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ARTICULOS ORIGINALES CORTOS/ SHORT ORIGINAL PAPERS

HUMAN β-NERVE GROWTH FACTOR EXPRESSED IN YEAST BY FUSION TO UBIQUITIN

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SUMMARY

An expression system has been established in *P. pastoris* to yield recombinant human-Nerve Growth Factor where the C-terminus of ubiquitin protein was fused to the N-terminus of β-NGF. The human Nerve Growth Factor gene was integrated into the chromosome of the *P. pastoris* under the control of the methanol-regulated alcohol oxidase promoter and fusioned to the ubiquitin gene. The fusion protein was expressed and processed *in vivo*. An ubiquitin C-terminal hydrolase cleaves the junction peptide bond between the ubiquitin and β-NGF. The identity of NGF was demonstrated by Western blot.

RESUMEN

Un sistema de expresión ha sido establecido en *Pichia pastoris* para producir NGF humano recombinante, donde el extremo C-terminal de la ubiquitina se fusionó al extremo N-terminal del β-NGF. El gen de NGF se integró en el cromosoma de *Pichia pastoris* bajo el control del promotor de la enzima alcohol oxidasa, el cual es fuertemente regulado por metanol. Esta proteína de fusión se expresó y procesó *in vivo*. Una enzima hidrolasa del sistema ubiquitina corta la fusión. Se demostró por Western blot la identidad del NGF

INTRODUCTION

Nerve Growth Factor (NGF) (1) is a member of the neurotrophin family, which also includes: brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5. NGF is required for the survival and development of the sympathetic and sensory neurons, and it also has trophic action on the cells of the adrenal medulla, on cholinergic neurons in the basal forebrain of the central nervous system, and on a number of tumor cell lines as well (2).

The *P. pastoris* heterologous gene expression system has been utilized to produce attractive levels of a variety of proteins. As a result of the fermentation development studies required for single-cell protein production, the growth characteristics of *P. pastoris* are well defined. The organism is routinely propagated in continuous culture at densities of approximately 130 g (dry weight) per liter for periods of up to 30 days in a 1.5 L fermentor. The culture medium is defined and inexpensive. Recent advances in the understanding and application of the system have improved its utility even further (3). Unlike the mouse-β-NGF which is synthesized in significant amounts in the mouse submaxillary gland, no sources of abundant hNGF synthesis have yet been identified. Several papers have appeared describing the expression of the hNGF gene by *E. coli* (4), *S. cerevisiae* (5), and CHO cell (6) but the amounts recovered and/or the biological activity of the rhNGF were low. Here we report the expression of human NGF gene using the methylotrophic yeast *P. pastoris.*

The NGF gene is driven by the methanol-controlled alcohol oxidase (AOX1) promoter and is fusioned to the ubiquitin gene. Ubiquitin is a small protein (76 amino acid) of high intracellular abundance.

The primary sequence of ubiquitin is of unparalleled conservation between species. Among its 76 amino acid residues only three differ between the human and the yeast protein. The fusion of genes to ubiquitin sequence greatly increase their yield (7) in heterologous expression system.

MATERIALS AND METHODS

Strains

E. coli MC1066 strain was used for the plasmid manipulation and amplification. *P. pastoriS* MP36 (his3-) (8) was used as host for expression of NGF.

