UTILIZATION OF DNA RECOMBINANT TECHNIQUES FOR THE PREPARATION OF RADIOIMMUNOASSAY REAGENTS FOR PITUITARY HORMONES*

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Abstract

UTILIZATION OF DNA RECOMBINANT TECHNIQUES FOR THE PREPARATION OF RADIOIMMUNOASSAY REAGENTS FOR PITUITARY HORMONES.

The complementary DNA (cDNA) of human growth hormone (hGH) and human prolactin (hPRL) have been cloned in bacteria after screening a library that was obtained starting directly from human pituitary mRNA. Recombinant hGH (rec-hGH) was also expressed and secreted in *E. coli* periplasmic space and subsequently purified through three different chromatographic steps. After testing its purity, potency and identity with purified hGH (pit-hGH), rec-hGH was used for the preparation of radioimmunoassay reagents. Its suitability for radioiodination and immunological equivalence to pit-hGH were confirmed, its relative immonological activity being 1.037 $\pm$ 0.063 in comparison with a well known reference preparation, NIDDK-hGH-RP-1, from the National Institute of Diabetics and Digestive and Kidney Diseases, Bethesda, MD, USA. The synthetic hPRL gene was also obtained from the same cDNA pituitary library, its identity to the published sequence coding for the human hormone being demonstrated. An expression vector is now being constructed, following a strategy similar to that already used for rec-hGH preparation, purification and testing, pursuing the same goal of radioimmunoassay reagents preparation.

1. INTRODUCTION

The difficulties of extracting and purifying human pituitary hormones e.g. growth hormone (hGH), prolactin (hPRL), thyroid stimulating hormone (hTSH), luteinizing hormone (hLH), follicle stimulating hormone (hFSH), are widely known and so are the difficulties of obtaining these glands from autopsy and the dangers related to their manipulation. Some of these hormones are present in the hypophysis

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in extremely limited amounts and, moreover, their similar physicochemical properties make their separation and purification very cumbersome. All these problems can be largely resolved by the use of deoxyribonucleic acid (DNA) recombinant techniques, through which high amounts of human proteins, and more recently also glycoproteins, can be produced by transformed prokaryotic or eukaryotic cells.

The present study describes the utilization of recombinant hGH, already prepared and tested in our laboratory mainly for therapeutic applications [1], for the additional purpose of radioimmunoassay reagents preparation. To date, although most of these pituitary hormones have been already obtained by DNA recombinant techniques, their utilization for in vitro diagnostic techniques has been mentioned only very seldom [2]. The cloning of human prolactin gene, for the same purpose, is also presented.

2. METHODS

2.1. Preparation of a cDNA pituitary library

This type of library was obtained starting from pituitary material and obtaining total RNA by the guanidinium caesium chloride centrifugation method. Poly $(A)^+$ RNA was obtained by purification on oligo (dT)-cellulose column (Sigma Chemical Co., St. Louis, MO, USA) and from this a double stranded cDNA was prepared usng the Amersham cDNA synthesis system and protocol (Amersham International, Amersham, United Kingdom). The cDNA cloning system in λgt 10, from the same manufacturer, was used to prepare the cDNA library.

2.2. Screening of the cDNA pituitary library

In both cases, for hGH and hPRL genes, the screening was carried out using two probes: a 21-mer synthetic oligonucleotide, hybridizing with a portion of the 5′ non-coding sequence of the cDNA and rat GH-cDNA or rat PRL-cDNA, which have a high degree of homology with the coresponding human cDNAs, kindly donated by Dr. Martial (University of Liège, Belgium). Sequencing was carried out using the method of Sanger et al. [3].

2.3. Preparation of the bacterial expression vector

This work was done following an original construction design (patent applied for) which led to *E. coli* periplasmic secretion of the cloned protein.

