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HUMAN CALCITONIN TETRAMERIC GENE: COMPARATIVE EXPRESSION IN YEAST AND TRANSGENIC POTATO PLANTS

H. Ofoghi[1], R. Mironova[3], N. Moazami[2], N. Domonsky[1] I. Ivanov[3]
Pasteur Institute of Iran, Molecular Biology Department, Tehran, Iran[1]
Iranian Research Organization for Science and Technology, Biotechnology Department, Tehran, Iran[2]
Institute of Molecular Biology, Bulgarian Academy of Science, Sofia, Bulgaria[3]

ABSTRACT

Human calcitonin (hCT) is a 32 amino acid peptide participating in the regulation of calcium and phosphorus metabolism in humans. It is used in clinics for treatment of diseases related to bone decalcification, such as Paget disease, osteoporosis imperfecta, parathyroid gland carcinomas, etc. A synthetic hCT tetrameric (hCT_t) gene is now cloned in binary vectors for gene expression in yeast and plants. The vector for expression in yeast is based on 2μ plasmid containing the α-factor leader sequence. The plasmid for expression in plants carries the 35S cauliflower mosaic virus promoter linked to the tobacco etch virus 5'-nontranslated leader sequence acting as a translational enhancer. Two strains of yeast cells (GRF18 and YY168) and one cultivar of potato (Solanum tuberosum Nevski) were transformed and expression of the hCT_t gene was studied by mRNA-DNA hybridization, RT-PCR, Northern-blot analysis, ELISA and RIA. The yield of recombinant hCT_t was estimated to be about 12-20 mg per liter of yeast culture for yeast and 0.02 % of the total soluble protein in transgenic potato plants.

Introduction

Calcitonin (CT) is a polypeptide hormone secreted by the thyroid gland in mammals and ultimobranchial glands in lower vertebrates. It expresses a strong hypocalcaemic activity and plays a key role in the utilization of calcium and phosphorous. For more than two decades CT is used in clinics for treatment of diseases related to bone decalcification, such as Paget disease, osteoporosis imperfecta, parathyroid gland carcinomas, etc. (1).

CT molecule is characterized as follows: a) all known calcitonins consist of 32 amino acids (aa); b) the 1st and the 7th aa are always cysteins; c) the 8th residue is a hydrophobic aa (usually methionine or valine); d) the 15th residue is a dicarboxylic aa; e) the 22nd residue is an aromatic aa; f) the 28th aa is always glycine; g) the C-terminal (the 32nd) residue is always proline and h) the C-termini of all known calcitonins are amidated (1).

CT activity is not species specific which makes it possible to use animal calcitonins (porcine, salmon and eel) for treatment of human patients. However, due to immunological reactions the prolonged application of animal calcitonins leads to a gradual decrease or loss of CT activity. That is why the long term treatment of human patients with CT preparations requires homologous (human) calcitonin (hCT).

hCT was first isolated from C-medullary thyrocarcinoma in 1968 and was synthesized soon after establishment of its primary struc-

ture. Although synthetic hCT is commercially available for a long time, its biological activity has been reported to be much lower (up to ten times) in comparison with that of the natural hormone. The lower activity is explained by amino acid isomerization and molecular occurring during the solid phase polypeptide synthesis (1).

