNAs which ensure expression of heterologic genes in a new genetic environment. In the case of a fibroblast human, interferon (IFBβ1), for ensuring expression of the gene IFNβ1 in cells of E.coli use is made of setting the coding portion of a mature interferon under the control of signals of transcription and translation of genes (tuf B, rec A) and operons (lac UV 5_{trp}) of E.coli and coliphages ($P_L\lambda$). A mature β1-interferon formed as a result of the microbiological synthesis (with a molecular mass of about 19,000 Dalton) is characterized by the absence of the carbohydrate component and by the availability of a formyl-methionine N-terminal residue instead of the methionine one. However, this distinction does not result in a change in the biological activity of a protein product of the gene IFNβ1 as compared to the natural glycoprotein produced in cells of fibroblasts. The β1-interferon synthesized by this microbiological method can be used both for studies of molecular mechanisms of interaction with cell receptors and in medical practice.

Known in the art are recombinant plasmid DNAs coding the synthesis of a fibroblastic human β1-interferon such as pC1857 and pPL c 245HFIF25 and the strain E.coli SG4044 containing these plasmids (Remaut E., Staussenes P., Fiers G., 1983, Nucl. Acida Res. 11, 4677-4688).

In the recombinant plasmid DNA pPL c245HFIF25 the transcription of the gene IFNβ1 is controlled by the regu-

latory unit P_LO_L of the bacteriophage λ and initiation of translation of the protein product of the gene is ensured at the account of engineering of a hybrid region of binding of ribosomes on the basis of the SD-sequence of the gene of replicase of the phage MS2. The maximum yield of human β1-interferon ensured by culturing of the above-identified strain is 4% of the total protein of plasmid-containing cells of E.coli.

The above-mentioned strain is characterized by that the controlled expression of the gene IFNβ1 ensured with the help of the P_L promotor necessitates an additional plasmid pC1857 with a positioned thereon temperature-sensitive gene regulating cIts857, of the phage λ . This results in a potential instability of the recombinant plasmid under conditions of the strain culturing on a large-scale production due to a recA-dependent recombination between the plasmids pC1857 and pPL c245HFIF25 at homological areas. Furthermore, the use of a relatively short SD-sequence of the gene of replocase of the phage MS2 for organization of a hybrid region of binding of ribosomes does not make it possible to fully realize the potential power of the translation apparatus of E.coli due to an insufficiently effective interaction with ribosomes upon initiation of translation of fibroblastic interferon. Besides, the absence of ρ- independent transcription terminators at the 3'-terminal field of the gene IFNβ1 in the plasmid pPL c 245HFIF25 results in a substantial reduction of replicability and even in a probable loss of the recombinant plasmid under conditions of depression of a highly-effective P_L promotor of the phage λ ensuring initiation of transcription of the gene IFNβ1.

All this does not allow to reach a high level of the biosynthesis of the product of the gene IFNβ1 and can result in a further lowering of the content ofβ1-interferon in a biomass of the producer strain upon its large-scale culturing, thus complicating recovery of the pure protein and lowering the yield of the desired product.

Disclosure of the Invention

The recombinant plasmid DNA pPR-INFβ1-13, the method of its engineering and the strain of bacteria Escherichia coli VNIIGenetika VL 903 (pPR-IFNβ1-13) containing the same are novel and hitherto unknown from the literature.

The present invention is directed to the provision of a novel recombinant plasmid DNA coding the synthesis of fibroblastic human β1-interferon, a method of its engineering and a novel highly-productive strain producing human β1-interferon containing the same which would ensure preparation of β1-interferon in a high yield.

This object is accomplished by that the recombinant plasmid DNA pPR-IFNβ1-13 coding the synthesis of human fibroblastic β1-interferon according to the present invention has the size of 3,900 b.p. and consists of the following units: BamHI-BglII fragment with the size of 3,4000 b.p. of the vector plasmid pPRI24B produced on the basis of pPR40 and pML24-EcorI-Sau3A fragment of the plasmid PINβ-trp7 with the size of 510 b.p. and has the following features:

- incorporates a region responsible for initiation of replication and its regulation - ColEI-replicon, a gene of the repressor cIts 857 of the phage λ, a gene of resistance to ampicillin Ap^r, a tandem of ρ-independent terminators of transcription of the phage fd, a regulatory field P_RO_R and a SD-sequence of the gene cro coding the sequence of a mature fibroblastic human interferon IFNβ1 with its own methionine codon;

