Volume 210, number 1, 56–60 FEB 04315 January 1987

Chemical synthesis and expression in *E. coli* of a human Val^8-calcitonin gene by fusion to a synthetic human interferon-γ gene

I. Ivanov, L. Gigova and E. Jay*

Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria and **Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick E3B 6E2, Canada*

Received 29 October 1986

A gene coding for human Val^8-calcitonin (Val^8-hCT) was synthesized by the solid-phase phosphite approach and fused to a synthetic human immune interferon-γ (IFN-γ) gene. The IFN gene was previously shown to be expressed at a very high level in *E. coli* [(1986) Gene, in press] due to the control of a strong synthetic promoter and strong ribosome binding site. The cells harboring the fused gene produced 100–150 μg per l of bacterial suspension of immunoreactive calcitonin in the form of hybrid IFN-γ–Val^8-hCT protein consisting of 140 amino acids. The Val^8-hCT can be released from this protein by CNBr treatment.

Gene expression; Gene synthesis; Gene fusion; Calcitonin; Interferon-γ; (*E. coli*, Human)

1. INTRODUCTION

Calcitonin (CT) is a 32 amino acid polypeptide secreted by the parafollicular cells of the thyroid in mammals. It plays an important homeostatic role by controlling calcium and phosphorus turnover. The main function of CT however, seems to be the protection of the skeleton against resorption during times of physiological stress such as pregnancy, lactation and growth [1,2]. CT has proven to be very effective in the treatment of various kinds of diseases, such as Paget's disease, osteoporosis imperfecta, postmenopausal osteoporosis, and bone metastases. [For details see Proc. Int. Symp. Calcitonin 1980 and 1984, Milan (Pecile, A. ed.) Elsevier.] CT exhibits low species specificity and this characteristic has made it possible to use animal CT (salmon, eel and porcine) for treatment of human patients in the absence of the availability of human CT (hCT). The species variations in the structure of these CTs however result in antibody formation upon repeated administration [3–7]. Since 1968 synthetic hCT has become available [8,9] but its activity appears to be inferior when compared with those of the natural preparations [10,11].

Correspondence address: I.G. Ivanov, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

The shortcomings outlined above of the CTs currently available for clinical use give momentum to the search for alternative approaches for preparing hCT for clinical applications. Recently, hCT genes coding for either Val^8-hCT [12] or Met^8-hCT [13] have been synthesized and expressed in *E. coli*. The Val^8-hCG gene has been fused to the gene of β-galactosidase [12] whereas the Met^8-hCT gene was expressed directly under a strong promoter and a strong synthetic ribosomal binding site (RBS) [13]. In both cases a low level of expression (low protein content) has been recorded.

Here we report the results on the chemical synthesis and expression in *E. coli* of another hCT gene coding for Val^8-hCT by fusing it to a synthetic IFN-γ gene which is expressed at a very high level and is under the control of a strong synthetic promoter and strong synthetic RBS [14].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

2.1. *Chemical synthesis, purification and phosphorylation of oligonucleotides*

All oligonucleotide fragments were synthesized by a modified solid-phase phosphite method as in [15]. The synthetic oligonucleotides were purified by HPLC on a 10SAX anion-exchange column. The high yield of the synthesis (40–60%) made it possible to avoid further purification on a second reversed-phase column. The purified and desalted oligonucleotides were phosphorylated at the 5′-terminus with [γ-^{32}P]ATP (5–10 Ci/mmol) and T_4 polynucleotide kinase. The labelled oligonucleotides were purified on a 20% polyacrylamide sequencing gel.

2.2. *Ligation and purification of the Val^8-hCT gene*

The phosphorylated oligonucleotides of 5 pmol aliquots each (except for the terminal fragments in which 8 pmol were used) were mixed in 40 μl of 100 mM Tris, pH 7.5/20 mM $MgCl_2$, heated to 90°C and slowly cooled to 12.5°C. The mixture was then adjusted to 50 mM Tris, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP and 10 U of T_4 DNA ligase. The ligation reaction was carried out at 12.5°C for 16 h. The T_4 DNA ligase was inactivated at 70°C for 10 min, *Bgl*II (10 units) added and the incubation continued overnight at 37°C. The reaction mixture was loaded on an 8% polyacrylamide gel (4 V/cm for 8 h) and the Val^8-hCT gene visualized by autoradiography and recovered as in [15].

