Hepcidin is the major regulatory hormone of iron metabolism, encoded by the HAMP (hepcidin antimicrobial peptide) gene. Hepcidin is expressed mainly in hepatocytes, but is also found in the blood in both a mature and prohormone form. Although, the function of mature hepcidin and the regulation of the HAMP gene have been extensively studied, the intracellular localization and the fate of prohepcidin remains controversial. In the present study, we propose a novel role for prohepcidin in the regulation of its own transcription. Using indirect immunofluorescence and mCherry tagging, a portion of prohepcidin was detected in the nucleus of hepatocytes. Prohepcidin was found to specifically bind to the STAT3 (signal transducer and activator of transcription 3) site in the promoter of HAMP. Overexpression of prohepcidin in WRL68 cells decreased HAMP promoter activity, whereas decreasing the amount of prohepcidin caused increased promoter activity measured by a luciferase reporter-gene assay. Moreover, overexpression of the known prohepcidin-binding partner, α-1 antitrypsin caused increased HAMP promoter activity, suggesting that only the non-α-1 antitrypsin-bound prohepcidin affects the expression of its own gene. The results of the present study indicate that prohepcidin can bind to and transcriptionally regulate the expression of HAMP, suggesting a novel autoregulatory pathway of hepcidin gene expression in hepatocytes.

Key words: α-1 antitrypsin (A1AT), autoregulation, hepcidin antimicrobial peptide promoter (HAMP promoter), iron metabolism, prohepcidin, signal transducer and activator of transcription 3 site (STAT3 site).

INTRODUCTION

Hepcidin is the key iron regulatory hormone produced mainly in the liver and secreted into the blood. It was originally described as LEAP-1 (liver-expressed antimicrobial protein 1), an antimicrobial peptide exhibiting consistent antifungal, but only weak antibacterial, activity [1,2]. Shortly after the discovery of hepcidin its fundamental role in iron homeostasis was realized [3,4]. The hormone acts by binding to the iron exporter ferroportin, triggering its internalization and intracellular degradation [5–7]. In the presence of hepcidin enterocytes release less iron into the portal system, resulting in down-regulation of the iron uptake through the intestines. Similarly, hepcidin negatively regulates macrophage iron export. Owing to these effects, hepcidin overexpression has been compellingly linked to microcytic anaemia [8,9].

Hepcidin is encoded by the HAMP (hepcidin antimicrobial peptide) gene and synthesized in hepatocytes as preprohepcidin [2,10]. Soon after the translation of this 84-amino-acid long precursor form it is processed to the 60-amino-acid prohepcidin, which is further cleaved to the biologically active 25-amino-acid peptide hormone hepcidin [11]. The proteolytic cleavage of prohepcidin to mature hepcidin is mediated by furin [11,12] and related proprotein convertases PC (proprotein convertase) 1/3, PC2, PC5/6, PC7/LPC (lymphoma PC) and PACE4 (paired basic amino acid cleaving system 4; H45/15-17). Both prohepcidin and bioactive hepcidin are present in the circulation [10,11].

Under physiological conditions the expression of the HAMP gene in the liver is modulated by numerous factors [13]. The known positive regulators are the hereditary HFE (haemochromatosis) protein, TIR2 (transferrin receptor 2), HJV (haemojuvelin) and BMPs (bone morphogenetic proteins) [14–18]. In addition, hepcidin expression can be regulated by factors independent of body iron levels, such as erythroid factors, hypoxia and inflammation [19–22]. One of the identified negative regulators of liver hepcidin expression is matriptase-2 encoded by the TMPRSS6 (transmembrane protease, serine 6) gene. Matriptase-2 is a transmembrane serine protease which inhibits the activation of hepcidin expression by interacting with membrane HIV and cleaving it into fragments [23–25]. SMAD7 was described as another potent inhibitor of HAMP gene expression [26]. SMAD7 is an inhibitory SMAD protein that mediates a negative feedback loop to both TGF-β (transforming growth factor β) and BMP signalling. The soluble BMPER (BMP-binding endothelial regulator) peptide was also identified as a negative regulator of the hepatic level [27].

Although both the regulation of the HAMP gene at the transcriptional level and the role of mature hepcidin peptide have been studied extensively, little is known about the fate of precursor prohepcidin within the hepatocytes and in the blood. Prohepcidin

Abbreviations used: A1AT, α-1 antitrypsin; A1ATD, A1AT deficiency; BMP, bone morphogenetic protein; CDMP1, cartilage-derived morphogenetic protein 1; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GdI5, growth differentiation factor 5; GFP, green fluorescent protein; GST, glutathione transferase; HAMP, hepcidin antimicrobial peptide; HCC, hepatocellular carcinoma; HJV, haemojuvelin; IPTG, isopropyl β-D-thiogalactopyranoside; NLS, nuclear localization signal; PC, proprotein convertase; PSSM, position-specific scoring matrix; RE, response element; RRR, relative response ratio; STAT3, signal transducer and activator of transcription 3; SVM, support vector machine; TGF-β1, transforming growth factor β1.

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was first described as a nuclear peptide with a predicted NLS (nuclear localization signal) sequence [3], but later it was found mainly in the Golgi compartment [28,29], or in the cytoplasm showing granular cytoplasmic localization [10,30].

