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Expression of recombinant human ceruloplasmin – an absolute requirement for splicing signals in the expression cassette

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Abstract We report the successful expression of recombinant human ceruloplasmin which was made possible by inclusion of splicing signals in the expression vector. Ceruloplasmin cDNA expressed from the vector pNUT in baby hamster kidney cells gave protein yields of 0.03 mg/l which increased to 15 mg/l with splicing signals present. The defect in expression from the intronless cDNA is due to complete retention of ceruloplasmin mRNA in cell nuclei. The block to cytoplasmic export is alleviated by splicing signals, allowing full expression of the mRNA.

Key words: Human ceruloplasmin; Intron; Expression cassette; mRNA export; In situ hybridization

1. Introduction

Ceruloplasmin (Cp) is the major copper binding protein of the serum and it has been suggested that one of its functions is to act as a ferroxidase, oxidising Fe^{2+} to Fe^{3+} for the efficient uptake of iron by transferrin [1]. Further support for the role of Cp in iron homeostasis is indicated in patients with hereditary Cp deficiency who have high iron deposition in liver and brain [2,3]. Moreover the *FET3* gene of yeast is required for high affinity transport of iron into yeast cells and has ferroxidase activity, with extensive sequence homology to multi-copper oxidases, located on the cell surface [4,5]. The primary site of Cp synthesis is the liver where it is synthesised as an apoprotein. Copper is then incorporated into the newly synthesised Cp, prior to its secretion from the hepatocytes into surrounding tissues [6]. This process is deficient in patients with Wilson's disease, who characteristically have an impaired ability to incorporate copper into their newly synthesised Cp, accompanied by a failure to excrete copper from the liver into the bile; this results in the accumulation of toxic amounts of copper in the liver, kidney and brain [7]. Although the liver is the major site for Cp synthesis, Cp is expressed in other tissues, including the mammary gland [8], synovial tissues [9], and lung [10,11].

Studies on the biochemical properties and biological functions of Cp have been hampered by its susceptibility to proteolytic degradation during isolation. Uncertainty as to the number and location of the copper atoms has recently been clarified by the solved X-ray structure of human serum Cp [12]. The structure reveals that Cp comprises six domains with three mononuclear sites, in domains 2, 4 and 6, and one trinuclear cluster at the interface of domains 1 and 6. With this new structural information on Cp, investigations into its role in both copper and iron metabolism can now be carried out using site-directed mutagenesis. However, successful expression of recombinant Cp is a prerequisite for such studies.

Although human Cp was cloned in 1986 [13] there have been no reports of production of recombinant Cp. However, factor VIII, which shares structural homology with Cp [14], has been expressed in baby hamster kidney (BHK), as well as Chinese hamster ovary (CHO) cells [15]. We have therefore explored the possibility of using the BHK expression system for the production of human Cp as BHK cells have already been used successfully by us for the expression of recombinant wild-type and mutants of the amino-terminal lobe of human serum transferrin [16]. The BHK expression system for transferrins utilises the vector pNUT, in which the cDNAs are under the control of an inducible metallothionein promoter and polyadenylation signals are derived from the last exon of the gene for human growth hormone [17]. We now report the successful expression of recombinant Cp by modification of the pNUT vector to incorporate splicing signals from the second intron of rabbit β-globin. This modification promotes cytoplasmic expression of Cp mRNA and stimulates protein production.

2. Materials and methods

2.1. Plasmid construction

All plasmids were constructed by standard recombinant DNA techniques [18]. The cDNA of human Cp (kindly provided by Professor J.D. Gitlin, Washington University School of Medicine, St. Louis, MO) was subcloned into pNUT to form pNUT-Cp. A 0.6 kb *Nhe*I-*Hin*dIII fragment containing the second intron of the rabbit β-globin gene was inserted upstream of the Cp cDNA to give pNUT-I-Cp. pcDNAΔ and pcDNA-βGI are modified versions of pcDNA1 (Invitrogen), in both of which the SV40 small-t intron has been excised out by digestion with *Dra*I; pcDNA-βGI has had the βGI inserted via the *Hin*dIII site on the vector. A 3.3 kb *Hin*dIII fragment containing the entire Cp cDNA was subcloned into the pcDNA vectors, downstream of the splicing signals in the case of pcDNA-βGI-Cp.

