BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 360–364 (1996)
Article No. 0063

Down-Regulation of *HCW9* mRNA in Rat Hepatocytes during Chemical Hypoxia Involves Both Transcriptional and Posttranscriptional Mechanisms[1]

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Received November 17, 1995

HCW9 cDNA encodes a rat protein with 95% homology to mouse phospholipase A_2 activating protein (PLAP). Its mRNA, which is substantially decreased in rat hepatocytes during chemical hypoxic injury, was found to be expressed in all rat tissues examined, including liver, heart, brain, spleen, lung, skeletal muscle, kidney, and testis. To elucidate the mechanisms responsible for this hypoxia-induced down-regulation of *HCW9* mRNA levels, the transcription rate and half-life of *HCW9* mRNA were measured. Nuclear run-off assays revealed a 54–57% inhibition in the transcription rate of *HCW9* gene during chemical hypoxic injury. The half-life of *HCW9* mRNA decreased from ≈15 min under normoxic conditions to ≈7 min during chemical hypoxic injury. These findings suggest that *HCW9* expression in rat hepatocytes is regulated at both the transcriptional and posttranscriptional levels during chemical hypoxia.

Hypoxia typically results from conditions where limited perfusion of tissues persists but insufficient oxygen is delivered to the tissue due to respiratory failure, tissue hypoperfusion, or a combination of the two. To investigate the molecular mechanisms that underlie hypoxic injury, we have recently employed subtractive cloning and have identified a set of genes whose expression levels are altered (up or down) during hypoxia (1,2). One of the cDNA clones whose mRNA level was substantially decreased in rat hepatocytes during chemical hypoxia was termed *HCW9* (1). Sequence analysis of *HCW9* cDNA revealed a 647-amino acid open reading frame encoding a protein with 96% identity to the published mouse phospholipase A_2-activating protein (PLAP) at the N-terminus (aa 1 − 255) (1,3), but overall more than 95% homology to the corrected PLAP amino acid sequence deduced from the mouse *PLAP* clone (1).

Here we report the characterization of the HCW9 protein. The complete coding region of *HCW9* λGEM-2 cDNA was subcloned into the plasmid vector pQE-30, and expressed in *E. coli* strain SG10039. In addition, the tissue distribution and mechanisms responsible for down-regulation of *HCW9* expression in rat hepatocytes during chemical hypoxia were also investigated.

MATERIALS AND METHODS

Cell culture and chemical hypoxia. Rat hepatocytes were isolated by collagenase perfusion of livers of male Sprague-Dawley rats (200–300g), and cultured in Waymouth's MB-752/1 containing 26.7 mM $NaHCO_3$, 2 mM L-glutamine, 5% fetal calf serum, 100 nM insulin and 100 nM dexamethasone as previously described (4). Chemical hypoxia with KCN (2.5 mM) plus iodoacetate (0.2 mM) in Krebs-Ringers-Hepes Buffer (KHB, pH7.4) containing 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM Na-Hepes buffer were used to mimic the ATP depletion and reductive stress of hypoxia as previously described (4). KCN inhibits mitochondrial cytochrome oxidase and causes reduction of mitochondrial electron transfer components, as in anoxia. Iodoacetate inhibits glycolysis and prevents ATP formation due to glycolytic metabolism of glycolytic substrates like fructose and glycogen. We have previously shown that this model of chemical hypoxia faithfully mimics true hypxoia (pO_2 < 0.1 torr).

Expression and purification of HCW9 protein. The 1944-bp coding region (nucleotide 144–2087) of *HCW9* clone (1) was amplified by PCR using primers (5′-CCC GC*G GAT CC*A TGC ACT GTA TGA GCG GCC, and 5′-CCC GC*A AGC TT*T

[1] This work was supported by Grant AG07218 from the National Institutes of Health.
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0006-291X/96 $12.00

CAA GGT CAG TGT AGC CAG) (6). After purification, the amplified cDNA insert was digested with *Bam*H1 and *Hin*dIII, subcloned into pQE-30, and subsequently transformed into *E. coli* strain SG13009(pREP4) (Qiagen, Chatsworth, CA, USA). High-level expression of HCW9 in *E. coli* from the recombinant plasmid was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG), the recombinant 6× His tag HCW9 protein was purified using the Ni-NTA purification system, and subsequently analyzed on a 4–15% SDS-polyacrylmide gel as instructed (Qiagen, Chatsworth, CA, USA).

Northern blot analysis. Total cellular RNA was isolated at the indicated times by the acid guanidium thiocyanate-phenol-chloroform extraction method (5) after various treatments of the cells and Northern blots were performed with a digoxigenin-11-dUTP labeled *HCW9* (nucleotide 1191–2080) probe as previously described (1). For determination of *HCW9* mRNA half-life, quantitative analysis of mRNA hybridization signal was performed by using an image scanner (Model Hewlett Packard Scan Jet II cx) and the NIH Image 1.57 software on a Macintosh. The mRNA half lives were determined from 'best-fit' regression lines obtained by using the least-square method ($r > 0.9$).