Homogeneous hCT preparations consisting of 100 % chiral amino acids can be prepared by the methods of genetic engineering only. This idea triggered numerous studies dedicated to the chemical synthesis (2-4) and expression of hCT gene in E. coli (2, 3, 5-8). The expression of small proteins in bacteria, however, is still a problem for the recombinant DNA technology. Our attempts to realize a direct expression of the hCT (monomeric) gene in *E. coli* failed to give satisfactory results and the low yield obtained was explained by the proteolytic instability of the recombinant product (2, 5). Two approaches have been employed so far for the stabilization of hCT in heterologous producers: a) fusion to highly expressed genes, such as the genes of human interferon gamma (3) or chloramphenicol acetyltransferase (7) and b) oligomerization of the hCT gene (4, 9). The fusion experiments showed that the hCT gene was a strong inhibitor of the expression of all genes used for fusion lowering the yield of corresponding protein by a factor of 100 to 1000. Higher, although unsatisfactory, results has been obtained with the hCT oligomeric genes (8). One of the most reliable constructs among a series of many oligomeric hCT genes turned out to be the hCT tetrameric (hCT_t) gene. Although this gene yielded several percent of recombinant protein in the form of insoluble inclusion bodies in *E. coli* cells (10) the microbial expression system demonstrated the following disadvantages: a) the increased expression of hCT obtained by oligomerization of the hCT gene strongly inhibited bacterial cell growth; b) the expression plasmids bearing oligomeric hCT genes showed a high segregation and genetic instability. The latter shortcomings, however, were stimulating for searching for eukaryotic hosts for the hCT gene expression.

Two eukaryotic expression systems, yeast and transgenic plants, have recently emerged for production of biologically active compounds of eukaryotic origin for clinical application (11). In this paper we describe the expression of a synthetic hCT_t gene in two yeast strains and one transgenic potato plants cultivar.

Materials and Methods

Microorganisms, plasmids and genes

Escherichia coli XL1-Blue (used for cloning) was purchased from Stratagene (USA) and *Agrobacterium tumefaciens* PGV3850 (used for plant transformation) was obtained from Prof. Piroozian (Institute of Molecular Genetics, Moscow). The yeast strains *S. cerevisiae* GRF18 (*MATa, leu2-3, leu2-12, his3-11, his3-15*) and VY168 (*MATa, srb1, ts1, leu2-3, leu2-12, his3-11, his3-15*) were used for hCT_t gene expression. The plasmid for yeast expression containing the yeast α-factor coding sequence and a portion of the plasmid pMA91 as a donor of both the yeast LEU2 gene and the 2μ origin of replication was constructed as described before (12). The plasmid pJP_1R_9-hCT_t (4) was used as a source of the synthetic hCT_t gene. Together with the plasmids pRTL (gifted by Prof. J. Carrington, Texas University) and pBin19 (provided by Prof. Piroozian, Institute of Molecular Genetics, Moscow) it was used to construct the plasmid for potato plant transformation.

Yeast transformation and growth

Yeast cells were transformed according to Ito *et al.* (13). Transformed GRF18 cells were cultured at 30 °C in a selective medium containing 1 % yeast nitrogen base, 4 % D-glucose and 80 μg/ml histidine under vigorous shaking. The VY168 cells were grown in the same medium supplemented with 10 % of sorbitol.

Plant cultivar and growth

A potato cultivar *Solanum tuberosum* Nevsky

was grown on a sterile MS medium under 18 hours light and 6 hours dark photoperiods. Potato minitubers were prepared from sterile *in vitro* grown potato shoots with 5-6 fully expanded leaves. After rooting on MS medium containing 6 % sucrose and 1 mg/l 6-benzylaminopurine (BAP), the plants were transferred to a dark place and grown at 4 °C for 7-10 days. Plants were then grown in a well shadowed place at 16-18 °C until minitubers were formed (14).

Plant transformation and regeneration

Agrobacterium tumefaciens pGV3850 cells were transformed with the recombinant binary vector pBin19-hCT$_t$ isolated from *E.coli* XL1-blue cells and used for plant transformation. *In vitro* grown potato minitubers (4 months old) were cut into 1-2 mm slices and transformed essentially as described by Ishida *et al.* (15). The tuber discs were washed with the same medium, dried between sterile filter paper sheets and transferred to new plates containing selective medium. The plates were incubated in dark at 26 °C until the regenerated shoots reached the length of 1.5-2 mm. The shoots were cut off, transferred to individual test tubes containing the same selective medium and incubated in the light at 26 °C. The plants developing roots under these conditions were assayed for transcription and translation of the hCT$_t$ gene.

