Published in final edited form as:

Biochim Biophys Acta. 2013 December ; 1829(12): 1257–1265. doi:10.1016/j.bbagr.2013.10.002.

Regulation of HFE expression by Poly(ADP-ribose) polymerase-1 (PARP1) through an inverted repeat DNA sequence in the distal promoter

Christopher Pelham[#], Tamara Jimenez[#], Marianna Rodova, Angela Rudolph, Elizabeth Chipps, and M. Rafiq Islam^{*}

Biochemistry Laboratory, Northwest Missouri State University, Maryville, MO 64468

[#] These authors contributed equally to this work.

Abstract

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder of iron overload among Caucasians of northern European descent. Over 85% of all cases with HH are due to mutations in the hemochromatosis protein (HFE) involved in iron metabolism. Although the importance in iron homeostasis is well recognized, the mechanism of sensing and regulating iron absorption by HFE, especially in the absence of iron response element in its gene, is not fully understood. In this report, we have identified an inverted repeat sequence (ATGGTcttACCTA) within 1700 bp (−1675/+35) of the HFE promoter capable to form cruciform structure that binds PARP1 and strongly represses HFE promoter. Knockdown of PARP1 increases HFE mRNA and protein. Similarly, hemin or FeCl₃ treatments resulted in increase in HFE expression by reducing nuclear PARP1 pool via its apoptosis induced cleavage, leading to upregulation of the iron regulatory hormone hepcidin mRNA. Thus, PARP1 binding to the inverted repeat sequence on the HFE promoter may serve as a novel iron sensing mechanism as increased iron level can trigger PARP1 cleavage and relief of HFE transcriptional repression.

Keywords

HFE; PARP1; Negative element; Inverted repeat; Cruciform; HAMP

1. Introduction

Hereditary hemochromatosis (HH) is an autosomal recessively inherited disorder of iron metabolism, affecting about 1 in 400 individuals of Northern European descent. The disease is characterized by excessive intestinal iron absorption and progressive iron overload throughout the body [reviewed in 1], leading to damaged liver. Feder et al [2] showed that homozygosity for mutation (C282Y, G>A) in the HFE gene was responsible for common phenotypic HH. This single mutation in the HFE accounts for approximately 85% of HH cases, although other rare mutations in the HFE coding region as well as mutations in few

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^{*}Corresponding author: Biochemistry Laboratory Northwest Missouri State University Maryville, MO 64468 Tel: 660-562-3118 islamr@nwmissouri.edu.

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other genes involved in iron metabolism such as transferrin receptor 2, hepcidin, ferroportin, and hemojuvelin, have also been associated with HH and abnormal iron profile [3-4]. Iron overload resulting from mutations of HFE or these genes occurs due to dysregulation of the iron-regulatory hormone, hepcidin encoded by HAMP gene, produced primarily in the liver. Hepcidin negatively regulates iron absorption and recycling by reducing surface levels of the iron export protein, ferroprotein on duodenal enterocytes and macrophages [46].

The *Hfe* knockout (*Hfe*^{-/-}) and C282Y knock-in *Hfe* mice develop iron overload, confirming that loss of HFE gene as well as the HFE-C282Y mutation confer abnormal iron metabolism [5-7]. HFE is an atypical member of the major histocompatibility complex class I proteins. Similar to other members of this family, HFE consists of a transmembrane domain and a short cytoplasmic tail [reviewed in 8]. However, unlike typical members of the family, it does not contain the peptide binding domain and is not involved in antigen peptide-presentation. Rather studies from HFE deficient patients and *Hfe*-knockout mice with iron overload suggest a role in HAMP expression [47]. Thus, importance of HFE in iron regulation became apparent from these and other studies with HH patients and murine models, the underlying mechanism by which HFE regulates iron homeostasis is only beginning to be understood. The data obtained thus far strongly suggest that modifier genes contribute to regulatory capacity of the HFE gene [5] and a signaling pathway that senses iron status may depend on the HFE gene.

Poly (ADP-ribose) polymerase-1 (PARP1) is a ubiquitous, abundant and highly conserved nuclear protein of ~116 kDa [9]. It is the founding member of the PARP family and accounts for more than 90% of catalytic activity in cells [10]. PARP1 is a multifunctional protein and has a number of biochemical activities. It possesses an NAD⁺-dependent catalytic activity that cleaves NAD⁺ into nicotinamide and ADP-ribose, then polymerizes the latter into negatively charged polymer called poly (ADP-ribose) or PAR on target proteins. In addition, PARP has been shown to be involved in the regulation of chromatin structure and transcription, DNA methylation, insulator activity, and chromosome organization [reviewed in 11].

PARP1 binds to DNA using an amino terminal, DNA binding domain with three zinc fingers, one of which is required for NAD⁺-dependent catalytic activity of PARP1. Its carboxyl-terminal catalytic domain catalyzes PARylation of target proteins [12-13]. Genome-wide search localized PARP1 to the promoters and enhancers of many actively transcribed genes, and the pattern correlates with active gene expression [14]. A cross-shaped DNA structure, called cruciform DNA, observable under the electron microscope, can be formed by complementary perfect or imperfect inverted repeats of 6 or more nucleotides in the DNA sequence. The sequence refolds into hairpin loops on opposite strand across from each other. Among the DNA binding proteins, PARP1 exhibits only a weak sequence preference but binds preferentially to cruciform structures. The order of PARP1's substrate preference has been shown to be: cruciform > loop > linear DNA [15]. PARP1 binding to cruciform structure in plasmid DNA results in relaxed plasmid DNA conformation [16].

