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A Plasmid Vector for Expressing the Human Insulin Gene in Nonendocrine Cells

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A method for cloning the insulin gene from total human DNA is developed on the basis of the polymerase chain reaction. Cloning of the insulin gene from the total DNA of human lymphocytes is performed to obtain a pNP-90 vector for subsequent expression of the insulin gene in nonendocrine cells for the purposes of gene therapy of diabetic angiopathies.

Key Words: *human insulin gene; polymerase chain reaction; subcloning of the insulin gene*

Diabetic angiopathies are one of the most serious diabetic complications [4]. Since angiopathies damage the small (micro-) and large (macroangiopathies) vessels of the heart (cardiopathy), brain (encephalopathy), kidneys (nephropathies), and eye (retinopathy), they frequently have a lethal outcome in diabetes. There are no suitable methods at present for preventing and treating micro- and macroangiopathies, i.e., direct injections of insulin are not able to compensate for insulin deficiency. In the organism insulin is synthesized by β-cells of the pancreas, and the gene coding for the amino acid sequence of preproinsulin, composed of three peptides (B, C, and A), plays a key role in insulin synthesis. Insulin maturation consists in splitting-off of the C-peptide under the influence of specific proteases, and this does not occur in other tissues.

We have previously shown that even in a case where arterial endothelium has been directly transformed with functional genes, expression of the model genes was observed during several months [10]. Therefore, gene therapy and genetic therapeutic agents [5,8] could provide a basis for the new strategy in the treatment of diabetes and different forms of diabetic angiopathies [1,13]. The creation of a plasmid vector capable of synthesizing biologically active insulin in human nonendocrine cells could be one approach to the prevention and treatment of diabetic angiopathies. Its subsequent transfection into endothelial cells would help maintain a constant, if not high, insulin level in the vessels of the eye, brain, heart, kidneys, and extremities for several months.

This paper desribes human insulin gene cloning on the basis of the polymerase chain reaction (PCR) and the design of a genetic construction for studying the expression of the human insulin gene in eukaryotic cells with a view to creating a therapeutic agent for the prevention and treatment of diabetic angiopathies.

MATERIALS AND METHODS

The native human insulin gene was obtained from the total DNA isolated from blood lymphocytes [6]. For this purpose 400 μl of whole blood were resuspended in 400 μl of buffer of the following composition: 10 mM Tris-HCl, pH 8.0; 10 mM NaCl; 10 mM EDTA, pH 8.0. The suspension was

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stirred, frozen at -20°C, and thawed at room temperature. Ten microliters of 10% sodium dodecylsulfate and 20 μl of an aqueous solution of proteinase K (10 mg/ml) were added to the thawed suspension and incubated at 58°C for 2 h. The mixture was treated once with phenol and twice with chloroform (the aqueous phase was separated by centrifugation). NaCl solution (5 M, 180 μl) was added to the aqueous phase and DNA was sedimented with 600 μl isopropanol. The DNA sediment was washed with 1 ml ethanol and dissolved in 20 μl tridistilled water.

The DNA obtained thus was treated with Bgl 1 restriction enzyme and the total hydrolysate was fractionated in 1% agarose gel during 10 h (horizontal electrophoresis). After fractionation of Bgl 1 fragments a gel strip containing DNA fragments with a length of 2500 to 3000 base pairs (b.p.) was cut from the gel. DNA fragments were transferred by electrophoretic blotting onto DE-81 paper, this being followed by elution of fragments with 1.2 M NaCl and sedimentation with ethanol [7]. As a result, we obtained a Bgl 1-enriched DNA fraction containing the human insulin gene [3].

Replication of DNA copies containing the human insulin gene was performed using PCR [3], which was carried out in a mixture of the following composition: PCR buffer; 50 mM $MgCl_2$; 2.5 mM 4dNTP (4-deoxynucleotide triphosphate); primer mixture; tridistilled water; Taq polymerase; DNA matrix. The total volume of the reaction mixture was 50 μl. The direct P1 primer 5'-d(TGGTCTAATGTGGAAAGTGG)-3', located in the region from 1866 to 1885 b.p. of the nucleotide sequence of the insulin gene (the EMBL bank of nucleotide sequences Human Gene for Preproinsulin from Chromosome 11), and the reverse P2 primer 5'-d(GCTTGGCCCAGGGCCCC)-3', located in the region from 3657 to 3641 b.p., were used as primers in DNA amplification.