RNA isolation and analysis

Total RNA was isolated from plant tissues by standard procedures and stored under 70 % ethanol.

Reverse transcriptase (RT) PCR reaction was carried out using a SuperScript^{tm} kit (Gibco-BRL) and two (forward and reverse) primers specific for both ends of the hCT gene.

Preparation of plant tissue extracts

Plant tissues frozen in liquid nitrogen were crushed into fine powder and extracted with 2 volumes of the following buffer: 20mM sodium phosphate, pH 7.0, 0.15 M NaCl/, 0.1 % Triton X-100 and 2 mM PMSF. The samples were clarified by centrifugation at 45 000 rpm for 45 in a Beckman TL-100 centrifuge and used for RIA or immunoaffinity purification.

Immunoaffinity purification of recombinant calcitonin

Rabbit polyclonal hCT antiserum was conjugated to CNBr-activated Sepharose 4B (Pharmacia) and packed into a glass column. Crude plant tissue extracts were loaded onto the column equilibrated with PBS buffer (0.14 M NaCl, 5 mM sodium phosphate pH 7.4) and after washing with PBS, the hCT$_t$ was eluted with 0.1 M acetic acid.

Radioimmunoassay

Radioimmunoassay (RIA) was based on the double-antibody approach for antigen precipitation using commercial kits (Diagnostic System Laboratories) and following the manufacturers instructions. RIA was applied for concentration measurements of hCT$_t$ in either yeast culture medium clarified by centrifugation or transgenic plant extracts.

Results and Discussion

In this study we describe the expression of a previously synthesized hCT$_t$ gene (4) in yeast and transgenic potato plants. The vector for yeast expression (YIMB-hCT$_t$) is shown in **Fig. 1A** and its creation was described earlier (12). The expression vector for potato plant cells (pBin19-hCT$_t$) is presented in **Fig. 1B** and its construction will be described elsewhere.

The transcription of the hCT$_t$ gene in transgenic potato plants was studied by RT-PCR. As shown in **Fig. 2**, the PCR test revealed a single band of about 100 base pairs (corresponding to the size of the hCT monomeric gene) in all transgenic plants tested whereas such a band was missing in the PCR products of the non-transformed (control) plants. The smaller size (100 bp) of the PCR fragments is due to the fact that the two primers used for PCR amplification are complementary to the ends of the hCT gene which is repeated four times in the hCT$_t$ gene.