2.2. Cell culture and transfection

BHK and COS cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum and antibiotics. Transient transfections were carried out using DEAE-dextran [19] for COS cells and using the LIPOFECTIN reagent (Gibco BRL) for BHK cells. Cells transfected with the pNUT constructs were induced 24 h after transfection with 80 μM zinc sulphate [16].

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Abbreviations: BHK, baby hamster kidney; Cp, ceruloplasmin; ISH, in situ hybridisation; DIG, digoxigenin; βGI, β-globin intron

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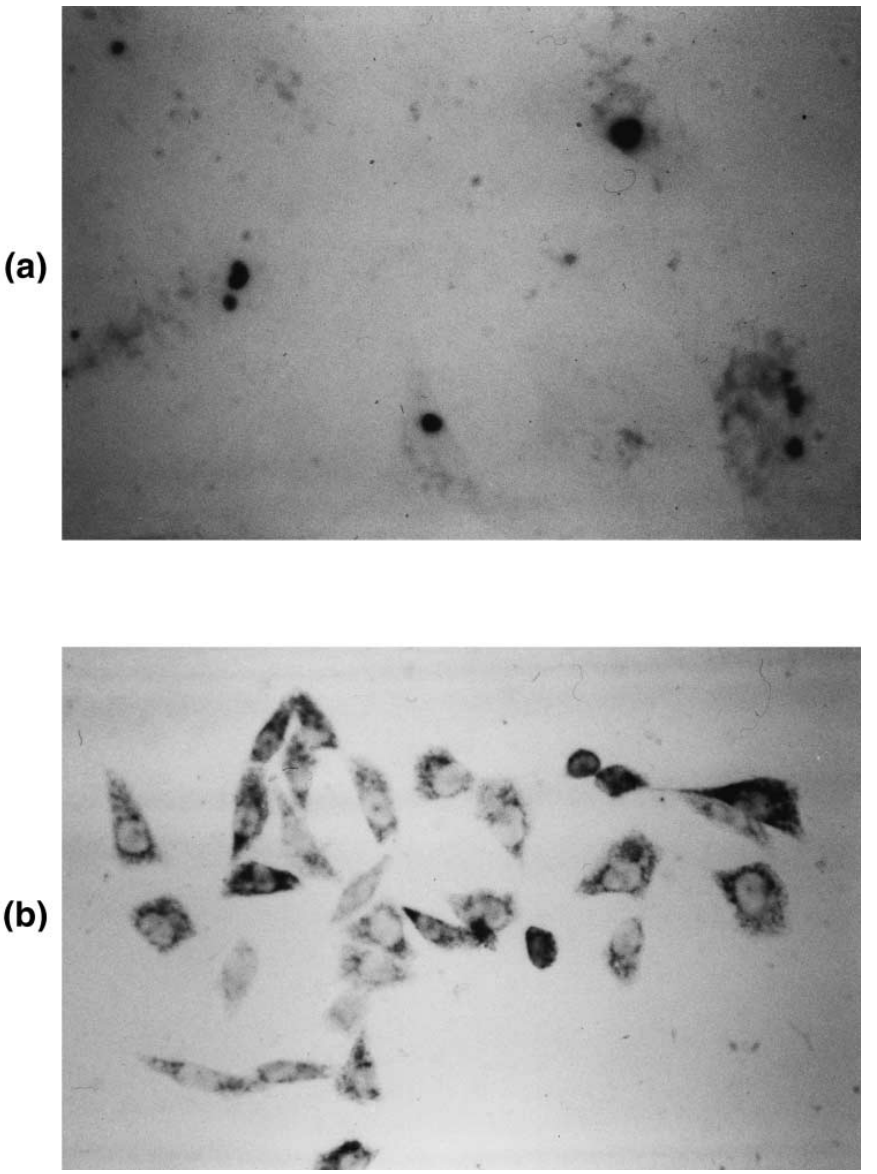

Fig. 1. Cellular localisation of Cp mRNA detected by ISA. Cp mRNA was expressed in BHK cells from (a) intronless pNUT-Cp, or (b) from vector with added splicing signals, pNUT-I-Cp.