- ligation of the regulatory field of the gene cro, coding portion of β1-interferon and terminators of transcription of the phage fd is effected so that before the gene IFNβ1 a hybrid region of combining ribosomes is formed which has the following nucleotide sequence: 5'-...TAAGGAGGTTGCATG...-3', wherein TAAGGAGGT - SD-sequence of the gene cro of the phage λ, ATG - methionine codon of fibroblastic interferon and, directly after the terminating codon of the gene IFNβ1 a tandem of ρ-independent terminators of transcription of the phage fd is located;

- has unique regions of recognition of restrictases cla I whose coordinate is the beginning of count (0), Pvu II (about 1,110), Bgl II (about 1,480), AccI (about 1,870), Pvu I (about 3,360); deposited on 12.01.87 at the Collection of Cultures of Microorganisms of the All-Union Research Institute of Antibiotics and registered under No. 1825.

The recombinant plasmid DNA pPR-IFNβ1-13 according to the present invention ensures expression of the gene IFNβ1 under the control of the regulatory field P_RO_R of the bacteriophage λ. The gene cIts857 - regulator of initiation of transcription from preceding promotors of the bacteriphage λ and the gene IFNβ1-13 which is under control of the P_R promotor are positioned in the same recombinant plasmid pPR-IFNβ1-13. This circumstance makes it possible to avoid a potential instability during culturing of the strain with the recombinant molecule of DNA, since in the structure according to the present invention no recA-dependent recombination originates due to the absence of an additional plasmid in the producer-strain.

The present invention also relates to a method of engineering the recombinant plasmid DNA pPR-IFNβ1-13 comprising cleavage of the vector plasmid pPR124B, built on the basis of the plasmids pPR40 and pML24, by means of the restriction endonucleases EcoRI and Bgl II and ligation of the greater of the formed fragments of the vector molecule with the EcoRI-Sau3A fragment of the plasmid pINFβ-trp7 containing the coding portion of a mature β1-interferon; in the thus-produced recombinant plasmid pPR-IFNβ1-123 there is effected nearing of the SD-sequence of the gene cro and of the first (methionine) codon of β1-interferon, wherefor the plasmid DNA is hydrolyzed by means of the restrictase BamHI, a portion of nucleotides is removed by means of the endonuclease activity of the DNA-polymerase I E.coli revealed under conditions of an incomplete set of nucleosidetriphosphates in the reaction mixture, the DNA is fermentatively cleaved by means of the restrictase EcoRI, treated with the SI-endonuclease to remove single-chain regions of the DNA, then the two-chain terminals are restored by means of a Klenov fragment of the DNA-polymerase

I E.coli and the formed linear DNA molecules are cyclized by means of the DNA-ligase of the phage T4; the resulting preparation is used to transform cells of E.coliC600 with selection of the transformants on a medium with ampicillin upon culturing of the cells at the temperature of 28 °C, followed by selection of the clones having a reduced growth rate at the temperature of 42 °C, whereafter from the thus-selected clones the recombinant plasmid pPR-IFNβ1-13 is isolated.

The use of the method for engineering of the recombinant plasmid pPR-INFβ1-13, wherein the longest (out of the known for the matrix RNA E.coli and coliphages) SD-sequence of the gene cro of the bacteriophage λ is connected with the coding portion of mature human β1-interferon, results in organization of a hybrid region of combining ribosomes of the gene IFNβ1 with the optimal primary and steric structure of the 5'-terminal area of the matrix RNA. In contrast to the known method, the method of engineering according to the present invention also ensures the presence of tandem of ρ-independent terminators of transcription in the 3'-non-translated area of the gene IFNβ1 on the plasmid pPR-IFNβ1-13, thus preventing interference between replication of the plasmid DNA and a highly-effective transcription of the gene of fibroblast interferon under the conditions of depression of the P_R promotor.

Apart from the above-said, the present invention also relates to a strain of bacteria Escherichia coli VNIIGenetika VL 903 (pPR-IFNβ1-13) producing human β1-interferon which incorporates the recombinant plasmid DNA pPR-IFNβ1-13 according to the present invention and obtained by the method of genetic engineering through introduction of the plasmid DNA pPR-IFNβ1-13 into bacteria Escherichia coli, deposited on 12.01.87 at the Collection of Cultures of Microorganisms of the All-Union Research Institute of Antibiotics and registered under No. 1825.

