Volume 234, number 1, 61–64 FEB 06018 July 1988

Over-expression of natural and variant human H-chain ferritins in *E. coli*

Deborah Prozzi, Robert R. Crichton and John Davison*

Unit of Biochemistry, Université Catholique de Louvain, Louvain-la-Neuve and **Unit of Molecular Biology, ICP, 75 Avenue Hippocrate, 1200 Brussels, Belgium*

Received 18 April 1988

The natural human H-chain ferritin was expressed in *E. coli* using a multi-copy expression vector containing the λ p_L promoter. A variant H-ferritin, having an altered N-terminus, was also produced. These proteins are overproduced (>30% of the soluble protein), correctly assembled into its 24-subunit shell, and able to bind iron. The identity of the products was confirmed using an antibody specific for H-ferritin.

recombinant DNA; H-ferritin; Expression vector; Iron metabolism; Protein engineering; (Human, *E. coli*)

1. INTRODUCTION

Iron is of biological importance for a great many enzymatic reactions, but its availability is limited by the extremely low solubility of Fe(III). In almost all organisms (animals, plants and bacteria), the problem of the insolubility of Fe(III) is solved by ferritin. Ferritin is a large (450 kDa) iron-storage protein, which has the role of maintaining iron in a soluble, non-toxic and biologically available form. It consists of a mineral core of hydrated ferric oxide [Fe(III)O-OH] surrounded by a spherical protein coat of 24 subunits (apoferritin). Human ferritins consist of a combination of heavy (21 kDa) and light (19 kDa) chain apoferritin subunits in various proportions which can be tissue-specific (reviewed in [1]). Recently, the full-length cDNAs coding for the human ferritin H-chain and L-chain were cloned in *E. coli* and their nucleotide sequences determined [2–4].

This report describes a strategy for the over-expression of the human ferritin H gene in *E. coli*. Such studies enable the production of ferritin composed only of H-chain subunits and open the way for protein engineering on this molecule.

Correspondence address: J. Davison, Unit of Molecular Biology, International Institute for Cellular and Molecular Pathology, 75 Avenue Hippocrate, 1200 Brussels, Belgium

2. MATERIALS AND METHODS

Plasmid pFR3, containing the complete human ferritin H-chain cDNA was obtained from R. Cortese [2]. *E. coli* λ p_L expression plasmid pAS1 and its host M5219 (carrying a thermosensitive λ cI857 repressor and a functional λ N gene) were obtained from M. Rosenberg [5]. Plasmid pIC20H containing a polylinker with multiple cloning sites was obtained from J.L. Marsh [6]. Plasmid pJRD184, which contains 49 unique restriction sites, has been described [7]. Recombinant DNA methods have been described in [8]. Enzyme-linked immunoassays using monoclonal antibodies for heart ferritin (H705) were kindly performed by M. Worwood (Cardiff), using the method of Flowers et al. [9]. Synthetic oligonucleotides were purchased from Eurogentech (Liège).

3. RESULTS AND DISCUSSION

Expression of a foreign gene in *E. coli* can be achieved by fusion of the cDNA coding sequence in-phase with the ATG codon of vector pAS1. In this vector, the λ cII ATG codon is preceded by its own ribosome-binding site and is immediately followed by a *Bam*HI site to facilitate gene cloning. The *E. coli* host M5219 carries a cryptic λ prophage which provides the thermosensitive

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

repressor at 32°C and the λ N gene product at 42°C. This latter prevents termination of transcripts initiated at the p_L promoter of pAS1 [5]. The in-phase fusion of the ATG initiator codon to the ferritin gene was accomplished as shown in fig.1. The first (ATG), second and third codons of the ferritin cDNA gene were removed by digestion with *Tth*111I (fig.1). The 5′-protruding nucleotide was made blunt-ended by repair with DNA polymerase I Klenow fragment. The DNA was cleaved with *Pst*I and the resulting 213 bp fragment subcloned between the *Eco*RV and *Pst*I sites in the polylinker of pIC20H. This step locates a *Bgl*II site just upstream of the *Eco*RV-*Tth*111I junction. Fusion of this *Blg*II site, via its compatible sticky end, to the *Bam*HI site of pAS1 yields an in-phase fusion with the λ cII initiator codon. The remainder of the ferritin gene was reconstituted by the addition of a 489 bp *Pst*I-*Sph*I fragment (*Sph*I being located on the cDNA downstream of the ferritin termination codon). In ferritin expression vector pDP219, the fusion maintains the 4th and all subsequent codons of natural ferritin:

MetAspLeuAspAlaSerThr... recombinant H-ferritin

MetThrThr AlaSerThr... natural H-ferritin

In a final step, the correct N-terminal sequence

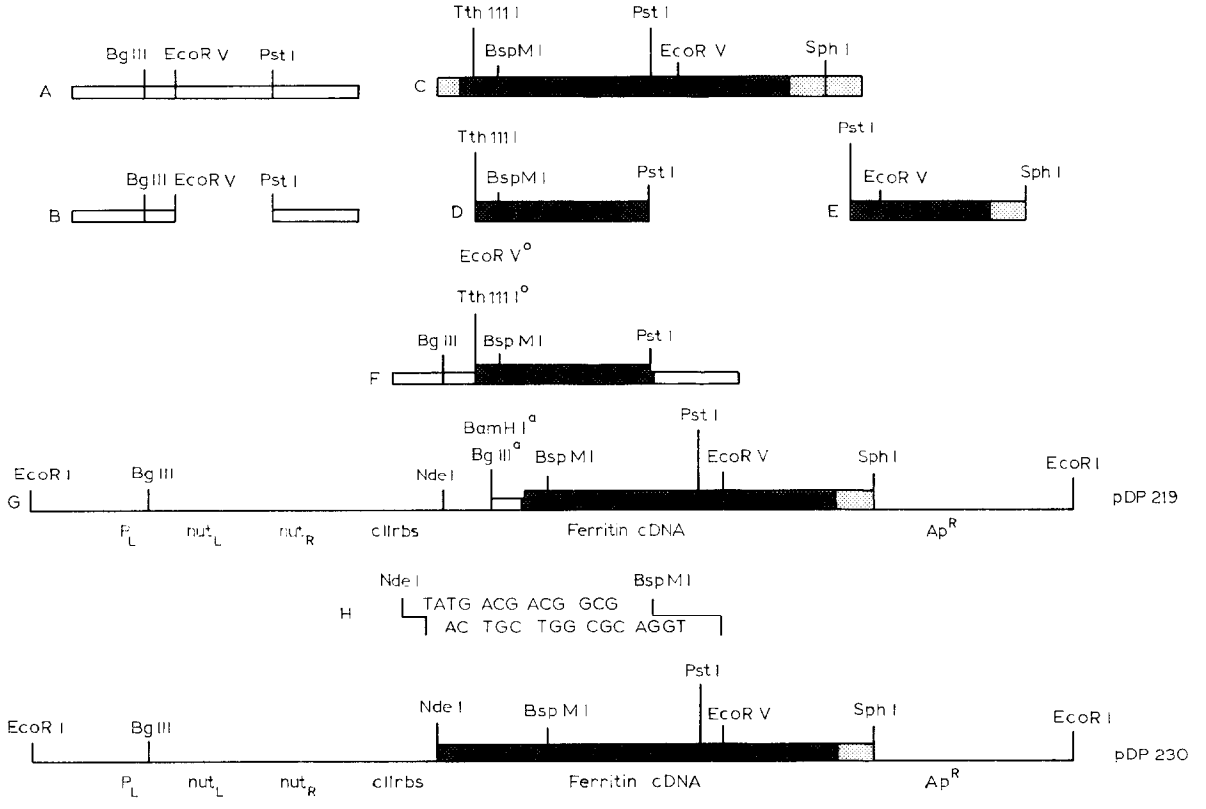

Fig.1. Construction of H-ferritin expression plasmid pDP219. The repaired *Tth*111I-*Pst*I fragment (D) of H-ferritin cDNA clone pFR3 (C) was cloned between the *Eco*RV-*Pst*I sites of pIC20H (A), thereby positioning a *Bgl*II site just upstream of the ferritin gene fragment (F). This *Bgl*II site was ligated to the *Bam*HI-*Sph*I cleaved pAS1 and the pDP219 recombinant plasmid (G) was completed by addition of the 3′-end of the ferritin cDNA on a *Pst*I-*Sph*I fragment (E). The plasmid pDP219 (G) carries the ferritin cDNA in-phase with the ATG codon of the λ cII gene of pAS1, but its N-terminus is different to that of native ferritin. A synthetic DNA fragment (H) was used to restore the native N-terminal sequence between the *Nde*I and *Bsp*MI sites of pDP219. The final plasmid pDP230 (I) carries the complete ferritin cDNA. Symbols: □, polylinker DNA of pIC19H; ■, coding region of the H-ferritin cDNA; □, non-coding region of H-ferritin cDNA; ——, pAS1 DNA (the plasmid is drawn as if linearized at its unique *Eco*RI site); p_L, lambda leftward promoter; nut_L and nut_R, sites of utilization of the λ N gene product [5]; cIIrbs, ribosome-binding site of the λ cII gene; Ap^R, resistance to ampicillin; °, denotes the loss of a previously existing restriction site.