DNA amplification was performed under the following temperature regime: 95°C 1 min, 56°C 1 min, and 72°C 1 min; 30 cycles. DNA amplification yielded a DNA fragment with a length of 1792 b.p., which contained the human insulin gene. This 1792 b.p. fragment was treated with HincII restriction enzyme. After hydrolysis we obtained a fragment with a length of 1608 b.p., which was cloned at the HincII site into a pGEM-3Z plasmid vector. The clones were tested for the presence of the insulin gene by the PCR method using two primers: Up 5'-d(ATCACTGTCCTTCTGCC)-3' located in the region from 2407 to 2423 b.p. and L2 5'-d(GGCTGCGGCTGGGTCA)-3'located in the region from 2595 to 2610 b.p. Recombinant clones, which after amplification yielded a DNA fragment of the insulin gene with a length of 203 b.p., contained the entire human insulin gene.

As a result, we obtained a pGEM-Ins recombinant vector. The structure of the insulin gene in the pGEM-Ins vector was determined using oligonucleotide primers by direct sequencing in both directions after Sanger *et al.* [12]. In sequencing of the first chain we used direct primers: UpU 5'-d(GTTTTCCCAGTCACGAC)-3'; Up (see above): Up1 5'-d(GTGAGCCAACCGCCCATTG)-3'; Up2 5'-d(TGGGGCAGGTGGAGCTG)-3'. In sequencing of the second chain we used reverse primers: LU 5'-d(CAGGAAACAGCTATGAC)-3'; L 5'-d(CTGCCAGGACGTGCCG)-3'; L1 5'-d(CCTGCAGGTCCTCTGC)-3'; L2 5'd (see above) (Fig. 1).

The insulin gene from the pGEM-Ins plasmid was isolated by hydrolysis with HincII and BamHI restriction enzymes. Treatment with HincII removes a DNA region from primer P1 to the HincII site, yielding a DNA fragment (1608 b.p.) which comprises 2 introns (IS1 and IS2), a capping site, a polyadenylation region (Poly A), signal peptide (SP), and the B-, C-, and A-peptides of insulin. The HincII, BamHI fragment of DNA, which contained the insulin gene, was subcloned into pNP-90 vector (kindly provided by G. Enikolopov). The pNP-90 vector comprises an RSV-promoter, a splicing signal region, and a polyadenylation region of the rabbit β-globin gene, which are separated by a small region containing the XbaI and BamHI restriction sites. The pNP-90 plasmid vector was treated with XbaI and BamHI. The XbaI restriction site was "blunted" with DNA polymerase I (Klenow fragment). By ligating the large fragment of the vector with the HincII, BamHI insulin fragment and by subsequent cloning we obtained a pNP-Ins plasmid vector, in which the insulin gene was controlled by the RSV-promoter.

RESULTS

The synthesis and transformation of preproinsulin are unique functions of endocrine cells [4]. The design of a genetic construction enabling nonendocrine cells to express and process insulin is an important task of molecular biology and is of special interest for the treatment of diabetic angiopathies. For the creation of such a genetic construction it was necessary to obtain the native human insulin gene. The structure of this gene has now been studied in full [3].

Basing ourselves on the structure of the insulin gene, we used the PCR method for rapid in-

