Transient overexpression of human H- and L-ferritin chains in COS cells

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The understanding of the *in vitro* mechanisms of ferritin iron incorporation has greatly increased in recent years with the studies of recombinant and mutant ferritins. However, little is known about how this protein functions *in vivo*, mainly because of the lack of cellular models in which ferritin expression can be modulated independently from iron. To this aim, primate fibroblastoid COS-7 cells were transiently transfected with cDNAs for human ferritin H- and L-chains under simian virus 40 promoter and analysed within 66 h. Ferritin accumulation reached levels 300–500-fold higher than background, with about 40 % of the cells being transfected. Thus ferritin concentration in individual cells was increased up to 1000-fold over controls with no evident signs of toxicity. The exogenous ferritin subunits were correctly assembled into homopolymers, but did not affect either the size or the subunit composition of the endogenous heteropolymeric fraction of ferritin, which remained essentially unchanged in the transfected and non-transfected cells. After 18 h of incubation with [^{59}Fe]ferric–nitrilotriacetate, cellular iron incorporation was similar in the transfected and non-transfected cells and most of the protein-bound radioactivity was associated with ferritin heteropolymers, while H- and L-homopolymers remained iron-free. Cell co-transfection with cDNAs for H- and L-chains produced ferritin heteropolymers that also did not increase cellular iron incorporation. It is concluded that transient transfection of COS cells induces a high level of expression of ferritin subunits that do not co-assemble with the endogenous ferritins and have no evident activity in iron incorporation/metabolism.

INTRODUCTION

Despite the large progress in understanding the regulation of cellular iron uptake and storage, very little is known about the pathway followed by iron once it crosses the endosomal membrane. Iron entering the cytosol finds a number of potential ligands, including ferritins, iron-regulatory proteins and other iron-binding molecules, which differ in binding affinity and capacity. The loosely bound iron pool is potentially toxic, and its size is thought to be tightly regulated by the concerted action of the iron-regulatory proteins, which act as sensors, and ferritins, which sequester the iron [1,2]. However, the actual physiological role of ferritin in the regulation of cellular iron metabolism is poorly understood and mainly deduced from *in vitro* studies. Ferritin is made up of 24 equivalent subunits which assemble in a protein shell with 4,3,2 point symmetry delimiting a large cavity in which iron is deposited in a mineral form [3,4]. Vertebrate ferritins are heteropolymers composed of variable proportions of H- and L-subunits, which have about 50 % amino acid sequence identity [3]. All ferritins react aerobically with Fe^{2+} ions and induce their mineralization inside the cavity. The H-chains have ferroxidase activity which promotes iron oxidation and incorporation [4,5]; L-chains promote iron nucleation inside the cavity more efficiently than H-chains [6]. Metal-binding sites located in the hydrophilic channels also take part in the mechanism of iron incorporation [7]. Iron incorporation appears to occur by a fast mechanism, mediated by the ferroxidase centre, and a slower one, by which oxidation occurs on the surface of the iron core [8,9]. Ferritin capacity to bind iron is high and in most cells its concentration exceeds the need for iron storage, as indicated by the observation that natural ferritins contain from a few up to 1000–2000 Fe atoms per molecule, which is far below the saturation limit of about 4000. However, ferritin affinity for iron varies greatly with iron speciation and chemical form, ferritin subunit composition, and possibly ferritin iron load [10,11], variables that are mostly unknown in the cells.

The following evidence from *in vivo* studies has indicated that the ferritin H-chain plays an active role in cellular iron metabolism: (i) its up-regulation induced by tumour necrosis factor α in myoblast cells caused a transient decrease in L-chain synthesis, probably the result of reduction of the regulatory iron pool [12]; (ii) mouse erythroleukaemic cells stably transfected with H-chain cDNA under the control of H-ferritin promoter have an iron-deficient phenotype and reduced globin synthesis [13]. Evidence that ferritin L-chains do not have a major role in iron metabolism comes from the observations of subjects with genetic hyperferritinaemia associated with cataract. Owing to defects of the iron-responsive element on the 5′-untranslated flanking region of L-chain mRNA, they exhibit constitutive up-regulation of ferritin L-chains, with levels in serum and tissue about 10-fold above normal, but no detectable abnormalities in iron metabolism [14,15].