The strain according to the present invention makes it possible to ensure a stable accumulation of human β1-interferon under conditions of a large-scale culturing and to attain a yield of the desired product of above 2×10^9 U/l

which corresponds to 5-10% of the total amount of the cell protein.

Best Mode for Carrying Out the Invention

The method for engineering the recombinent plasmid pPR-IFNβ1-13 according to the present invention is carried out in several stages.

In the first stage the vector plasmid pPRI24 is engineered.

To this end, deletion by means of the endonuclease Bal31 is effected in the DNA of the plasmid pPR40 (Molekulyarnaya Biologiya, 1987, vo. 21 No. 5, Moscow, p. 1309-1321) in the region of recognition of the restrictase Bgl II.

In this manner, the plasmid pPRI00 is obtained, wherein the region of recognition BamHI is located directly after the area of combining of ribosomes of the phage λ so that in the region of the initiating AUG codon a sequence is formed:

AUGGATCC

BamHI

Afterwards, using conventional procedures of genetic engineering, the smallest PstI-BamHI fragment of the plasmid pPRI00 is ligated with the greatest PstI-BamHI fragment of the plasmid pML24 with the formation of the plasmid pPRI24, whereinto a region of recognition of the restructase Bgl II is introduced by means of an oligonucleotide linker. The plasmid pPRI24B is thus obtained.

The an intermediate plasmid pPR-IFNβ1-123 is engineered.

The thus-produced vector plasmid pPRI24B is cleaved by means of restriction endonucleases EcoRI and Bgl II and ligation of the greater fragment out of the formed fragments of a vector molecule with the EcoRI-Sau3A fragment incorporating the coding portion of mature β1-interferon from the plasmid pINβ-trp7 is effected to give the intermediate recombinant plasmid pPR-IFNβ1-123.

Then the third stage is conducted - engineering of the plasmid pPR-IFNβ-13. To this end, in the intermediate plas-

mid pPR-IFNβ1-123 the nearing of the SD-sequence of the gene cro and of the first codon of β1-interferon, wherefor the plasmid DNA is hydrolyzed by means of the restrictase BamHI, a portion of nucleotides is removed by means of the endonuclease activity of the DNA-polymerase I E.coli, the DNA is cleaved by means of the restrictase EcoRI, treated with the SI-endonuclease, followed by a complete restoration of the two-chain terminals by means of a Klenov fragment of the DNA-polymerase I E.coli and cyclization of the resulting linear DNA molecules is ensured by means of the DNA-ligase of the phage T4. The thus-obtained preparation is used to transform the cells of E.coli C600, the transformants are selected on a medium with ampicillin upon culturing of the cells at the temperature of 28 °C, followed by selection for a lowered rate of growth at the temperatured of 42 °C. From the thus-selected clones the plasmid DNA pPR-IFNβ1-13 is isolated.

The strain of bacteria Escherichia coli VNIIGenetika VL 903 (pPR-IFNβ1-13) according to the present invention is produced by transformation of the recipient strain with the recombinant plasmid pPR-IFNβ1-13, followed by selection of the recombinant clones on a medium with ampicillin at the temperature of 28 °C and determination of the activity of fibroblastic β1-interferon in extracts of the transformant cells after depression of the P_R-promotor ensured by culturing of the strain for 1-2 hours at the temperature of 42 °C. As the recipient the strains E.Coli C600 and other derivatives of E.coli K12 can be used.

The strain E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13) is characterized by the following features.

Morpholigical features. Cells straight, bacilliform (1.2-1.6)x(2.0-6.) um, low-mobile, capable of producing thread-like forms, gram-negative, non-sporiferous.

Cultural features. Cells grow well on dense and liquid ordinary synthetic, semi-synthetic and complex media. When grown on an agarized Hottinger's broth or L-broth they form mucus-coated, round, slightly matted colonies. When grown in liquid media such as L-broth or M9 with casamine acids they form a uniform suspension.

Physiologo-biochemical features. Cells are capable of growing at a temperature within the range of from 5 to 40 °C (optimum 35 °C) at a pH of from 6.5 to 7.5. As the source of carbon aminoacids and carbohydrates (for example, saccharose) are used. The source of nitrogen can be represented by mineral salts in their ammonium form, as well as organic compounds in the form of peptone, tryptone, yeast extract and aminoacids.

Resistance to antibiotics. The strain is resistant to ampicillin in a concentration of up to 100 mg/l upon growing in liquid and agarized nutrient media.