2.3. In situ hybridisation

The intracellular distribution of Cp mRNA was monitored by ISH using riboprobes as described [20]. Cells grown on sterile coverslips were fixed 72 h after transfection in 2% paraformaldehyde (BDH Chemicals) for 15 min at room temperature. Cells were then dehydrated through a graded series (50, 70 and 95%) of ethanol solutions before hybridisation overnight with DIG-labelled antisense probes. Following treatment with RNase A and stringent washing to remove non-specifically bound probes, bound riboprobes were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer-Mannheim) and alkaline phosphatase activity was stained according to the manufacturer's instructions.

2.4. RNA isolation and slot blot analysis

Total RNA was isolated from cells 72 h after transfection by the acid guanidinium thiocyanate-phenol-chloroform method [21]. For hybridisation analysis of the RNA, 500 ng of total RNA was immobilised on an N^+ Hybond nylon membrane (Amersham), using a slot blotting manifold. Each slot was further washed with 0.5 ml of 10×SSC. The membranes were baked at 80°C for 2 h and pre-hybridised at 65°C for 1 h before probing overnight with a full-length, DIG-labelled antisense Cp riboprobe. Filters were washed stringently and bound probes were detected as above. After colour development, relative levels of Cp-specific RNAs were determined by scanning densitometry, using an Oast Tech Gel Analysis System. These values were corrected for differences in RNA transfer efficiency by probing blots with riboprobes specific for β-actin.

2.5. Sandwich ELISA

A primary anti-human Cp antibody (Binding Site) was bound onto a multiwell plate, washed, then blocked overnight at 4°C. Cp from the expressed media was detected using a second anti-human Cp antibody labelled with horseradish peroxidase (HRP) (Binding Site) [22]. Colour was developed using *o*-phenylenediamine reagent (Sigma) and the absorbance read at 450 nm. The same principle for the quantitative determination of recombinant transferrin was used, a primary anti-human transferrin antibody (Binding Site) was used and detection using a second anti-human transferrin antibody labelled with horseradish peroxidase (HRP) (Binding Site).

2.6. Purification of Cp

Recombinant Cp was partially purified from 200 ml of expressed medium from the BHK cells using a Sepharose-ethyleneimine column [23]. Purified human Cp was isolated from out-dated human plasma using affinity chromatography, on a Sepharose-ethyleneimine column,

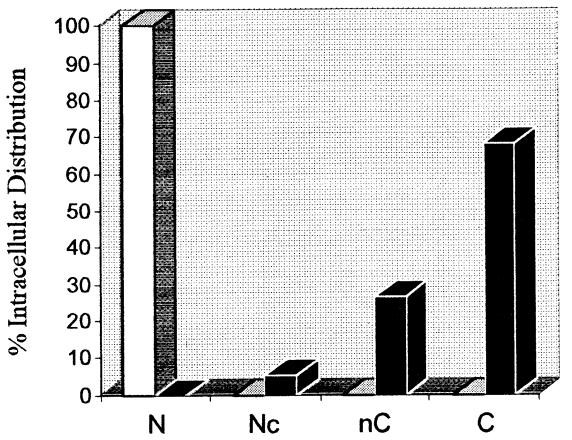

Fig. 2. Requirement of splicing signals for efficient cytoplasm expression of Cp mRNA. The intracellular distributions of Cp mRNA expressed from pNUT (□) or pNUT-I (■) in BHK cells were analysed semi-quantitatively. The localisation of Cp mRNA was categorised to reflect a movement from nucleus to cytoplasm. N, staining almost exclusively in the nucleus; Nc, more nuclear than cytoplasmic staining; nC, more cytoplasmic than nuclear staining; and C, staining almost exclusively in the cytoplasm. A minimum of 100 cells have been scored for each transfection, for at least three transfections per plasmid, by three persons. The data has been normalised and represented as percentage distributions between the four categories.

and ion-exchange chromatography on a Mono Q column (Pharmacia).

2.7. Western blotting

Detection of Cp was carried out by resolving samples on 10% SDS-PAGE, transferring proteins electrophoretically to nitrocellulose membrane, blocking with 3% BSA in PBS containing 0.01% Tween 20 and incubated with anti-human Cp antibody labelled with HRP. The blot was washed three times with PBS containing 0.05% Tween 20 and the resulting immune complexes detected with the diaminobenzidine reagent (Sigma).