Isolation of hepatocytes nuclei and run-off transcription assay. At 30 min after initiation of chemical hypoxia, nuclei from 5×10^7 normoxic or hypoxic cells were prepared as described [6]. Transcription reactions were performed for 30 min at 37°C with 100 μl of the isolated nuclei in a final volume of 150 μl in the presence of [α-^{32}P]UTP and unlabeled nucleotides. Elongated RNA transcripts were treated with 10 μg/μl RNase-free DNase I for 15 min at 37°C, and were then extracted with 65°C phenol. The precipitated RNA (at equivalent amounts of radioactivity, i.e., 2×10^6 cpm/sample) was hybridized to cDNAs (2μg) immobilized on nylon filters in hybridization buffer (250 mM NaCl, 10 mM Tris-HCl, pH 8.0, 4% SDS, 50% formamide, 1 mM EDTA, 50 μg/ml yeast tRNA, 50 μg/ml polyadenylic acid, and 100 μg/ml salmon sperm DNA) at 48°C for 18 h. The filters were washed in 2 × SSC/0.1% SDS three times at 25°C and in 0.1 × SSC/0.1% SDS three times at 65°C with shaking. Washed filters were exposed to Kodak X-OMAT AR film at −70°C with intensifying screens for 2–3 days, and hybridization signals were quantitated as described [1].

RESULT AND DISCUSSION

Expression and purification of HCW9 protein. To begin the characterization of rat HCW9 protein in mammalian cells, we subcloned the *HCW9* coding region (1944 bp) downstream from an IPTG-inducible promoter in pQE-30 in order to express HCW9 protein in *E. coli* strain SG13009 containing repressor plasmid pREP4 (3.74 kb). Agarose DNA gel analysis revealed the presence of an insert of the correct size (1956 bp) in pQE-30 (*HCW9*) (Fig. 1A, Lane 2). Utilizing the QIAexpress Ni-NTA expression and purification system, the recombinant 6× His tag HCW9 protein was overexpressed, purified under severe denaturing conditions (8M urea), and subsequently analyzed on a 4–15% SDS-polyacrylmide gel. While no proteins were retained from the Ni-NTA agarose from *E. Coli* lacking the *HCW9* plasmid (Fig. 1B, Lane 2), a variety of truncated protein products ranging from 28 to 74 kDa were observed in SG13009 cells containing

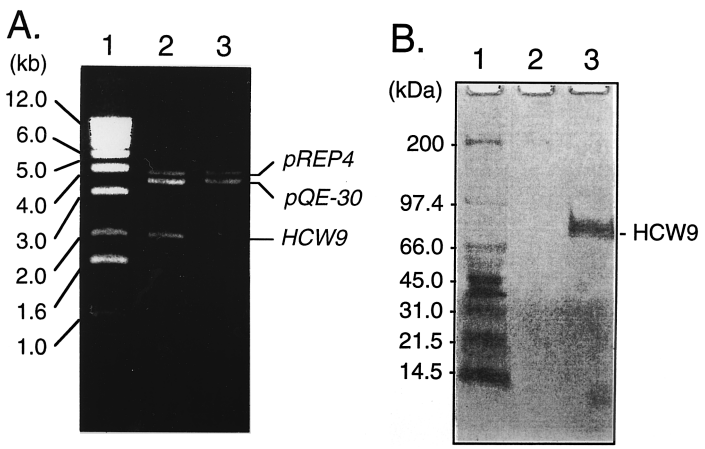

FIG. 1. Gel analysis of recombinant pQE-30 (*HCW9*) plasmid and 6× His-tag HCW9 protein **(A).** Agarose gel of recombinant pQE-30 (*HCW9*) plasmid containing *HCW9* insert (lane 2), vector pQE-30 only (lane 3), as well as the *E. coli* host *lac* repressor plasmid pREP4 (lanes 2 and 3). Lane 1, DNA size markers (GibcoBRL, Grand Island, NY, USA). **(B).** SDS–PAGE of purified recombinant 6x His tag HCW9 protein. Lane 1, broad range protein size markers with indicated molecular weights (Bio-Rad Lab., Hercules, CA, USA). Lane 2, proteins purified from SG13009 containing vector pQE-30 only. Lane 3, purified recombinant 6 × His tag HCW9 protein.