Stability of the plasmid.. Upon storage of cells on an agarized medium (for a period of up to 1 month) with a series of successive reinoculations (for at least 6 months) and during a deep culturing in a liquid medium with an antibiotic no loss or restructuring of the plasmid occurs.

The thus-prepared strain E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13) is a highly-efficient producer of fibroblastic human β1-interferon and can be used for a commercial-scale production of β1-interferon.

The present invention is further illustrated by examples of particular embodiments of the method for engineering of the plasmid, the method for producing the strain, its culturing with reference to the accompanying Figures, wherein:

Fig. 1 - physico-genetic chart of the recombinant plasmid pPR-IFNβ1-13;

Fig. 2 - diagram of the preparation of the plasmid pPR--IFNβ1-13 according to the present invention.

Example 1

The recombinant plasmid pPR-IFNβ1-13 (Fig 1) is produced in several stages. Stage I - engineering of the vector plasmid pPRI24B.

To this end, the plasmid pPRI"@ is engineered in the following manner. 3 μg of the plasmid pPR40 are cleaved by means of the restrictase Bgl II in 60 μl of a buffer for restriction-I containing 10 mM of tris-HCl, pH 8.0, 6 mM of $MgCl_2$, 6 mM of 2-mercaptoethanol, 150 mM of NaCl. The DNA is repricipitated with 2 volumes of ethanol. The precipitate

is dissolved in 20 µl of H_2O. The treatment of the DNA with the endonuclease Bal 31 is effected for 3 minutes at the temperature of 30 °C in 30 µl of a buffer containing 3 mM NACl, 60 mM of $CaCl_2$, 60 mM of $MgCl_2$, 100 mM of tris-HCl, pH 8.0, 5 mM of ethylenediaminetetracetic acid. The DNA is precipitated with 2 volumes of ethanol. The precipitate is dissolved in 10 ul of H_2O. The treatment with the restrictase BamHI is effected in a 30 ul sample containing the above-mentioned buffer for restriction-I. The completion of the single-chain 3'-terminal regions of the DNA is effected by treatment with a Klenov fragment of the DNA-polymerase I E.coli in a 20 µl sample containing 10 mM of tris-HCl, pH 8.0, 10 mM of $MgCl_2$, 2 µg of the DNA preparation, by 30 µM of each of desoxyribonucleoside triphosphates, 5 units of the enzyme. The DNA from the reaction mixture is repricipitated with ethanol and dissolved in 100 µl of H_2O. The ligation of the linearized plasmid DNA is effected at the temperature of 16 °C for 12 hours by means of the DNA-ligase of the phage T4 in a sample containing a buffer for ligation (60 mM of tris-HCl, pH 7.6, 10 mM of $MgCl_2$10 mM of 2-mercaptoethanol, 0.4 mM of adenosinetriphosphate) and 1 ug of the DNA. The resulting mixture is used for transformation of cells of E.coli C600. The efficiency of transformer is $5x10^6$ colonies per 1 µg of the native plasmid pPR40. The clones resistant to ampicillin (100 µg/ml), the plasmid DNA is recovered therefrom by a modified method suggested by Birnboim and Doli and then employed for a restriction analysis. As a result, the plasmid pPRI00 is obtained which contains a unique region of cleavage BamHI. Thereafter, the plasmid pPRI24 is engineered.

To this end, 2 µg of the DNA of the plasmid pML24 are jointly cleaved by means of the restrictases BamHI and PstI in 40 µl of a buffer for restriction-I. The ligation is effected by means of the DNA-ligase of the phage T4 at the temperature of 0 °C with the grafments obtained in hydrolysis of the DNA of the plasmid pPRI00 with the restrictases BamHI and PstI. The resulting DNA preparation is used for transformation of cells E.coli C600 with selection of trans-

