Expression of human α_1-antitrypsin in *Escherichia coli*

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Received 3 November 1983

Complementary DNA coding for human α_1-antitrypsin has been placed under the control of the λP_R promotor carrier by the expression vector pCQV2 [1]. In conditions which allow transcription from this promotor (thermoinactivation of the repressor), *Escherichia coli* cells harbouring the recombinant plasmid pULB1114 express human α_1-antitrypsin ($\pm$ 9000 molecules/cell). The product has a M_r of 44000, corresponding to mature unglycosylated α_1-antitrypsin.

Human α_1-antitrypsin *Plasma protein* *Recombinant DNA* *Cloning of human cDNA* *Bacterial expression of human cDNA* *Immunoassay for α_1-antitrypsin*

1. INTRODUCTION

α_1-Antitrypsin, one of the major components of plasma proteins, is synthesized in the liver and secreted into the plasma [2]. The enzyme functions mainly as an inhibitor of elastase, a protein involved in the degradation of lung tissues [3]. Hereditary deficiencies of α_1-antitrypsin are relatively frequent [4]; when associated with environmental factors, they result in cumulative pulmonary damage known as emphysema [5]. It has been reported [6] that human emphysematic conditions can be relieved by intravenous injection of partially purified α_1-antitrypsin. It thus appears worthwhile to try to produce sufficient quantities of human α_1-antitrypsin through recombinant DNA technology to assess its curative value in animal models and possibly in human therapy. In this context, we reported [7] the cloning of full length cDNA coding for human α_1-antitrypsin and its expression in bacteria as part of a fusion with β-lactamase.

We here describe the construction of a recombinant plasmid, pULB1114, where the DNA sequence coding for α_1-antitrypsin is placed under the control of a strong promotor and we show that bacteria harbouring this plasmid express a 44-kDa protein corresponding to mature unglycosylated α_1-antitrypsin.

2. MATERIALS AND METHODS

Restriction endonucleases, T4 DNA polymerase and T4 DNA ligase were purchased from Boehringer-Mannheim and used as recommended by the manufacturers. *E. coli* K-12 strain 294 (*end*A, *thr*$^-$, *hsr*$^-$, *hsm*$_K^+$) was used for bacterial transformations [7]. Plasmids used in this work, pCQV2 and pULB1523, have been described in [1,7].

LB medium for *E. coli* growth was as in [8] with the addition of 50 μg/ml ampicillin for the selection of plasmid transformants. DNA fragments were purified from polyacrylamide gels by electroelution followed by two phenol extractions. Colony hybridization experiment follows the procedure in [9].

Cell extracts for immunodetection of α_1-antitrypsin were prepared as follows: a 20-ml culture in LB medium of the clone to be analyzed was incubated at 30°C up to an A_{620} of 0.15; half the culture was then quickly heated to 42°C by the addition of 1 vol. hot medium (80°C) and further incubated for 2 h at 42°C. The remaining half of

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00145793/84/$3.00

the culture was treated similarly at 30°C. Cells were collected by centrifugation, resuspended in 1 ml of lysis buffer (50 mM Tris–Cl, pH 7.5; 60 mM EDTA, 25% sucrose, 500 μg/ml lysozyme) incubated for 30 min at 4°C, then treated for 15 min at 4°C with 0.01% Triton X-100. The resulting extracts were centrifuged for 20 min at 10000 rev./min and frozen at −20°C.

Immunodetection of α_1-antitrypsin in cell extracts was done using the ELISA system described in [7].

Characterization of the α_1-antitrypsin synthesized in bacteria was done by SDS–PAGE [10] followed by western blotting on nitrocellulose sheets [11]. Blots were saturated with phosphate saline buffer (PBS) pH 7.5, containing 0.1% Tween 80, 10% horse serum and 1% bovine serum albumin (4 h at room temperature). Sheets were then washed 3 times with PBS buffer containing 0.1% Tween 80 and incubated overnight at 20°C in saturation buffer supplemented with mouse antihuman α_1-antitrypsin serum (dilution 1/1000). After 5 washes with PBS/Tween buffer, the blots were incubated in the same buffer supplemented with peroxidase-labelled goat antimouse serum (GAM-POD, 10 μg/ml). Following 5 washes with PBS/Tween buffer, the nitrocellulose sheets were exposed to the chromogenic substrate (25 min in 100 mM Tris–Cl, pH 7.6; 10 mg diaminobenzidine, 100 μl of a 10% urea peroxide solution) and finally rinsed with distilled water.

