

# Expression of E-Cadherin and Other Paracellular Junction Genes Is Decreased in Iron-Loaded Hepatocytes

John P. Bilello,\* Edward E. Cable,\* and Harriet C. Isom\*<sup>†</sup>

From the Departments of Microbiology and Immunology\* and Pathology,<sup>†</sup> Milton S. Hershey Medical Center, The Penn State College of Medicine, Hershey, Pennsylvania

**Iron overload in the liver may occur in the clinical conditions hemochromatosis and transfusion-dependent thalassemia or by long-term consumption of large amounts of dietary iron. As iron concentrations increase in the liver, cirrhosis develops, and subsequently the normal architecture of the liver deteriorates. The underlying mechanisms whereby iron loading of hepatocytes leads to the pathology of the liver are not understood. Similarly, a direct relationship between the expression levels of paracellular junction genes and altered hepatocellular physiology has been reported; however, no relationship has been identified between iron loading and the expression of paracellular junction genes. Here, we report that the expression of numerous paracellular junction genes was decreased in iron-loaded hepatocytes, leading to increased cellular permeability, increased baculovirus-mediated gene transfer, and decreased gap junction communication. Iron loading of hepatocytes resulted in decreased E-cadherin promoter activity and subsequently decreased E-cadherin mRNA and protein expression. The data presented in this study describe a clear relationship between iron overload and decreased expression of paracellular junction genes in hepatic cells of rat and human origin. (*Am J Pathol* 2003, 162:1323–1338)**

Iron is essential for life and yet, inorganic unbound iron is toxic to all living cells. Iron transport and storage are regulated by well-defined mechanisms.<sup>1</sup> When the level of iron exceeds the capacity of these mechanisms, cells become iron-loaded. Apoferritin in cells enables them to survive the toxicity of free iron by binding iron. Ferritin regulates, by storage and release on demand, the amount of iron in the cell. However, most cells, other than erythroid precursors and macrophages, can only tolerate a few ferritin particles in the cytoplasm before they are considered to be iron-overloaded.<sup>1</sup> Iron overload is

associated with liver pathogenicity in several human diseases, including hereditary hemochromatosis (HH) and transfusion-dependent thalassemia. HH is an autosomal recessive disorder that afflicts nearly one million Americans of European descent, making HH the most common genetic disorder among this population.<sup>2–5</sup> HH is a clinical condition in which excessive amounts of iron are sequestered in organs, primarily the liver.<sup>6,7</sup> HH is caused by a homozygous defect in the HFE gene;<sup>8</sup> however, the precise mechanism(s) that leads to iron deposition into hepatocytes has not been elucidated.

Iron deposition in hepatocytes of HH patients begins early in life and progresses at a constant rate, leading to liver fibrosis and cirrhosis as the normal architecture of the liver deteriorates.<sup>7,9</sup> Compared to normal cells, fibrotic and cirrhotic cells have an altered cellular morphology and histological differentiation, which is accompanied by changes in intercellular adhesion. Intercellular adhesion is mediated by paracellular junctions that include the calcium-dependent tight and adherens junctions, desmosomes, and calcium-independent gap junctions.<sup>10</sup> Tight junctions, partially composed of the intercellular occludin protein and the zona occludens (ZO)-1 plaque protein, are the apical-most paracellular junction complexes of an epithelial cell and, as such, they form continuous contacts that limit paracellular movement of molecules between adjacent cells.<sup>11–13</sup> The adherens junction is composed of the cadherin family of homophilic intercellular adhesion receptors.<sup>10,14,15</sup> E-cadherin, a 120-kd transmembrane glycoprotein that is highly expressed during development and thereafter in the epithelium, is the most predominant cadherin of the liver epithelium. The cytoplasmic domain of E-cadherin is directly associated with  $\alpha$ - and  $\beta$ -catenin and through these proteins, E-cadherin is indirectly associated with several proteins including  $\alpha$ -actinin and radixin that link the transmembrane E-cadherin to the actin cytoskeleton.<sup>13</sup> In the absence of E-cadherin, epithelial cells are not capable of stable intercellular adhesion.<sup>10</sup>

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Address reprint requests to Harriet C. Isom, Ph.D., Department of Microbiology and Immunology, H107, Penn State College of Medicine, Penn State Milton S. Hershey Medical Center, P.O. Box 850, 500 University Dr., Hershey, PA 17033-0850. E-mail: hisom@psu.edu.