The development of cell model systems in which ferritins can be up-regulated independently of iron levels is important for the study in more detail of the biological roles of ferritin. To this end we analysed monkey fibroblastoid COS cells which can be induced to express large amounts of exogenous proteins and may allow the study of the functionality of ferritin H- and L-chain homopolymers as well as heteropolymers. The cells were transiently transfected to overexpress human H- and L-ferritin chains alone or together and were analysed for ferritin content and structure and for the capacity to incorporate radioactive iron.

MATERIALS AND METHODS

Plasmid construction

The cDNAs for the entire coding sequences of human H- and L-ferritin chains generated by PCR were cloned into the plasmid

Abbreviations used: FeNTA, ferric–nitrilotriacetate complex; DFO, desferroxamine.

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pSG5 (Stratagene) under the control of simian virus 40 promoter. The pSG5 constructs were further modified by inserting the L-chain 5′-untranslated region upstream of the corresponding coding region and the H-chain 3′-untranslated region downstream of the corresponding coding region.

Cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco). All media were supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM glutamine and 10 % fetal calf serum (Gibco). Transfection experiments were performed as described by Jordan et al. [16]. The plasmid DNAs (10 μg) in 500 μl of 0.25 M $CaCl_2$ were incubated for 30 min with 500 μl of 2 × HBS buffer (1.4 mM Na_2HPO_4, 12 mM D-glucose, 275 mM NaCl, 10 mM KCl, 40 mM Hepes, pH 7.05) and then with COS-7 cells (1×10^6) seeded the day before. After 24 h the medium was replaced with a fresh one and the cells were incubated for further 48 h at 37 °C. They were then washed twice with PBS, mixed with 1.5 ml of TEN buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 7.5, 150 mM NaCl) and harvested after 5 min on ice. The cells were recovered by centrifugation, resuspended in 100–200 μl of 0.25 M Tris/HCl, pH 7.4, and the cytosolic cell extracts prepared by repeated freezing and thawing.

Quantification of ferritin and protein in cell extracts

Ferritin content in cell extracts was measured by ELISA based on monoclonal antibodies specific for H-ferritin (rH02) and L-ferritin (LF03) [17] and calibrated using the corresponding recombinant homopolymers expressed in *Escherichia coli*. Protein content was determined by the BCA method (Pierce) calibrated using BSA. Immunoblot experiments were performed as described by Santambrogio et al. [10].

Ferritin purification and characterization

Ferritin was purified from 7×10^6 transfected cells by following essentially the method of Levi et al. [5]. Briefly, the cells were sonicated in 500 μl of 20 mM Tris/HCl, pH 7.4, containing 0.2 mM PMSF for 3 min at 0 °C. After centrifugation, the supernatants were treated with Benzon-Nuclease (Merck) and heated at 75 °C for 10 min. The soluble fractions were incubated in 4 M urea for 1 h, dialysed against Tris-buffered saline and concentrated with Centricon-100 (Amicon). The purified protein (0.5 mg/ml; 0.1 μM) was incubated with 0.1 mM ferrous ammonium sulphate in 0.1 M Hepes, pH 7.0, for 2 h at room temperature. After incubation, the samples were electrophoresed on 7.5 % polyacrylamide non-denaturing gel and stained with Prussian Blue.