2.4. Purification of rec-hGH

The purification process was carried out in three chromatographic steps, using Octyl Sepharose CL-4B, diethylaminoethyl (DEAE), Sepharose Fast Flow and Sephadex G-100, as described by Lefort and Ferrara [4]. The required battery of identity, potency and purity tests was carried out on the purified product.

2.5. Radioiodination

The ^{125}I labelling was carried out using the technique described by Biscayart et al. [5], employing 0.5–0.7 mCi[1] of radioisotope (Medgenix Diagnostics, Wevelgem, Belgium). The tracer purification was performed by gel filtration on Sephadex G-100.

2.6. Radioimmunoassay

Assays were carried out in a classical manner, with simultaneous addition of all reagents (tracer, reference preparation and first antibody) incubating for 24 hours and using a liquid phase second antibody separation technique. Pit-hGH reference preparation, NIDDK-hGH-RP-1, from the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA, used for the standard curve, was kindly donated by the National Hormone and Pituitary Program (Baltimore, MD, USA).

3. RESULTS

Figure 1 shows a schematic representation of the main steps involved in the preparation of rec-hGH and rec-hPRL. In Fig. 2 the three chromatographic purification steps utilized for rec-hGH preparation are presented. Transformed *E. coli* cells were grown and submitted to an osmotic shock [6] in order to obtain the desired protein mainly in the periplasmic fluid (hGH, determined by radioimmunoassay, represented approximately 15–20% of the total periplasmic protein). This was then purified by hydrophobic interaction chromatography on Octyl Sepharose CL-4B, obtaining a specific activity increased by about 4–5 times. The fractions containing hGH were pooled and loaded directly onto a DEAE Sepharose Fast Flow ionic exchange chromatographic column after having determined the protein and hGH radioimmunoassay content, and obtaining a purity of more than 90%. The final purification was carried out on Sephadex G-100, obtaining a practically pure

[1] 1 Ci = 37 GBq.

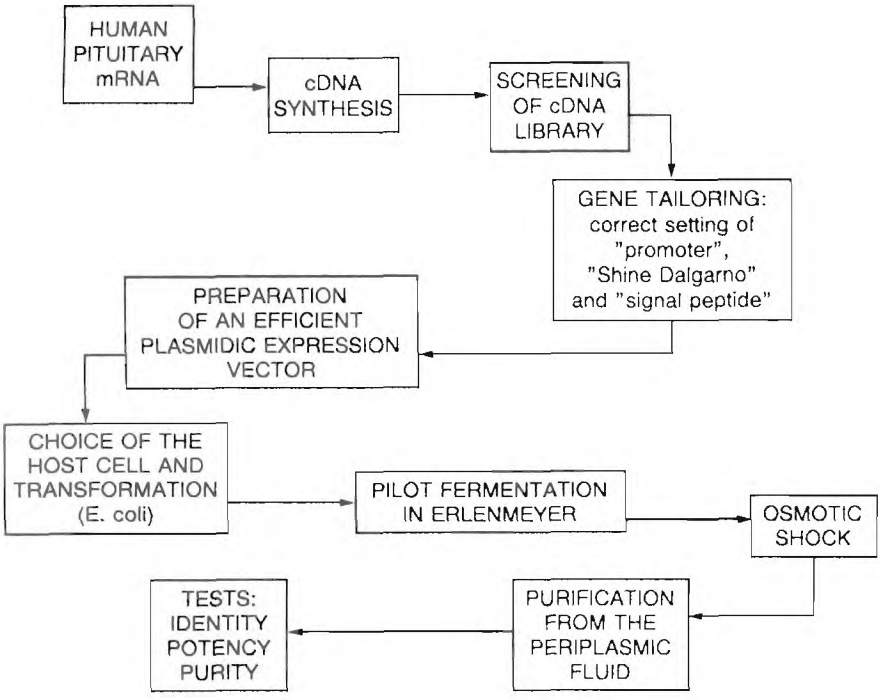

FIG. 1. Preparation of hGH and hPRL by DNA recombinant techniques.

product. The quality of the hormone obtained was controlled by reducing and non-reducing sodium diodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, size exclusion and reverse phase high pressure liquid chromatography (HPLC), isoelectric focusing, tryptic mapping and biological assay in hypophysectomized rats.