Our laboratory previously reported that primary rat hepatocytes, plated on collagen-coated plastic dishes and fed chemically defined medium supplemented with dimethyl sulfoxide (DMSO), can be maintained in a differentiated state for longer than 1 year.<sup>16,17</sup> The morphological and biochemical characteristics of these cells are similar to those of hepatocytes in rat liver *in vivo*. Hepatocytes in culture secrete albumin and retain steady-state mRNA expression of liver-specific and common genes. As in the uninduced *in vivo* liver, the level of DNA synthesis in cells in these hepatocyte cultures is low and the hepatocytes do not undergo cell division,<sup>16</sup> but retain the capacity to synthesize DNA and divide.<sup>18,19</sup> During the first 3 days after plating, primary rat hepatocytes in this culture system form a single-cell monolayer. Shortly thereafter, the hepatocytes move on the collagen-coated dish into multicellular islands (HC Isom, unpublished data).<sup>16,17</sup> This dynamic motility is observed throughout the lifetime of the culture. As the cultures age, no space remains between hepatocytes within the islands and paracellular junctions between adjoining cells can be observed by electron microscopy.<sup>16,20</sup>

Primary rat hepatocytes in long-term DMSO culture have been used to study the molecular mechanisms of albumin expression,<sup>17,21</sup> altered growth control including induction of DNA synthesis and proliferation,<sup>22</sup> immortalization and transformation,<sup>23–25</sup> as well as programmed cell death.<sup>26</sup> More recently, we have demonstrated that hepatocytes in long-term DMSO culture function as an ideal model system for studying chronic iron loading of hepatocytes.<sup>19,20,27</sup> The efficacy of various forms of iron to donate iron to long-term primary rat hepatocytes was examined and 3,5,5-trimethylhexanoyl ferrocene (TMH-ferrocene) was determined to be the most efficient iron donor of the compounds tested.<sup>20</sup> TMH-ferrocene is a chemically stable, nonionic form of iron that does not catalyze oxygen reactions under routine tissue culture conditions. TMH-ferrocene is lipophilic, and therefore readily diffuses into cultured hepatocytes, where a cytochrome p450-dependent reaction metabolizes the organic portion of the molecule and releases iron into the hepatocyte.<sup>19</sup> TMH-ferrocene was shown to load iron into hepatocytes in long-term DMSO culture in a concentration-dependent manner.<sup>20</sup> Iron from TMH-ferrocene was incorporated into ferritin cores within primary hepatocytes and ferritin protein levels were elevated in a physiological manner.<sup>20</sup> Changes in iron content, ferritin protein levels, and the expression of genes associated with iron regulation after chronic iron loading by TMH-ferrocene have also been studied using this system (E. E. Cable and H. C. Isom, unpublished results). Human hepatoma cells (Huh7s) are more difficult to iron-load than primary rat hepatocytes because of their rapid proliferation rates. However, acute responses to iron can be achieved by using the ferric nitrilotriacetate (FeNTA) iron donor. It has been previously demonstrated in human hepatic cell lines, that FeNTA induces the synthesis of ferritin by a transferrin-independent mechanism.<sup>28–30</sup> In the current study, the effect of iron loading on the expression of paracellular junction genes, specifically that of

E-cadherin, was examined in iron-loaded hepatic cells of rat and human origin.