In the present study we describe a novel role of prohepcidin in the regulation of HAMP gene expression. Our results suggest that prohepcidin is located in the cytosol as well as in the nucleus of hepatocytes. In the nucleus prohepcidin can regulate its own gene expression by binding to the HAMP promoter. The single STAT3 (signal transducer and activator of transcription 3) site within the proximal 942 bp of the HAMP promoter is important for the binding of prohepcidin. The overexpression of prohepcidin decreased promoter activity, whereas inhibition of prohepcidin expression or its sequestration by the known prohepcidin binding partner A1AT (α-1 antitrypsin) [31] caused increased promoter activity, suggesting an important role for non-A1AT-bound prohepcidin in the regulation of hepcidin expression.

MATERIALS AND METHODS

Construction of plasmids and probes

Human full-length prohepcidin cDNA was amplified by PCR using the primers 5′-AAAGAATTCGGCACTGACTCCAGATG-3′ (sense) and 5′-AACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (antisense) and prohepcidin antisense DNA was amplified using the primers 5′-AAAGAATTCGACTCGTACCTCCAGA-3′ (sense) and 5′-AAAAAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (antisense), then cloned into the pcDNA3.1 vector (Invitrogen). Prohepcidin, A1AT, and STAT3 cDNAs were amplified by PCR using the primers 5′-AAAGAATTCGCACTGACTCGTACCCAGA-3′ (prohepcidin sense), 5′-AACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (prohepcidin antisense), 5′-AAAGAATTCGCTAGCTGACTCGTACCCAGA-3′ (A1AT sense), 5′-AACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (A1AT antisense), 5′-AAAAAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (STAT3 sense) or 5′-AAACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (STAT3 antisense) which were inserted into the pTriex3-Neo expression vector (Novagen, EMD4Biosciences).

A 942-bp fragment of the HAMP promoter was amplified by PCR using the primers 5′-AAAGAATTCGCACTGACTCGTACCCAGA-3′ (sense) and 5′-AAAAAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (antisense) and subcloned into the pGL3 basic vector containing the firefly luciferase reporter gene (Promega). The S1 and S2 fragments were amplified by PCR using primers 5′-AACTCTGAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (S1 sense) and 5′-AAAAAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (antisense), 5′-AACTCTGAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (S2 sense) and 5′-AAAAAGCTTCTAGCCTGGCAGCACAATCCACA-3′ (S2 antisense). The STAT3 mutant probe (mS1S) and the BMP-RE (response element) mutant probe (mS1B) were made by deletion of the STAT3-binding site (-64 to -72) and the proximal BMP-binding site (-80 to -85) respectively, and each was cloned into pGL3 basic vector. A 220-bp fragment of the MMP3 matrix metalloproteinase 3; also known as transin) promoter was amplified using the primers 5′-TATAAGAATAAACCAGGCCCATG-3′ (sense) and 5′-CAAGAATTCGTACCTGGCAGCACAATCCACA-3′ (antisense). Chromosomal DNA isolated from human blood or human liver cDNA libraries was used as a template for the cloning.

The mutagenesis of the prohepcidin NLS was created by nucleotide substitution at the nucleotide triplet of arginine (AGG) at amino acid position 57 to threonine (ACG) using the primers 5′-TTCAGAGGGCGAAGGGAGGAGGAC-3′ (sense) and 5′-AACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (antisense) for the first PCR, and 5′-AAAGAATTCGCACTGACTCGTACCCAGA-3′ (sense) and 5′-AACTCTGAGCTTCTGGCAGCACAATCCACA-3′ (antisense) for the second PCR. The mutagenesis was confirmed by sequencing, and the mutated prohepcidin cDNA was cloned into pTriex3-Neo or mCherry plasmids.

Indirect immunofluorescence

Immunofluorescence was performed as described previously [32], with the following modifications: anti-prohepcidin antibody (Alpha Diagnostics) was used as the primary antibody at a 1:100 dilution overnight and Cy3 (indocarbocyanine)-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) was used as the secondary antibody at a 1:300 dilution for 1 h. We used Hoechst 33258 staining to visualize the cell nuclei. Cells were imaged using an Olympus Fluoview FV1000 laser-scanning confocal microscope system with an UPlanSapo 60 × 1.35 N.A. (numerical aperture) oil-immersion objective. Cells labelled with only the secondary antibody were used to set the detector gain so that the signal was on the limit of visibility.

DNA binding prediction

We used the SVM (support vector machine)-based prediction algorithm developed by Kumar et al. [33] to predict binding of DNA to prohepcidin. Taking into account the relatively short sequence of prohepcidin, we performed the SVM model trained on the main dataset (DNASET) that is more suitable for protein chains or domains as input sequences. To ensure the highest specificity and to minimize the number of false positives we chose the highest available threshold (1.0). The method determines the SVM score for the input sequence and compares it with the set threshold: if the SVM score is higher than the threshold the protein is predicted to bind DNA. We performed the analysis using the 60-amino-acid prohepcidin as well as the 25-amino-acid hepcidin as the input sequence, on the basis of both amino acid composition and a PSSM (position-specific scoring matrix).