Metabolic labelling and immunoprecipitation

The experiments were performed 24–70 h after transfection. Cells (10^5) were grown for 1 h in methionine-free minimal essential medium (Gibco) and then labelled for 2 h with 25 μCi/ml [^{35}S]methionine. They were washed and then lysed on the plate with 500 μl of lysis buffer (20 mM Tris/HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA, 0.5 % Nonidet P40). Total radioactivity associated with the soluble proteins was determined by trichloroacetic acid precipitation. For immunoprecipitation studies, 2×10^6 c.p.m. of cytosolic lysates were incubated with 3 μl of a non-specific rabbit polyclonal antibody, then with 30 μl of Protein A–Sepharose (50 %) and centrifuged. The supernatants were subjected to immunoprecipitation with 10–15 μg of monoclonal antibodies for ferritin H-chain (rH02) or L-chain (LF03), incubated for 1 h at 4 °C and precipitated as above. In sequential experiments the supernatants, after immunoprecipitation with antibody for one ferritin type, were subjected to further immunoprecipitation with the antibody for the second ferritin type. The precipitated beads were resuspended in SDS buffer, boiled for 10 min and subjected to SDS/PAGE (15 % gels). The gels were treated with an autoradiography image enhancer (Amplify; Amersham), dried and exposed. The intensity of ferritin subunit bands was quantified by densitometry (Molecular Dynamics).

Cellular ^{59}Fe uptake

At 2 days after transfection, 10^5 cells were grown for a further 18 h in minimal essential medium containing 0.5 % fetal calf serum and 0.5 % BSA supplemented with 2 μCi/ml [^{59}Fe]ferric–nitrilotriacetate (^{59}FeNTA) and 150 μM ascorbic acid. The cells were washed and lysed as above, the homogenates centrifuged at 4500 ***g*** (13000 rev./min) for 5 min at 4 °C, and the radioactivity associated with the soluble and insoluble fraction measured on a γ-counter (Packard). The extracts were analysed by native PAGE (6 % gels) or subjected to immunoprecipitation experiments, as above. The pellets were resuspended in SDS loading buffer containing 4 M urea, subjected to SDS/PAGE (6 % polyacrylamide gel containing 4 M urea) and then autoradiography. Under these conditions, ferritin is not denatured and iron remains associated with the assembled molecule.

Immunofluorescence and immunogold staining

The transfected cells (4×10^4/slice) were fixed with 3 % paraformaldehyde, pH 7.5, and permeabilized with 0.5 % Triton X-100 in 3 mM sucrose/50 mM NaCl/0.3 mM $MgCl_2$ for 3 min. After a wash with PBS, the slices were incubated at 37 °C with 2 μg/ml primary anti-ferritin monoclonal antibody in PBS/1 % BSA for 40 min and then for 40 min with TRITC–anti-mouse IgG rabbit antibodies (Dako). The fluorescence stain was visualized by fluorescence microscopy (Zeiss). For immunogold staining, cell culture monolayers were fixed for 30 min at 4 °C with 4 % paraformaldehyde/0.25 % glutaraldehyde in 125 mM sodium phosphate buffer, washed with buffer, detached by scraping, suspended in the phosphate buffer and centrifuged at low speed. The pellets were infiltered with polyvinylpyrrolidone, frozen in a 3:1 (v/v) mixture of propane and cyclopentane cooled with liquid nitrogen, and transferred to Reichert F4 cryokit apparatus mounted on an ultracut ultramicrotome. Ultrathin cryosections (50–100 μm thick) were collected over nickel grids and covered with 2 % gelatin. After treatment with 125 mM sodium phosphate buffer, pH 7.4, supplemented with glycine, they were exposed for 2 h at 37 °C to the first antibody diluted in phosphate/glycine buffer, and then washed with the buffer and decorated with IgG-coated gold particles (5 μm), diluted 1:60 in the same buffer [18]. The immunodecorated grids were then processed as recommended by Keller et al. [19]. The cryosections were examined in an Hitachi H-7000 electron microscope.

RESULTS

Ferritin overexpression in COS cells

The amino acid sequence of monkey ferritin is at present not known; it is probably more similar to human ferritin than the other mammalian ferritins so far sequenced, which have about 90 and 85 % identity with the corresponding human H- and L-chains respectively [3]. The immunological cross-reactivity be-

Table 1 Ferritin accumulation in transfected COS cells

COS-7 cells were treated for 24 h with calcium phosphate in the absence (COS-7) or presence of 10 μg of H-chain plasmid DNA (H-Transfectant) or 10 μg of L-chain plasmid DNA (L-Transfectant). After 30 h rest, 100 μM FeNTA or 100 μM DFO was added and the cells were incubated for a further 18 h. Cells were harvested and the homogenates analysed for H- and L-ferritin content by ELISA. The results are expressed as ng of ferritin/mg of soluble protein or as fold change over the untransfected controls. Values are means + S.D. from three independent experiments.