## Materials and Methods

### Cell Culture

Primary rat hepatocytes were maintained in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with insulin (0.06  $\mu\text{g/ml}$ ), glucagon (0.04  $\mu\text{g/ml}$ ), dexamethasone (0.4  $\mu\text{g/ml}$ ), transferrin (100  $\mu\text{g/ml}$ ), epidermal growth factor (25 ng/ml; Sigma Chemical Co., St. Louis, MO), 1  $\mu\text{mol/L}$  [ $+$ ]- $\alpha$ -tocopherol (Sigma) and 2% DMSO (Sigma) in a humidified incubator at 37°C with 5% CO<sub>2</sub>.<sup>16,17</sup> At 7 days after seeding and thereafter, primary rat hepatocyte cultures were maintained in Dulbecco's modified Eagle's medium/F12 supplemented as above (referred to as control medium) or control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene.<sup>31–33</sup> For iron chelation experiments, primary rat hepatocytes that were maintained in either control medium or control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days were thereafter maintained in either control medium or control medium supplemented with 500  $\mu\text{mol/L}$  of desferrioxamine (DFO). Huh7 human hepatoma cells were maintained in Dulbecco's medium supplemented with 10% fetal calf serum. Huh7 cells were rinsed in phosphate-buffered saline (PBS) and fed serum-free medium before treatment with medium supplemented with 100  $\mu\text{mol/L}$  of FeNTA.

### Infection of Primary Rat Hepatocytes with Recombinant CMV-lacZ Baculovirus

The CMV-lacZ baculovirus (AcMNPV; *Autographa californica* multiple nucleopolyhedrovirus) was a kind gift of FM Boyce (Massachusetts General Hospital, Boston, MA) and baculovirus stocks and titers were prepared in Sf21 insect cells as described in Delaney and Isom.<sup>34</sup> Primary rat hepatocytes from male Fischer F344 rats (180 to 200 g; Charles River Breeding Laboratories, Wilmington, MA) were isolated and plated as described in Hu and colleagues.<sup>21</sup> At designated days of treatment with 20  $\mu\text{mol/L}$  of TMH-ferrocene, duplicate plates of cells were trypsinized, and viable cell number was determined with a hemocytometer using Trypan blue exclusion. Average cell counts were calculated and used to determine the volume of high-titer CMV-lacZ baculovirus stock necessary to infect cells at the indicated multiplicity of infection. Baculovirus was diluted in Dulbecco's modified Eagle's medium/F12 (control medium) or control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene to a final volume of 0.5 ml. Baculovirus was absorbed to primary rat hepatocytes for 1 hour at 37°C with gentle rocking every 15 minutes to ensure even distribution of the inoculum. After infection, cultures were washed twice in PBS, then refed with control medium or control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene.

### In Situ $\beta$ -Galactosidase ( $\beta$ -gal) Staining with X-gal

At 24 hours after infection with CMV-lacZ baculovirus, primary rat hepatocyte cultures were washed once with PBS containing 2 mmol/L of  $MgCl_2$ . Cultures were then fixed for 5 minutes in PBS containing 2% formaldehyde and 0.05% glutaraldehyde. Cultures were washed twice in PBS. Substrate/stain solution (PBS containing 5 mmol/L of potassium ferricyanide, 5 mmol/L of potassium ferrocyanide, 2 mmol/L of  $MgCl_2$ , and 1 mg/ml of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal; Fisher Biotech, Fair Lawn, NJ) solubilized in dimethyl formamide was applied and cultures were incubated in a 37°C humidified incubator containing 5%  $CO_2$  for appropriate times to allow the appearance of  $\beta$ -gal activity. Cultures were then rinsed once in PBS and fixed at room temperature for 10 minutes in 10% phosphate-buffered formalin. After fixation, cultures were rinsed once in PBS and stored at 4°C in PBS containing 0.2% sodium azide. Cultures were observed for  $\beta$ -gal activity on an inverted microscope.