was restored by the use of a synthetic DNA sequence, 17 bp long, defined at its 5′-end by an *Nde*I site and at its 3′-end by a *Bsp*MI site. This DNA fragment was used to substitute the sequence between the *Nde*I site (1 bp upstream of the ATG codon) and the *Bsp*MI site (12 bp downstream of the ATG codon) of pDP219. To do this, the oligomer was first joined to a 243 bp *Bsp*MI-*Eco*RV ferritin fragment in the restriction site bank vector pJRD184 cleaved with *Nde*I and *Eco*RV. The resulting plasmid was then cleaved with *Nde*I and *Eco*RV, and the 260 bp fragment subcloned between the *Nde*I and *Eco*RV sites pDP219 in a triple ligation using an intermediate *Bgl*II site; this step is necessary because of the multiple *Nde*I sites in pAS1. This ferritin expression vector was named pDP230.

The ability of the ferritin clones to express H-chain ferritin was investigated by temperature induction (4 h) of the cryptic λ cI857 lysogen M5219, followed by SDS-polyacrylamide gel electrophoresis. The results (fig.2) show that pDP219 and pDP230 code for a polypeptide of 21 kDa, corresponding to the known size of H-chain apoferritin. This protein comprised >20% of the total protein, and >30% of soluble protein, as determined by densitometry of the Coomassie-blue-stained gel. Non-denaturing gels (fig.3A) indicate that the H-chain subunits are correctly assembled into complete ferritin (± 450 kDa); however, the pDP219-ferritin migrates somewhat faster than pDP230-ferritin, probably because of the increased positive charge due to the 2 aspartic acid residues at the N-terminus.

The ability to bind iron was verified by staining the gel with Prussian blue (fig.3B). The recombi-

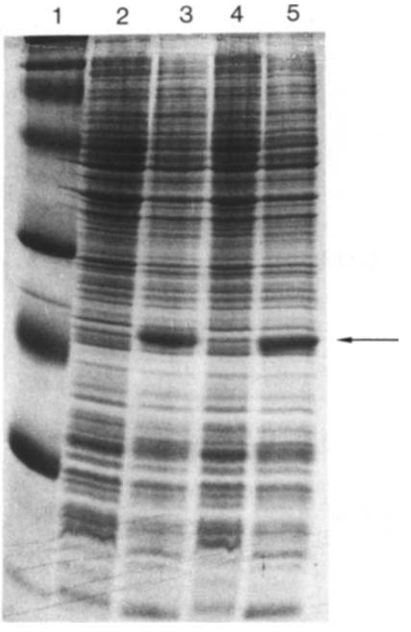

Fig.2. Electrophoresis in an SDS-17.5% polyacrylamide gel of polypeptides made from *E. coli* M5219 containing pDP219 or pDP230. Cultures of M5219 containing pDP219 (lanes 2,3) or pDP230 (lanes 4,5) were grown at 32 and 42°C, respectively. Only whole cell extracts are shown. Lane 1 contains molecular mass markers (Amersham Rainbow proteins: 14.3, 21.5, 30.0, 46.0, 69.0, 92.5 and 200 kDa from bottom to top of the gel). The apoferritin band is indicated by an arrow.

