P/CAF/p300 complex binds the promoter for the heavy subunit of ferritin and contributes to its tissue-specific expression

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We analysed the role of the nuclear protein P/CAF in regulating the transcription of the gene for human heavy (H) ferritin in given cell types. P/CAF is a histone acetylase, recruited to specific promoters via interaction with the co-activator molecule p300/CREB-binding protein (CBP). Histone acetylation promoted by P/CAF destabilizes the nucleosome structure, thus contributing to activation of transcription. The transcription of the H ferritin gene is regulated by the transcription factor B-box-binding factor (Bbf), which bridges RNA polymerase II via p300/CBP. Northern blot analyses of RNA species from various human tissues and cell lines demonstrate that the H ferritin gene is expressed at high levels in cells containing high levels of the P/CAF transcript. Moreover, transient overexpression of

P/CAF in cells constitutively expressing low levels of this protein activates transcription driven by the region of the H promoter interacting with Bbf. The involvement of p300/CBP in the possible P/CAF-mediated regulation of H promoter was also explored by evaluating the phenomenon in the presence of the oncoprotein E1A. The results of these experiments demonstrate that P/CAF activates the H promoter also in the presence of limited amounts of p300/CBP. We argue that P/CAF is a component of the basal transcription apparatus of the H ferritin gene and that the relative amounts of the P/CAF protein in different cell types could account for the cell-specific control of the H ferritin gene transcription.

INTRODUCTION

The ferritin molecule consists of 24 subunits of the heavy (H) and light (L) types. The heavy chain is the acidic subunit and has a molecular mass of 21 kDa; the light subunit is basic and has a molecular mass of 19 kDa [1]. The H and L subunits can polymerize in different proportions, producing a family of immunologically and biochemically distinct isoforms. Different cell types display different isoform patterns: L-rich isoforms are present in liver and spleen, whereas H-rich isoforms are abundant in heart and kidney [2]. The molecular mechanism(s) of the tissue-specific expression of ferritin genes are still unknown.

Ferritin gene expression is controlled at the translational and transcriptional levels. Translation of the H and L mRNA species is stimulated by iron [3] and cytokines [4], whereas transcription of the two genes is selectively modulated by hormones [5] and drugs [6], as well as during cell differentiation [7,8]. The mRNA levels of both H and L ferritin differ remarkably depending on the cell type, probably because of a balanced regulation of transcription efficiency and RNA turnover rate [9]. Regulation of the H gene promoter seems to be controlled mainly by proximal elements, one of which, the so-called 'B-box element', interacts with the B-box-binding factor (Bbf) [10]. We recently demonstrated that Bbf bound to the B-box element forms a complex *in vivo* with the nuclear protein p300 [11]. p300 is a high-molecular-mass protein that mediates the crosstalk between TFIIB and a variety of transacting factors, such as c-Jun, MyoD, cAMP response element-binding protein and YY1, thus functioning as a general co-activator molecule [12]. The p300-associated protein P/CAF has histone acetylase activity and it has been proposed

that the acetylation of chromatin mediated by P/CAF associated with p300 contributes to the transcriptional activation of specific genes [13]. P/CAF exists in all human tissues analysed, but its mRNA level varies significantly between cell types [13].

We noticed that P/CAF mRNA is expressed at high levels in tissues in which H-rich isoforms are most abundant. This alerted us to the possibility that P/CAF might be involved in regulating H ferritin gene transcription. Indeed we found a good correlation between H ferritin mRNA and P/CAF mRNA levels in both human tissues and human cell lines of different origins and degrees of differentiation. Here we also show that the efficiency of the H gene promoter is enhanced in cells transiently overexpressing P/CAF, thereby demonstrating that P/CAF has a functional role in H-gene transcription.

MATERIALS AND METHODS

Cell lines

The human cell lines were cultured as monolayers in Dulbecco's modified Eagle's medium (Flow Laboratories), supplemented with 10% (v/v) foetal calf serum (Hyclone; Logan), 2 mM glutamine (Flow Laboratories), 100 i.u./ml penicillin and 100 µg/ml streptomycin (Sigma). Cells were grown at 37 °C under an air/CO₂ (19:1) atmosphere. The cell lines used were: (1) HeLa cells derived from an epithelioid carcinoma of the cervix, (2) HepG2 and Hep3B cells derived from a hepatocellular carcinoma, (3) neuroblastoma cells SKN and (4) K562 cells derived from chronic myelogenous leukaemia. Caco-2 human cells are a cell line that spontaneously differentiates into enterocyte-like cells under standard culture conditions. The

Abbreviations used: Bbf, B-box-binding factor; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; H, heavy; L, light; RSV, Rous sarcoma virus.