The efficiency of translation of the hCT$_t$

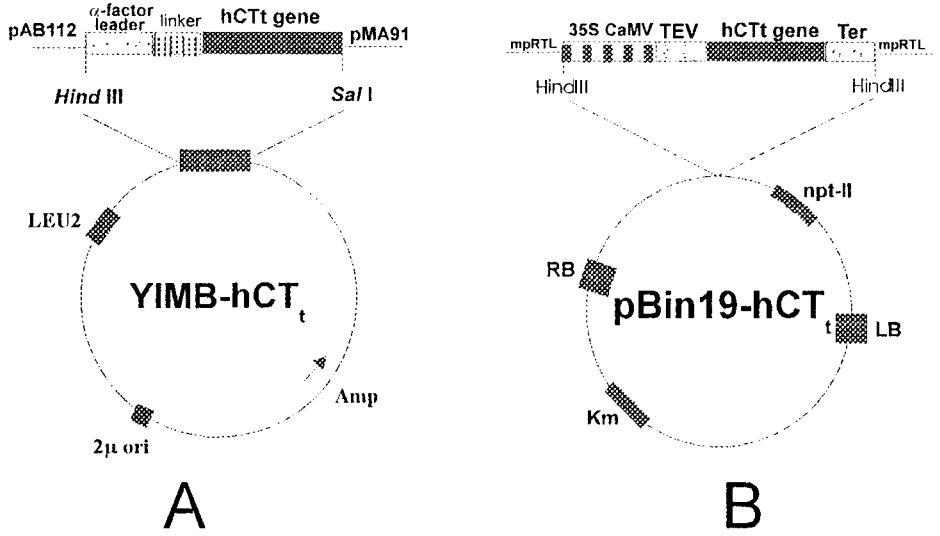

Fig. 1. Structure of the plasmids for expression of the hCT_t gene in yeast (A) and potato plants (B).
Abbreviations in (A): pAB112 and pMA91, portions of the two plasmids used for construction of the expression plasmid YIMB-hCT_t; LEU2, a gene for selection; Amp, ampicillin; 2μ ori, origin of replication borrowed from the wild yeast plasmid 2μ. Abbreviations in (B): RB and LB, right and left border sequences of the Ti-plasmid; npt-II, kanamycin (Km) resistance marker; hCT, human calcitonin monomeric or tetrameric gene; mpRTL, portions of the plasmid pRTL; TEV, a portion of the tobacco etch virus, modified as shown at the bottom of the figure; Ter, transcription terminator and a polyadenilation site; Km, kanamycin.

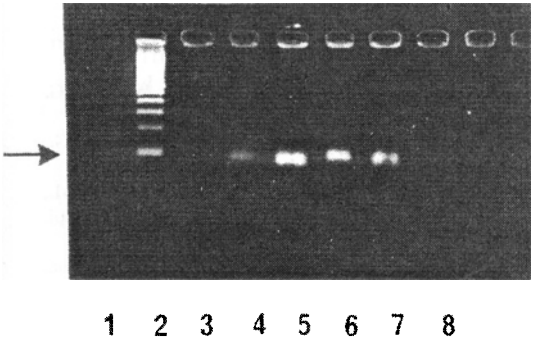

Fig. 2. Identification hCT_t gene transcripts in transgenic potato plants by RT-PCR. Lanes: 1, a 100 base pair DNA ladder (Gibco-BRL); 2, non-transgenic potato plant (negative control); 3-6 different regenerates transformed with the expression plasmid pBin-hCT_t.

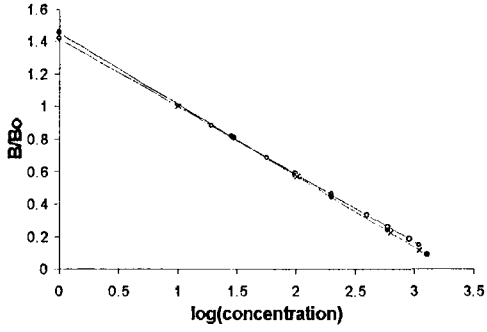

Fig. 3. Radioimmunoassay (RIA) for binding of unlabelled hCT_t isolated from yeast growth medium (empty circles) and hCT_t isolated from transgenic potato plant leaves (full circles) with hCT specific antiserum in the presence ^{125}I labeled standard hCT. Bo is binding of labeled hCT in the absence of unlabeled competitor (considered as 100 %) and B is the binding of labeled calcitonin in the presence of unlabeled calcitonin. Antigen concentration is given on the abscissa. The third line (marked by crosses), represents a standard curve expressing the competition with homologous (standard) unlabeled hCT.

mRNAs in yeast and transgenic plants was studied by radioimmunoassay (RIA). As shown in **Fig. 3**, the recombinant hCT_t protein isolated from both yeast growth medium and transgenic potato plants competed very well with the ^{125}I-labeled standard hCT for

TABLE

Yield of hCT_t produced by recombinant yeast and transgenic potato plants

Producer	Yield of hCT_t[a]	
	mg/l[b]	ng/mg protein[c]
Yeast GRF18	20.0 ±0.5	
Yeast VY168	12,0 ±0.6	
Potato leaves		2.0±0.3
Potato roots		1.0±0.2
Potato tubers		0.7±0.2

[a] The yield of hCTt is measured by RIA in either clear (with no yeast cells) growth medium ([b]) or plant tissue extracts ([c]).