Luciferase reporter assay

WRL68 human hepatic cells (Health Protection Agency Culture Collections) grown in six-well plates (10⁶ cells/well) were transiently cotransfected with 1 µg/well pGL3/HAMP promoter and with 1 µg/well of one of the following constructs: pcDNA3.1/preprohepcidin, pcDNA3.1/preprohepcidin antisense or pTriex3-Neo/A1AT, using 2 µl of Transfectin reagent per 1 µg of DNA (Bio-Rad Laboratories). Cells were also transfected with PSV-β-galactosidase control vector (Promega) for normalization of transfection efficiency. In this manner, the PSV-β-galactosidase vector acts as an internal control for transient expression assays. After 24 h the cells were lysed in 150 µl of Luciferase Cell Culture Lysis reagent (Promega) and cellular extracts were analysed for luciferase activity by a Lumat 9507 Luminometer (Berthold Technologies) using the Luciferase Assay system (Promega). Cellular protein concentrations were measured with the DC Protein Assay kit (Bio-Rad Laboratories). The β-galactosidase activities of transfected cells were determined using the β-Galactosidase Enzyme Assay system (Promega) according to the manufacturer’s protocol. The luciferase activity was determined as described in the Luciferase Assay System.
Immunoprecipitation from bacteria

Escherichia coli BL21(DE3) cells were transformed with prohepcidin/pTriex3-Neo or A1AT/pTriex3-Neo constructs. Prohepcidin–His or A1AT–His protein expression was induced with 0.5 mM IPTG (isopropyl β-D-thiogalactosidase) for 2 h at 30°C. Bacteria were harvested and resuspended in TBST buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.5 % Triton X-100], then were lysed by sonication at 4°C. The supernatant was gently mixed in 5 ml of binding buffer with 30 μl of anti-(His tag) IgG-coupled CNBr-activated Sepharose 4B beads for 30 min at 4°C. The beads were collected and washed three times for 10 min in 5 ml of binding buffer. The 50 % suspension was used for the PCR-based protein–DNA binding assay, and 10 μl of elute was dotted on to a nitrocellulose membrane.

ChIP (chromatin immunoprecipitation) assay

ChIP analysis was performed according to the protocol of Carey et al. [34] with minor modifications. WRL68 cells (8 × 10⁷) were transiently transfected with prohepcidin/pTriex3-Neo plasmid DNA or empty pTriex3-Neo plasmid DNA for 24 h. Genomic DNA from WRL68 cells was fragmented by sonication to an average size of 400–1000 bp. Immunoprecipitation was carried out using CNBr-activated Sepharose 4B beads and monoclonal antibodies against the His tag (Penta-His, Qiagen). Anti-mouse IgG (Dako) was used as a negative control for the experiment. The eluted DNA was purified using the QiaQuick PCR purification kit (Qiagen). The PCR was performed using iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories) with 2 μl of purified DNA using the HAMP promoter (sense, 5'-AAAGTACTCATCGGACCTGATG-3' and antisense, 5'-AAAGTACGACTGGTTTATGGGG-3') and transin promoter-specific primers (sense, 5'-TATAAGATAAAAACCGACCCACCAT-3' and antisense, 5'-CAAAGAATTTCCAGAGTTCAAA-3').

PCR-based protein–DNA binding assay

Immunoprecipitated peptide-coated beads (10 μl) were mixed in 10 μl of 5 x Ziff buffer [50 mM Hepes (pH 7.4), 50 % glycerol, 500 mM NaCl and 5 mM EDTA] with 100 ng of ssDNA (single-stranded DNA), 1 μg of poly(dI/dC) and 0.1 pmol of HAMP, S1S2, S1, S2, S3, mS1S, mS1B or transin promoter DNA. The final volume was brought to 50 μl. The mixture was incubated for 30 min at room temperature and then the beads were washed with 800 μl of 1 x Ziff buffer three times for 10 min. The protein–DNA complex was eluted from the surface of the beads using 100 μl of glycine (100 mM, pH 2.5) and finally 20 μl of Tris/HCl (1 M, pH 8.0) was added to the reaction mixture for neutralization. A 1 μl aliquot of the eluted sample was used as a template for PCR. The PCR was carried out using iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories) in 20 μl of total reaction volume using the HAMP, S1S2, S1, S2, S3, mS1S, mS1B or transin promoter-specific primers. After the PCR 3 μl of the mixture was loaded on to a 3 % agarose gel and visualized by SYBRGold nucleic acid gel staining (Invitrogen). The gel images were obtained by using a Dark Reader (Clare Chemical Research) and a MultiGenius Bioimaging system (Syngene). To verify the specificity of the interaction the following control experiments were performed. The nuclear extract was omitted from the sample to exclude the non-specific binding of the beads to DNA. An immunoprecipitated sample prepared from WRL68 cells expressing only the His tag was used to prove that the beads do not bind non-specifically to other DNA-interacting proteins and that the His tag alone cannot bind to the HAMP promoter
probes. Finally, a 220-bp region of the transin promoter was used instead of the HAMP promoter to examine whether binding is specific to the HAMP promoter sequence.