2.3. *Cloning of Val^8-hCT gene into pJP$_1$R$_9$–IFN-γ expression vector*

The purified Val^8-hCT gene containing two terminal *Bgl*II sites was ligated using standard conditions to a 4-fold excess of pJP$_1$R$_9$-IFN-γ pretreated with *Bgl*II and calf intestinal alkaline phosphatase. The ligation mixture was used to transform competent cells (*E. coli* LE392). Recombinant clones were selected by colony hybridization using ^{32}P-labelled synthetic oligonucleotides as specific probes.

2.4. *Sequence analysis of DNA cloned in pJP$_1$R$_9$–IFN-γ vector*

The primary structure of the synthetic Val^8-hCT gene was verified after cloning by the method of Maxam and Gilbert [16].

2.5. *RNA dot hybridization*

To detect the clones with the CT gene in the correct orientation, and under the control of the transcription unit of the IFN gene, total cell RNA was isolated from 14 different clones selected by colony hybridization and probed with (5-^{32}P)-labelled synthetic oligonucleotides of the non-coding DNA strand of the CT gene. RNA was prepared from 1.5 A_{590} units of recombinant bacteria grown in LB containing 10 μg/ml tetracycline at 37°C to A_{590} = 2.0. The cells were pelleted and lysed in 300 μl of 300 mM sodium acetate, pH 6.2/1% SDS. An equal volume of phenol was added and the mixture heated for 10 min at 65°C. The aqueous phase was further deproteinized and mixed with 3 vols ethanol. The precipitate was dissolved in 200 μl of 50 mM Tris, pH 7.4/10 mM $MgCl_2$, treated with 10 μg/ml DNase I (RNase-free), deproteinized as above and precipitated with ethanol. RNA was dissolved in 50 μl of 3 × SSC/10% formaldehyde, heated at 65°C for 10 min and dotted onto two nitrocellulose filters. Each sample was loaded separately as two aliquots (6 and 18 μl, respectively). The filters were dried in a vacuum oven for 15 min at 80°C, prehybridized in 1 M NaCl/0.2% SDS/100 mM Tris, pH 7.0/100 μg/ml homomix I (a partial alkaline hydrolysate of yeast tRNA) [24] for 2 h at 60°C. Hybridization was carried out in 10 ml of the same solution containing 2–5 × 10^7 cpm of end-labelled oligonucleotide at 42°C for 2 h. The filters were washed with 1 M NaCl/0.2% SDS twice for 15 min at the temperature of incubation and once at 50°C for 15 min.

2.6. *Radioimmunoassay and protein analysis*

Goat hCT antibodies and standard synthetic hCT were purchased from Milab (Sweden) and the RIA test was carried out as recommended by the producer.

Total bacterial protein was analysed by Laemmli [17] electrophoresis. For this purpose 2 A_{590} units of recombinant cells were harvested and lysed in 200 μl of 50 mM Tris, pH 6.8/2% SDS/2% β-mercaptoethanol/10% glycerol/0.01% bromophenol blue in a boiling water bath for 5 min.

Samples of 5–15 μl were loaded on a 15% polyacrylamide gel.

3. RESULTS AND DISCUSSION

Manufacturing of eukaryotic, biologically active peptides of low M_r by the recombinant DNA approach is still not a trivial task for genetic engineering. The low-M_r proteins are presumably recognized as foreign substances and are usually degraded extensively by the bacterial cell [18,19].

To study the stability of recombinant hCT against proteolysis in bacteria we have constructed and expressed in *E. coli* a series of synthetic genes coding for hCT in either monomeric or multimeric form. It was found that the hCT is stable in *E. coli* cells when synthesized as a tetramer or larger, i.e. when the polypeptide chain has increased to 132 amino acids in length [20]. These results encouraged us to construct a hybrid IFN-γ–hCT gene coding for a protein of approximately the same molecular size. The gene of IFN-γ was chosen for this construction since this gene was recently synthesized and expressed (under the strong synthetic promoter P_1 and the strong synthetic RBS R_3) in *E. coli* at a very high level (> 15% of the total cell protein), confirming its compatibility with the *E. coli* cell [14,15]. Our experiments on the direct expression (without fusion) of monomeric and multimeric hCT genes in *E. coli* under the same expression system resulted in a low yield (as compared with that of IFN-γ) and inhibition of cell growth [20]. Taking into account these observations, it would be interesting to determine whether the fusion of the hCT gene to the IFN-γ gene would favor both the expression of the hybrid CT protein and the normal growth of the recombinant bacteria.