Some studies on transcriptional regulation by PARP1 provided evidence that the enzymatic activity of PARP1 is required [17-18], whereas others have indicated that it is not [19-21]. Thus, transcriptional regulation by PARP1 may or may not require its enzymatic activity.

In this report, to gain new insight into human HFE expression, we focused on a 1700 bp HFE promoter including the transcription start site and have identified a negative element, an inverted repeat sequence, in the distal HFE promote. We further demonstrated that nuclear protein, PARP1 bound to this sequence negatively regulating HFE expression.

Diminishing cellular PARP1 by means including iron treatment increases HFE. We speculate that, in the absence of iron response element, HFE utilizes PARP1 in its own expression in response to excess iron.

2. Materials and Methods

2.1 Plasmid constructs

A 1,674 bp human HFE promoter fragment (−1638 to +37) in the promoterless luciferase reporter vector pGL3-Basic (Promega) was PCR generated using primer pairs H1m and H4 (Table 1), digested and cloned at Sma I and Kpn I sites of the vector. Promoter deletion constructs, H870 bp and H210 bp, in pGL3-Basic were cloned using Sac I and Hind III sites of the vector, respectively. The sequential deletion constructs between H871 and H214 were created by PCR cloning using a forward primer harboring the designed Kpn I site in combination with the reverse primer GL3R in the vector (Table 1). Following PCR, the fragment of varying sizes were restricted and cloned into Kpn I site of the vector. The orientations of the inserts were determined by Hind III digestion. The sequences were verified by sequencing.

All PCRs were performed in similar fashion: following a denaturation step at 95°C for 3 min, 30 cycles of 94°C for 30 sec, respective annealed temperature (53–60°C) for 45 sec, and 72°C for 45 sec, with a final extension of 7 min at 72°C.

The putative repressor binding site in H810 was deleted using the primer pairs H72d1 and d2 (Table 1) and the QuikChange site-directed mutagenesis kit (Stratagene) following the Supplier's instruction. The back slashes within the primer sequence represent the deletion site. Following verification by sequencing, the deleted fragment was subcloned into pGL3 vector. After 5'-phosphorylation, duplex H72d1 and d2 primers was cloned at Sma I site, 5' to the H210 construct (dH210) as well as to the 200 bp proximal PKD1 promoter (dPKD). Wild-type duplex primers (hfee11 and 12) in the same region was also cloned at Sma I site, 5' to the H210 construct (pH210) as well as to the 200 bp proximal PKD1 promoter (pPKD). Orientations of these constructs (d or p) were confirmed by PCR.

2.2 Cells, transfections and reporter assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose, HCT116 p53^{−/−} cells were maintained in McCoy's media, HepG2 in and HeLa in Eagle's minimal essential medium with 2 mM L-glutamate, and 1 mM pyruvate. All media are supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotic (100 IU/ml penicillin, 100 µg/ml streptomycin). All cells were grown at 37°C supplied with 5% CO₂. Following overnight culturing in six-well (or 12-well) plates, the cells were transfected using either the calcium phosphate method (HEK293T and HCT116 cells) or with Lipofetamine (Gibco) (HepG2, HeLa cells) according to the manufacture's instruction. A β-galactosidase expression plasmid (50 ng) was included to monitor transfection efficiency. After 6 hr, the DNA-containing medium was removed, and the cells were incubated with growth media containing, and harvested at 40 hours as described earlier [22]. Luciferase assays were carried out in 1x reporter lysis buffer (Promega) using Luminol (Promega) as a substrate and β-galactosidase assays were performed using o-nitrophenyl-β-D-galactopyranoside (Sigma) as a substrate. When desired, protein concentrations were determined with the BCA protein assay kit (Pierce). The measured luciferase activity in each sample was normalized to either β-galactosidase activity. These normalized luciferase activities (RLU) were plotted using Microsoft Excel as average ± SD of triplicate samples from typical experiments. Experiments were repeated at least three times.

For caspase 3/7 assay 293T cells were grown in 24 well plate in 0.4 ml growth media overnight prior to treatment with FeCl₃, Hemin, TNF α and vehicle citrate. After 24 hr 0.3 ml of the media were removed and 100 μ l of the Caspase 3/7 substrate containing buffer was added following the supplier's instructions.

Transfection with siRNA was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. Briefly, 80 pmol of duplex siRNA for scramble, human Dicer, human Sp1 or human PARP1 in FBS and antibiotic-free DMEM was added to cells and incubated for 6 hr. Following that, serum was added to 1% and incubated overnight at 37°C. Equal volume of fresh growth media (with 10% serum) was added next morning and harvested at 40 h.

2.3 Endogenous expression

HEK293T cells were transfected with the control vector or antisense RNAs for 40 hr. A set of cells was also treated with 75 μ M Hemin in 0.1 M NaOH. Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol, and the samples were treated with DNase I (Ambion). Reverse-transcription was carried out with 1 μ g of total RNA using random hexamer at 42°C for 1 hr using a Promega kit. PCR of the HFE and L7 was performed for 30 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec, with a final extension of 7 min at 72°C. While PCR of the hepcidin was performed for 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, with a final extension of 7 min at 72°C. Amplified PCR fragments were electrophoresed on 2% agarose gels containing ethidium bromide. Specific primers for ribosomal protein, L7 as control (L7-1 and -2) giving a 400 bp product, h63df and hh4r for HFE giving a 300 bp product and hpcdn3 and hpcdn2 giving a product of 190 bp product.