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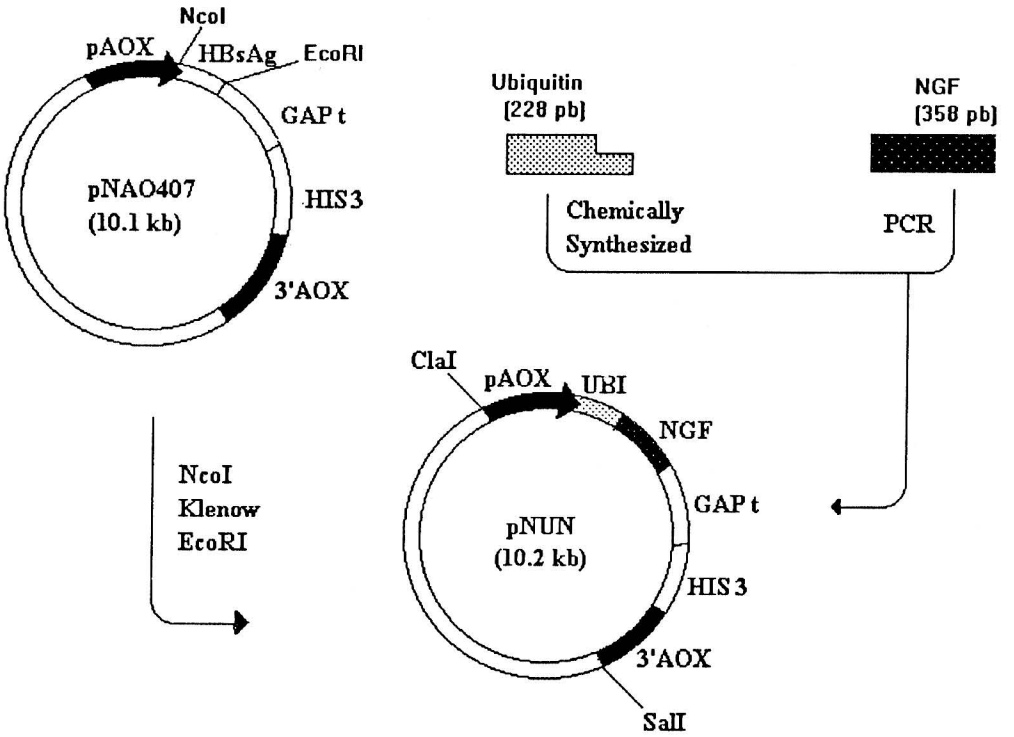

Fig. 1 Construction of Ubiquitin-NGF expression vector, pNUN.

Standard molecular techniques

Molecular genetic techniques were performed essentially as described by Sambrook *et al.* (9). The ubiquitin gene was synthesized according to Jimenez *et al.* (10).

Transformation and culture medium

Transformation of *P. pastoris* was performed according to a procedure described by Martinez *et al* (11). Culture medium was YNB (0.17% YNB, 0.5% $(NH4)_2SO_4$) supplemented with 2% glycerol and 1% yeast extract.

Culture conditions

Five mililiters of a single transformant were used to inoculate 2 L flask containing 500 mL YNB medium. This flask was shaked at 250 rpm for 12 hours at 30°C. These 500 mL of culture were inoculated in a 5 L fermentator. Fermentation conditions were 700 rpm, 1 vvm of aeration, 30°C and pH 5.5 Methanol was added for induction at a flow of 2 mL/Lh, beginning when the methanol was depleted.

Protein analysis

SDS-PAGE was done as described by Laemmli (12). Western blot was performed as described by Towbin *et al* (13) using an anti-mouse Nerve Growth Factor 2.5 S developed in rabbit (Sigma Immuno Chemicals).

RESULTS AND DISCUSSION

The vector construction is described in figure 1. The plasmid pNUN contains the expression cassette including: the methanol regulated *P. pastoris* alcohol oxidase I promoter (pAOX), the UBI-NGF gene fusion, the *S. cerevisiae* GAP transcriptional termination signal (GAP t), the *S. cerevisiae HIS3* gene, which provided a selectable marker for transformation of *P. pastoris* MP36, and a 2.1 Kb DNA fragment from the downstream region of the chromosomal *P. pastoris AOX1* gene, which together with the AOX1 promoter provide the chromosome-homologous ends needed for integration.

The ubiquitin gene was chemically synthesized (10). The plasmid pNGF with the same integration cassette but without the ubiquitin gene was constructed. *Pichia pastoris* MP36 was transformed with ClaI-SalI digested pNUN and pNGF plasmids.

The transformants were selected by histidine prototrophy (His^+). Stabilitity tests were made to define integration to chromosomal DNA, according to its growth in non selective medium, at the end of fifty generation (14). More than 98% of stability was obtained.