Cells	Treatment	H-ferritin (ng/mg)	Fold change	L-Ferritin (ng/mg)	Fold change	H/L ratio
COS-7	—	263 ± 22	—	72 ± 3	—	3.7
	FeNTA	690 ± 128	2.6	300 ± 22	4.2	2.3
	DFO	< 20	< 0.08	< 20	< 0.27	—
H-Trans-fectant	—	54 037 ± 2865	205	31 ± 10	0.43	1.7×10^3
	FeNTA	56 517 ± 5119	214	46 ± 16	0.64	1.2×10^3
	DFO	54 000 ± 4011	205	< 20	< 0.27	$> 2.7 \times 10^{-3}$
L-Trans-fectant	—	274 ± 21	1.0	35 051 ± 2668	487	7.8×10^{-3}
	FeNTA	393 ± 4	1.5	30 025 ± 2687	417	13×10^{-3}
	DFO	< 20	< 0.08	33 040 ± 2585	459	$< 6 \times 10^{-4}$

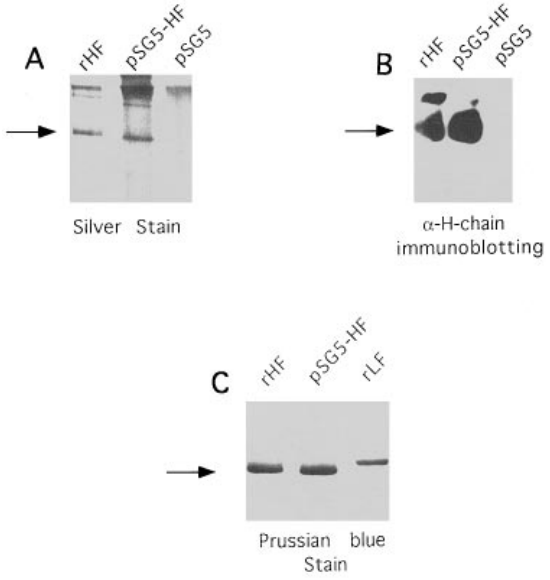

Figure 1 Non-denaturing PAGE (7.5% gel) of ferritins overexpressed in COS cells

Cells transfected with H-chain cDNA in pSG5 vector (pSG5-HF) or with pSG5 empty vector (pSG5) were collected, homogenized, run on the gels in parallel with purified recombinant H-chain hompolymer from *E. coli* (rHF) and stained with silver stain (**A**) or with anti-H-ferritin monoclonal antibody after blotting (**B**). (**C**) The ferritin from H-transfectants was partially purified by heating at 75 °C and incubated with 1000 Fe atoms per molecule (ferrous ammonium sulphate) at pH 7.0 in parallel with purified recombinant H-ferritin from *E. coli* (rHF) loaded on the gel and stained with Prussian Blue. rLF is a control of recombinant L-ferritin from *E. coli* preloaded with iron.

tween the human and monkey proteins allowed us to measure H- and L-ferritin concentrations in untransfected fibroblast COS-7 cells using ELISA assays for human ferritin. They increased 2–5-fold after supplementation with Fe(III) and decreased below assay sensitivity after 18 h of iron deprivation with desferrioxamine (DFO) (Table 1). About 48 h after transfection with 10 μg of human H- or L-ferritin cDNAs, the ferritin levels in COS cells reached 30–60 μg of ferritin/mg of total protein, i.e. an increase over background of up to 200-fold for H-ferritin and 500-fold for L-ferritin, which has a lower basal level (Table 1). The levels of transfected ferritin types remained constant for a further 24 h after transfection, and were not affected by iron supplementation with FeNTA or by chelation with DFO (Table 1). In contrast, in the same transfected cells the concentration of the non-transfected ferritin type decreased in DFO-treated and increased in FeNTA-treated cells, although by less than in the untransfected cells (Table 1). In addition we found that H-ferritin overexpression caused a consistent 2-fold decrease in endogenous L-ferritin, whereas L-ferritin overexpression had little effect on H-ferritin accumulation (Table 1).