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differentiation process starts at confluence, which in our culture conditions was around day 6, and reaches a maximum at around day 14 [7].

Northern blot analysis

Total RNA species were extracted from cell lines with the phenol/chloroform method [14]. Samples of 5 µg of total RNA were size-fractionated on a 2.2 M formaldehyde/1.5% (w/v) agarose gel, transferred to a nitrocellulose filter (Nytran; Schleicher and Schuell) and probed as described previously [7]. The filter with the poly(A)⁺ RNA species from various human tissues was provided by Clontech. The ³²P-labelled probes were: a 528 bp *Pst*I fragment of the H ferritin cDNA (H Fer) [15] and a 2850 bp *Eco*RI–*Kpn*I fragment from the human P/CAF cDNA (P/CAF), kindly provided by Dr. Y. Nakatani [13].

DNA transfections and chloramphenicol acetyltransferase (CAT) assays

The H ferritin promoter/CAT construct 5' H H/A, Bbf/TATAA and Bbf_{mut}/TATAA, the p300-expressing plasmid (p300), the Rous sarcoma virus (RSV)/CAT construct and the E1A-expressing plasmid (E1A 13S) are described elsewhere [11]. The pCX-Flag-P/CAF construct [13] was kindly provided by Dr. Nakatani [13]. HeLa and HepG2 cells were transiently transfected with these constructs by the calcium phosphate co-precipitation method [16]. For each experiment, 20 µg of total DNA was transfected. CAT activity was measured and normalized for transfection efficiency by including in each transfection the luciferase gene under the control of the cytomegalovirus enhancer (CMV-Luc). CAT and luciferase assays were performed as described [11,17]. The values reported in the figures are the means of three independent transfection experiments.

Immunoprecipitation and Western blot analysis

HeLa cells were extracted with 10 ml of lysis buffer [25 mM Hepes/KOH (pH 7.2)/150 mM potassium acetate/2 mM EDTA/1 mM dithiothreitol/1 mM PMSF/10 µg/ml aprotinin/10 µg/ml leupeptin/1 µg/ml pepstatin/20 mM sodium fluoride/0.1% NP40] [13]. HeLa cell extracts (1 mg) were incubated with 50 µl of anti-Flag M2 affinity gel for 1 h at 4 °C. The beads were collected by centrifugation, washed four times with lysis buffer and resolved by SDS/PAGE [12% (w/v) gel]. The proteins were transferred to a nitrocellulose filter. After the addition of blocking mixture [1% (v/v) Tween/5% (w/v) non-fat dried milk], the membrane was incubated with a 1:1000 dilution of mouse anti-Flag for 1 h at room temperature. Bound antibodies were detected by using horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:5000) and enhanced chemiluminescence (Amersham).

RESULTS

Relative abundance of H ferritin and P/CAF mRNA species in human tissues and cell lines

The mRNA for the human histone acetylase P/CAF is expressed in all human tissues, but with remarkable differences in the steady-state levels depending on cell type: it is abundant in heart and muscle and at a lower relative abundance in liver and kidney [13]. The ferritin H subunit also displays a tissue-specific preferential expression, the H-rich isoforms being abundant in heart and almost undetectable in liver [2].

The first step of our investigation of the role of P/CAF in H ferritin transcription was to examine by Northern blot analysis

	HFer mRNA %	P/CAF mRNA %	Ratio
Heart	97	76	1.27
Brain	40	50	0.80
Placenta	48	30	1.6
Lung	97	20	4.85
Liver	48	39	1.23
Muscle	100	100	1.0
Kidney	70	45	1.5
Pancreas	69	55	1.25

Figure 1 Correlation between H ferritin and P/CAF mRNA levels in different human tissues

Expression profile of H mRNA and P/CAF mRNA in various human tissues: H, heart; B, brain; Pl, placenta; Lu, lung; Li, liver; M, muscle; K, kidney; Pa, pancreas. Northern blot analysis was performed as described in the Materials and methods section. An RNA blot from different human tissues (Clontech) was hybridized with a radiolabelled H ferritin cDNA and with a human P/CAF cDNA. Bottom panel: H ferritin mRNA and P/CAF mRNA contents, determined by scanning densitometry of the Northern blot, expressed as percentages of those found in skeletal muscle, arbitrarily set at 100%. The third column shows the ratio of the abundances of the two mRNA species.

the relative abundance of the two mRNA species in several human tissues and cell lines. Both the H ferritin and P/CAF transcripts were highly expressed in human heart, skeletal muscle, kidney and pancreas, and less represented in brain, placenta and liver (Figure 1). Two major species hybridized with the P/CAF probe in the RNA sample from lung. The most abundant transcript did not correspond to the size of the P/CAF mRNA in any of the samples tested. This transcript might represent the product of alternative splicing of the P/CAF transcript or it might result from partial degradation of the RNA. With the exception of the lung, our results confirm those reported for P/CAF expression in human tissues [13].