Real-time (Quantitative) PCR reactions were run with 5 ng of original input RNA (in 5 μ l), 10 pmol of each primer for HFE or L7 and GoTaq qPCR master mix (Promega) in a total reaction volume of 20 μ l and the products were detected by syber green in a Bio-Rad CFX96 cycler. The products were confirmed by melting temperatures. The qPCR condition for L7 and HFE amplification was: 95°C for 3 min and then 40 cycles of three steps: 30 sec at 95°C, 62 sec at 62°C and 45 sec at 72°C. The qPCR condition for L7 and Hepcidin amplification was: 95°C for 10 min and then 60 cycles of two steps: 30 sec at 95°C, 20 sec at 51°C and 45 sec at 72°C. The "cycle threshold" C_t values were selected from the linear part fluorescence signal between 300-400 RFU. Δ C_t was determined as: Δ C_t = Avg. PKD1 C_t - Avg. L7 C_t, and gene-fold expression was calculated according to the Livak method, $2^{-\Delta\Delta C_t}$.

2.4 Western Immunoblotting

Expression of various proteins in cells was confirmed by 10 % SDS-PAGE, followed by immunoblotting using respective primary antibodies: mouse monoclonal anti-PARP1 (SantaCruz, sc-74469, 1:1,500), mouse monoclonal anti- β -actin (Sigma, A5316, 1:10,000), rabbit anti-HFE (sc133654, 1:500). Alkaline phosphate-conjugated secondary anti-rabbit (Sigma, A8025) or anti-mouse (Sigma, A7434) antibodies were used at 1:10,000 dilution.

The membrane was incubated with substrate CDP-Star (Amersham) for 5 min following equilibration in chemiluminescent buffer (0.1 M diethanolamine, 1 mM MgCl₂, pH 9.5) and exposed to film (RPI). In case of re-probing, the blots were stripped off primary and secondary antibodies using 20 mM Tris-HCl, pH 6.8 containing 1% SDS and 100 mM 2-mercaptoethanol at 55°C for 60 min with shaking at 125-135 rpm, followed by extensive washing and blocking in 5% non-fat dry milk in TBST at least for 2 h.

2.5 Chromatin immunoprecipitation-PCR

Chromatin immunoprecipitation (ChIP) of untransfected or transfected HEK293T cells were carried out as described in (Islam et al, 2010) [22-23] using supernatants rabbit preimmune antisera (Sigma), anti-Dicer, anti-Parp or anti-Sp1 antibodies. PCR of the input and specific and nonspecific immunoprecipitated DNA was performed for 35 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, with a final extension of 7 min at 72°C using the primers hfee11 and H7r for endogenous promoter, and GL3F and H7r for exogenous promoter.

2.6 Nuclear Extract, DNA-pull down and EMSA

Nuclear extracts was prepared from HEK293T cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, 78833) following the manufacturer's instruction. Biotin labeling by A-tailing, binding reaction, gel separation, and detection were carried out as described earlier [24]. For pull down, following binding with pre-cleared nuclear extracts, the reaction mixture was diluted with 250 µl of 1x PBS containing 0.5% NP40, 0.5% TX-100, 0.1% SDS, 1 mM EDTA and protease inhibitor cocktail. Streptavidin-agarose (30 µl) was added followed by rotation on a wheel for 4 h at 6°C. Agarose-bound streptavidin was collected by centrifugation for 1 min at 16,300 g. The pull-down product was washed 3 times with 10 mM Tris-HCl, pH 8.0 containing 150 mM NaCl, 0.5% NP40, 0.5% Tx-100, 0.1% SDS and 1 mM EDTA and boiled for 5 min in SDS-sample buffer prior to SDS-PAGE. The band cut out and analyzed by LC-MS/MS at the Mass Center of the University of Kansas Medical Center.

For electromobility shift assay (EMSA) binding reactions with nuclear extracts in the absence or presence of annealed primer (for competition, 2 x of probe) or mouse monoclonal anti-PARP1 (SantaCruz, sc-74469, 0.5 µg) were loaded on a 6% non-denaturing polyacrylamide gel in 0.5x Tris-borate-EDTA (TBE) buffer and the gel was run at 120 V for 55 min and wet-transferred to Biotodyne B (Pall Life Sciences) membrane for 50 min in 0.5x TBE at 300 mA on ice. The Biotodyne B membrane was air-dried at room temperature for 5 min, and then baked at 80°C for 1 h and exposed to UV light for 2 min. After blocking, the membrane was incubated with Alkaline Phosphatase-conjugated ExtrAvidin (Sigma Chemical; 1:1000 dilution) exposed to film. The binding reactions ran on agarose gel were detected by ethidium bromide staining.

3. Results

3.1 Identification of a strong repressor element in HFE distal promoter

In a report, Mura and coworkers have been shown that proximal HFE promoter harbors a TATA-like sequence and four GC box containing Sp1 binding sites as well as multiple transcription initiation sites [25]. These features are typical for core promoters of housekeeping genes [26]. However, tissue distribution and expression level of HFE varies markedly. In addition, at least nine variants of HFE, ranging in sizes from 8.8 kDa to 40 kDa, have been identified [27]. Tissue distribution and expression level of each of these variants differ significantly. Not all of these variants are generated by exon skipping or alternate splicing [27-28]. In order to investigate transcription regulation of HFE gene, a 1675 bp fragment (H1675) comprising 1637 bp upstream and 37 bp downstream of the reported transcription start site was PCR amplified from human genomic DNA and inserted into pGL3 Basic luciferase-reporter vector. Since this fragment did not produce any reporter luciferase activity in transient transfection in HEK293T cells, two other constructs differing in insert size (H210 and H810) were prepared using unique restriction sites (Fig.1A). When tested in HEK293T cells, only H210 produced significant luciferase activity- 3-fold over the control vector. Although the expression level was slightly different, the result was essentially similar in three other human cultured cell lines (HeLa, HepG2, and HCT116) of

different origin. H810 produced no luciferase activity; rather it reduced the background level in the vector to almost zero level, indicating a strong silencing element between –833/–173 region upstream of the HFE transcription start site (Fig.1B). To further localize the repression site, several sequential deletion constructs were made in pGL3 vector using PCR cloning. Transient transfection experiments revealed the presence of a strong repressor element in fragment between –773/–708 (H810 and H745 constructs) (Fig.1C).