formants on a medium with ampicillin, followed by selection of Cm^r clones on a medium with 300 ug/ml of chloramphenicol upon culturing of the cells at the remperature of 42 °C. From the thus-selected clones the plasmid DNA is isolated and studied by means of a restriction analysis. As a result, the plasmid pPRI24 is obtained which contains a unique region by means of the restrictase BamHI. Thereafter 3 μg of the DNA of the plasmid pPRI24 are cleaved by means of the restrictase XbaI in 70 ul of a buffer for restriction-I containing 10 mM of tris-HCl, pH 7.9, 6 mM of $MgCl_2$, 6 mM of 2-mercaptoethanol, 150 mM of NaCl. The DNA is reprecipitated with 2 volumes of ethanol. The precipitate is dissolved in 15 μl of H_2O. The completion of thesingle-chain 3'-terminal regions of the DNA is effected by treatment with a Klenov's fragment of the DNA-polymerase I of E.coli in a 20 μl sample containing 10 mM of tris-HCl, pH 8.0, 10 mM of $MgCl_2$, 2 μg of the DNA preparation, by 30 μM of desoxyribonucleisidetriphosphates, 5 units of the enzyme. The DNA from the reaction mixture is precipitated with ethanol and dissolved in 100 μl of H_2O. The ligation of the linearized plasmid DNA with BglII linkers is effected at the temperature of 16 °C for 12 hours by means of the DNA-ligase of the phage T4 in a sample containing a buffer for ligation (60 mM of tris-HCl, pH 7.6, 10 mM of $MgCl_2$, 10 mM of 2-mercaptoethanol, 0.4 mM of adenosinetriphosphate, 2 μg of the plasmid DNA and 0.5 ug of the linkers. After the ligation the DNA from the reaction mixture is precipitated with ethanol, dissolved and subjected to an enzymatic hydrolysis by means of the restrictase BglII in a 40 μl sample containning a buffer for restriction-2 (6 mM of tris-HCl, pH 7.6, 6mM of $MgCl_2$, 6 mM 2-mercaptoethanol, 50 mM of NaCl). The DNA is again precipitated with ethanol, dissolved and cyclization of the DNA molecules is ensured; to this end, 1 μg of the plasmid DNA preparation in 240 μl of a buffer for ligation are treated with the DNA-ligase of T4. The resulting mixture is used to transformer cells of E.coli C600. The transformation efficiency is up to $5x10^6$ colonies per 1 μg of the native plasmid pPRI24. The clones resistant to

ampicillin are chosen (100 μg/ml), the plasmid DNA is isolated therefrom following a modified procedure suggested by Birnboim and Doli and this DNA is used for a restriction analysis. As a result, the plasmid pPRI24B is obtained, in contrast to the starting vector molecule pPRI24, has a unique of restriction Bgl II instead of the previously available sequence of recognition XbaI (Fig. 2).

Stage 2 - engineering of the intermediate plasmid pPR-IFNβ1-123. Into the vector pPRI24B the coding sequence of fibroblastic human interferon is integrated from the plasmid pINβ-trp 7. To this end, 10 μg of the DNA pINβ-trp 7 are treated jointly with the restrictases EcoRI and Sau 3A in a 100 μl sample containing a buffer for restriction-2. The resulting preparation is applied onto a gel of a 1.1% low-melting agarose and subjected to electrophoresis in a tris-acetate buffer system. The gel strip containing the DNA fragment 510 b.p. long is cut out and the DNA is eluted from the gel. The thus-produced DNA fragment (about 1 μg) is integrated into the plasmid pPRI24B. To this end, 1 μg of the DNA pPRI24 is cleaved by means of the restrictases EcoRI and BglII in a 20 μl sample in a buffer for restriction-2. The resulting preparation is subjected to a phenolic deproteinization, the DNA is precipitated with ethanol and dissolved in 10 μl of H_2O. 0.5 μg of the cleaved plasmid pPRI24B is combined with 1 ug of the isolated fragment of the plasmid pINβ-trp 7 and treated with the DNA-ligase of T4 in a 30 μl sample in a buffer for ligation. The thus-produced DNA preparation is used for transformation of cells of E.coli C600 with selection of transformants on a medium with ampicillin, followed by selection of Cm^S-clones on a medium with 200 μg/ml of chloramphenicol upon culturing of the cells at the temperature of 42 °C. From the thus-selected clones the plasmid DNA is isolated and studied by way of a restriction analysis. In the resulting plasmid the EcoRI-BglII fragment coding the C-terminal portion of chloramphenicolacetyltransferase in the vector pPRI24 is replaced with the coding sequence of mature human β1-interferon. This plasmid is denoted as pPR-IFN 1-123 and it is used at the next stage of the engineering

process.