### RNase Protection Assay

The RNase protection assay (RPA) was performed as described by Cable and colleagues.<sup>27</sup> RNA probes were synthesized from double-stranded polymerase chain reaction (PCR) products to ensure that each RNase probe was of a single and correct size before use in the RPA. The reverse PCR primer was designed to contain the bacterial T7 RNA polymerase promoter (5'-TAATACGACTCATATAGGAGGP-3'). The underlined nucleotides correspond to the first five nucleotides of nascent RNA, whose presence has been shown to produce a highly efficient reaction.<sup>35</sup> The non-underlined sequence is the T7 RNA polymerase promoter. The bold P represents the sequence of the reverse PCR primer. RNA was transcribed from the PCR template according to the manufacturer's instructions (Life Technologies, Inc.). The primers used to produce the specific templates were: for rat E-cadherin, forward 5'-TTCAAAGTGTCTACAGATGGCGTC-3' and reverse 5'-T7CCTCAAAGACCTCTGGATAAACTC-3'; for rat protocadherin, forward 5'-AGTATCGCAAGAGTCAGT-GCCAC-3' and reverse 5'-T7CGGAGGAGTTCCTTTGTCT-TTAGCC-3'; for rat junctional adhesion molecule (JAM), forward 5'-CCAGATCAGATTCCCTATGCAGAC-3' and reverse 5'-T7ATGCGTACAGCCTCTGACCTCATG-3'; for rat radixin, forward 5'-CACCTTGCCAAAGCACTCATACC-3' and reverse 5'-T7TGATCCGTGTGACACCAGAATTAC-3'; for rat  $\alpha$ -actinin, forward 5'-TTCATAGCCAGCAAAGGGGTC-3' and reverse 5'-T7TCCGACGATGTCTTCTGCATCCAG-3'; for rat caveolin, forward 5'-CATGGCAGACGAGGTGAATGAG-3' and reverse 5'-T7TGGACGTAGATGGAATAGACCGG-3'; for rat  $\alpha$ -catenin, forward 5'-CCACGCAGGCAACATA-AACTTC-3' and reverse 5'-T7AGCATGGATCATCTGCA-AACTCC-3'; for rat  $\beta$ -catenin, 5'-TGGACCACAAGCAGAGT-GCTGAAG-3'; and reverse 5'-T7GGTTTCGGATCAATCCA-ACAGTTG-3'; for rat cyclophilin, forward 5'-ATGGTCAAC-CCCACCGTGTCTTC-3' and reverse 5'-T7ATGCCAGGAC-CTGTATGCTTCAGG-3'; and for rat glutaraldehyde phos-

phate dehydrogenase (GAPDH), forward 5'-TCTTCCACCTT-TGATGCTGGG-3' and reverse 5'-T7TTGTTATGGGGTC-TGGGATGG-3'. The T7 sequence described above was added to each reverse primer to transcribe the anti-sense RNA. The probe templates were amplified from total rat liver RNA using the reverse transcriptase-PCR. The sizes of each of the PCR products were: rat E-cadherin, 583 nucleotides; rat protocadherin, 537 nucleotides; rat JAM, 459 nucleotides; rat radixin, 425 nucleotides; rat  $\alpha$ -actinin, 393 nucleotides; rat caveolin, 366 nucleotides; rat  $\alpha$ -catenin, 338 nucleotides; rat  $\beta$ -catenin, 538 nucleotides; rat cyclophilin, 290 nucleotides; and rat GAPDH, 269 nucleotides.

A total of 100  $\mu$ Ci (33.3 pmol) of [<sup>32</sup>P]CTP (NEN Life Science Products, Inc., Boston, MA) was used to label the E-cadherin, protocadherin, JAM, radixin,  $\alpha$ -actinin, caveolin,  $\alpha$ -catenin,  $\beta$ -catenin, and cyclophilin RNA probes, whereas 50  $\mu$ Ci was used to label the GAPDH RNA probe. Cold nucleotides were added to each reaction to a final concentration of 500  $\mu$ mol/L for each probe, except for CTP, which was added to a concentration of 175  $\mu$ mol/L. After incubation at 37°C for 1 hour, the DNA template was removed from the reaction mixture by treatment with RNase-free DNase (Life Technologies, Inc.) and the nascent RNA was precipitated by the addition of 3 vol of ethanol/acetate and the addition of 20  $\mu$ g of tRNA as a carrier. The precipitate was resuspended in formamide gel buffer and the probes purified using a 4% preparative urea-polyacrylamide gel electrophoresis gel. The portion of the gel containing the major product was excised from the gel and the RNA was extracted in 500  $\mu$ l of  $dH_2O$  by incubating at room temperature overnight. Contaminating urea and proteins were removed from the aqueous phase using standard phenol/chloroform extractions. The final product was again ethanol/acetate precipitated and redissolved in 100  $\mu$ l of  $dH_2O$ . A 1- $\mu$ l aliquot was counted in a scintillation counter. Typically, a total of  $1 \times 10^7$  to  $5 \times 10^7$  cpm were obtained for each reaction product. The probes were stored at -80°C until used.