Electrophoretic analysis in non-denaturing 7.5% polyacrylamide gels showed that the cytosolic homogenates of transfectant cells contained assembled ferritin detectable with silver stain or with immunoblotting with the specific antibodies (Figures 1A and 1B), but undetectable with Prussian Blue stain. Under these conditions the ferritin in untransfected cells was undetectable (Figures 1A and 1B). The transfected ferritins had electrophoretic mobilities similar to those of the purified recombinant ferritins from *E. coli* (Figure 1), and were resistant to heating at 75 °C for 10 min (not shown). This facilitated the purification. When purified recombinant H-ferritin from COS cells or from *E. coli* were incubated with 1000 Fe atoms per molecule they incorporated similar amounts of iron (Figure 1C).

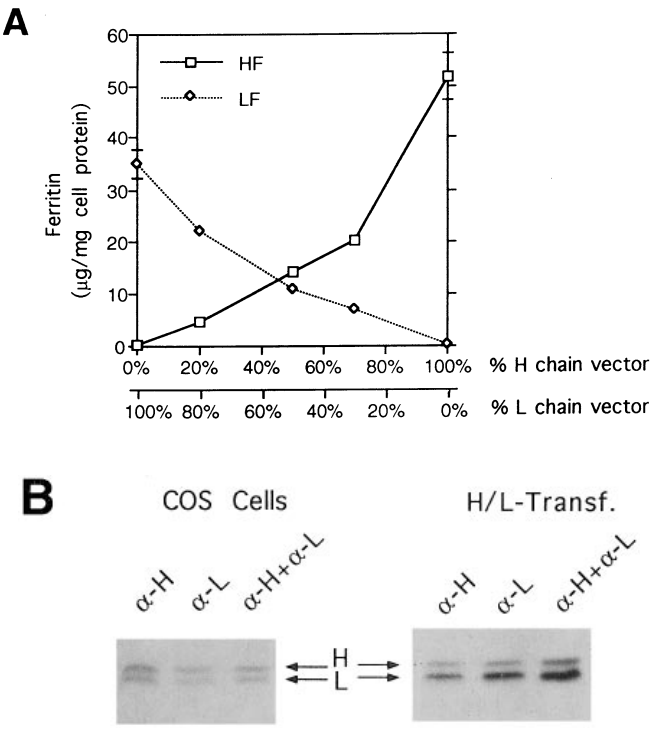

Figure 2 Co-expression of human H- and L-ferritin chains

(**A**) COS cells were transfected with 10 μg of purified plasmid DNA composed of the indicated proportion of vectors encoding the H- and L-chains. After 66 h, cells were homogenized and H- and L-ferritin concentrations in the cytosolic extracts measured by ELISA. Means of two independent experiments are shown. (**B**) Cytosolic homogenates of cells metabolically labelled with [^{35}S]methionine were immunoprecipitated with saturating amounts of anti-H (α-H) or anti-L (α-L) antibodies, or the two together (α-H + α-L), separated by SDS/PAGE and autoradiographed. The left panel shows the results with untransfected cells (COS Cells) and the right panel (H/L-Transf.) the results with cells transfected with equivalent amounts of H-chain and L-chain cDNAs. The mobilities of H- and L-chains are indicated by arrows.