To construct the fused gene an hCT gene was synthesized as designated in fig.1. This gene is composed of 16 oligonucleotides (underlined in the figure) comprising both strands of the synthetic DNA. The gene codes for 33 amino acids (32 corresponding to the mature CT molecule plus one additional terminal methionine). The nucleotide sequence of the synthetic gene was derived from the structure of the human pre-CT mRNA as determined by Craig et al. [21] except that the Val^8 codon was substituted for the native Met^8 codon. This substitution was necessary to obtain an hCT free of methionine in order to treat the hybrid protein with CNBr to provide the Val^8-hCT. The following arguments were taken into account when designing this substitution: (i) most of the animal CTs contain Val^8 instead of Met^8 [2] and are both more active and more stable (since Met tends to be oxidized) [22]; (ii) the Val^8 substitution should not change the antigenic properties of the CT molecule since only the amino acids between 11 and 32 are responsible for the antigenicity of CT [23].

The synthetic Val^8-hCT gene is flanked by two *Bgl*II sites. Its 5′-end is constructed such that its coding sequence is in the same reading frame as that of the IFN-γ gene in the fused gene (see fig.2). The principle scheme for cloning of the Val^8-hCT gene into the expression plasmid pJP_1R_9–IFN is shown in fig.3. This plasmid has a unique *Bgl*II site located between amino acids 105 and 106 of the IFN-γ gene (the N-terminal Met not included). Upon insertion of the hCT gene the last 40 codons of the IFN-γ gene (of the total 147 codons) are switched off from translation and 33 new CT codons are added. Since the insertion of the Val^8-hCT gene is possible in two orientations, recombinant clones containing the gene in the correct orientation had to be identified. As seen in fig.1, the synthetic gene is devoid of any appropriate restriction endonuclease sites and therefore restriction analysis cannot be applied to test for the gene's orientation. Because of that we

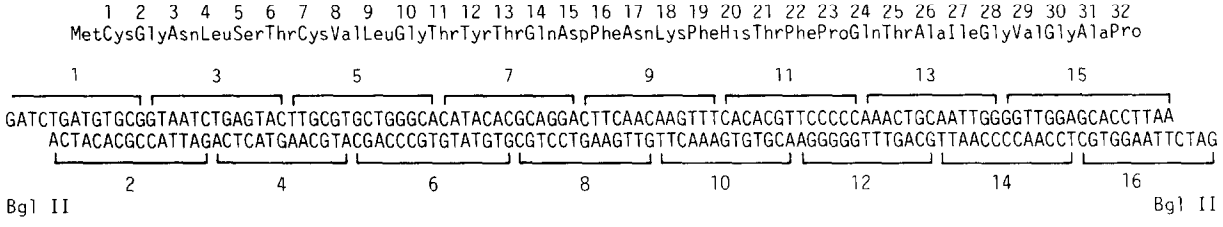

Fig.1. Nucleotide sequence of the synthetic Val^8-hCT gene.

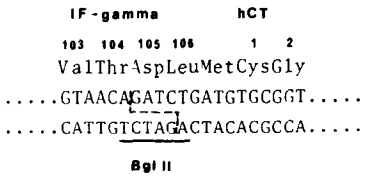

Fig.2. Cloning of the synthetic Val[8]-hCT gene in pJP_1R_9-IFN-γ expression plasmid.

selected clones with the hCT in the correct orientation by RNA-oligonucleotide hybridization. Total cell RNA was hybridized with labelled synthetic oligonucleotides belonging either to the coding (fig.4B) or to the opposite (fig.4A), non-coding strand of the gene. Only those clones giving a positive signal with gene fragment nos 2 and 6 (fig.4B) and a negative signal with oligonucleotide no.15 (fig.4A) were used in subsequent experiments.

The efficiency of transcription of the fused Val[8]-hCT gene was tested using the same hybridization procedure described above and it was found that the maximum accumulation of CT mRNA in the *E. coli* cells occurred when the density of the bacterial suspension reached 1.7–2.0 A_{590}.