RNA was harvested from a single 60-mm dish of primary hepatocytes using the method of Chomczynski and Sacchi<sup>36</sup> Ten  $\mu$ g of total RNA was used for each reaction mixture. The RNA was hybridized to 16,000 cpm of each probe in a final volume of 12  $\mu$ l of 5 mol/L of guanidine and 100 mmol/L of ethylenediaminetetraacetic acid (pH 7.4). The mixture was heated to 65°C for 5 minutes and incubated overnight (>16 hours) at room temperature. Unhybridized probe was removed by treating the hybridization mixture with RNase A (0.125 U) and RNase T1 (5 U; Ambion, Austin, TX) at 37°C for 1 hour in 350  $\mu$ l of RNase buffer containing 10 mmol/L of HEPES (pH 7.3), 5 mmol/L of ethylenediaminetetraacetic acid, and 300 mmol/L of NaCl. The RNases were inactivated by treatment of the mixture with 10  $\mu$ l of 20% sodium dodecyl sulfate (Sigma) and 0.06 U of proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 15 minutes. Protein was removed from the reaction mixture using phenol/chloroform and chloroform washes. The remaining protected RNA probes were precipitated using ethanol/acetate with Glycoblue (Ambion) as a co-precipitate. Standard positive and negative controls were in-

cluded in each reaction set. The difference between the protected and unprotected probes was five nucleotides for all probes because of the inclusion of the GGAGG sequence. The protected probes were separated using a 4% urea-polyacrylamide gel electrophoresis sequencing gel and the fragments visualized and quantitated using a Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of protected probe in relation to the GAPDH internal control was calculated using the volume measurements from the Phosphorimager. Amounts of protected probe are expressed as fold difference compared to control medium.

### *Gene Array Analysis*

Gene array analysis was performed by Incyte Genomics (Palo Alto, CA). The array used was the Rat Toxicology Chip (RatGem3). The array contains 8951 elements with 8456 unique genes.

### *Immunolocalization*

After 21 days of treatment with TMH-ferrocene, cultures were fixed in 1% phosphate-buffered paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 10 minutes at room temperature. Cultures were rinsed twice in PBS and permeabilized with either 0.2% (ZO-1, occludin, and connexin32) or 0.5% (E-cadherin) Triton X-100 (Sigma) for 10 minutes at room temperature. Cultures were then incubated for 1 hour in a block solution containing 10% bovine serum albumin and 0.1% Triton X-100 in PBS. Primary anti-ZO-1 antibody (a kind gift of Dr. T. W. Gardner, Penn State University College of Medicine, Hershey, PA) specific for rat, anti-E-cadherin antibody (Transduction Labs, Lexington, KY), anti-connexin32 (Chemicon International, Temecula, CA), or anti-occludin (Zymed Laboratories Inc., San Francisco, CA) was applied and incubated at room temperature for 2 hours. After five rinses in PBS and 0.1% Triton X-100, cultures were incubated for 1 hour in a Cy-2-conjugated secondary antibody specific for either rat (ZO-1) or mouse (E-cadherin, connexin32, and occludin) IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in PBS and 0.1% Triton X-100. Cultures were rinsed three times in PBS and 0.1% Triton X-100 and overlaid with coverslips. Immunolocalization for all proteins was observed by fluorescence microscopy.

### *Immunoblot Analysis*

Protein was isolated from FeNTA-treated Huh7 cells or primary rat hepatocyte cultures maintained in control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days (day 28 after seeding). Cultures were rinsed in triplicate with PBS and lysed in either membranous extraction buffer [150 mmol/L NaCl, 25 mmol/L Tris-Cl (pH 7.4), 2% Triton X-100 (Sigma), 2  $\mu\text{g/ml}$  bovine serum albumin, and 50  $\mu\text{l/ml}$  Protease Inhibitor Cocktail for Mammalian Cell Extracts (Sigma)] (E-cad-