To further narrow down and map the sequence responsible for the repression, additional deletion constructs were made (Fig.2A) using PCR cloning (Table 1) between –773 and –708 and tested in transient transfection assay using three different cell lines (Fig.2B). The results pointed out that the core sequence of the repressor element is located between –751/–745 bp upstream of the transcription start site (Fig. 2A), which is an inverted repeat sequence. All constructs containing this sequence have drastically reduced transcriptional activity (constructs H751 and longer). Deletion of 14 bp encompassing this sequence (H810Δ construct) resulted in restoration of activity similar to the H745 construct (Fig. 2C, top) lacking the repressor site.

To test whether the sequence retains its repressor activity at a heterologous location, we cloned hfee11/12 duplex probe containing the repressor and its flanking sequence (Table 1) 5' to H210, the most active 210 bp HFE gene core promoter, to produce pH210 and heterologous promoter, polycystic kidney disease-1 200 bp proximal core promoter (pPKD1 construct described in 23). For comparison, flanking sequence without the 14 bp repressor sequence (72d1/d2) was also cloned 5' to these promoters (ΔH210 and ΔPKD1 constructs). As shown in Fig. 2C (middle and bottom), the pH210 and pPKD1 constructs with the silencer sequence produced markedly reduced activity compared to the respective construct without the silencer sequence (ΔH210/ΔPKD1). These results confirmed that the 14 bp sequence possesses strong repressor activity even if placed in heterologous environment.

3.2 PARP1 binds the repressor site in vitro and in vivo

To determine if the repressor sequence is able to bind nuclear proteins, electrophoretic gel mobility shift (EMSA) assay was carried out using biotinylated hfee11/12 duplex (Table 1) as DNA probe. As illustrated in Fig.3A, that this probe is shifted when incubated with nuclear extract, but not with BSA in polyacrylamide gel following transfer to membrane and detection by streptavidin. The protein binding to the probe was specific as 10-fold molar excess of duplex probe efficiently outcompeted the biotinylated probe in complex formation (Fig. 3A, lane 4). The protein-probe band is further shifted in the presence of anti-Parp1 antibody. The EMSA experiments clearly demonstrated protein binding ability of the repressor sequence.

In order to identify the protein participating in formation of DNA-protein complex and reveal its size, we took advantage of the biotinylated probe used in EMSA experiments above as a tag to pull down uv-crosslinked protein(s). Fig.3C shows the Coomassie staining of the SDS-PAGE gel. A major protein with molecular mass of ~116 kDa was observed. In contrast, this protein was no longer present with hfee14/15 duplex probe, a sequence in the HFE promoter upstream of the repressor site. This protein band was excised, digested with trypsin and the resulting peptide fragments were extracted and analyzed by LC-MS/MS. Results presented in Fig. 3C shows the set of matching peptides obtained in MS analysis that revealed identity of the protein as PARP1. In order to confirm Parp1 binding to the repressor sequence EMSA was performed in the presence or absence of anti-Parp1 antibodies. As presented in Fig. 3D, the Parp1-probe complex band is further shifted (supershift) in the presence of anti-Parp1 antibody (lane 3 vs lane 2). A diffused doublet appeared below the original shifted band possible due to a different preparation of nuclear extract. This in fact aided in distinguishing between the shifted and supershifted bands.

To further verify whether PARP1 can directly bind endogenous or exogenous HFE promoter *in vivo*, we performed ChIP assay. Formaldehyde cross-linked chromatin from HEK293T cells transfected with H810 or H745 constructs was immunoprecipitated with specific antibodies against PARP1 and analyzed by PCR using the primer-pair, GL3F/H7r (Table 1) flanking the repressor site to selectively detect PARP1 bound promoter transcribed by the plasmid. A 270 bp PCR band similar in size to input was amplified from ChIP DNA of H810 transfected cells (Fig. 3E). In contrast, this band was barely visible from ChIP DNA of H745 transfected cells (Fig. 3E) as well as when chromatin was precipitated using preimmune sera or unrelated anti-Dicer antibodies. In line with this, qPCR analyses of the immunoprecipitated ChIP DNA showed ~4-fold increased products in cells transfected with H810 versus cells transfected with H745, when normalized with respective input and preimmune amplified products (Fig. 3F). PARP1 binding to endogenous HFE promoter was also investigated in these cells transfected with H810 and H745 using primer-pair Hfee1/H7r. Comparison indicated a significantly reduced PARP1 binding in H810 transfected cells compared in H745 transfected cells. Although this result seems contradictory to previous findings, it can simply illustrate competitive binding of PARP1 to the exogenous repressor site in H810 transfected cells compared to endogenous HFE promoter. Supporting this notion are the RT-qPCR (Fig. 3G) results, which showed ~3-fold increase in HFE mRNA in cells transfected with H810 construct compared to cells transfected with H745. As mentioned above, this is due to the exogenous repressor in H810 competing with the endogenous HFE repressor for PARP1 binding, thus relieving some repression of the endogenous promoter. Taken together, these data indicated that PARP1 binds the repressor sequence located at the distal region of HFE promoter both *in vitro* and *in vivo*, and that this repression can be relieved once PARP1 is displaced from the repressor site.