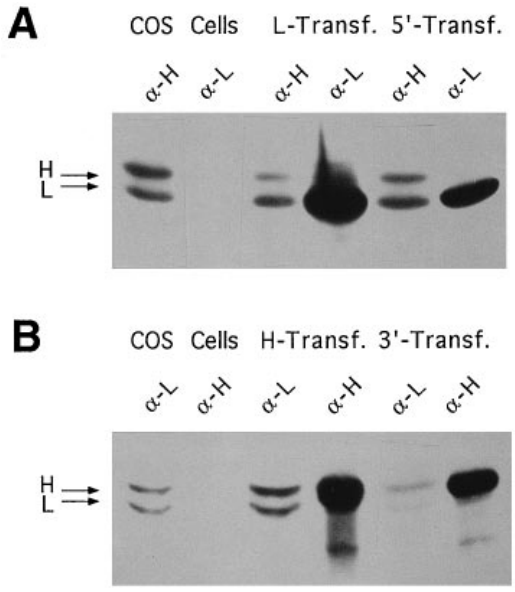

Figure 3 Analysis of newly synthesized ferritin hetero- and homo-polymers

The cytosolic homogenates of cells metabolically labelled with [^{35}S]methionine were immunoprecipitated with saturating amounts of anti-H (α-H) or anti-L (α-L) antibodies, separated by SDS/PAGE and autoradiographed. (**A**) Experiments in which L-tranfectants with (5′-Transf.) or without (L-Transf.) the 5′-untranslated regions were immunoprecipitated first with anti-H-ferritin antibody, to separate the heteropolymeric ferritin fraction, and then with anti-L-ferritin antibody to precipitate the homopolymeric fraction. (**B**) Similar experiments on H-transfectants with or without the 3′-flanking region precipitated first with anti-L-ferritin and then with anti-H-ferritin antibodies. COS cells are the untransfected cell controls. The arrows indicate the mobility of the two ferritin chains.

Table 2 Uptake of ^{59}Fe by COS cells

Cells were incubated in serum-free medium with 2 μCi of ^{59}FeNTA/150 μM ascorbic acid for 18 h, harvested, washed and analysed. Radioactivity incorporated into the cells is expressed as percentage of that incorporated in the matched controls. Values are means $\pm$ S.D. from four independent experiments. The exogenous ferritin concentration in the ^{59}Fe-incorporation experiments was determined as indicated in Table 1 and expressed as μg/mg of soluble protein. UTR, untranslated region.

Chain transfected	Cellular ^{59}Fe incorporation (%)	Exogenous ferritin (μg/mg)
None	100%	—
H-chain	139% ($\pm$40)	43
H-chain with 3′-UTR	102% ($\pm$42)	20
L-chain	127% ($\pm$22)	33
L-chain with 5′-UTR	106% ($\pm$40)	11
H + L-chains	124% ($\pm$25)	27

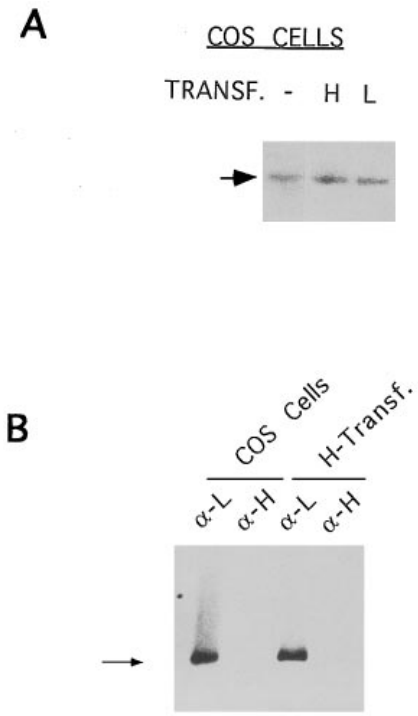

Figure 4 *In vivo* incorporation of ^{59}Fe into ferritin

Transfected and untransfected cells were incubated with 2 μCi/ml ^{59}Fe for 18 h, as in Table 2, and the cytosolic homogenates analysed. (**A**) Autoradiography of a native polyacrylamide gel of extracts from an equal number of cells untransfected (—), or transfected with 10 μg of H- and L-chain cDNAs (H and L respectively). (**B**) The homogenates of untransfected and H-transfected cells were sequentially precipitated first with the anti-L-chain antibody to separate the heteropolymers, and then with anti-H-chain antibody to recover H-chain homopolymers. Ferritin was solubilized by incubation at 20 °C in 0.1% SDS/4 M urea, loaded on SDS/6% polyacrylamide gels containing 4 M urea, and subjected to autoradiography. The arrows indicate the mobility of assembled ferritin.