Stage 3 - engineering of the plasmid pPR-IFNβ1-13. 30 ug of the DNA of the plasmid pPR-IFNβ1-I23 are cleaved by means of the restrictase BamHI in 150 μl of a buffer for restriction-I, the DNA is subjected to a phenolic deproteinization and precipitated with ethanol. The precipitate is dissolved in 40 μl of H_2O. For the limited degradation of the DNA by means of $3^I \rightarrow 5^I$ exonuclease activity of the DNA-polymerase-I. E.coli, the resulting DNA preparation is subjected to incubation in a 150 μl sample containing 50 mM of tris-HCl (pH 8.0), 10 μM of ehtylenediaminetetracetic acid, 5 mM of $MaCl_2$, 100 μM of desoxyadenosinetriphosphate (and then desoxyguanosinetriphosphate) and 20 units of the DNA-polymerase. The reaction is stopped, the DNA is precipitated and the precipitate is again dissolved in 40 μl of H_2O. 10 μg of the resulting preparation are treated with the restrictase EcoRI in 50 μl of a buffer for restriction--2, followed by a phenolic deproteinization and precipitation of the DNA with ethanol; the precipitate is dissolved in 30 μl of H_2O.

The removal of the single-thread terminal regions of the resulting DNA preparation is effected using the SI-endonuclease in a 100 μl sample containing 30 mM of CH_3COONa (pH 4.4), 4.5 mM of $ZnSO_4$, 250 mM NaCl, 10 μg of the DNA, 200 units of the SI-endonuclease. The reaction is conducted at the temperature of 20 °C, whereafter the mixture is subjected to a phenolic treatment and precipitation of the nucleic acid with ethanol. The precipitate is dissolved in 20 μl of H_2O. 4 μg of the thus-obtained DNA preparation are treated with a Klenov fragment of the DNA-polymerase I E.coli under the above-described conditions to complete probable existing single-chain molecule terminals to the two-chain ones. The reaction is stopped by a phenolic depoteiniziation, the nucleic acids are precipitated with ethanol and dissolved in 20 μl of H_2O.

The ligation of the linear DNA molecules with the two-chain terminals is conducted at the temperature of 16 °C in a 50 ul sample containing 60 mM of tris-HCl (pH 7.6),

10 mM of $MgCl_2$, 10 mM of dithiothreitol, 4 mM of adenosine-triphosphate, 8% by mass of polyethylene glycol-6000, 4 ug of the DNA, 100 units of the DNA-ligase of T4. On completion of the reaction the sample is diluted by 4 times and precipitation of nucleic acids is effected by means of ethanol. 2 ug of the DNA dissolved in water are treated with the restrictase Bgl II in 30 ul of a buffer for restriction-2, the DNA is precipitated from the reaction mixture with ethanol, dissolved in water and treated with the DNA-ligase of T4 in 200 ul of a buffer for ligation. The resulting DNA preparation is used for transformation of cells of E.coli C600 as it has been described hereinbefore, followed by selection of the transformants on a medium with ampicillin upon culturing of the cells for one day at the temperature of 28 °C. Out of the thus-prepared transformants the ones having a reduced ability for growth at the temperature of 42 °C are singled. From these clones the plasmid DNA is isolated as described hereinabove and subjected to a restriction analysis.

To identify the primary structure of the hybrid region of combining r bosomes before the gene IFN-1 in the thus-produced plasmid pPR-IFN-1-13, 30 ug of the corresponding DNA are treated with the restrictase Hind II in 100 ul of a buffer for restriction-2, the preparation is applied onto a gradient (4-12%) polyacrylamide gel and subjected to electrophoresis in a tris-acetate buffer system. The DNA fragment about 190 b.p. long is eluted from the gel using the Maxam Gilbert method; it is then extended, by means of the DNA-ligase of T4, with BamHI linkers in a manner similar to the above-described procedure of the addition of Bgl II-linkers to the linearized plasmid pPRI24. The DNA is precipitated from the reaction mixture by means of ethanol and the precipitate is dissolved in 20 ul of H_2O, cleaved by means of the restrictase BamHI in 30 ul of a buffer for restriction-1, the DNA is subjected to a phenolic deproteinization, again precipitated with ethanol and dissolved in H_2O.

The molecular cloning of the DNA fragment in the replicative form of the DNA of the bacteriophage M13mpI0

formed upon cleavage with BamHI, selection of recombinant phages on an indicator medium containing 5^{I}-bromo-4-chloro--3-indolyl- -D-galactoside, isolation of the single-chain phage DNA and determination of the primary structure of the cloned fragment by the method of a limited matrix copying (method suggested by F.Senger) are carried out following standard procedures. The nucleotide sequence of the hybrid region of combining ribosomes found by the Senger method in the plasmid pPR-IFNβ1-13 is the following: 5^{I}-... TAAGGAGGTTGCATG...-3^{I}, wherein TAAGGAGGT - SD-sequence of the cro of the phage λ and ATG - methionine codon of β1-interferon.