Next, we examined whether the repression of the HFE promoter will be affected by inhibition of PARP1 enzymatic activity. Preventing PARylation activity by treating with iodoacetamide had no effect (data not shown) on the promoter activity in HEK293T cells transfected with H810 containing the repressor site, suggesting that HFE promoter repression by PARP1 does not utilize its enzymatic activity.

3.3 PARP1 affects endogenous HFE expression

To determine how depletion of PARP1 will affect HFE in mRNA and protein expressions, we used HEK293T cells transfected with siRNAs targeting specifically PARP1. In contrast to nonspecific siRNA and unrelated dicer siRNA, a significant reduction in PARP1 using specific RNA interference and concomitant increase in HFE expression both at the mRNA (Fig. 5A-B) and protein level (Fig. 5C) were observed. Consistent with the siRNA data, we further found elevated level of HFE mRNA in cells with genetically ablated Parp1 (Fig. 5D). Taken together, these results further support that PARP1 represses HFE expression *in vivo*.

Since importance of HFE in iron homeostasis is well recognized, we set out to examine any effect of iron supplement to HFE expression. RT-PCR and qPCR analyses of the isolated total RNA from HEK293T cells treated with or without hemin, a source of iron, and FeCl₃ are presented in Fig. 5 A-B. The results showed a slight increase of HFE mRNA in FeCl₃ treated cells, and a robust increase of HFE in hemin treated cells. Consistent with these results, treating cells transfected with repressor containing promoter, H810 with iron agents (hemin, FeCl₃) relieved some repression. In both experiments, much greater response was recorded with hemin compared to FeCl₃, most likely due to the presence of other components in hemin that induces greater breakdown of PARP1, reducing nuclear pool of PARP1 and hence increasing HFE expression. To examine this possibility and to determine probable cause(s) for the upregulation by iron supplement, we investigated PARP1 protein level in the nucleus of the treated cells. As shown in Fig. 5D-E, PARP1 level is significantly

reduced in both FeCl₃ and hemin treated cells, more in hemin than FeCl₃ treated, suggesting these treatments can reduce nuclear PARP1 pool. The increase of PARP1 fragments (Fig. 5F), especially 89 and 24 kDa and increase of caspase 3/7 (Fig. 5G) activity in the treated HEK293T cells compared to untreated cells seem to indicate PARP1 cleavage by apoptotic pathway indeed occurred by iron agents treatment. Cleavage of PARP1 can occur by either apoptosis and/or necrosis responses and recently its cleavage induced by iron agents hemin and ferric protoporphyrin has been reported by others [44, 48-50]. Taken together these results imply that iron loading can activate HFE transcription and translation, at least in HEK293T cells, most likely via PARP1 cleavage resulting in relieve of PARP1 mediated repression.

3.4 Iron treatments increase hepcidin mRNA

Significant decrease in hepcidin, a hormone primarily produced in the liver and involved in iron homeostasis, expression was observed in both HFE-deficient patients and Hfe-knockout mice despite significantly increased iron overload suggesting HFE play an important role in hepcidin expression [47]. Subsequently, it was demonstrated that transferrin bound HFE translocate to the nucleus and activate hepcidin expression. Since FeCl₃ and hemin treatments increased HFE expression (Fig. 5A-B) in HEK293T cells, we investigated whether this HFE increase has any effect on HAMP expression. Expression level of HAMP is unknown in HEK293T cells, so we compared its mRNA levels between HEK293T cells and HepG2 cells. As shown in Fig. 6A, in usual 30 cycles with a final 7 min extension, HAMP mRNA in HEK293T cells is significantly lower than in HepG2 cells. Ribosomal protein L7 was used as control (even in the control with no HAMP primers to eliminate sample contamination). In the iron treated cells (Fig. 6B-C), HAMP mRNA is significantly increased (1.5 to 2.0-fold) compared to control, implying iron treatments increased HAMP expression, most likely due to increase in HFE protein.

4. Discussion

The goal of this study was to characterize HFE gene promoter and its regulation. We identified within the genomic sequence upstream of the transcription start site presence of a repressor region that reduced transcription of HFE ~1.7 kb promoter drastically. Through sequential deletion analysis, the sequence responsible for this repression was identified as ATGGTcttACCTA located in the distal promoter between -931/-918. Subsequently, by pull-down with a probe containing the repressor sequence followed by mass spectrometry analysis, we identified PARP1 as the binding protein. Several reports have implicated PARP1 in transcriptional regulation of a number of genes including CXCL1, cTnT, iNOS, a-synuclein and Tcigr1 based on direct binding to DNA in sequence specific manner [17, 29-33]. PARP1 has been shown to bind TGTTG sequence in the cTnT gene promoter and TTCCCACAGC in Tcigr1 gene promoter. Although the sequence in the distal HFE promoter is quite different from these two sequences, the palindrome-like sequence in ATGGTcttACCTA with 3 bp loop can form cruciform-like secondary structure. It is well-documented that PARP1 preferentially binds to hairpin-like, cruciform DNA [15-16, 34]. It has been shown that PARP1 down-regulates its own promoter by binding to cruciform structures [31]. The sequence in the distal HFE promoter has five nucleotides in the perfect repeat, one fewer than six usually found in the cruciform structures. However there are instances where 5-nucleotide cruciforms are stabilized by protein binding [15]. It is possible that this inverted repeat sequence may not form cruciform structure, but PARP1 has been shown also to bind non-cruciform sequences as mentioned above in cTnT and Tcigr1 genes. Transient transfection data of deletion promoter constructs demonstrated strong repressor activity of this sequence in the HFE promoter, even when it was placed at different location (pH210) (Fig. 2D) or at heterologous promoter (pPKD1) (Fig. 2E). The repression was not

relieved by treating the cells with iodoacetamide, which inhibits PARP1 enzymatic activity, suggesting PARylation process is not involved in the repression of HFE promoter. This is similar to down-regulation of its own (PARP1) promoter [32], but different from regulation of *Tcigr1* and other promoters where catalytic activity is shown to be needed. Thus, transcriptional regulation of HFE by PARP1 presents another example of negatively regulated gene that does not depend on PARylation activity of PARP1. Recently, another iron regulatory gene human hemojuvelin has been shown to be down-regulated by tumor necrosis factor alpha via a novel response element within its promoter [35].