3. Results

3.1. Assessment of intracellular distribution of Cp mRNA by ISH

Using ISH to detect the presence and localisation of Cp mRNA, it was found that Cp mRNA expressed from pNUT-Cp was completely retained in the nuclei of BHK cells (Fig. 1a). In contrast, inclusion of splicing signals in the expression vector (pNUT-I-Cp), allowed efficient export of Cp mRNA into the cytoplasm (Fig. 1b). The patterns of Cp mRNA distribution (Fig. 2) were evaluated semi-quantitatively [24] and the results substantiate the above finding that export of mRNA was enhanced when expressed from pNUT-I-Cp.

When the experiments were repeated with pcDNA-βGI-Cp and pcDNAΔ-Cp in COS cells, we found that once again most of the Cp mRNA expressed from the intronless vector pcDNAΔ was retained inside the nucleus, while 99% of the signal from pcDNA-βGI-Cp was mainly or exclusively in the cytoplasm (data not shown).

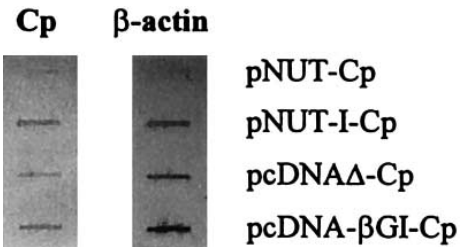

Fig. 3. The level of Cp mRNA expressed from each construct. Total RNA from COS cells transfected with the indicated plasmid was measured by slot blot analysis. Both Cp (left) and β-actin (right) mRNAs were assessed as described in Section 2.

3.2. Quantification of Cp mRNA by slot blot analysis

Total Cp mRNA levels were measured by slot blot analysis (Fig. 3). It was found that the total amount of Cp mRNA produced from each pair of vectors was fairly similar, irrespective of the presence of splicing signals (Table 1).

3.3. Quantification and characterisation of Cp by an ELISA and Western blotting

The amounts of Cp protein expressed from BHK and COS cells was quantified by an ELISA and are shown in Table 1. BHK cells transfected with pNUT-I-Cp produced 15 mg/l of protein, a 500-fold increase compared with cells transfected with pNUT-Cp. COS cells produced similar amounts of protein from pNUT-I-Cp, 16–31 mg/l, but levels expressed from pNUT-Cp were below the limits of detection of the ELISA.

Western blotting showed that the partially purified recombinant Cp expressed in BHK cells had a major band at 132 kDa corresponding to intact human Cp (Lane 2, Fig. 4). This compared with the pattern obtained in a crude human serum

Table 1
The distribution and expression levels of Cp mRNA and protein in BHK/COS cells transfected with different constructs

Construct	Cell line	mRNA localisation[a]	Relative levels of Cp mRNA[b]	Protein levels (mg/l)[c]
pNUT-Cp	BHK	N	–	0.03
	COS	N	1.0	N.D.[d]
pNUT-I-Cp	BHK	C	–	15
	COS	C	1.0	16–31
pcDNAΔ-Cp	COS	Nc	1.1	N.D.[d]
pcDNA-βGI-Cp	COS	C	1.4	100
pNUT-Tf	BHK	C	–	7

[a]Intracellular localisation of Cp mRNA categorised as described in the legend to Fig. 2.
[b]The relative efficiency of Cp mRNA production by different constructs was determined by taking the value obtained from pNUT-Cp as a reference point and the values from other constructs are expressed as a ratio of the intensities to that of pNUT-Cp; values are normalised to the signal from β-actin.
[c]Protein levels were assayed by an ELISA, which has been performed in duplicates, repeated at least twice.
[d]Not detectable.