Example 2

The strain E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13) - producer of fibroblastic human 1-interferon is produced in the following manner.

The plasmid pPR-IFNβ1-13 coding the synthesis of fibroblastic human interferon is introduced, by transformation in a manner similar to that described in Example 1 hereinabove, into cells of the strain E.coli VNIIGenetika VL 903 (deposited at the All-Union Collection of Industrial Microorganisms of the All-Union Research Institute of Genetics and Selection of Industrial Microorganisms and registered under No. BKIM B-3546). The efficiency of transformation of cells of E.coli VNIIGenetika VL 903 is about 10^6 clones per μg of the native DNA of the plasmid pPR-IFNβ1-13. The transformants are selected on a medium containing ampicillin (100 μg/ml) after culturing of the cells for one day at the temperature of 28 °C. From the selected clones the plasmid DNA is recovered and its identity with the DNA preparation pPR-IFNβ1-13 is proven by means of a restriction analysis. The strain E.coli VNIIGenetika VL 903 (pPR-IFB 1-13) is thus obtained.

For the determination of productivity of the strain E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13) the plasmid-containing cells are grown at the temperature of 28 °C on a slant agarized standard Hottinger medium containing 50 ug/ml of ampicillin for 14 hours. The biomass grown on

the slants is used for the preparation of the inoculation material. To this end, the cells are transferred into 750 ml Erlenmeyer flasks with 100 ml of the Hottinger medium containing 100 μg/ml of ampicillin and grown at the temperature of 28 °C on a shaker at 240 r.p.m. for 6 hours. The optical density of the inoculation culture is 1.5-2.5 units.

The fermentation is carried out in a fermenter provided with systems for controlling pH, temperature, stirring and aeration rate. For the fermentation a Hottinger medium with 100 μg/ml of ampicillin and 10 g/l of glucose is used. The inoculation culture is introduced in an amount of 5-10% by mass. The culturing is conducted at a pH of 6.6-6.8 while maintaining this level by supplying ammonia water. The first part of the fermentation is conducted at the temperature of 28 °C till the optical of 3.5 at 550 nm, whereafter thermoinduction is effected by elevating temperature to 42-45 °C for 5 minutes, then the fermentation is continued for 2 more hours at the same temperature.

On completion of the process, to determine the activity of interferon, cells from 1 ml of the cultural liquid are precipitated by centrifugation, the precipitate is suspended in 1 ml of a 1% solution of sodium dedecylsulphate in a 0.02M phosphate buffer with the pH of 7.2 containing 1% of 2-mercaptoethanol and heated for 2-4 minutes at the temperature of 100 °C. Afterwards, the precipitate is separated by centrifugation and the activity of interferon contained in the supernatant fraction is determined by standard procedures or by protection of human diploid fibroblasts from the cytopathic effect of the virus of vesicular stomatitis or by the method of an immuno-enzymatic analysis. As the standard references preparations of β-interferon ("Torey", Japan) are used which are titrated against the standard leukocytic interferon MPC B 69/19 (Great Britain).

The activity of fibroblastic human interferon synthesized in cells of the strain E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13) determined by various methods is more than

2×10^9 international units/litre of the bacterial culture.

For the determination of the share of the synthesized fibroblastic interferon in the total of the cell protein, the cells from 1 ml of the culture liquid are precipitated by centrifugation and treated ad described hereinbefore; the preparation is separated by way of electrophoresis in the presence of 0.1% of sodium dodecylsulphate in a 15% polyacrylamide gel following a standard procedure. The proteins separated in the polyacrylamide gel are dyed in a solution Kumassi R-250 "Serva" (West Germany) following a standard procedure. The quantitative content of proteins in the zones is determined after scanning the gel in an automated lase densitometer. The identification of the zone corresponding to the mature fibroblast interferon synthesized in the cells (19 kD) is effected on the basis of comparison with the electrophoretic mobility of the marker proteins, as well as by means of immunoblotting of separated protein fractions onto nitrocellulose filters and identification of the zone of interferon by treating the filters in solutions containing mive monoclonal antibodies against β-interferon and anti-mice rabbit antibodies conjugated with peroxidase from horse radish. After a corresponding dyeing procedure the zone of interferon appears as a dark brown band against a non-dyed background of the nitrocellulose filter.