pQE-30(*HCW9*) induced with IPTG for long periods of time (4 hours) at high temperature (37°C) (data not shown). This suggested that the recombinant HCW9 protein was either not stable and partially degraded within *E. coli*, or very long and subject to premature termination of translation. Further investigation of HCW9 expression in *E. coli* pointed to the first possibility, as decreasing the time period of IPTG induction (from 4 hr to 1 hr) as well as the induction temperature (from 37 to 30°C) resulted in the production of an approximately 74 kDa polypeptide, albeit with substantially decreased yield (Fig. 1B, Lane 3). The molecular weight of the expressed HCW9 protein was estimated to be approximately 73 kDa, which is in close agreement with the predicted molecular weight of the 645-aa HCW9 protein (1). However, the size of HCW9 protein was not the same as the molecular weight of the 28 kDa PLAP protein isolated from bovine endothelial cells, murine smooth muscle cells, and gouty synovial fluid. However, the 28 kDa PLAP protein was isolated on the basis of its reactivity with antibodies specific to bee venom melittin (7,8,9). Future structural and functional characterization of HCW9 protein in mammalian cells will have to await the availability of antibodies directed against purified HCW9 protein which are currently under production.

Tissue distribution of HCW9 transcripts. Rat HCW9 is highly homologous to mouse PLAP, a protein that is expressed in various tissues including smooth muscle, endothelial cells and T cells. The regional expression of *HCW9* was investigated by multiple tissue Northern blot analysis using a fragment of *HCW9* as probe. Consistent with previously published results (1), the size of *HCW9* transcript in all rat tissues was equivalent (2.5 kb) (Fig. 2A). Moreover, a minor transcript slightly larger than 4.5 kb was also observed in many tissues with the exception of liver and spleen. The same blot was stripped and rehybridized with a constitutively expressed house-keeping gene, *IB15*, revealing equivalent loading of mRNA in all lanes (Fig. 2B). While the *HCW9* transcript was expressed in all of the tissues examined, differences in mRNA abundance among the different tissues was noticed (Fig. 2A). HCW9 mRNA was expressed most abundantly in heart and liver, least in brain and spleen. The fact that rat HCW9, a protein homologous to mouse PLAP containing four WD-40 repeats at the N-terminus (10), is expressed in many tissues, may signify its importance in fundamental cellular activities important to a variety of tissues.

Transcriptional Activity of the HCW9 Gene. We previously reported that rat *HCW9* mRNA levels were substantially decreased in rat hepatocytes during chemical hypoxia (1). This chemical hypoxia-induced decrease in *HCW9* mRNA levels could result from decreased rate of gene tran-

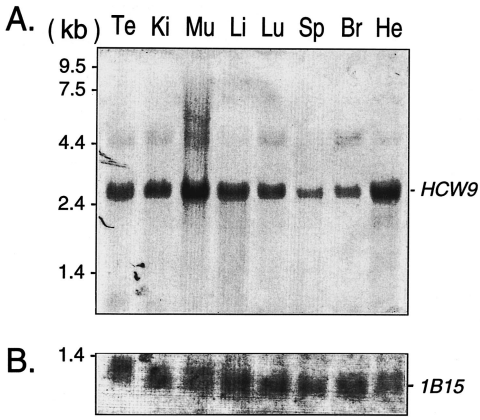

FIG. 2. Tissue distribution of *HCW9.* **(A).** hybridized with *HCW9* cDNA probe. Rat multiple tissue Northern blot that contained 2 μg of poly(A)$^+$ RNA per tissue was purchased from Clontech (Clontech Lab., Palo Alto, CA, USA). Blot hybridization of RNA was carried out with a digoxigenin-11-dUTP labeled *HCW9* cDNA probe corresponding to nucleotide 1191–2080 as described under Materials and Methods **(B).** hybridized with house-keeping gene *IB15* which served as a loading control.

scription, accelerated rate of RNA degradation, or a combination of both. To determine whether changes in the level of *HCW9* mRNA after chemical hypoxia was due to a variation in the transcriptional rate of the *HCW9* gene, we measured the transcriptional rate of *HCW9* during chemical hypoxia. As shown in Fig. 3, the transcription rate of*HCW9* gene decreased to 43–46% of the control (average of three independent experiments, determined by scanning densitometry) at 30 min after initiation of chemical hypoxia, whereas the transcription rate of the house-keeping gene *IB15* (as a control) as well as a recently cloned cDNA, *CLP-36* (2), remained unchanged. The decrease in *HCW9* transcription therefore accounts, at least in part, for the down-modulation of *HCW9* mRNA in rat hepatocytes during chemical hypoxia. *PLAP* transcriptional activity has been shown to be regulated by cytokines (IL-1) in T cells (11), or LTD_4 in smooth muscle and endothelial cells (3,8), suggesting an important role in chronic immune and inflammatory process (12). Experiments to determine whether hypoxia-induced alterations in hepatocyte cytokine levels are responsible for altered *HCW9* expression levels are currently underway.