Immunoprecipitation (Fig. 3E-F) of the repressor region with anti-PARP1 antibodies from chromatin, and increased HFE mRNA expression in HEK293T cells transfected with PARP1 siRNA provided evidence that HFE gene is *in vivo* negatively regulated by PARP1. PARP1 has been shown to be involved in transcriptional regulation using different mechanisms: modification of chromatin structure, or function as an enhancer, co-regulator, or insulator [reviewed in 38]. Its catalytic activity is not always necessary in regulating promoter activity, however, in all cases intact PARP1 is involved: PARP1 binds to *CXCL1* gene promoter and blocks NF- κ B binding, but auto-PARylation leads to its replacement with NF- κ B and relieves PARP1 mediated repression on *CXCL1* promoter [39]. It also binds to intronic regulatory element in *BCL6* gene to inhibit its expression [40]. The down-regulation of *Tcigr1* promoter is relieved upon RANKL mediated proteolytic cleavage of PARP1 [29]. Consistent with these findings, only intact PARP1, but none of its cleaved (89, 42 or 24 kDa) fragments, was pulled down with the the biotinylated repressor sequence from nuclear proteins (Fig.3B), indicating only intact PARP1 is capable of binding this repressor sequence in the HFE promoter.

Although considered a house-keeping gene, HFE shows quite extensive variation in expression [28]. Another complexity is its existence of at least nine different variants, the mechanism of their synthesis and physiological roles are not fully known [27]. Regulation of HFE expression by PARP1 is quite novel and interesting as PARP1 cleavage is a hallmark of apoptotic process [36], necrosis [37] or other cellular stress [42]. Apoptosis induced caspase-3 cleaves PARP1 into two fragments of 89 and 24 kDa [36], while necrosis induced cathepsin B or G generates a 42 kDa fragment [37]. Since PARP1 binds HFE promoter as an intact protein, we speculated that cleavage of PARP1 by either apoptosis or necrosis can induce HFE promoter. Such mechanism of de-repression by PARP1 cleavage has been described for *MMP-9* and *Tcigr1* gene promoters, which are inhibited by intact PARP1: PARP1 dependent repression on *Tcigr1* is overcome upon RANKL mediated cleavage of PARP1 [29]. Similarly, PARP1 mediated repression *MMP-9* gene promoter was relieved in apoptotic cells upon PARP1 cleavage [43]. Increase in HFE expression (Fig. 5A-B) associated with a decrease of nuclear PARP1 (Fig. D-E), increase in both apoptosis (Fig. 5G) and PARP1 cleavage (Fig. 5F) upon FeCl_3 and hemin treatment suggest a similar mechanism might be in play with the HFE promoter. Induction of apoptosis and PARP1 cleavage by iron donors such as hemin, ferric protoporphyrin have been shown recently by others [44, 48-50]. In consistent its activity is stabilized by iron chelators like deferoxamine [51].

Iron is a necessary cellular component involved in energy metabolism, oxygen transportation, and DNA synthesis. On the other hand, excess iron is toxic and leads to generation of reactive oxygen species and highly reactive radicals. Depending upon the damage, these events may result in apoptosis or necrosis as demonstrated earlier [44, 48-50] and Fig. 5FG). HFE plays important role in human iron homeostasis which is controlled in the liver. HFE is bound with Transferrin Receptor 1 on the surface of hepatocytes and is dislodged by excess of iron bound ferritin. Unbound HFE then binds Transferrin Receptor 2, and this complex is transported into nucleus to trigger transcription of hepcidin gene *HAMP*,

a hormone predominantly produced in the liver and involved in iron homeostasis by regulating its absorption. Thus, HFE is a key component involved in iron sensing and regulation mechanism [reviewed in 4, 41]. Further importance of HFE in iron sensing came from the studies showing reduced HAMP expression in both human HFE patients and Hfe-knockout mice despite significant iron overloading in the liver [47]. Our results in Fig. 5 show that excess iron induced HFE expression (Fig. 5A-B) most likely via inducing apoptotic PARP1 cleavage (Fig. 5 F-G), thus relieving PARP1 mediated repression on the HFE promoter. This HFE increase in turn upregulated HAMP mRNA (Fig. 6). We were unable to find direct in vivo data relating the effect of iron overload on HFE expression. However, Gardenghi et al. [52] showed a correlation of liver iron content and HFE expression in β -thalassemia mice: in young (2 months) β -thalassemia intermedia mice (*th3/+*) as the body iron levels are still too low reduced level of *Hamp1* are associated with low level of *Hfe*. In older (12 months) thalassemia intermedia mice liver, however, with increased iron content relatively elevated expression of *Hamp1* associated with Hfe was noticed compared with control.

This potential pathway may also present a link between iron metabolism and HFE expression compensating for the lack of IRE in HFE promoter. IRE is found in most genes controlling iron homeostasis [45]. PARP1 binding site in human HFE promoter is not conserved over mammalian evolution, which implicates that PARP1 regulation of HFE expression is newly created mechanism for tighter control of human iron homeostasis. The importance of PARP1 binding site in HFE expression and, as a consequence, function in iron homeostasis implicates this site as another potential source of pathogenic mutations resulting in malfunctioning of iron metabolism, even though no iron responsive element (IRE) motifs have been found in the HFE mRNA.