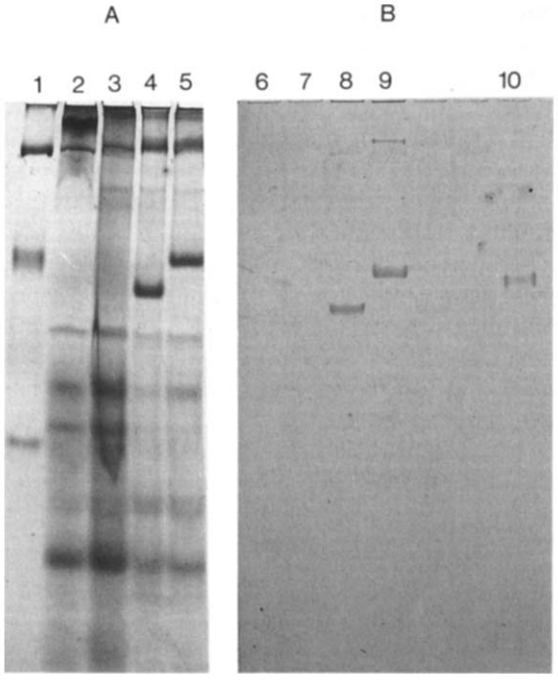

Fig.3. Electrophoresis in a 6% polyacrylamide gel of soluble proteins (extracted by sonication and centrifugation) of *E. coli* M5219 containing pDP219 or pDP230. (A) Samples extracted from cultures of M5219 containing pDP219 or pDP230, grown at 32°C (lanes 2,3, respectively) and at 42°C (lanes 4,5, respectively) and stained with Coomassie blue. (B) Prussian blue staining of the 42°C cultures of M5219 containing pDP219 or pDP230 (lanes 6,7, respectively). Lanes 8,9 contain the same samples, pretreated with ferrous ammonium sulfate. Lanes 1,10 contain molecular mass markers (Pharmacia high molecular mass calibration kit: 232, 440 [horse spleen ferritin] and 669 kDa, from bottom to top of the gel). The band staining with Prussian blue in lane 10 is the horse spleen ferritin.

nant ferritins contain little iron, as judged by the weak staining. However, when soluble cell extracts had been preincubated with 1 mM ferrous ammonium sulfate, a dense Prussian blue stained band appeared, indicating that the recombinant ferritins are able to take up iron from the medium. The identity and the quantity of the over-expressed protein as H-ferritin were also confirmed by using a monoclonal antibody specific for H-ferritin. These assays indicate that ferritin comprises about 35% of the soluble protein of *E. coli*. Recently, similar results on the synthesis of H-ferritin in *E. coli*, using a different construction method, have been reported [10].

These results open the way for the production of large quantities of this rare human protein for use in structural studies involving X-ray crystallography and protein engineering.

Acknowledgements: We gratefully acknowledge the cooperation of Drs R. Cortese, J.L. Marsh and M. Rosenberg in providing plasmids used in this study, and Dr M. Worwood for the immunological identification of H-ferritin. D.P. is grateful to Dr M. Heusterspreute for useful discussions. This work benefited from a 'Fonds du Développement Scientifique' grant from the University of Louvain.

REFERENCES

[1] Crichton, R.R. and Charloteaux-Wauters, M. (1987) Eur. J. Biochem. 164, 485–506.
[2] Costanzo, F., Santoro, C., Colantuoni, V., Bensi, G., Raugei, G., Romano, V. and Cortese, R. (1984) EMBO J. 3, 23–27.
[3] Dörner, M.H., Salfeld, J., Will, H., Leibold, E.A., Vass, J.K. and Munro, H.N. (1985) Proc. Natl. Acad. Sci. USA 82, 3139–3143.
[4] Boyd, D., Jain, S.K., Crampton, J., Barrett, K.J. and Drysdale, J. (1984) Proc. Natl. Acad. Sci. USA 81, 4751–4755.
[5] Rosenberg, M., Ho, Y.-S. and Shatzman, A. (1983) Methods Enzymol. 101, 123–138.
[6] Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) Gene 32, 481–485.
[7] Heusterspreute, M., Ha-Thi, V., Emery, S., Tournis-Gamble, S. and Davison, J. (1985) Gene 39, 299–304.
[8] Davison, J., Heusterspreute, M. and Brunel, F. (1988) Methods Enzymol. 153, in press.
[9] Flowers, C.A., Kuizon, M., Beard, J.L., Skikne, B.S., Covell, A.M. and Cook, J.D. (1987) Am. J. Hematol. 23, 141–151.
[10] Levi, S., Cesareni, G., Arosio, P., Lorenzetti, R., Soria, M., Sollazzo, M., Albertini, A. and Cortese, R. (1987) Gene 51, 269–274.