Regulation of HCW9 mRNA Half-Life by Chemical Hypoxia. Since the marked decline of *HCW9* mRNA level in rat hepatocytes during chemical hypoxia could also result from post-transcriptional modifications, we measured the half-life of *HCW9* mRNA in the absence or presence of KCN (2.5 mM) plus IAA (0.2 mM) using actinomycin D (5 μg/ml) to block transcription. The disappearance of *HCW9* transcripts was assayed by Northern blot analysis over a 60-min period. The half-life of *HCW9* mRNA declined from 15 min to ≈7 min during chemical hypoxia (average of three experiments, Fig. 4). The half-life of *IB15* mRNA in both normoxic and chemical hypoxic cells was >60 min, (data not shown), consistent with the previously described lack of effect of chemical hypoxia on *IB15* mRNA levels (2). The mechanism responsible for post transcriptional regulation of *HCW9* mRNA stability in rat hepatocytes during chemical hypoxia is not known, although the presence of two AUUUA sequences in the 3′-nontranslated region could be responsible for destabilization of *HCW9* mRNAs (1,13).

Our findings indicate that the level of *HCW9* mRNA in rat hepatocytes during chemical hypoxic

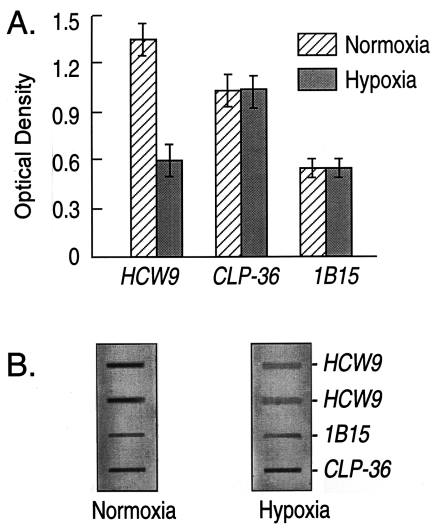

FIG. 3. Effect of chemical hypoxia on transcriptional rate of *HCW9* mRNA. **(A).** Quantitative determination of transcription rate of *HCW9* and *IB15* in the absence or presence of KCN plus IAA. Slot blot was scanned with a image scanner (Model Hewlett Packard Scan Jet II cx), and optical band density was quantitated by using NIH Image version 1.57 software on a Macintosh. The data is representative of three independent experiments. **(B).** Representative blot hybridization of ^{32}P-labeled nascent RNAs with various cDNAs. The transcriptional level of *IB15* and *CLP-36* were also determined. *IB15* is constitutively expressed and its mRNA level remains constant during chemical hypoxia. *CLP-36* mRNA level is down-regulated during chemical hypoxia (2).

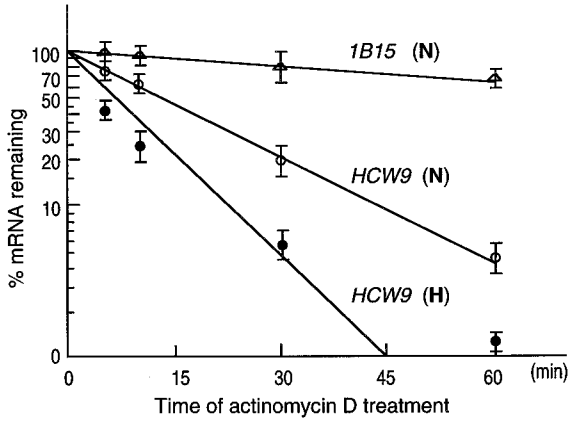

FIG. 4. Effect of chemical hypoxia on stability of *HCW9* mRNA. Cells were treated with actinomycin D (5 μg/ml) to block transcription before chemical hypoxia (0 min). At the indicated times after initiation of actinomycin treatment, total RNA was extracted, Northern blot was performed, and Northern blot signals were analyzed by video densitometric scanning as described under 'Materials and Methods.' The half-life of *HCW9* mRNA was calculated by lineal estimation from the best fit of each line on a logarithmic plot. Data in the figure represent mean ± S.E. of three separate experiments, where the normalized values were plotted as percentage of the mRNA level observed before actinomycin D treatment. The 'best-fit' regression lines (mean of the three experiments) are represented on the figure with standard deviation (bars).

injury is determined both by the rate of transcription and stability (half-life) of *HCW9* mRNA, suggesting that its expression in rat hepatocytes during chemical hypoxia is regulated at both transcriptional and post-transcriptional levels. Numerous studies suggest that alterations in the activity of pH-dependent phospholipases may contribute to alterations in membrane structure that occur during hypoxic injury and contribute to cell death (1,2,14–16). Hypoxia-induced alterations in the levels of *HCW9*, which displays 95% homology to PLAP, may underlie in part the altered levels of phospholipase A_2 activity observed and believed to contribute to hypoxic injury.

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