Figure 3 shows typical radioiodination of rec-hGH to a relatively low specific activity: 40 μCi/μg. A comparison of the distribution coefficient (K_d) with previously pooled data [7] indicates the presence of the monomeric form only, with practically no aggregate. The usual presence of a quite large peak of bovine serum albumin (BSA) carried ^{125}I and of unreacted ^{125}I can also be observed.

In Fig. 4 the two standard curves obtained with a pituitary reference preparation, NIDDK-hGH-RP-1, and with rec-hGH are presented. While the reference preparation was plotted in terms of the declared content (ng/mL), the unknown preparation was plotted in terms of microlitres of undiluted solution of rec-hGH used in the assay. A good parallelism between the two curves can be observed while, on

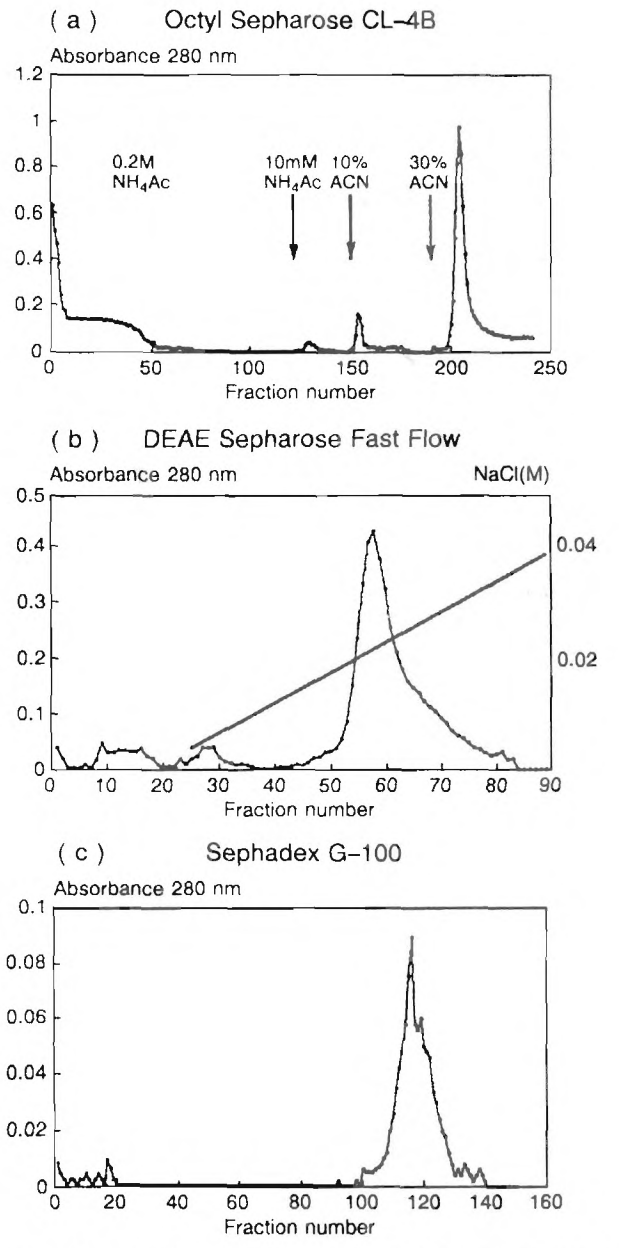

FIG. 2. Purification of rec-hGH from E. coli *periplasmic fluid. (a) Octyl Sepharose CL-4B: column size, 13 × 1.25 cm; flow rate, 30 mL/h; wash with 0.2M ammonium acetate (NH_4Ac); elution with 0.01M NH_4Ac, 0.01M NH_4Ac + 10% acetonitrile (ACN), 0.01M NH_4Ac + 30% ACN. (b) DEAE Sepharose Fast Flow: column size, 10 × 1.25 cm; flow rate, 30 mL/h; elution in 0.01M NH_4Ac; gradient 10–50mM NaCl. (c) Sephadex G-100: column size, 83 × 1.25 cm; flow rate, 12 mL/h; elution in ammonium bicarbonate 0.05M.*