**Determination of antibody specificity**

Preprohepcidin and hepcidin cDNA sequences were cloned into the pGEX-4T-1 expression vector (GE Healthcare). GST (glutathione transferase), preprohepcidin–GST or hepcidin–GST proteins were produced in *E. coli* BL21(DE3) cells transformed with the empty pGEX-4T-1 plasmid, preprohepcidin-GST/pGEX-4T-1 or hepcidin-GST/pGEX-4T-1 constructs after induction with 0.5 mM IPTG for 2 h at 30 °C. Cells were harvested, lysed in Laemmli buffer and the total cell lysates were subjected to Western blotting using an anti-prohepcidin antibody. The preprohepcidin–GST sequence was amplified by PCR using the preprohepcidin-GST/pGEX-4T-1 plasmid and the primers 5′-AAAGAATCTTATGTCCTCAACTAAGTTAT-3′ (sense) and 5′-AACCTCGAGGCTTGCCTTGACAGCACTCCACA-3′ (antisense) and then inserted into the pTriex3-neo plasmid. WRL68 cells were transiently transfected with empty pTriex3-neo plasmid or preprohepcidin-GST/pTriex3-neo plasmid for 24 h then were collected and resuspended into 100 μl of lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.1% Triton X-100]. The samples were subjected to Western blotting. The presence of prohepcidin–GST was detected by an anti-prohepcidin antibody. β-Actin was used as a loading control (Cell Signaling Technology). The preprohepcidin/pET-42a construct was expressed in Origami2 DE3 pLacI bacteria after induction with 0.5 mM IPTG for 2 h at 30 °C. Cells were harvested, resuspended into STE buffer [10 mM Tris/HCl (pH 8), 150 mM NaCl and 1 mM EDTA] and lysed by mild sonication at 4 °C in STE buffer with a final concentration of 1.5% sarcosyl. The supernatant was gently mixed with STE-washed glutathione–Sepharose 4B beads (GE Healthcare) at 4 °C for 1 h. Preprohepcidin–GST protein bound to beads was collected, washed three times for 10 min in 5 ml of STE buffer, eluted and then the presence of protein was detected by Western blotting using the anti-prohepcidin antibody. The eluted protein was used for the anti-prohepcidin antibody preabsorption. The anti-prohepcidin antibody was incubated with preprohepcidin–GST for 2 h at 4 °C and then was used in prohepcidin detection.

**RESULTS**

**Prohepcidin is located in the nucleus and in the cytoplasm of hepatocytes**

Intracellular localization of prohepcidin was studied by using indirect immunofluorescence and mCherry tagging. WRL68 human hepatic cells were labelled with anti-prohepcidin antibody and imaged using a laser-scanning confocal microscope to allow imaging of cellular cross-sections. In addition to the granular cytoplasmic staining, a fraction of prohepcidin was found in the nuclear region of hepatocytes (Figure 1A), although the fluorescence signal was relatively weak, probably owing to the low intracellular level of the endogenous peptide. To enhance the signal we overexpressed prohepcidin by transiently transfecting WRL68 cells with the pTriex3-Neo plasmid containing the HAMP gene. In this case we observed a similar pattern, but with a more pronounced nuclear localization (Figure 1B). We also examined the localization of prohepcidin in live WRL68 cells transiently expressing mCherry-tagged prohepcidin. Confocal images showed strong nuclear localization with a granular cytoplasmic pattern (Figure 1C and Supplementary Movie S1 at http://www.biochemj.org/bj/451/bj4510301add.htm).

To confirm the ability of prohepcidin to bind to DNA, we performed a ChIP assay. Formaldehyde-cross-linked prohepcidin-bound DNA fragments were immunoprecipitated from WRL68 cells overexpressing the prohepcidin–His peptide using an anti-His antibody. The ChIP assay revealed significant amounts of the HAMP promoter bound to prohepcidin (Figure 2), raising the possibility of an interaction between prohepcidin and the promoter region of its own gene.

To obtain independent support for the DNA-binding property of prohepcidin, we developed a PCR-based promoter binding assay and we further analysed the interaction between the peptide and the promoter region of the HAMP gene. The promoter-binding assay was performed using WRL68 cells overexpressing the prohepcidin–His peptide. The peptide was purified from the nuclear extract using the anti-His antibody-coated CNBr-activated Sepharose beads, then the washed beads were incubated with the HAMP promoter. The peptide–DNA complex was eluted from the surface of the beads, and the eluate was used as a template in a PCR with promoter-specific primers for the detection of the prohepcidin-bound HAMP promoter. As our results show (Figure 3A), we detected promoter binding only in the sample in which prohepcidin–His was incubated with the HAMP promoter. We did not observe promoter binding in case of controls where: (i) the nuclear extract was omitted from the sample; (ii) the nuclear extract of cells expressing only the His tag was used; or (iii) transin promoter was used instead of the HAMP promoter. These controls confirmed that: (i) the anti-His antibody or the CNBr-activated
Prohepcidin autoregulates its own expression

Figure 1  Prohepcidin is located in the cytoplasm and in the nucleus of WRL68 human hepatic cells