We next analysed the two mRNA species in human cell lines of different origins. The Northern blot analysis reported in Figure 2 shows that the levels of P/CAF mRNA are well correlated with those of H ferritin in HeLa, HepG2 and Hep3B cells, as demonstrated by the ratio of expression of the mRNA species. In the other two cell lines, SKN and K562, both the transcripts were expressed at very low levels. This probably prevented an accurate determination of the ratio between the two mRNA species; nevertheless the result of the experiment suggests the existence of a close relationship between high levels of P/CAF mRNA and high levels of H ferritin mRNA on the one hand, and between low levels of P/CAF mRNA and low levels of H ferritin mRNA on the other.

Figure 2 Correlation between H ferritin and P/CAF mRNA levels in different human cell lines

Expression profile of H mRNA and P/CAF mRNA in different human cell lines: in HeLa cells derived from an epithelioid carcinoma of the cervix, in HepG2 and Hep3B cells derived from a hepatocellular carcinoma, in neuroblastoma cells SKN, in K562 cells derived from chronic myelogenous leukaemia and in Caco-2 cells that spontaneously differentiate into enterocyte-like cells under standard culture conditions. Caco-2 cell mRNA species were prepared at 3 days (undifferentiated) and at 15 days (differentiated) after plating. Northern blot analysis was performed as described in the Materials and methods section. Bottom panel: H ferritin mRNA and P/CAF mRNA contents, determined by scanning densitometry of the Northern blot, expressed as percentages of those found in HeLa cells, arbitrarily set at 100%. The third column shows the ratio of the abundances of the two mRNA species.

A similar correlation was found during the differentiation process of Caco-2 cells. Caco-2 cells differentiate spontaneously into enterocyte-like cells as a function of cell density alone. Under our culture conditions this process was accomplished within 15 days. In the fully differentiated Caco-2 cells, harvested after 15 days of continuous culture, P/CAF mRNA was approx. 3-fold more abundant than in undifferentiated cells harvested after 3 days (Figure 2). In the differentiated cells the H ferritin mRNA was about 3-fold more abundant than in undifferentiated cells. Moreover, both H ferritin and P/CAF mRNA levels increased linearly with the time of culture as shown by the analysis of RNA species from cells harvested daily from day 2 to day 15 (results not shown).

These results demonstrate that the expressions of P/CAF and H ferritin mRNA species occur at comparable levels in the same cell types, as well as in a given cell under different differentiation conditions.

P/CAF overexpression increases transcription driven by H ferritin promoter in HepG2 cells

The H ferritin promoter efficiency was several-fold higher in HeLa cells than in HepG2 cells and the levels of the H ferritin

and P/CAF mRNA species were consistently higher in HeLa cells than in HepG2 (see Figure 2). Therefore these two cell lines represent a good model system for evaluating whether P/CAF affects transcription driven by the H promoter. HeLa and HepG2 cells were transiently transfected with plasmid fusions carrying regions of ferritin promoter upstream of the CAT reporter gene together with increasing concentrations of a P/CAF-expressing plasmid. The ferritin/CAT construct 5' H H/A contains the $-100/+1$ region of the H promoter. This ferritin promoter segment harbours the *cis*-element B, which is recognized by the transcription factor Bbf and is involved in both basal and cAMP-induced stimulation of H transcription [10,11]. A 20 bp oligonucleotide spanning the wild-type B-site was fused to the ferritin TATAA box in the Bbf/TATAA construct, whereas the Bbf_{mut}/TATAA contained a mutated version of the oligonucleotide, not recognized by Bbf [11]. CAT activities were normalized for transfection efficiency by including in each transfection a plasmid carrying the luciferase gene under the control of the cytomegalovirus promoter (CMV-Luc). The expression of exogenous P/CAF in the cells transfected with the expression vector was controlled by Western blot analysis (Figure 3A).