the hCT specific antibodies. This was an indication for the immunological identity of the recombinant proteins synthesized in both eukaryotic producers. The latter was a reason for the application of RIA for quantitative analysis of the expression of hCT genes in recombinant yeast and transgenic potato plants. As shown in **Table**, the yield of hCT_t in the yeast culture medium was about 12 mg/l for the yeast strain VY168 and 20 mg/l for the strain GRF18. As for the transgenic plants our study showed that the yield of hCT in the tissues was variable and depended on both the plant organ used and the stage of plant development (Table). In spite of that the recombinant calcitonin was detected in all plant tissues, its content in the green parts was twice higher than that of the roots and tubers. The tissue and organ dependence of the yield of recombinant hCT_t protein could be explain by the differential activity of the 35S CaMV promoter in plant organs (16).

To determine the size of recombinant protein produced by yeast and transgenic potato plants, protein preparations were purified from either yeast growth medium or plant tissue extracts by affinity chromatography using hCT specific polyclonal antibodies. The SDS-polyacrylamide gel electrophoresis followed by a Northern-blot analysis showed that the molecular masses of the immunoreactive proteins was about 13 KDa which corresponded to the size of the hCT tetrameric protein (data not shown).

The results presented in this study indicate that the yield of recombinant hCT is not satisfactory high either in yeast or in transgenic potato plants. Bearing in mind the unsuccessful expression of the hCT gene in *E. coli* (see above), it seems to us that the hCT gene is in principle a poorly expressed gene in all kinds of heterologous hosts tested so far. In spite of that, the fact that both yeast and transgenic potato plants can tolerate and express hCT genes even at low levels, give us reason to continue our efforts to this direction. We have recently started a new series of experiments aiming to improve the expression of the hCT gene in plant tissues using other (tissue specific) promoters and the new results will be a subject of another publication.

REFERENCES

1. **Turner C.D., Bagnara J.T.** (1976) In: General Endocrinology, Saunders, W.B. Co, Philadelphia, 225-257.
2. **Ivanov I.G., Tam J., Wishart P., Jay E.** (1987a) Gene, **59**, 223-230.
3. **Ivanov I., Gigova L., Jay E.** (1987b) FEBS Lett., **210**, 56-60.
4. **Ivanov I.G.** (1990) Analyt. Biochem., **189**, 213-216.
5. **Alexciev K., Usheva A., Ivanov I.G.** (1989) Curr. Microbiol., **18**, 5-9.
6. **Alexciev K., Usheva A., Pavlova M., Ivanov I.G.** (1989) Int. J. Biochem., **21**, 987-996.
7. **Bachvarov D., Wishart P., Usheva A., Ivanov I.G., Jay E.** (1990) Biol. Zentralblat, **109**, 113-117.
8. **Gigova L., Wishart P., Uscheva A., Ivanova M., Bardarov S., Jay E., Ivanov I.** (1989) Biotechnol. Appl. Biochem., **11**, 401-412.
9. **Ivanov I.G., Christov C., Alexciev K.** (1990) Gene, **95**, 295-299.
10. **Petrov P., Jay E., Ivanov I.** (1997) Biology of the Cell, **61**, 1-4.
11. **Kusnadi A.R., Nikolov Z.L., Howard J.A.** (1997) Biotechnol. Bioengin., **56**, 464-473.
12. **Mironova R., Philipova D., Marekov L., Venkov P., Ivanov I.** (1991) FEMS Microbiol. Lett., **83**, 23-28.
13. **Ito H., Fukufa Y., Murata N., Kimura A.** (1983) J. Bacteriol., **153**, 163-168.
14. **Akita M., Takayama S.** (1994) Plant Cell Tissue & Organ Cultures, **36**, 177-182.
15. **Ishida B.K., Snyder Jr.G.W., Belknap W.R.** (1989) Plant Mol. Biol., **8**, 325-328.
16. **Stefanov I., Fekete S., Bogre L., Pauk J., Feher A., Dudits D.** (1994) Plant Sci., **95**, 175-186.