Intracellular localization of endogenous (A) and overexpressed (B) prohepcidin in WRL68 cells determined by indirect immunofluorescence using an anti-prohepcidin antibody followed by indocarbocyanine (Cy3)-conjugated anti-rabbit secondary antibody (left-hand panels). The sample labelled with only the secondary antibody was used to set the detector gain so that the signal was on the limit of visibility. Hoechst staining was used to visualize cell nuclei (middle panels). Merged images are shown in the right-hand panels. (C) Intracellular localization of the mCherry tag alone, prohepcidin–mCherry, and NLS mutant prohepcidin–mCherry within live WRL68 cells visualized by confocal microscopy. Scale bar, 20 μm. Figures are representative of three biological replicates and the observed localization pattern was always more that 85 % of the transfected cells. (D) Amino acid sequence of native and NLS mutant prohepcidin. Putative NLS sequences (PMFQRTRR and RRRR) are marked with lines and furin cleavage sites (RXRRX) are framed. The position of amino acid substitution (arginine to threonine) within the prohepcidin sequences are shown in bold. (E) The specificity of the anti-prohepcidin antibody was determined by Western blot analysis of total lysate of WRL68 cells not expressing or expressing prohepcidin (prophepcid)-GST using an anti-prohepcidin antibody, with and without pre-incubating the antibody with control peptide before immunoblotting. β-Actin served as a loading control. Molecular mass is given on the left-hand side in kDa.

Sepharose beads alone do not bind to the HAMP promoter; (ii) the anti-His antibody does not capture other HAMP promoter-binding proteins and the His-tag alone is not able to bind DNA or HAMP promoter-binding proteins; and (iii) the interaction between the HAMP promoter and prohepcidin is specific.

In a similar experiment we first pre-incubated the HAMP promoter with the nuclear extract of WRL68 cells overexpressing the prohepcidin–His peptide and then isolated the HAMP–prohepcidin–His complex using anti-His CNBr-activated Sepharose beads. Similarly to our previous result, we detected promoter binding only from the sample in which the prohepcidin–His peptide and the HAMP promoter were present (results not shown). These results clearly suggest a specific interaction between prohepcidin and its own promoter.

However, it is possible that prohepcidin did not bind to the beads alone, but as part of a larger complex that might contain other...
DNA-binding protein(s). The presence of such protein(s) could also explain the positive result of the HAMP promoter-binding assay. To find out whether prohepcidin can bind to the promoter region of the HAMP gene by itself or as a member of a complex, we expressed prohepcidin–His in BL21 bacterial cells in order to prevent the potential formation of a complex with nuclear DNA-binding proteins. The immunoprecipitated peptide was subjected to the promoter-binding assay using exactly the same method as in previous experiments. The results of the present study show that we could amplify the HAMP promoter region only from the sample which contained the bacterially expressed prohepcidin–His peptide, whereas DNA binding was not detected in the control samples (Figure 3B). These results confirm that prohepcidin alone is able to bind to the HAMP promoter.

Prohepcidin binds to the single STAT3 site within the HAMP promoter

To narrow down the boundaries of the prohepcidin-binding region within the HAMP promoter we first divided the 942-bp long promoter sequence into three smaller regions, S1 (+1 to −175), S2 (−175 to −350) and S3 (−350 to −942) (Figure 4A), and then performed the binding assay with these smaller regions. We found that the S1 region alone was sufficient for the binding of prohepcidin (Figure 4B).

The +1 to −942 interval of the HAMP promoter contains many transcription factor-binding motifs (Figure 4A), but the single STAT3 site (TCTTTGAAA, between positions −64 and −72) is the only one which is found exclusively in the S1 region. To examine whether this site is responsible for the binding, we constructed a deletion mutant lacking this nine-nucleotide-long sequence (mS1S) and repeated the HAMP promoter-binding assay using this sequence. Deletion of the STAT3 site resulted in the loss of binding to prohepcidin, as well as to the transcription factor STAT3 which was used as a control (Figure 4C, lanes 4 and 5). In contrast, when the BMP-RE site adjacent to the STAT3 site was deleted (mS1B), the DNA–prohepcidin interaction remained intact (Figure 4D). This suggests that the nine-nucleotide-long STAT3 site within the S1 region is important for the binding of prohepcidin.

We repeated the assay with the NLS mutant prohepcidin. Since the NLS mutant prohepcidin was found to localize predominantly in the cytoplasm (Figure 1C) whole-cell extract of WLR68 cells expressing NLS mutant prohepcidin was used (Figure 4E, lanes 1–3). According to our results, the NLS mutant prohepcidin did not bind to the HAMP promoter. No bound DNA was detected in our negative control either, where the nuclear extract was used (Figure 4E, lanes 4–8). Since the His-tagged NLS mutant did not bind DNA, we can exclude that binding of His-tagged prohepcidin to DNA occurs via the His tag.

Prohepcidin down-regulates its own gene expression

Having identified prohepcidin as a HAMP promoter-binding peptide, we turned our attention to confirming whether this interaction has a HAMP gene regulatory effect. To determine the effect of prohepcidin binding on the HAMP gene expression we compared the HAMP promoter activity at different intracellular prohepcidin levels.