herin, ferritin, and caveolin) or RIPA buffer [50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, 12 mmol/L deoxycholic acid, and 50  $\mu\text{l/ml}$  Protease Inhibitor Cocktail for Mammalian Cell Extracts (Sigma)] ( $\beta$ -catenin, connexin32, and occludin). Lysates were centrifuged at  $6400 \times g$  for 10 minutes at 4°C. Protein concentrations were determined by the bicinchoninic acid assay<sup>37</sup> using bovine serum albumin protein standards (Sigma). Ten  $\mu\text{g}$  of protein were diluted in Laemmli buffer<sup>38</sup> and analyzed by sodium dodecyl sulfate-polyacrylamide (8 or 12%) gel electrophoresis under reducing conditions (3%  $\beta$ -mercaptoethanol). Proteins were transferred by electroblotting onto a nitrocellulose membrane (Osmonics, Minnetonka, MN) and stained with Ponceau S (Sigma) to verify equal loading of the gels. Blots were blocked in 5% milk in Tris-buffered saline-Tween for 1 hour at room temperature. Blots were incubated in primary anti-E-cadherin (Transduction Labs), anti- $\beta$ -catenin (Cell Signaling Technology, Inc., Beverly, MA), anti-occludin (Zymed Laboratories, Inc.), anti-connexin32 (Chemicon International), anti-caveolin (Transduction Labs), or anti-ferritin (DAKO Corporation, Carpinteria, CA) antibody for 1 hour at room temperature. After three washings with Tris-buffered saline-Tween, secondary antibody conjugated to horseradish peroxidase was applied for 1 hour at room temperature, followed by an additional three washes in Tris-buffered saline-Tween. Bound antibodies were visualized by chemiluminescence.

### *Measurement of Gap Junction Intercellular Communication (GJIC)*

GJIC was measured by the scrape-loading and dye-transfer technique as described previously.<sup>39,40</sup> Briefly, cultures maintained in control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days (day 28 after seeding) were rinsed in triplicate. Two  $\mu\text{l}$  of 0.05% Lucifer yellow and 0.1% rhodamine dextran (Molecular Probes, Inc., Eugene, OR) dissolved in PBS were added to the cells and scrape-loaded using a rubber policeman. After a 2-minute incubation, the dye solution was removed and the cultures were rinsed four times with PBS to remove detached cells and excess dye. Subsequently, PBS was added to the cultures and to prevent drying. Because of its large size (molecular weight = 10,000), rhodamine dextran will only enter cells damaged by scrape-loading; however, the small size of Lucifer yellow (molecular weight =  $\sim$ 450) allows it to freely diffuse through intact gap junctions of adjoining cells. The diffusion of the Lucifer yellow dye as compared to the nondiffusible rhodamine dextran was examined as a measure of GJIC by fluorescence microscopy.

### *Paracellular Junction Integrity/Membrane Permeability Assay*

Loss of paracellular junction integrity and membrane permeability were measured by the inability of the primary rat hepatocytes in culture to retain the fluorescein metabolic



product of fluorescein diacetate (FDA). Calcium-depleted or control cultures were washed four times in control medium. Cultures were then incubated for 45 minutes in Dulbecco's modified Eagle's medium/F12 containing 2 ng/ml of FDA (Molecular Probes) at 37°C. Cultures were then washed five times with PBS and overlaid with coverslips using Prolong Antifade mounting medium (Molecular Probes). Cultures were observed for fluorescein retention by fluorescence microscopy.

### Reporter Assays

Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. Control and TMH-ferrocene-treated primary rat hepatocyte cultures or control and FeNTA-treated Huh7 cultures were transfected using the Effectene transfection reagent (Qiagen, Inc., Valencia, CA) with either: 1) CMV-*renilla* plasmid alone (a kind gift of Dr. B. Wigdahl, Penn State University College of Medicine, Hershey, PA); 2) CMV-*renilla* and pGL3 (Promega Corp., Madison, WI) plasmids; or 3) CMV-*renilla* and pGL3/PECAD (containing the firefly luciferase gene under control of the human E-cadherin promoter; a kind gift of Dr. Antonio Garcia de Herreros; Universitat Pompeu Fabra, Barcelona, Spain) plasmids. At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/*renilla*) luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corp.).