In some experiments, the cells were transfected with a constant amount of DNA made of different proportions of H- and L-chain cDNAs. Analysis of the cell homogenates showed that both ferritin types can be expressed together over a larger range of concentrations and in predetermined proportions (Figure 2A). It also appeared that the transfected subunits co-assembled into heteropolymers, since ferritin precipitated with either anti-H- or anti-L-subunit antibodies had similar subunit composition (Figure 2B).

The expression of a vast excess of one subunit type in cells transfected with a single cDNA can lead to either a shift in the subunit composition of all the ferritin molecules or the production of homopolymers in addition to the endogenous heteropolymers. To distinguish between the two possibilities, we set up sequential immunoprecipitation experiments in which [^{35}S]methionine-labelled proteins were first precipitated with saturating amounts of anti-L-chain antibody and then with anti-H-chain antibody. The first precipitates from control and transfected cells consistently contained similar and equivalent proportions of H- and L-chains. This means that the subunit composition of ferritin heteropolymers was not strongly affected by the overexpression of either of the two subunit types (Figures 3A and 3B). The second precipitates from the untransfected cells contained no detectable ferritin, whereas that from the transfected cells contained a single and large band of the expected subunit type (Figures 3A and 3B), indicating that most of the exogenous subunits accumulated as homopolymers. Addition of the 5′-untranslated region to the L-chain-coding region or of the 3′-untranslated region to the H-chain sequence did not increase the proportion of the transfected subunit in the heteropolymeric fraction of the transfected cells (Figures 3A and 3B). Incubation of the H-transfectants with FeNTA increased the relative proportion of ferritin heteropolymers without affecting their subunit composition (not shown). Thus the ferritin heteropolymeric fraction in transfected and untransfected cells was similarly regulated by iron.

Iron incorporation

We performed cellular iron-incorporation experiments with the various tranfectants using ^{59}FeNTA. The amount of iron taken up by cells transfected with H- and L-chains, either alone or together, and with or without the untranslated flanking regions was essentially the same as that taken up by the untransfected cells (Table 2). Non-denaturing PAGE analysis of the soluble fraction of cellular homogenates revealed that more than 90 % of protein-bound radioactivity was associated with the ferritin molecule, and, more importantly, that the mobility and intensity of the radioactive bands did not vary in the controls and H- or L-chain transfectants (Figure 4A). The cytosolic homogenates of cells metabolically labelled with ^{59}Fe were subjected to sequential immunoprecipitation experiments with anti-L-ferritin and anti-H-ferritin antibodies followed by PAGE analysis under conditions that do not denature the ferritins [20]. The results show that ferritin heteropolymers containing L-chains incorporated radioactive iron in both the control and H-transfected cells, whereas the more abundant H-chain homopolymers remained unlabelled (Figure 4B).

Immunostaining studies

Immunofluorescent staining of the cells with the specific antibodies clearly distinguished the transfected strongly stained cells from the untransfected weakly stained cells. Cell counting from four independent experiments showed that transfected cells accounted for about 40 % of the total cell population. Immunogold staining and electron-microscopic analysis indicated that endogenous and transfected ferritins have a similar cytoplasmic distribution (not shown).

DISCUSSION

The present data show that H- and L-ferritin cDNAs under the control of simian virus 40 promoter are efficiently expressed in COS cells as fully assembled proteins. A plateau of expression was reached about 48 h after transfection, with an increase in ferritin levels of 200–500-fold over background. About 40 % of cells overexpressed the ferritin, thus the single cell can accumulate ferritin up to a concentration 1000-fold above background without evident signs of toxicity. Most of the exogenous ferritins assembled into homopolymers, which are electrophoretically and functionally analogous to the same homopolymers produced in *E. coli*. Only a minor amount of the ferritin was found in the culture medium, about 100 ng/ml, probably originating from cell damage.