To increase the constitutive level of prohepcidin in WRL68 cells, we transiently transfected the cells with a pcDNA3.1 expression plasmid containing preprohepcidin cDNA. In addition, the intracellular prohepcidin level was reduced by transfecting the cells with pcDNA3.1 plasmid containing preprohepcidin antisense DNA. To help with the quantification of the HAMP promoter activity we cloned the 942-bp fragment of the HAMP promoter as a transcriptional fusion with firefly luciferase, resulting in the pGL3/HAMP promoter construct that was cotransfected with the pcDNA3.1/preprohepcidin or the antisense construct into WRL68 cells. We measured the expression levels of pre-prohepcidin mRNA by real-time PCR, the expression levels of prohepcidin peptide by ELISA and the activation of the promoter by luciferase assay.
Prohepcidin autoregulates its own expression

At a normal prohepcidin level the HAMP promoter–luciferase reporter vector exhibited basal activity in WRL68 hepatocytes (Figure 5A, bar 1). The activity of the HAMP promoter decreased to 52% of the original level in cells harbouring the overexpression construct (Figure 5A, bar 2), where the preprohepcidin mRNA level was 435-fold higher and the prohepcidin peptide levels in cells and in the medium were more than 3- and 1.5-fold higher respectively (Table 1). An even more significant effect was detected upon diminishing the amount of prohepcidin by transiently transfecting WRL68 cells with preprohepcidin antisense DNA. When the intracellular prohepcidin mRNA level decreased to 63% and the amount of prohepcidin was decreased to 66% of the normal level (Table 1), the activity of the HAMP promoter increased up to 5.2-fold compared with the original level (Figure 5A, bar 3). The expression of the NLS mutant prohepcidin did not change the activity of the HAMP promoter (Figure 5A, bar 4).

To confirm the importance of the single STAT3 site in prohepcidin-mediated regulation, we examined the effect of altered prohepcidin levels on the STAT3 site-deleted HAMP promoter (mS1S). We detected neither decreased promoter activity upon prohepcidin overexpression (Figure 5A, bars 6 and 7) nor elevated promoter activity upon preprohepcidin antisense treatment (Figure 5A, bars 6 and 8), suggesting that the regulatory effect of prohepcidin is lost if the STAT3 site within the HAMP promoter is deleted.

Next we verified whether the propeptide region alone is sufficient to influence the activity of the HAMP promoter. We created a fusion protein containing the propeptide region of prohepcidin fused to a cytoplasmic enzyme, GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Overexpression of the preproGAPDH protein in WRL68 cells did not change the activity of the HAMP promoter (Figure 5A, bar 5), indicating that the propeptide region alone is not sufficient for the effect on promoter activity. Finally, the elevated or decreased level of prohepcidin had no effect on the activity of the promoter region of the transin gene (Figure 5A, bars 9 and 10), indicating that the interaction between prohepcidin and the HAMP promoter is specific. These observations suggest that prohepcidin regulates its own gene expression by down-regulating its promoter activity.
The activity of the HAMP promoter is affected by the level of prohepcidin or A1AT

(A) The intracellular level of prohepcidin was increased or decreased by transiently transfecting WRL68 cells with a preprohepcidin expression vector (bars 2, 7 and 9) or the preprohepcidin antisense construct (lanes 3, 8 and 10). The 942-bp fragment of the human HAMP promoter (bars 1–5), STAT3 site-deleted S1HAMP promoter region (bars 6–8) or transin promoter fragment (bars 9 and 10) as a transcriptional fusion with firefly luciferase was co-transfected into WRL68 cells. After 24 h the luciferase activity of cell extracts was measured and compared with the activity of WRL68 cells with an unaltered prohepcidin level, defined as 100% (bar 1). (B) WRL68 cells were cotransfected with the A1AT expression vector and the HAMP promoter transcriptional fusion with the firefly luciferase gene. Luciferase activity of cell extracts was compared with the activity of WRL68 cells with unaltered A1AT levels, defined as 100%. Results are means ± S.E.M. for three independent experiments.

Table 1 Changes in prohepcidin mRNA and peptide levels for overexpression and prohepcidin antisense DNA treatment

<table>
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<th>Normalized prohepcidin mRNA</th>
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Sequestration of prohepcidin by A1AT prevents down-regulation of HAMP gene expression

We have previously shown that although mature hepcidin does not, prohepcidin specifically binds to A1AT in the cell and in the serum [31], indicating the role of A1AT in the post-translational modification of prohepcidin. In order to find out if this interaction has any influence on HAMP gene expression, we increased the amount of A1AT in WRL68 cells and analysed its effect on HAMP promoter activity using a luciferase assay. When we overexpressed A1AT, the activity of the HAMP promoter increased up to 3.3-fold compared with HAMP promoter activity in cells with a normal level of A1AT (Figure 5B). The elevated promoter activity suggests that the A1AT-bound prohepcidin may not be able to down-regulate HAMP gene expression.

To study the effect of A1AT on the binding of prohepcidin to the HAMP promoter, we overexpressed A1AT in WRL68 cells, purified the protein by immunoprecipitation and analysed the sample with dot blot analysis using anti-hepcidin antibodies. As expected, we could detect A1AT-bound prohepcidin, confirming the interaction of the two proteins (Figure 6A). Next the immunoprecipitated A1AT–prohepcidin complex was subjected to a HAMP promoter-binding assay. Our observation was that neither prohepcidin-free A1AT (bacterially expressed A1AT) nor A1AT-bound prohepcidin (from WRL68 cells) were able to bind to the HAMP promoter (Figure 6B).

Taken together, the results of the present study suggest that only the non-A1AT-bound prohepcidin binds to the promoter site, and, as a consequence, down-regulates HAMP gene expression.