3. RESULTS

3.1. *Construction of the recombinant plasmid pULB1114*

The plasmid vector pCQV2 [1] contains a strong promoter λP_R, the *cro* ribosomal binding site and the thermosensitive repressor for the P_R promotor; in addition a convenient *Bam*H1 site allows insertion of foreign genes downstream of an ATG codon. To construct the recombinant plasmid pULB1114, we first linearized the pCQV2 DNA with the enzyme *Sal*I, treated the DNA with T4 DNA polymerase to obtain blunt ends and then cut with the enzyme *Bam*H1 to generate a plasmid molecule suitable to accept any foreign DNA fragment having a 5′ *Bam*H1 cohesive end and a 3′-blunt end (fig.1a).

Plasmid pULB1523 carries the full length cDNA

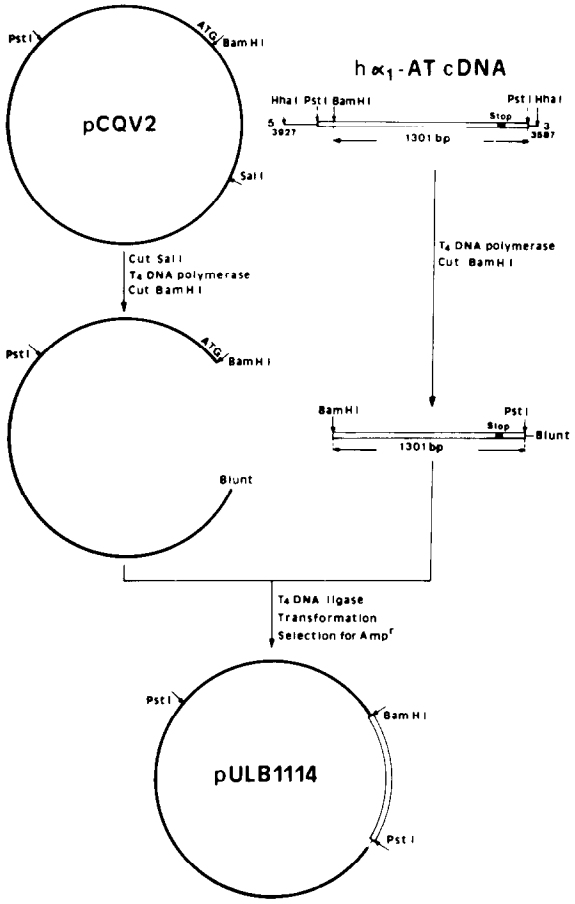

Fig.1. Construction scheme of the recombinant plasmid pULB1114. A 1322 bp DNA fragment coding for mature α_1-antitrypsin [7] was ligated to the *Bam*H1 site adjacent to an ATG codon in plasmid pCQV2 [1]. Hybrid molecules were used to transform competent *E. coli* cells and transformants were selected on ampicillin-containing medium.

coding for human α_1-antitrypsin [7]; we excised from this plasmid the *Hha*I fragment encompassing the α_1-antitrypsin sequence, made it blunt end with T4 DNA polymerase, then cut it with the enzyme *Bam*H1. This procedure generates a DNA fragment having a 5′ *Bam*H1 cohesive end, located at position 76 in the α_1-antitrypsin DNA sequence (second amino acid of the mature protein) and a 3′-blunt end downstream to the stop codon (fig.1b). The treated pCQV2 DNA was then ligated to the α_1-antitrypsin DNA fragment and the resulting hybrids were used to transform competent *E. coli* MM294 cells. Transformants were

selected at 30°C for ampicillin resistance and strains carrying the appropriate insert were identified by colony hybridization [9] using a nick-translated human α_1-antitrypsin cDNA probe [7]. Clones positive in this assay (not shown) were analyzed by restriction digestions to verify the presence of a typical 1301 bp fragment (*Bam*H1–*Pst*I) encompassing the sequence starting at nucleotide 76 (second amino acid of mature α_1-antitrypsin) and ending 120 bp downstream to the stop codon (fig.1,2). One clone satisfying to this condition, pULB1114, was chosen for expression analysis.

3.2. *Expression of human α_1-antitrypsin in clone pULB1114*

Cells carrying either the control plasmid pCQV2 or the pULB1114 plasmid were grown at 30°C then quickly heated at 42°C in order to inactivate the λ repressor and allow induction (see section 2). Pelleted bacteria were lysed with lysozyme and Triton X-100 and the resulting extracts were analyzed in an amplified immunosorbant assay