The integration pattern in the chromosomal DNA of one clone per construction with the highest percent of stability was analyzed by Southern blot for each construction (figure 2). In this experiment, each of the DNA samples was digested with EcoRI and, after agarose gel electrophoresis and transfer to a nitrocellulose filter was hybridized with a labelled probe composed of the pAOX-NGF fragment.

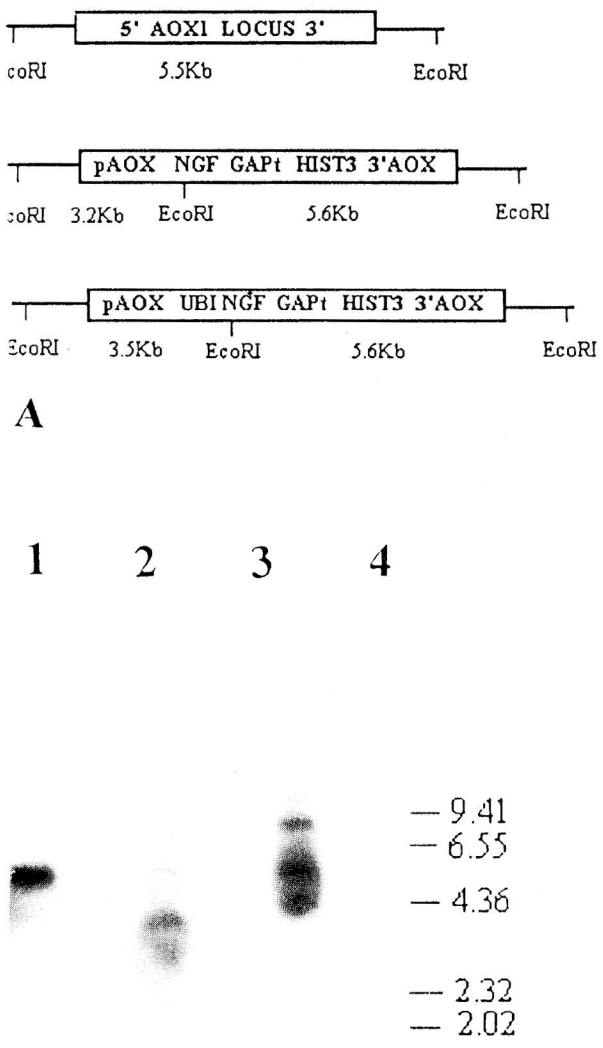

Fig. 2 Integration patron in the chromosomal DNA. A: Diagram of replacement of the AOX1 locus, *P. pastoris*. B: Southern blot analysis. 1, 2, 3: each 5 μg of EcoRI-digested genomic DNA from the following *P. pastoris* strains, respectively: MP36, MP36(pNGF), MP36(pNUN). 4: HindIII-λ DNA.

The EcoRI-digested DNA from the untransformed host MP36 (figure 2B, lane 1) showed a band corresponding to AOX1 locus, 5.5 Kb (see the AOX1 locus in figure 2A). Lane 2 of figure 2B shows the correct integration pattern of the genetic construction (MP36 (pNGF)) Integration of the 9.2 Kb ClaI-SalI fragment from pNUN at the AOX1 locus of MP36 (figure 2B, lane 3) resulted in the appearance of three bands (9.2 Kb, 5.6 Kb, 3.5 Kb). The higher band may be due to a partial digested DNA. The MP36(pNUN) and MP36(pNGF) yeast strains with the correct integration pattern were fermented as described in materials and methods.

Growth curves for these two yeast strains in YNB plus yeast extract selective medium are shown in figure 3.