The expression of recombinant P/CAF in HeLa cells only slightly increased the transcription of the reporter gene driven by the entire ferritin promoter region. Conversely, in HepG2 cells P/CAF stimulated the transcription driven by the $-100/+1$ promoter segment in a dose-dependent manner, leading at maximal concentrations to a 4-fold increase over the basal level. Very high P/CAF expression resulted in inhibition of the H-gene promoter, probably as a consequence of a squelching phenomenon (results not shown). The results shown in Figure 3(B) were obtained with the Bbf/TATAA construct and with its mutated version Bbf_{mut}/TATAA. The exogenous expression of P/CAF stimulated, in HepG2 cells, the transcription of the reporter gene, driven by the B element of the ferritin promoter, but not by the mutated version of this element. Taken together, these findings indicate that P/CAF is able to trans-activate the ferritin H promoter in HepG2 cells, and that the target site is the B element, in position -62 . The functional role of the B element is confirmed by the results obtained with the inactive Bbf mutant. The Bbf_{mut}/TATAA construct had a basal activity comparable to that given by the ferritin TATAA-box only (results not shown) [11] and did not respond to exogenous P/CAF.

The stimulatory effect of P/CAF on the H ferritin promoter seems to be specific because RSV promoter activity (RSV/CAT construct) was unaffected by the co-transfection of P/CAF-expressing plasmid both in HeLa and HepG2 cells (see Figure 3C).

In HeLa cells P/CAF rescues the E1A-inhibited transcription driven by the H promoter

P/CAF was originally identified as a cellular p300-associated factor [13]. We have recently demonstrated that the transcription factor Bbf binds p300 *in vivo* [11]. Because the results of P/CAF stimulation of ferritin promoter demonstrated that the B-element has a functional role, we examined whether p300 constitutes the link between P/CAF and Bbf.

To address this question we transiently expressed the E1A oncoprotein in HeLa cells to see whether P/CAF was able to rescue the H promoter activity. Previous studies indicated that the E1A protein strongly decreases the transcription efficiency driven by the H promoter by titrating p300 from the Bbf/p300 active complex [11]. In E1A-expressing HeLa cells the exogenous expression of P/CAF, controlled by Western blotting (results not shown), completely restored the transcription efficiency of the 5'

Figure 3 P/CAF overexpression affects H ferritin promoter activity

(A) CAT activity driven by the $-100/+1$ region of the H ferritin promoter (5' H H/A construct). For each experiment 3 pmol of 5' H H/A construct (10 μ g) were transiently co-transfected in HeLa or in HepG2 cells without or with increasing amounts of the pCX-Flag-P/CAF-expressing plasmid (0.33, 0.67 and 1 pmol, corresponding to 1.50, 3 and 4.7 μ g respectively). The total amount of transfected DNA was taken to 20 μ g by using the plasmid pUC19 as carrier DNA. The values are means for three independent transfection experiments and are expressed as percentages of conversion of the [14 C]chloramphenicol into its acetylated forms. The lower panel shows the Western blot analysis with anti-Flag antibody of total extracts from HeLa and HepG2 cells mock-transfected (P/CAF $-$) or transfected with the pCX-Flag-P/CAF expression vector (P/CAF $+$). (B) CAT activity driven by the Bbf/TATAA construct containing a 20 bp oligonucleotide spanning the wild-type B-site and by Bbf_{mut}/TATAA, which contains a mutated version of the oligonucleotide. For each experiment 3 pmol of the two constructs (12 μ g) were transiently co-transfected in HeLa and HepG2 cells with 1 pmol of the pCX-Flag-P/CAF expression vector (4.7 μ g). The total amount of transfected DNA was taken to 20 μ g by using the plasmid pUC19 as carrier DNA. The values are means for three independent transfection experiments and are expressed as percentages of conversion of the [14 C]chloramphenicol into its acetylated forms. (C) CAT activity driven by the RSV promoter (3 pmol of RSV/CAT construct, corresponding to 10 μ g) in HeLa and HepG2 cells transiently co-transfected without or with two different amounts of the pCX-Flag-P/CAF expression vector (0.67 and 1 pmol, corresponding to 3 and 4.7 μ g respectively). The total amount of transfected DNA was taken to 20 μ g by using the plasmid pUC19 as carrier DNA. The values are means for three independent transfection experiments and are expressed as percentages of conversion of the [14 C]chloramphenicol into its acetylated forms.