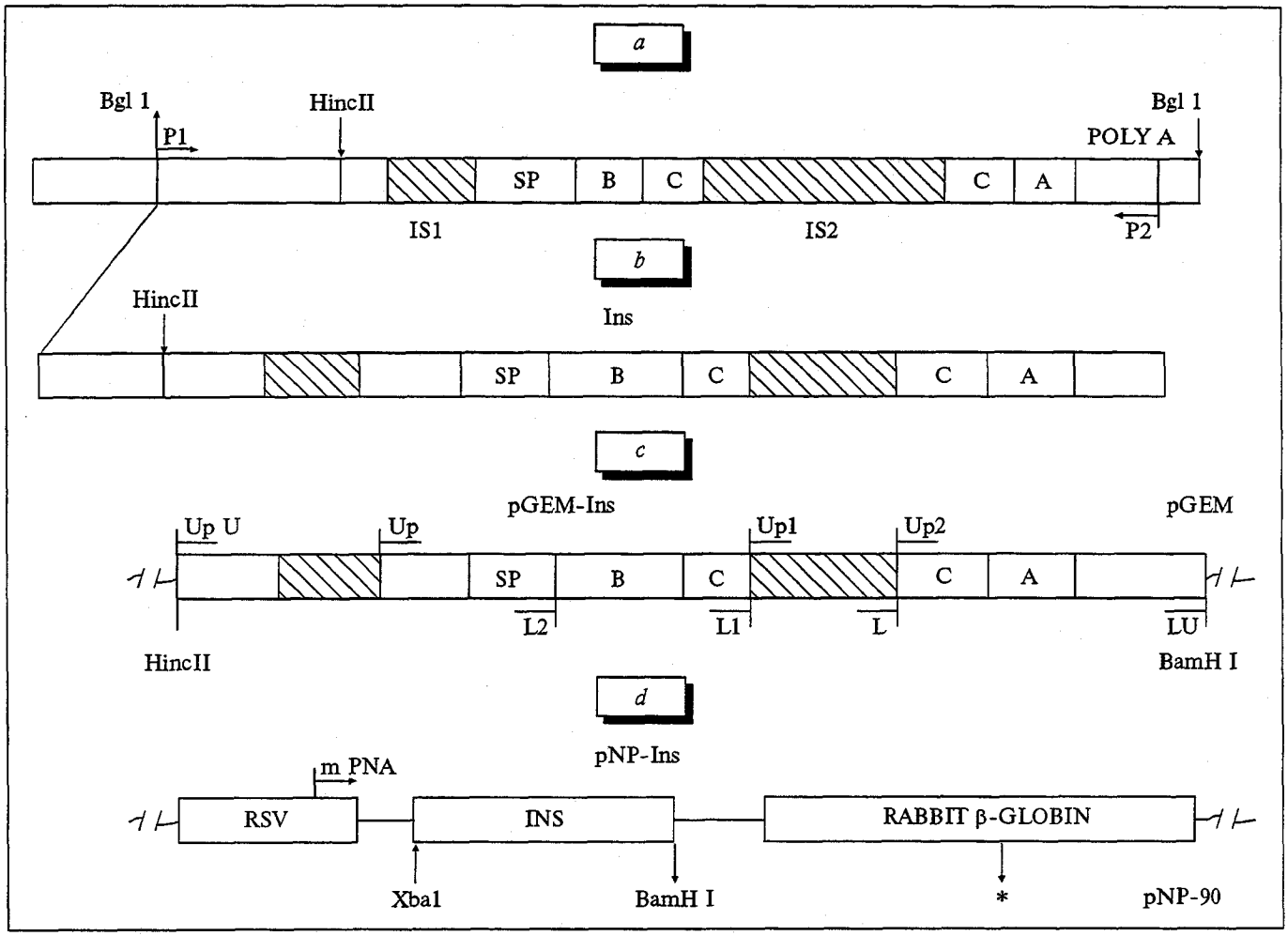

Fig. 1. Scheme for the design of a vector for insulin expression in nonendocrine cells. *1*) Bgl 1–enriched DNA fraction (2711 b.p., including the insulin gene) obtained from total DNA of human lymphocytes (IS1 and IS2 are introns, POLY A is the polyadenylation region, SP is the signal peptide, and B, C, and A are the B–, C–, and A–peptides of insulin) [3]. *2*) PCR product (1792 b.p.) obtained by amplification of the Bgl 1 fraction using P1 and P2 primers (Ins is the human preproinsulin gene). *3*) Subcloning of PCR product (1608 b.p.) treated with HincII into pGEM–3Z plasmid (pGEM–Ins is the plasmid vector containing the insulin gene) and sequencing strategy for the insulin gene (designations of primers in MATERIALS AND METHODS). *4*) pNP–Ins vector for insulin gene expression in nonendocrine cells (an asterisk denotes the splicing and polyadenylation regions).

sulin gene cloning from total human DNA. In order to exclude nonspecific synthesis during PCR as much as possible, the total DNA isolated from blood lymphocytes was treated with Bgl 1. It is evident from the structure of the gene that the Bgl 1 fragment of DNA with a length of 2711 b.p. (Fig. 1) includes the entire insulin gene. Therefore, Bgl 1 fragments of DNA with a length close to 2711 b.p. (from 2500 to 3000 b.p.) were isolated from the total Bgl 1 hydrolysate fractionated in 1% agarose gel.