5. Conclusion

In conclusion, as depicted in Fig. 7, our data reveal a novel regulatory mechanism of HFE expression by a repressor site in the distal HFE promoter, where intact PARP1 binds. Excess iron, as in the case of hemin and FeCl_3 treatment, causes proteolytic cleavage of PARP1 via caspases and abolishes PARP1 binding to this site, thereby releasing its repression on HFE gene. Increased HFE in turn leads to increase in iron regulatory hormone HAMP mRNA. This unique mechanism is thus compensating the absence of IRE in the HFE gene, which is serving as an iron sensor. Identification of PARP1 as a transcriptional regulator of the HFE gene provides a new avenue of therapeutic treatment/research for hereditary hemochromatosis.

Acknowledgments

This work was supported by NIH grants R15 DK069897 (M.R.I.). We thank Dr. Vogelstein (John Hopkins University Medical Center) for HCT116 p53 null cells and Dr. William Sly (St. Louis University Medical Center) for critical review of the manuscript and for providing some resources. *Parp*^{-/-} cells were the contribution of Dr. Craig Thomson (Abrahamson Family Cancer Research Institute, University of Pennsylvania).

Abbreviations

HH	hemochromatosis
PARP1	poly(ADP-ribose) polymerase-1
HFE	hemochromatosis protein

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Highlights

- An inverted repeat sequence within 1700 bp of the HFE promoter acting as a repressor was identified
- PARP1 via binding this site repressed HFE transcription, for which activity was not required
- Excess iron increased HFE expression by reducing nuclear PARP1 pool via apoptotic induced cleavage
- Excess iron also increased iron regulatory hormone HAMP mRNA correlated to HFE increase
- Thus, PARP1 binding to the HFE promoter may serve as a novel iron sensing mechanism

Figure 1.

Mapping of the repressor site within 1.7 kb human HFE promoter region. Schematic representation of sequentially deleted HFE promoter-luciferase reporter constructs between -1638 and +37 (A). The black box represents the repressor site. Transcriptional activity of the HFE promoter constructs (1.0 ug) created using unique restriction sites (B), or serially truncated by PCR cloning (C) [between -833 (H870) and -173 (H210) region] in transiently transfected HEK293T, HeLa, and HepG2 cells. Measured luciferase activities were normalized to β -galactosidase activity used as a control for transfection efficiency. The normalized luciferase activities are expressed relative to pGL3 vector, which was set at 1.0. The data for this and following experiments are representative of three independent experiments and are represented as RLU-fold mean $\pm$ SD.

Figure 2.

Identification of the repressor sequence in the distal HFE promoter. (A) Schematic representation of sequentially deleted HFE promoter-luciferase reporter constructs between -773 (H810) and -708 (H745) region. The black box represents the repressor site. (B) Transcriptional activity of the serially truncated promoter constructs (1.0 ug) between -733 and -708 region created by PCR cloning in transiently transfected HEK293T, HeLa, and HepG2 cells. The sequence represented by the black box is shown underneath. (C) Transcriptional activity of the promoter with (H810) or without (HΔ810, H745) the repressor sequence in HEK293T cells. (D, E) Transcriptional repression mediated by the negative element (Hfee11/12, Table 1) when placed 5' to H210 construct (pH210) or PKD1 proximal promoter (pPKD1) in HEK293T cells. In ΔH210 and ΔPKD1, duplex 72d1/d2 (Table 1), missing the repressor sequence, substituted hfee11/12. Measured luciferase activities were normalized and presented as in Fig. 1 (***, $p < 0.001$ compared to vector control).

Figure 3.

PARP1 binds the repressor sequence. Electrophoretic mobility shift assays using biotinylated hfee11/12 probe (10 pmol) containing the repressor site and BSA (10 ug) or nuclear extracts of 293T cells (10 ug) is resolved in polyacrylamide gel (A): 1, probe; 2, probe with BSA, 3, probe with nuclear extracts and 4, probe with nuclear extracts and excess unlabeled probe. (B) Pull down with biotinylated probe (100 pmol) and precleared nuclear extracts from 293T cells (100 ug): 1, with hfee11/12 probe; 2, with hfee14/15 probe near the repressor site. (C) Fifteen peptides of human PARP1 with matching probability obtained by MS analysis of the cut out band. (D) Supershift in the presence of anti-Parp1 antibody: 1, probe; 2 probe with nuclear extracts and 3, probe with nuclear extracts in the presence of mouse monoclonal anti-PARP1 antibodies (0.5 μ g). (E) ChIP-PCR analysis of chromatin from HEK293T cells transfected either with (H810) or without (H745) repressor containing constructs immunoprecipitated prior to PCR using preimmune sera (lane 3), anti-Dicer (lane 4), or anti-PARP1 (lane 5) or input chromatin (lane 2). The fragment amplified using the primer set (GL3F/H7r) towards the 5' end (0.21 kb for H745, 0.27 kb for H810) of the cloned HFE promoter was analyzed by gel electrophoresis. The PCR products from H745 transfected cells (H745) were run 15 min prior to loading of the PCR products from the H810 transfected cells (H810) onto the same gel. (F) qPCR analysis of the ChIP DNA isolated in (E) using primer pair GL3F/H7r for exogenous (transfected) and primer pair hfee1/H7r for endogenous HFE promoter. Each amplified product was normalized with product from input and preimmune, and the normalized product from H745 transfected cells was set to 1.0. (G) RT-qPCR of the total RNA isolated from cells transfected with H745 or H810 promoter constructs. Ribosomal protein L7 mRNA was used as reference

Figure 4.