### Statistical Analyses

All RNase protection and luciferase reporter experiments used at least triplicate samples for each treatment condition studied and statistical analyses were performed with JMP 3.0.2 (SAS Institute, Cary, NC) using analysis of variance, then Dunnett's test versus control, or Tukey-Kramer test for multiple comparisons as appropriate. The null hypothesis was rejected for *P* values less than or equal to 0.05.

## Results

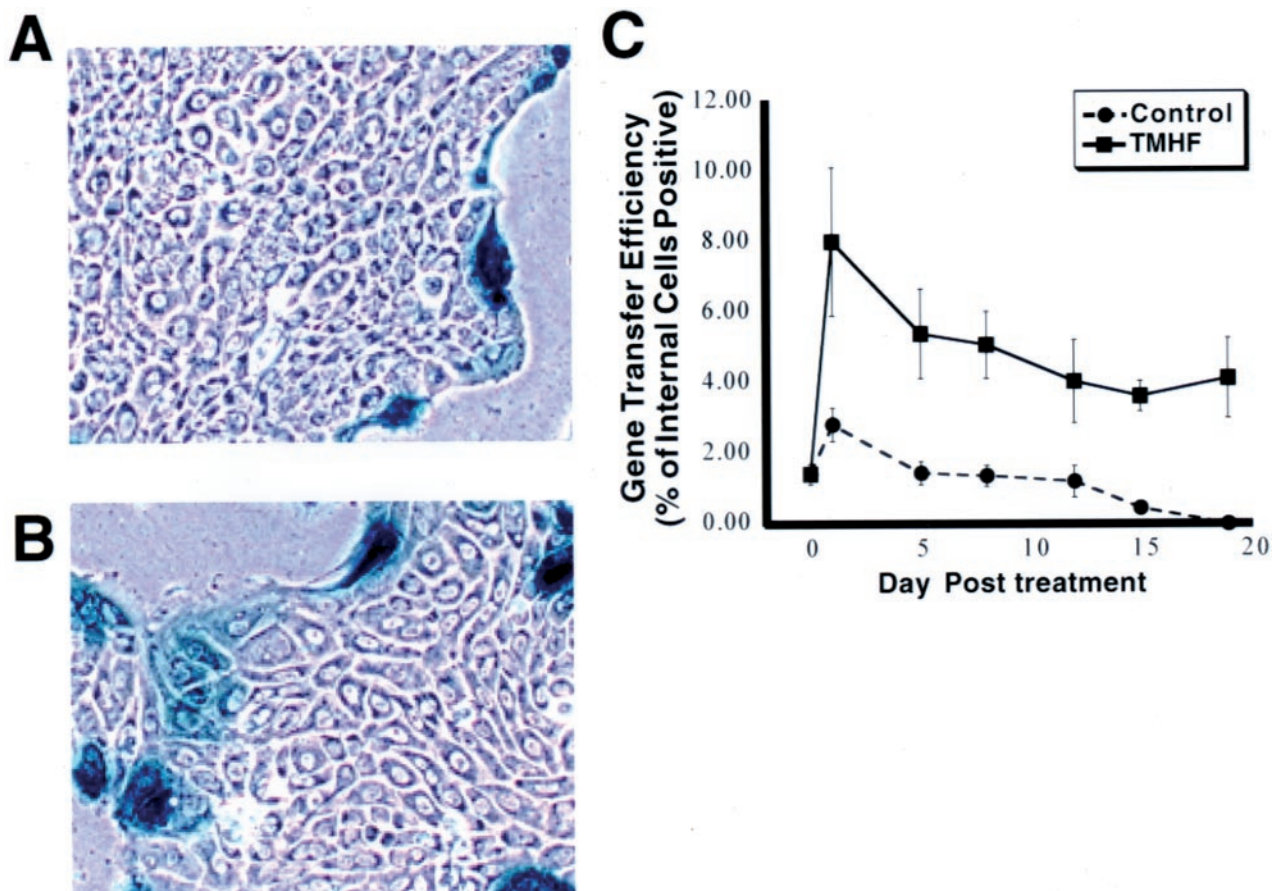
### *Baculovirus-Mediated Gene Transfer Efficiency to Iron-Loaded Hepatocytes*