To analyse ferritin composition of the cells, we developed a method to separate ferritin homopolymers from heteropolymers based on sequential immunoprecipitation with antibodies for L- and H-chains. These assays showed that ferritin homopolymers were present only in the transfected cells and accounted for a large proportion of total ferritin (Figure 3). More significant was the finding that the newly synthesized heteropolymers had similar H/L ratios in both transfected and non-transfected cells. These heteropolymers probably correspond to the endogenous ferritin detected by ELISA (Table 1), the level of which is only marginally modified by the overexpression of H- or L-ferritin chains. Thus the endogenous ferritin heteropolymers and the exogenous ferritin homopolymers appear to form two distinct pools, which do not cross-hybridize. This is probably related to the heterogeneity of the cell population, being made up of transfected cells (about 40 %), expressing large amounts of homopolymers, and untransfected cells (about 60 %), expressing the endogenous ferritin heteropolymers. Alternatively, the transfected and the endogenous subunits may be synthesized in different subcellular compartments. However, the introduction of either the 5′ non-coding region of L-chain mRNA or the 3′ non-coding region of H-chain mRNA to the respective coding sequences in the pSG5 constructs did not favour co-assembly of exo- and endogenous subunits (Figures 3A and 3B). It should be noted that ferritin chains from various mammalian species co-assemble *in vitro* [21] and during co-expression in *E. coli* [22], and there are no apparent structural reasons why monkey and human ferritins should not co-assemble *in vivo* or *in vitro*.

The major and most significant findings were that, despite the high efficiency of transfection and expression, the indices of iron metabolism in the transfectants were only marginally modified and that the recombinant H- and L-chain homopolymers did not incorporate detectable ^{59}Fe within the cell (Figure 4). Accordingly, the presence of a massive excess of homopolymers did not interfere with the iron-mediated translational regulation of the endogenous ferritin synthesis of heteropolymers, which were similarly regulated by iron or DFO in both transfected and non-transfected cells (Table 1). The lack of any functional activity of L-ferritin in the transfectants was expected, since L-chain homopolymers have no ferroxidase activity, do not incorporate iron when expressed in *E. coli* [23] and are extracted from tissues as iron-poor proteins [24]. In addition, it has been recently shown that subjects expressing L-chain at levels about 10-fold above normal have no evident abnormalities of iron metabolism [14,15]. More surprising was the finding that H-chain overexpression had no evident effects, since H-chain homopolymers have ferroxidase activity, readily take up iron *in vitro* (Figure 1C) and, when expressed in *E. coli*, incorporate iron [5].

We considered two hypotheses to explain these findings. First, their unnatural homopolymeric structure may not be functional *in vivo* since ferritins need L-chains for iron deposition in the core. This is compatible with our *in vitro* studies showing that H- and L-chains co-operate in iron storage and uptake [25] and is supported by *in vivo* studies of stably transfected cells that overexpress ferritin H-chain. In these cells, the endogenous subunits co-assemble with the exogenous ones to generate 90 % H-chain-rich isoferritins which take up iron and induce an iron-deficient phenotype in the cells [13]. Saturation of cellular iron-incorporation capacity may explain why all cells took up similar amounts of iron, including those co-transfected with H- and L-chains which overexpress functional heteropolymers (Table 2). An alternative, but not mutually exclusive, hypothesis is that the apparent lack of ability to accumulate iron in the exogenous H-ferritins reflects the limits posed by the transient expression system. Ferritin is a long-living protein with half-life of 1.3–3.6 days [26], and the iron-incorporation experiments were terminated, at the latest, less than 3 days after transfection, a period not sufficient to eliminate completely the pre-existing endogenous ferritin. It is conceivable that iron-free exogenous ferritins do not compete with the iron-containing endogenous ones to incorporate iron, as has been shown to occur *in vitro* [27].

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