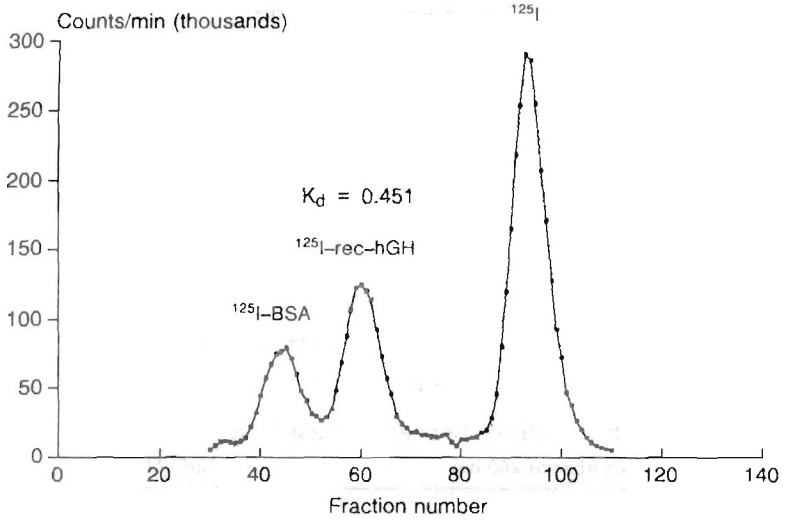

FIG. 3. Example of purification of radioiodinated rec-hGH on Sephadex G-100. Column size, 40 × 2.5 cm; flow rate, 12 mL/h.

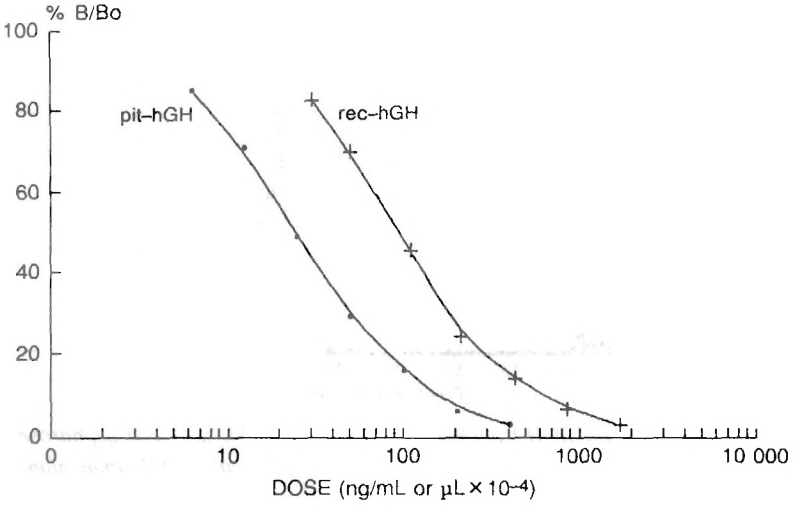

FIG. 4. A comparison between RIA curves of pit-hGH (NIDDK-hGH-RP-1) in ng/mL and rec-hGH (National Nuclear Energy Commission, Brazil) in µL.