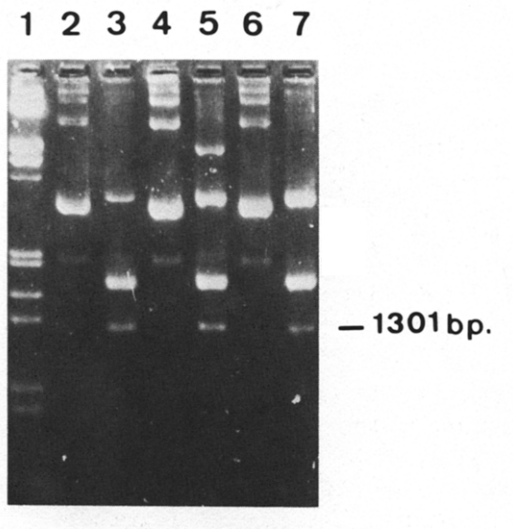

Fig.2. Restriction analysis of the plasmid pULB1114. Plasmid DNA was digested with the enzymes *Bam*H1 and *Pst*I and analyzed on 1% agarose gel. M_r standards are fragments of λ DNA cleaved with *Eco*RI and *Hin*dIII. The arrow points to the expected 1301 bp fragment derived from the digestion of pULB1114 with *Bam*H1 and *Pst*I. (1) M_r standards λ *Eco*RI *Hin*dIII; (2,4,6) pULB1114 DNA undigested; (3,5,7) pULB1114 DNA digested with *Bam*H1 and *Pst*I.

Table 1

Expression of human α_1-antitrypsin in bacterial extracts

Clone	Growth at (°C)	α_1-Antitrypsin (ng/ml extract)
pCQV2	30	<3
	42	<3
pULB1114	30	<3
	42	3400 (8854 molecules/cell)

Procedures to prepare cell extracts and to dose α_1-antitrypsin are described in section 2

described in [7]. This assay which allows the detection of low levels of α_1-antitrypsin (down to 1 ng/ml) was calibrated using a standard human α_1-antitrypsin preparation then performed on cell extracts derived from cultures at 30°C and 42°C. As seen in table 1, expression of human α_1-antitrypsin could be detected only in the clone pULB1114 when thermoinduction conditions were used (8854 molecules of α_1-antitrypsin/cell). The expressed product was characterized by SDS–PAGE, western blotting and immunodetection on the nitrocellulose blots. Fig.3 shows that a

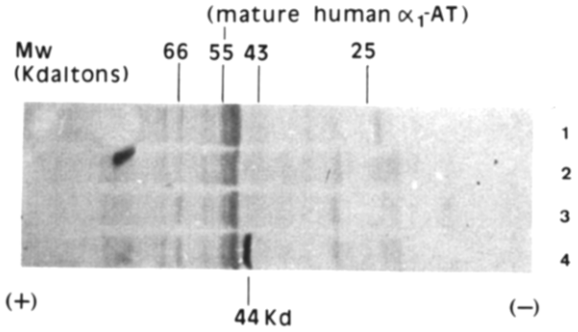

Fig.3. Western blot of bacterially synthesized α_1-antitrypsin. Protein extracts were prepared for electrophoresis on 15% polyacrylamide gels as described in section 2. Nitrocellulose blots were reacted with mouse antihuman α_1-antitrypsin serum followed by peroxidase-labelled goat antimouse serum and the chromogenic substrate (section 2). Source of protein samples: (1) pCQV2, control plasmid at 30°C; (2) pCQV2, at 42°C; (3) pULB1114 at 30°C; (4) pULB1114 at 42°C. M_r standards (bovine serum albumin, ovalbumin, chymotrypsinogen, lysozyme) and native human α_1-antitrypsin were run on the same gel, electroblotted and stained with Coomassie blue.

single product is expressed at 42°C in clone pULB1114. This polypeptide has a molecular mass of 44000, consistent with the known molecular mass of mature unglycosylated human antitrypsin [12].

4. DISCUSSION

The cDNA sequence coding for mature human α_1-antitrypsin has been fused to the ATG codon carried by the plasmid pCQV2 [1]. In thermoinduction conditions, *E. coli* cells carrying the recombinant plasmid express a polypeptide specifically recognized by anti human α_1-antitrypsin serum. The molecular mass of the bacterially-produced α_1-antitrypsin is consistent with the known M_r (44256) of human mature unglycosylated α_1-antitrypsin [12]. The level of expression, nevertheless, remains relatively low when compared to levels obtained using vector pCQV2 to produce other proteins [1]. This could result from internal foldings of the mRNA, to fortuitous termination sites in the DNA or to mRNA and/or protein instability. Solving these problems is obviously a prerequisite to obtain higher expression levels.

Attempts to demonstrate biological activity of the bacterially-synthesized α_1-antitrypsin have been unsuccessful so far. This failure could be due either to inactivation of the molecule in the extracts or to a lack of sensitivity of the assay we used (inhibition of trypsin activity). To solve this problem, it might be necessary to use protease-deficient bacteria and/or to purify at least partially the α_1-antitrypsin produced in *E. coli*.

ACKNOWLEDGEMENTS

We thank Dr C. Queen for providing us with the vector pCQV2. This work has been carried out under a contract between the Région Wallonne (CGCT) and the University of Brussels.

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