PARP1 depletion up-regulates HFE mRNA and protein. (A) RT-PCR analysis of the total RNA isolated from HEK293T cells transfected with PARP1 siRNA (Parp), Dicer (Dic) siRNA or non-specific (NS) siRNA. (B) RT-qPCR analysis of the total RNA samples in (A) normalized with ribosomal protein L7 mRNA. HFE mRNA in NS is set to 1.0. (C) Protein expression analysis by Western blot of the HEK293T cells transfected as in (A). Scanned HFE over loading control actin is shown below. (D) RT-qPCR analysis of the total RNA isolated from genetically ablated PARP1 (Parp^{-/-}) MEF cells compared with wild type (Parp^{+/+}) MEF cells normalized with ribosomal protein L7 mRNA. The latter is set to 1.0. The data for the qPCR experiments are expressed as the mean $\pm$ SD (**, $p < 0.005$, ***, $p < 0.001$) normalized with ribosomal L7 mRNA.

Figure 5.

Hemin and FeCl₃ treatments decrease PARP-1 and increase HFE mRNA expression. (A) RT-PCR of the total RNA isolated from HEK293T treated with FeCl₃ (10 uM) or hemin (75 uM) for 40 hr using primers for HFE and L7, (B) RT-qPCR analysis was performed to quantify HFE mRNA in the samples in (A). The data for the qPCR experiments are expressed as the mean $\pm$ SD of three determinations (*, $p < 0.05$; **, $p < 0.005$) normalized with ribosomal L7 mRNA. (C) Ectopic expression of H810 in HEK293T cells in the presence of FeCl₃ or hemin compared to untreated cells presented as mean RLU fold $\pm$ SD (*, $p < 0.05$). (D) PARP1 is somewhat diminished in the nucleus of the treated cells. Actin is used as a loading control. (E) The ratio of PARP1 over actin in the treated cells is shown as the mean $\pm$ SD (*, $p < 0.05$). (F) breakdown of PARP1 in the cell extracts of the treated and control cells shown in (A). 30 ug of total proteins were loaded and actin is used as a loading control. (G) Apoptosis was measured using Caspase 3/7 substrate in these cells.

Figure 6.

Hemin and FeCl₃ treatments increase Hepcidin mRNA expression. (A) RT-PCR of the total RNA isolated from HEK293T or HepG2 cells using primers for L7 alone or together with hepcidin. (B) RT-PCR of the total RNA isolated from HEK293T treated with FeCl₃ (10 uM) or hemin (75 uM) for 40 hr using primers for hepcidin and L7. (C) RT-qPCR analysis was performed to quantify hepcidin mRNA in the samples in (A). The data for the qPCR experiments are expressed as the mean $\pm$ SD of three determinations (*, $p < 0.05$; **, $p < 0.005$) normalized with ribosomal L7 mRNA.

Figure 7.

Proposed mechanism of how excess iron and PARP1 regulate expression of HFE. PARP1 binding to the repressor site reduces HFE protein. While excess iron induces cleavage of PARP1 resulting in relieving PARP1 mediated HFE repression. Increase in HFE protein regulates iron uptake.

Table 1

Sequences of the primers used and their purposes.

Primers	Constructs	Sequence	Purpose
H1m	H1675	forward: 5'-tgactccaacccgggcaatag-3'	PCR cloning of
H4		reverse: 5'-ctctctacagcagaaggtacc-3'	1.7 kb promoter
GL3R		reverse: 5'-gctagcacgcgtaagagctcg-3'	Reverse primer for PCR cloning
H5	H555	5'-gatatttgggtaccgactttac-3'	Forward primers for PCR cloning with GL3R at Kpn I site
H7	H612	5'-gccaccttaggtaccctccactg-3'	
H73	H810	5'-gtatctgggtaccaggatgatg-3'	
H72	H745	5'-gtgcatgggtggtacctataattg-3'	
H71	H670	5'-ctattataatggtaccatgatgaactgggg-3'	
H72.3	H795	5'-gctatacaagggtaccattaaactgtgc-3'	
H72.2	H770	5'-caatctaaagggtaccctgtgcatggtc-3'	
H72.1	H751	5'-ctaaaattaacggtacctgtcttacc-3'	
H72d1	HΔ810, ΔH210, ΔPKD	5'-caatctaaaataaactgtg/aatttgaagaaaagc-3'	deletion, cloning
H72d2		5'-gcttttctaacaatt/cacagtttaatttagattg-3'	
hfee11	pH210, pPKD	5'-caatctaaaataaactgtgcatggtcttacctat-3'	EMSA, pull-down, cloning
hfee12		5'-ataggtaagaccatgcacagtttaatttagattg-3'	
hfee14		5'-actgtgcatggtcttacctat-3'	EMSA, pull-down
hfee15		5'-ataggtaagaccatgcacagt-3'	
h63df		forward: 5'-ctcagagcaggaccttggtc-3'	HFE mRNA
hh4r		reverse: 5'-ataccgtacttccagtagccct-3'	
L7-1		forward: 5'-gcttcgaaggcaaggagggaagc-3'	Ribosomal L7 mRNA- control
L7-2		reverse: 5'-tctccatgcagatgatg-3'	
Hpcdn3		forward: 5'-catgttccagaggccaag-3'	Hepcidin mRNA
Hpcdn2		reverse: 5'-caagacctgaattctggggcagc-3'	
H7r		reverse: 5'-cctgtcttccaagttcacc-5'	ChIP
Hfee1	with H7r	forward: 5'-gagtgacaggatgatgttattga-3'	ChIP endogenous
GL3F	with H7r	forward: 5'-ctagcaaaataggctgtccc-3'	ChIP exogenous