The DNA fraction enriched in such a manner was used as a matrix during PCR. P1 (direct) and P2 (reverse) primers located inside the "insulin" Bgl 1 fragment and entirely flanking the insulin gene were used for priming. Amplification yielded a PCR product with a length of 1792 b.p. (Fig. 1).

For confirmation of the structure of the insulin gene by sequencing, a DNA fragment with a length of 1792 b.p. was cloned into the pGEM-3Z plasmid at the HincII site (Fig. 1). Clones containing the insulin gene were tested for the presence of a DNA fragment with a length of 203 b.p. using the PCR method. The Up (direct) and L2 (reverse) primers flanking the region from the first intron to the end of the signal peptide (Fig. 1) were used for priming in PCR. The clone yielding a PCR product 203 b.p. long was designated as pGEM-Ins.

The identity of the nucleotide sequence containing the insulin gene obtained after amplification of the Bgl 1 fraction was verified by direct sequencing of the pGEM-Ins plasmid. Sequencing was performed along the two chains using the chain termination method [12]. Sequencing of the

first chain was performed using the direct primers UpU, Up, Up1, and Up2, and of the second chain using the reverse primers LU, L, L1, and L2 (Fig. 1). UpU and LU are universal primers for the pGEM-3Z plasmid vector. The other primers located at the junctions between introns and exons were identified and synthesized by us.

We did not discover any disturbances or point mutations in the nucleotide sequence of the insulin gene obtained by the PCR method vis-a-vis the known nucleotide sequence [3]. The human insulin gene cloned into the pGEM-3Z vector was subcloned into the pNP-90 vector at the Xba1 and BamHI sites (Fig. 1). These sites are located between the RSV promoter and the splicing and polyadenylation sites of the rabbit β-globin gene. Proper orientation of the insulin gene with respect to the RSV promoter was determined by the cloning conditions (the BamHI site is the end of the insulin gene). The presence of the powerful eukaryotic RSV promoter and of splicing and polyadenylation regions provides for constitutive synthesis of insulin in human nonendocrine cells.

Thus, using the insulin gene, we showed that cloning a gene with a known structure can be rapidly and effectively performed using the PCR method. This renders such an approach useful for cloning of the subtilisinlike peptidase gene into a particular expressing vector [2,11], which is necessary for insulin maturation in nonendocrine cells.

REFERENCES

1. R. I. Zhdanov, I. E. Kovalev, M. Yu. Beburov, and V. I. Fedchenko, in: *Abstracts of the First Russian Congress "Man and Drug,"* Moscow (1992), p. 223.
2. P. J. Barr, O. B. Mason, K. E. Landsberg, *et al., DNA and Cell Biology,* **10**, 319-328 (1991).
3. G. I. Bell, R. I. Pictet, W. J. Rutter, *et al., Nature,* **284**, 26-32 (1980).
4. P. Cautrecasas and S. Jacobs, "Insulin", in: *Handbooks of Experimental Pharmacology,* Vol. 92, Springer (1990).
5. P. L. Felgner and G. Rhodes, *Nature,* **349**, 351-352 (1991).
6. B. Lindblom and G. Holmlund, *Gene Anal. Tech.,* **5**, 97-101 (1988).
7. T. Maniatis, E. E. Fritsch, and J. Sambrook, *Molecular Cloning,* CSH Laboratory Press (1991).
8. A. Meager and E. Griffin, *Biotechnology,* **12** (4), 108-113 (1994).
9. K. B. Mullis and F. A. Faloona, *Meth. Enzymol.,* **255**, 335-350 (1987).
10. E. G. Nabel, G. Plautz, and G. J. Nabel, *Science,* **249**, 1285-1288 (1990).
11. A. M. W. van den Ouweland, J. J. M. van Groningen, A. J. M. Roebroek, *et al., Nucleic Acid Res.,* **17**, 7101-7102 (1989).
12. F. Sanger, S. Miklen, and A. R. Coulson, *Proc. Nat. Acad. Sci. USA,* **74**, 5463-5467 (1977).
13. R. I. Zhdanov, V. I. Fedchenko, S. O. Guriev, *et al., NATO ASI Series. Pharmacokinetics: from Theory to Practice,* Ed. A. Thakur, New York (1994).