The content of protein in the zone corresponding to β1-interferon is about 10% of the total protein of the plasmid-containing cells of E.coli VNIIGenetika VL 903 (pPR-IFNβ1-13).

Industrial Applicability

The recombinant plasmid DNA pPR-IFNβ1-13 according to the present invention coding the synthesis of fibroblastic human β1-interferon is useful in the preparation of strains producing human β1-interferon with a high activity.

The strain of bacteria Escherichia coli VNIIGenetika VL 903 (pPR-IFNβ1-13) according to the present invention - a producer of human β1-interferon, is useful in the microbiological and medical industries.

C L A I M S:

1. A recombinant plasmid DNA pPR-IFNβ1-13 coding the synthesis of fibroblastic human β1-interferon characterized in that it has the size of 3,900 b.p. and consists of the following units:

- BamHI-Bgl II fragment with the size of 3,400 b.p. of the vector plasmid pPRI34B produced from pPR40 and pML23;

- EcoRI-Sau3A fragment of the plasmid pINβ-trp 7 with the size of 510 b.p. and having the following features:

- incorporates a region responsible for initiation of replication and its regulation - Col EI-replicon, a gene of repressor cIts 857 of the phage λ, a gene of resistance to amicillin Ap^r, a tandem of ρ-independent terminators of transcription of the phage fd, a regulatory area P_RO_R and a SD-sequence of the gene λcro coding the sequence of a mature fibroblastic human interferon IFNβ1 with its own methionine codon;

- the ligation of the regulatory area of the gene λ cro, coding part of β1-interferon and terminators of transcription fd is effected so that before the gene IFNβI a hybrid region of combining ribosomes is formed which has the following nucleotide sequence:

5^I-...TAAGGAGGTTGCATG...-3^I, wherein TAAGGAGGT - SD-sequence of the gene cro of the phage λ ATG - methionine codon of interferon and directly after the terminating codon of the gene IFNβ1 a tandem of ρ-independent terminators of the transcription of the phage fd is located;

- has unique regions of recognition of the restrictases ClaI whose coordinate is the beginning of count (0), Pvu II (abount 1,110), Bgl II (about 1,480), AccI (about 1,870), Pvu I (about 3,360); deposited at the Collection of Cultures of Microorganisms of the All-Union Research Institute of Antibiotics and registered under No. 1825.

2. A method foe engineering a recombinant plasmid DNA pPR-IFNβ1-13 according to Claim 1, characterized in that the vector plasmid pPRI24B engineered on the basis of the plasmids pPR40 and pML24 is cleaved by means of the res-

triction endonucleases E.coRI and BglII and ligation of the greater of the formed fragments of the vector molecule with the EcoRI-Sau3A fragment of the plasmid pINβ-trp 7 containing the coding portion of mature human 1-interferon; in the thus-produced recombinent plasmid pPR-IFNβ1-123 nearing of the SD-sequence of the gene cro and the first (methionine) codon of 1-interferon is effected, wherefor the plasmid DNA is hydrolyzed by means of the restrictase BamHI, a portion of nucleotides is removed by means of the exonuclease activity of the DNA-polymerase I of E.coli manifesting itself under conditions of an incomplete set of nucleosidetriphosphates in the reaction mixture; the DNA is enzymatically cleaved by the restrictase EcoRI, treated with the SI-endonuclease to remove single-chain DNA regions, then the two-chain terminals are restored by means of a Klenov fragment of the DNA-polymerase I of E.coli and the resulting linear DNA molecules are cyclized by means of the DNA-ligase of the phage T4; the resulting preparation is used to transfer cells of E.coli C600 with selection of transformants on a medium with ampicillin upon culturing of the cells at the temperature of 28 °C, followed by selection of clones exhibiting a reduced rate of growth at 42 °C, whereafter from the selected clones the recombinant plasmid DNA pPR-IFNβ1-I3 is isolated.

3. A strain of bacteria Escherichia coli VNIIGenetika VL 903 (pPR-IFNβ1-I3) a producer of human β1-interferon containing the recombinant plasmid DNA pPR-IFNβ1-I3 according to Claim 1, produced by the method of genetic engineering by way of introducing the recombinent plasmid DNA pPR-INFβ1-I3 into the bacteria Eschericia coli, deposited on 12.01.87 at the collection of cultures of microorganisms of the All-Union Research Institute of Antibiotics and registered under No. 1825.

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