As it can be observed, MP36 (pNGF) strain has a growth curve similar to MP36 strain but MP36(pNUN) strain stops to growth at 48 h. Due to the poor growth of the MP36(pNUN) strain, we studied the expression at 48 h and at the end of the fermentation. The identity of NGF is shown by Western blot in figure 4. The results obtained by Western blot shown a 14 kD migratory band (lanes 2, 3) for the MP36(pNUN) strain at the same level than the murine NGF (lane5). As it has been reported (15) the fusion protein was processed *in vivo* by the C-terminal ubiquitin hydrolases. The MP36(pNGF) strain did not express NGF even at 120 h of induction (figure 4, lane 4).

Additional clones of the same construction with a correct integration pattern were studied with a similar result (not shown).The behaviour of growth curve of MP36(pNUN) could be explained by a toxic effect of NGF expression to cells.

The expression of NGF only as a fusion protein could confirm, that the ubiquitin fusion technology at their N-termini increases the yield of unstable or poorly expressed proteins (7). In addition, the ubiquitin molecule, well known for its resistance to proteases, might protect the fused protein from N-terminal proteolytic attack. Besides, the ubiquitin may facilitate proper folding of the fused protein, as a result the protein may remain

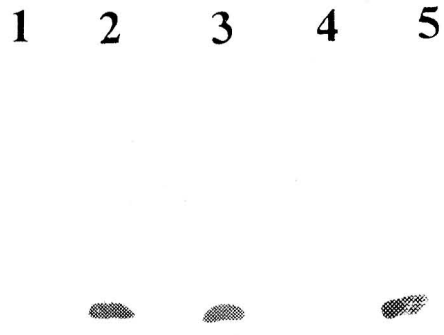

Fig. 3 Western blot. Expression analysis.
1: total cellular proteins from MP36(pNUN) before induction. 2, 3: after induction for 48 h and 120 h with methanol, MP36(pNUN). 4: after induction with methanol, 120 h, MP36(pNGF). 5: murine NGF.

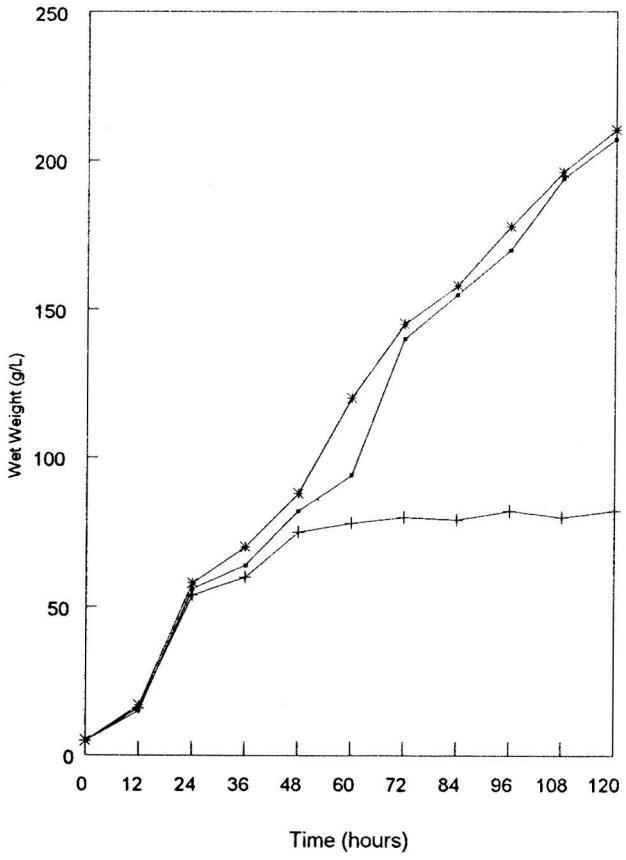

Fig. 4 Growth curves in YNB supplemented with 2% glycerol and 1% yeast extract. MP36: *, Mp36(pNGF): +, MP36(pNUN): ◦

soluble (this is the role of ubiquitin in some cell surface receptors, it keep these hydrophobic proteins soluble and facilitate their translocation to the cell membrane).

On the other hand, ubiquitin, nature's most conserved protein, might have evolved the best codon usage for eukaryotes, this ensure efficient translation initiation. Using this strategy of cloning, 2-3% of NGF expression of the total protein was obtained.

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