The efficiency of translation of the hybrid gene was studied both by gel analysis according to Laemmli [17] and by RIA testing. Our results

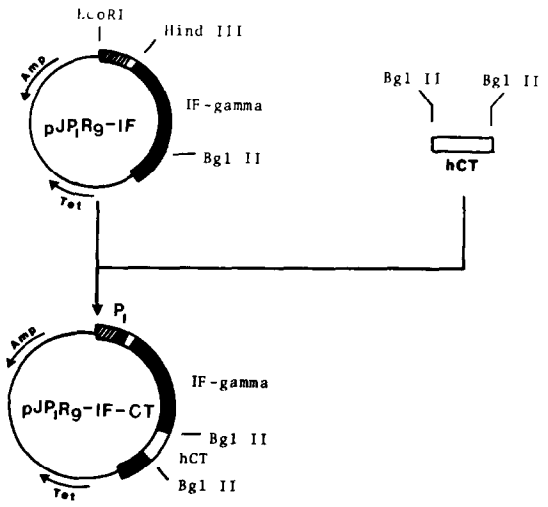

Fig.3. The site of fusion between the two synthetic genes.

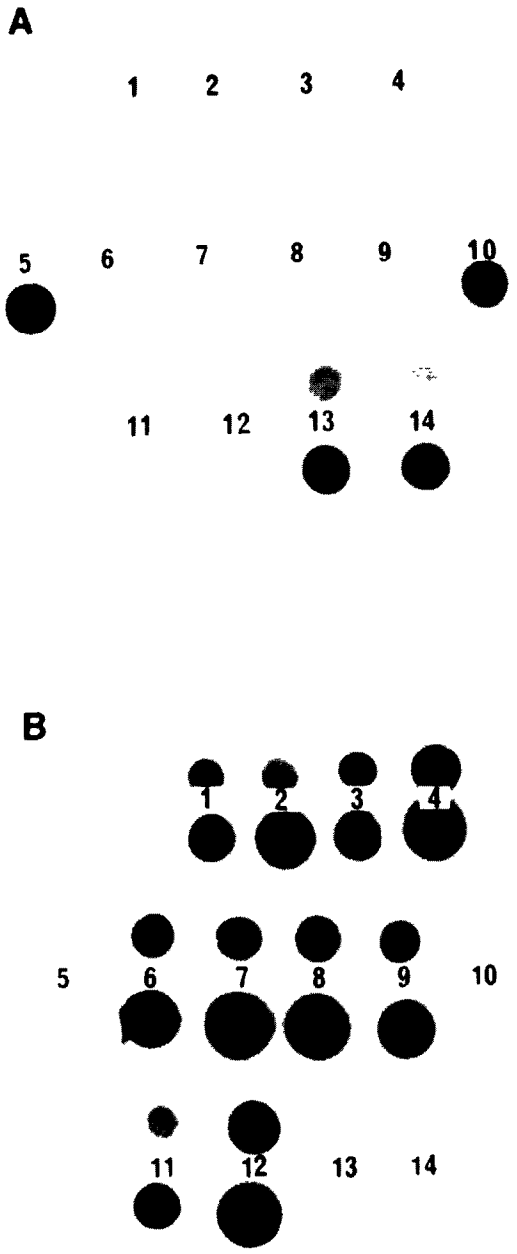

Fig.4. Screening for clones harboring the Val[8]-hCT gene in the correct orientation. Total cell RNA was isolated as described in section 2 and dotted onto two nitrocellulose filters. Filter A was hybridized with oligonucleotide no.15 (as marked in fig.1) and filter B with a mixture of oligonucleotide nos 2 and 6.

clearly showed that the fusion of the Val^8-hCT gene to the gene of IFN-γ led to a dramatic decrease in translational efficiency. The yield of the hybrid protein was below 1% of the total cell protein (vs 15% for the IFN-γ gene) as judged by SDS electrophoresis method. This decrease cannot be due to a change in the initiation of translation, but results from either a decrease in the stability of the hybrid protein or the hybrid in mRNA.

The immunoreactive recombinant hCT was quantitated by RIA and a value of about 100–150 μg CT per l of bacterial culture (at a cell density of 1.7–2.0 A_{590}) was registered. This value however might be underestimated since the recombinant hCT is not amidated, whereas the antibodies used for RIA have been prepared against synthetic amidated hCT.