DISCUSSION

In the present study we present data supporting that prohepcidin is localized not only to the cytoplasm, but also to the nucleus of hepatocytes. Our in vitro binding results indicate that the
peptide located in the nucleus has the potential to bind the HAMP promoter. Consistent with these results, we showed that the overproduction of prohepcidin in WRL68 cells decreased HAMP promoter activity, whereas a decrease in prohepcidin expression enhanced expression from the HAMP promoter. The presence of the STAT3 site was essential for the binding of prohepcidin and the regulation of promoter activity. Moreover, the known interaction between A1AT and prohepcidin prevents this autoregulatory effect.

Since the discovery of prohepcidin, its intracellular localization has been studied in different cell lines by several research teams. In an initial report Pigeon et al. [3] had already shown that the nuclear localization of the GFP (green fluorescent protein)-tagged murine prohepcidin, but the fact that the GFP tag was placed to the N-terminus of prohepcidin raised the possibility of an aberrant processing and trafficking of the peptide [28]. This seems to have confused the control experiment by Pigeon et al. [3] in which the putative NLS sequence was deleted from preprohepcidin and the resulting mutant was fused to GFP. In this configuration the peptide was detected predominantly in the cytosol, confirming the functional importance of the NLS sequence and raising the possibility that the nuclear localization of the peptide has a biological function. Wallace et al. [28,29], studying the intracellular localization of mouse and human hepcidin, have found that the peptide behaves as a secretory protein. Using indirect immunofluorescence analysis they showed that in HEK (human embryonic kidney)-293 cells the human hepcidin is located predominantly in the Golgi apparatus. In an independent study the immunohistochemistry of human and guinea pig livers showed hepcidin staining only at the basolateral membrane of hepatocytes, mainly around the portal triads [10]. However, in the same study, and in a later publication as well [30], the detection of hepcidin in HepG2 cells by immunofluorescence microscopy showed strong granular cytoplasmic distribution.

For the present study we chose WRL68, a human hepatic cell line exhibiting morphology similar to hepatocytes and hepatic primary cultures [36]. On the basis of our immunofluorescence and mCherry-tagging data, which showed granular cytoplasmic prohepcidin distribution and nuclear localization, we assumed that prohepcidin may have a role in gene expression. We studied prohepcidin–DNA interaction by performing a conventional ChIP assay and we developed a PCR-based promoter-binding assay. The latter assay combined the immunoprecipitation of the peptide with an EMSA (electrophoretic mobility-shift assay)-like protein–DNA binding reaction, followed by the detection of the bound DNA with PCR. Using this assay we proved that prohepcidin exhibits a strong binding to the single STAT3 site within the HAMP promoter (between positions −64 and −72), indicating a possible role of prohepcidin in the regulation of its own synthesis. To confirm this hypothesis, we studied the effect of altered levels of prohepcidin on the activity of the HAMP promoter in vivo. We found that both increasing and decreasing intracellular prohepcidin levels changed HAMP promoter activity. When we decreased the level of prohepcidin using an antisense construct, the HAMP promoter activity increased significantly, whereas overexpression of prohepcidin reduced the promoter activity to a lesser, but still significant, extent. These results support the autoregulatory effect of prohepcidin. However, the details of the in vivo binding mechanism remain to be elucidated. It is possible that in hepatocytes this peptide binds to its own promoter alone or as a member of a larger protein complex, in which the role of prohepcidin might be the recognition of and binding to the HAMP promoter site.

Although the nucleus seems to be an unlikely destination for a secreted protein, it is not unexampled. One well-documented example of a protein shown to be located in the nucleus before its secretion is the high molecular weight isoform of FGF-2 (basic fibroblast growth hormone). Other examples include BMP2, BMP4 and Gdf5 (growth differentiation factor 5)/CDMP1 (cartilage-derived morphogenetic protein 1) [37]. All three belong to the family of BMPs, the largest group of the TGF-β superfamily. BMPs originally have been recognized only as secreted growth factors. Their fate within the cell shows similarity with that of hepcidin. Just like hepcidin, BMP2, BMP4 and Gdf5/CDMP1 are synthesized as inactive proproteins. Their translation is directed to the rough endoplasmic reticulum by their N-terminal signal peptides, where the proproteins are cleaved at their convertase-recognition sites by prohormone convertases such as furin, releasing the C-terminal mature peptides which are then secreted from the cell [38,39]. Previously the nuclear variants of these three proteins were detected in a variety of cell lines [37]. The nuclear variants of BMP2, BMP4 and Gdf5/CDMP1 (nBMP2, nBMP4 and nGdf5/CDMP1) carry a functioning bipartite NLS overlapping with their proprotein convertase-recognition sites. Similarly, the amino acid sequence analysis of human prohepcidin performed by the PSORTII program predicted a putative NLS that overlaps with the furin convertase-recognition site (Figure 1D). The results of the present study confirmed the existence of a functional NLS within the sequence of prohepcidin. The intracellular pattern of the NLS-mutant prohepcidin was dramatically different from that of the wild-type peptide and the mutant peptide had no HAMP gene regulatory effect at all. This leads to the possibility of a strong connection between NLS and the DNA-binding ability.