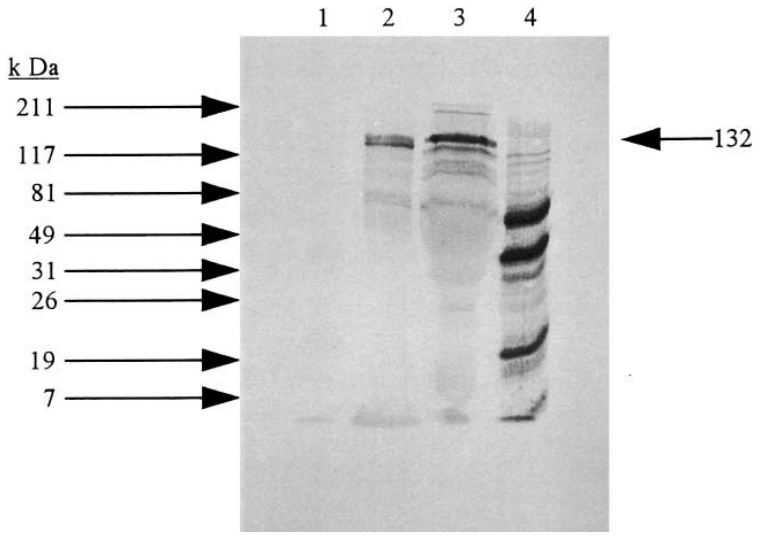

Fig. 4. Western blot analysis of Cp. Lane 1, MW markers; Lane 2, partially purified recombinant Cp; Lane 3, normal serum sample; Lane 4, purified serum Cp. The arrow shows the location of the 132 kDa band of ceruloplasmin.

sample (Lane 3, Fig. 4). Human Cp purified from out-dated human plasma contained very low levels of full-length protein (Lane 4, Fig. 4).

4. Discussion

Although we have obtained only very low levels of Cp production in cells transfected with pNUT-Cp we have demonstrated, using ISH, that this is due to retention of Cp mRNA in the nucleus. Export of Cp mRNA into the cytoplasm, which is accompanied by enhanced levels of protein expression, can be achieved by inclusion of splicing signals in the expression vector. However, as the levels of mRNA produced from vectors with and without splicing signals are similar, the enhanced expression of Cp protein cannot be attributed to any significant extent by the effects of the intron on transcription [25,26]. Nor does it seem likely that the lack of cytoplasmic mRNA expressed from the intronless constructs is due to a very rapid turnover of cytoplasmic RNA – the mRNAs expressed from the vectors with the βGI are almost identical in sequence to those expressed from the intronless counterparts, except for the inclusion of 53 bp of spliced β-globin mRNA upstream of the Cp reading frame. β-Globin has been used in studies of mRNA turnover and is relatively stable, but can be induced to turn over rapidly if part of a chimeric transcript containing destabilising signals from another gene [27,28], indicating that β-globin mRNA does not possess the ability to stabilise otherwise rapidly turned over mRNA. Instead, the effect of splicing signals in the vectors seems to be at the level of nucleocytoplasmic export of mRNA into the cytoplasm. The same patterns of mRNA distributions from experiments performed in two different systems, pNUT/BHK and pcDNA/COS (Table 1), demonstrate that the nuclear retention of unspliceable mRNA, and the enhancement of nucleocytoplasmic export by splicing signals, are not an artefact of the plasmid or the cell line used. In the case of Cp there seems to be an absolute requirement of splicing signals for cytoplasmic expression of mRNA. Other studies have documented enhanced efficiency of expression of cytoplasmic mRNA by the inclusion of splicing signals [29–32] and the requirement seems to be general for most cDNAs derived from genes containing introns. What is striking in the case of Cp is that so little protein is expressed without the splicing signals which contrasts strongly with human transferrin where significant amounts of protein are expressed from the intronless form of pNUT (Table 1). Interestingly, ISH reveals that transferrin mRNA can be exported to a significant degree to the cytoplasm of BHK cells without the need for splicing. In addition, amongst a survey of mRNAs expressed from cDNAs of genes which normally contain introns varying degrees of cytoplasmic expression were observed (Fu, Suen, Waseem and White, unpublished results), but uniquely Cp mRNA was exclusively retained in the nucleus. The complete lack of nucleocytoplasmic export of Cp mRNA in the absence of splicing signals is suggestive of a specific retention of the RNA in the nucleus. Such a mechanism has been demonstrated for the aberrant nuclear localisation of a tRNA harbouring a single point mutation [33]. Further work is required to characterise the putative retention sequence of Cp mRNA and determine whether or not such retention plays a role in the regulation of expression of the Cp gene.

It is well known that human Cp is susceptible to proteolytic attack during its isolation from serum and this has been the major obstacle in the study of the physicochemical characteristics of the protein. However, the recombinant Cp expressed in BHK cells appears to be intact, as very little degradation was observed upon analysis by SDS-PAGE. Work is now in progress to assess the physicochemical properties of the recombinant protein ahead of the use of site-directed mutagenesis to explore structure-function relationships in Cp.

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