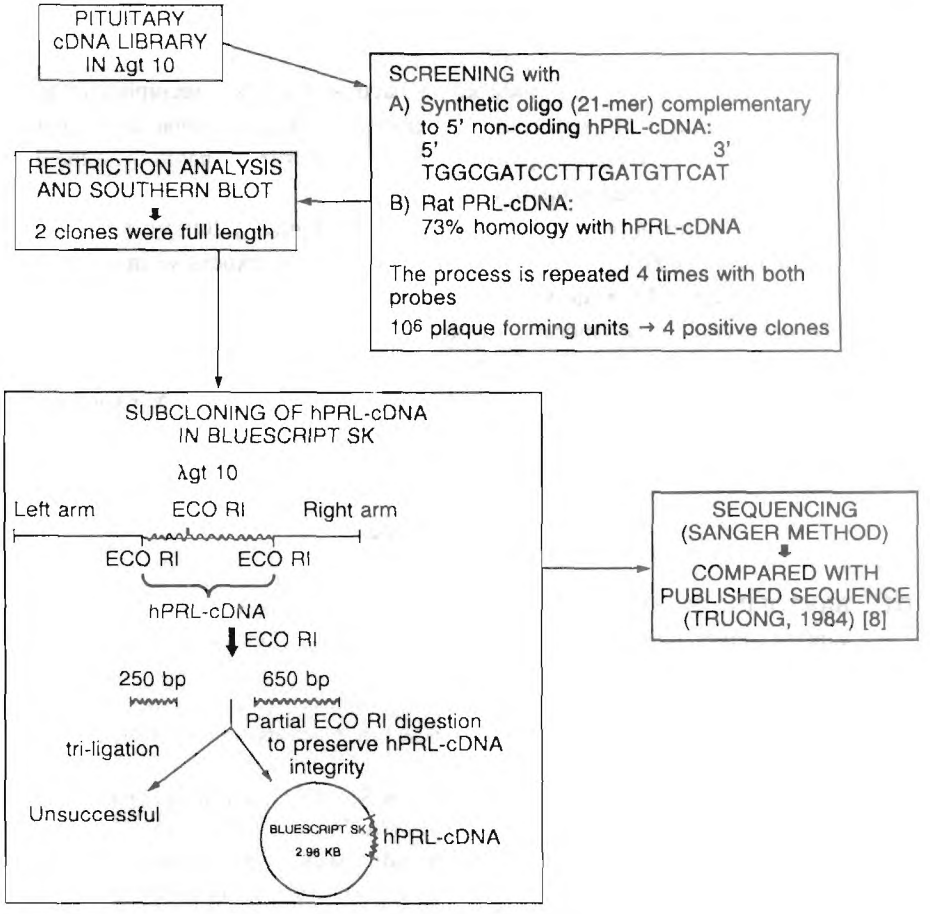

FIG. 5. Cloning and sequencing of full length hPRL-cDNA.

the basis of the determined protein content, the recombinant preparation presented a relative activity of 1.037 ± 0.063 (CV = 6.1% for n = 4 independent assays) in terms of NIDDK-hGH-RP-1.

The strategy of cloning, subcloning and sequencing of hPRL-cDNA is presented in Fig. 5. A problem had to be resolved in the subcloning process. Considering that the pituitary library had been cloned in the λgt10 Eco RI site and that hPRL-cDNA also includes one of these sites, the prolactin gene was, unfortunately, split into two fragments. Milder digestion conditions were set up, decreasing incubation time, in order to preserve the integrity of hPRL-cDNA.

4. DISCUSSION

Human growth hormone, produced in bacteria by DNA recombinant techniques, was successfully utilized in the preparation of radioimmunoassay reagents. Both radioiodinated and reference preparations were indistinguishable from the corresponding pituitary derived preparations.

Human prolactin, a protein which is present in human pituitary in extremely limited amounts (50–100 μg/gland) and therefore extremely expensive in its purified form, is being prepared for the same purpose.

The next step, in collaboration with an experienced laboratory, will be the preparation of recombinant hTSH, a hormone which is formed by two different subunits and which, because of its glycoproteic composition, has to be expressed in eukaryotic cells.

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