When cultivating recombinant bacteria harboring the IFN-γ–Val^8-hCT gene, it was also found that they grew more slowly than the host cells or cells carrying the IFN-γ gene only. However, they grew better than the cells expressing hCT gene(s) directly (without fusion). Based on these observations and keeping in mind the hydrophobic character of hCT, one might propose that the cell growth inhibition (and probably the poor expression as well) is due to some interaction between CT and the inner surface of the cell membrane. This offers an interesting model for studying the effects of amino acid substitutions on the stability of heterologous proteins in bacteria and their effect on bacterial cell functioning in addition to that on the activity of the modified calcitonin protein.

ACKNOWLEDGEMENTS

The work was partially supported by a grant from NSERC of Canada to E.J. The procedure for RNA colony hybridization was established by D. MacKnight.

REFERENCES

[1] Stevenson, J.C., Hillyard, C.J., MacIntyre, I., Cooper, H. and Whitehead, M.I. (1979) Lancet ii, 769–773.
[2] MacIntyre, I. and Stevenson, J. (1981) in: Proc. Int. Symp. Calcitonin 1980, Milan (Pecile, A. ed.) pp.1–10, Elsevier, Amsterdam, New York.
[3] De Rose, J., Singer, F.R., Avramides, A., Flores, A., Dziadiw, R., Baker, R.K. and Wallach, S. (1974) Am. J. Med. 56, 858–866.
[4] Haddad, J.C. and Caldwell, J.G. (1972) J. Clin. Invest. 51, 3133–3141.
[5] Singer, F.R., Aldred, J.P., Neer, R.M., Krane, S.M., Potts, J.T. and Bloch, K.J. (1972) J. Clin. Invest. 51, 2331–2338.
[6] Woodhouse, N.J.Y., Mohamedally, S.M., Saed-Nejad, J. and Marthin, T.J. (1977) Br. Med. J. 2, 927–929.
[7] Hosking, D. (1981) in: Proc. Int. Symp. Calcitonin 1980, Milan (Pecile, A. ed.) pp.365–378, Elsevier, Amsterdam, New York.
[8] Riniker, B., Neher, R., Maier, R., Kahnt, F.W., Byfield, P.G.H., Gudmunsson, T.V., Galante, L. and MacIntyre, I. (1968) Helv. Chim. Acta 51, 1738–1745.
[9] Sieber, P., Brugger, M., Kamber, B., Riniker, B. and Rittel, W. (1968) Helv. Chim. Acta 51, 2057–2063.
[10] Rivaille, P. and Milhaud, G. (1972) Helv. Chim. Acta 52, 102–107.
[11] Ontjes, D.A., Roberts, J.D., Hennessy, J.F., Burford, H.J. and Cooper, C.W. (1973) Endocrinology 92, 1780–1785.
[12] Rubtsov, P.M., Chernov, B. and Skryabin, K. (1985) Dokl. Akad. Nauk USSR 283, 1507–1509.
[13] Ivanov, I. and Jay, E. (1986) Gene, in press.
[14] Jay, E., Rommens, J., Cloney, L., MacKnight, D., Lutze-Wallace, C., Wishart, P., Harrison, D., Liu, W., Asundi, V., Dawood, M. and Jay, F. (1984) Proc. Natl. Acad. Sci. USA 81, 2290–2294.
[15] Jay, E., MacKnight, D., Lutze-Wallace, C., Harrison, D., Wishart, P., Liu, W., Asundi, V., Cloney, L., Rommens, J., Eglington, L., Pawlak, J. and Jay, F. (1984) J. Biol. Chem. 259, 6311–6317.
[16] Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560–564.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Lin, S. and Zabin, I. (1972) J. Biol. Chem. 247, 2505–2511.
[19] Goldschmidt, R. (1970) Nature 228, 1151–1154.
[20] Ivanov, I. and Jay, E. (1986) Gene, in press.
[21] Craig, R.L., Hall, M., Edbrooke, M., Allison, J. and MacIntyre, I. (1982) Nature 295, 345–347.
[22] Guttmann, S. (1981) in: Proc. Int. Symp. Calcitonin 1980, Milan (Pecile, A. ed.) pp.11–24, Elsevier, Amsterdam, New York.
[23] Dietrich, F. and Rittel, W. (1970) Nature 225, 224–225.
[24] Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1974) Nucleic Acids Res. 1, 331–353.