Table 1 P/CAF overexpression counteracts the E1A inhibition of H ferritin transcription

CAT activity driven by the $-100/+1$ region of the H ferritin promoter (3 pmol of 5' H H/A construct, corresponding to 10 μ g) co-transfected in HeLa cells with 1 pmol (2.5 μ g) of 13S E1A-expression vector (E1A) and/or with 1 pmol (4.7 μ g) of pCX-Flag-P/CAF expression vector (P/CAF) and/or with 1.5 pmol (6 μ g) of p300 expression vector (p300) or with both P/CAF and p300 expression vectors. The total amount of transfected DNA was taken to 23 μ g by using the plasmid pUC19 as carrier DNA. The values reported in the table are means $\pm$ s.d. for three independent transfection experiments and are expressed as percentages of conversion of the [14 C]chloramphenicol into its acetylated forms.

5' H H/A	+	+	+	+	+
P/CAF	-	-	+	-	+
E1A	-	+	+	+	+
300	-	-	-	+	+
CAT activity (%)	19.8 $\pm$ 1.49	3.8 $\pm$ 0.25	25.54 $\pm$ 0.77	16.6 $\pm$ 1.4	18.8 $\pm$ 0.80

H H/A construct (Table 1). The same effect was obtained by p300 expression alone and by the contemporary expression of p300 and P/CAF. This result shows that P/CAF trans-activates the H ferritin promoter also in the presence of limited amounts of p300.

DISCUSSION

We have analysed the role of the human histone acetylase P/CAF in the control of transcription efficiency driven by the H ferritin promoter. P/CAF is a recently identified p300-associated nuclear protein, with intrinsic histone acetylase activity. It has been suggested that P/CAF, once bound to specific promoters via interaction with p300, could locally promote an open chromatin configuration that is transcriptionally active [13].

The expression of P/CAF varies widely in several human tissues [13]. Because the expression of the H ferritin gene also varies between tissues [18], we first looked for a correlation between the cell-specific pattern of expression of the two genes and found that P/CAF and H ferritin mRNA species accumulate in the same cells. Moreover, the spontaneous differentiation of Caco-2 cells *in vitro* was accompanied by a parallel increase in the two transcripts. The correlation between the P/CAF and H ferritin mRNA levels suggests that local chromatin remodelling, mediated by P/CAF activity, facilitates the transcription of the ferritin gene. Another possibility is that the transcription of the two genes might be regulated by the same factor(s), without a cause-effect relationship between their expression levels.

To test the two possibilities we overexpressed P/CAF in HepG2 cells and analysed the transcription of a reporter gene driven by the ferritin promoter. The transfection experiments demonstrated that P/CAF is a cell-specific regulator of ferritin promoter. In HepG2 cells, which have a low P/CAF mRNA basal level, its exogenous expression determines a remarkable stimulation of H promoter activity. Conversely, in HeLa cells, which have a high basal level of P/CAF mRNA, its overexpression does not have a strong effect on ferritin promoter (Figure 3A).

The ferritin promoter site responsive to P/CAF is the B-site in position -62 (Figure 3B). Bbf, stably bound to its DNA element, uses p300 as a molecular bridge to contact the RNA polymerase II [11]. The E1A oncoprotein titrates p300 from the active Bbf/p300 complex and down-regulates the H transcription [11]. Because P/CAF associates with p300, its activation of the ferritin

promoter could be mediated by p300 associated with Bbf. We show in E1A-expressing HeLa cells that P/CAF overexpression is itself able to restore transcription driven by the ferritin promoter. Moreover, overexpression of p300 together with P/CAF did not further stimulate the transcription.

This observation suggests that P/CAF is able to contact Bbf also in the presence of limiting amount of p300 or perhaps even in the absence of this protein. We suggest that the H ferritin promoter is regulated at the level of the B-box element by a multimeric complex that includes the transcription factor Bbf, the co-activator molecule p300 and the histone acetylase P/CAF. Furthermore it was recently demonstrated that P/CAF interacts directly with NF-Y [19], which has been suggested to bind the B-box element of the H ferritin promoter [20].

Despite the large number of studies, the molecular basis of the tissue-specific expression of ferritin genes is unknown. We propose a regulatory mechanism of ferritin gene transcription on the basis of the different abundances of the P/CAF factor in different tissues, cell types and during the different phases of cell differentiation. This hypothesis could be extended to other genes, considering that most of the so-called 'housekeeping genes' are transcribed with very different efficiencies in different tissues. Histone acetyltransferase could be differently expressed in the various tissues as shown for P/CAF, but also their activity could be regulated in a tissue-specific fashion leading to a similar effect on the transcription. Furthermore the existence of histone deacetylase activities suggests that the efficiency of a promoter might be regulated either positively or negatively depending on the equilibrium between histone acetyltransferase and histone deacetylase activities.

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