The autoregulatory effect observed in the case of prohepcidin is also supported by known examples. Transcription factors have been known to act as repressors or activators of their own transcription including the hepatocyte-enriched trans-activator LFB1/HNF1 (hepatic nuclear factor 1) [40], the homeodomain Oct4 (octamer 4) [41] or Pax6 (paired box 6), the transcription factor essential for normal development of the eyes and the nervous system [42]. The tumour suppressor protein p53 has also been shown to autoregulate its own transcription by numerous autoregulatory loops, including direct binding to its own promoter [43]. However, there is no agreement on whether wild-type p53 trans-activates or down-regulates its own transcription [44–47].

The results of the present study also suggest an important role for A1AT in HAMP gene expression through the regulation of free prohepcidin level. We have previously shown that the serine protease inhibitor A1AT interacts with prohepcidin in vivo [31]. In the present work we used A1AT as a direct internal agent to reduce the free prohepcidin pool. When the free prohepcidin level was decreased owing to the overexpression of A1AT, the activity of the HAMP promoter was elevated approximately 3-fold. Additionally, the HAMP promoter-binding assay using A1AT-bound prohepcidin did not detect its interaction with the HAMP promoter. Accordingly, the elevated activity of the HAMP promoter owing to the overexpression of A1AT can be explained by the sequestration of free prohepcidin by A1AT, which, as a consequence, loses its ability to down-regulate HAMP gene expression. Taken together, these results confirm that only free prohepcidin has an autoregulatory effect.

These data have led to a hypothetical model in which prohepcidin is not just a precursor of the iron regulatory hormone hepcidin, but it behaves as a biologically active peptide with a distinct function: influencing HAMP transcription (Figure 7). Soon after being transcribed, the 84-amino-acid long preprohepcidin is cleaved to a 60-amino-acid long prohepcidin. Free prohepcidin may undergo the final cleavage step made by furin and other proprotein convertases creating the biologically
active hormone hepcidin, or it may translocate into the nucleus where it directly inhibits its own gene activity. Although the exact molecular mechanism is not deciphered yet, it seems probable that A1AT plays an important regulatory role by binding to prohepcidin and preventing its final maturation together with blocking its nuclear translocation. Nevertheless, it would be interesting to assess if this phenomenon exclusively belongs to cancer-derived cell lines or it is a general property of primary hepatocytes as well.

Although the regulation of hepcidin at the transcriptional level has been intensively investigated, considerable evidence strengthens the importance of post-translational control as well. In a recent study the quantification of hepcidin indicated that the amount of hepcidin in serum and urine significantly, but only slightly, correlates with the levels of liver transcript in the whole population [48]. Another study found that the serum hepcidin concentration does not correlate with the expression of HAMP mRNA in either cancerous or non-cancerous liver tissue of patients suffering from HCC (hepatocellular carcinoma) [49]. The autoregulatory effect of prohepcidin may be a new component of the hepcidin level-regulating apparatus, providing a mechanism where changes at the post-translational level may influence transcriptional-level processes. For example, in case of an effect that could inhibit or retard the prohepcidin–hepcidin conversion, this autoregulation may provide a negative-feedback mechanism preventing the excessive intracellular accumulation of prohepcidin. Nevertheless, the factors influencing the activity of this autoregulatory system remain to be elucidated.

Unraveling the molecular mechanisms of prohepcidin autoregulation may also help the elucidation of pathophysiological processes. For example, the presence of a mutant A1AT allele is frequently associated with disturbance of iron homeostasis. Point mutations within the A1AT gene cause perturbation in protein structure followed by polymerization and intracellular accumulation of A1AT in the endoplasmic reticulum of hepatocytes, and, as a consequence, hereditary A1ATD (A1AT deficiency). One of the possible clinical manifestations of A1ATD is HCC, although the association is not clarified yet [50]. Notably, a study demonstrated that the expression of HAMP mRNA is suppressed in cancerous, but not in non-cancerous, liver tissue of patients with HCC [49]. The link between A1ATD and HCC with suppressed HAMP expression raises the possibility of an altered HAMP regulation owing to the mutant A1AT. This may lead to lower hepcidin levels and, as a consequence, iron overload, a major risk factor in the development of HCC [51]. Further studies will require the clarification of the importance of prohepcidin autoregulatory mechanisms influenced by A1AT in vivo, which may contribute to a better understanding of the association of mutations in A1AT and various disorders of iron homeostasis.

**Figure 7** Hypothetical model showing the relation of the autoregulatory effect of prohepcidin and its sequestration by A1AT to the expression and maturation pathway of hepcidin
29 Pinson, J., Simpson, T. I., Mason, J. O. and Price, D. J. (2006) Positive autoregulation of the transcription factor Pax6 in response to increased levels of either of its major isoforms, Pax6 or Pax6(5a), in cultured cells. BMC Dev. Biol. 6, 25
33 Pinson, J., Simpson, T. I., Mason, J. O. and Price, D. J. (2006) Positive autoregulation of the transcription factor Pax6 in response to increased levels of either of its major isoforms, Pax6 or Pax6(5a), in cultured cells. BMC Dev. Biol. 6, 25
SUPPLEMENTARY ONLINE DATA
Prohepcidin binds to the HAMP promoter and autoregulates its own expression

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Figure S1 The specificity of the anti-prohepcidin antibody was determined by Western blot analysis of total lysate of BL21 cells expressing GST, hepcidin–GST or prohepcidin–GST proteins using the anti-prohepcidin antibody, with and without pre-incubating the antibody with control peptide before immunoblotting.

Molecular mass is given on the left-hand side in kDa.

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