We recently reported that baculovirus-mediated gene delivery to long-term (>20 days) primary rat hepatocytes was markedly increased when cultures were treated with calcium-free medium supplemented with 100  $\mu\text{mol/L}$  of EGTA before and during infection with baculovirus.<sup>41</sup> Under control medium conditions, baculovirus-mediated transfer of the *lacZ* reporter gene was limited to peripheral cells of multicellular hepatocyte islands and hence, baculovirus-mediated gene transfer efficiency was extremely low. However, examination of  $\beta$ -gal staining in cultures after calcium-depletion by treatment in calcium-free medium or calcium-free medium supplemented with

EGTA revealed that gene transfer was no longer limited to peripheral cells and was homogeneous throughout the hepatocyte islands.<sup>41</sup> For example, at a multiplicity of 1600 pfu/cell, the percent  $\beta$ -gal-positive internal cells increased from 1.2% in medium containing calcium to 75.3% in calcium-free medium supplemented with 100  $\mu\text{mol/L}$  of EGTA.<sup>41</sup> Detailed analyses demonstrated that depletion of extracellular calcium enhanced baculovirus entry into internal hepatocytes and subsequent gene transfer by disrupting paracellular junction integrity. Paracellular junction integrity was restored at 12 hours after the readdition of calcium to the cultures, therefore conferring no permanent effects on the cultures.<sup>42</sup>

As discussed above in the introduction, hepatocytes in long-term DMSO culture can be chronically overloaded with iron. Because it is important to be able to introduce exogenous genetic information into a high percentage of iron-loaded hepatocytes, we designed experiments to determine whether transient depletion of calcium had the same effects on baculovirus-mediated gene delivery to iron-loaded primary rat hepatocytes as we had observed for control hepatocyte cultures. The concentration of TMH-ferrocene and time point selected were based on data obtained from previous studies in our laboratory.<sup>19,20,27</sup> Specifically, when hepatocytes were treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene, a linear increase in total iron content was observed for 21 days (E. E. Cable and H. C. Isom, unpublished data). Compared to untreated control cultures, total iron content in cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days was increased by >20-fold. Cultures treated for 21 days with 20  $\mu\text{mol/L}$  of TMH-ferrocene were morphologically indistinguishable from control cultures (Figure 1, compare A and B). No rounding of cells or cytopathology was observed as a result of treatment with 20  $\mu\text{mol/L}$  of TMH-ferrocene. In addition, total intracellular protein levels were not significantly different in iron-loaded compared to control cultures (data not shown).

Control cultures and cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 20 days were infected with 400 pfu CMV-*lacZ* baculovirus/cell. At 24 hours after infection, cultures were fixed and stained for  $\beta$ -gal activity (Figure 1, A and B), demonstrating that baculovirus-mediated gene delivery could be achieved in iron-loaded primary rat hepatocytes. However, we made the unexpected observation that although the majority of gene transfer was restricted to the peripheral cells,  $\beta$ -gal activity was also observed in internal iron-loaded hepatocytes. Indeed, the pattern of gene transfer in iron-loaded cultures was reminiscent of what was observed for control cultures pretreated and infected in calcium-free medium.<sup>41</sup> A detailed time-course analysis showed that by day 2 after treatment with TMH-ferrocene, the percentage of internal hepatocytes expressing the transgene was higher in iron-loaded cultures compared to control cultures (Figure 1C). At day 20 after treatment with TMH-ferrocene, the percentage of  $\beta$ -gal-positive internal hepatocytes was ~5%, whereas in control cultures, no internal hepatocytes expressed the  $\beta$ -gal transgene. Indeed, the pattern of increased baculovirus-mediated gene transfer after iron treatment mimicked the effects of calcium depletion.



**Figure 1.** Internal hepatocytes stained positive for baculovirus-mediated gene transfer in iron-loaded cultures. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. Primary rat hepatocytes maintained in control medium or control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene were infected for 1 hour with 400 pfu of CMV-lacZ baculovirus/cells at the indicated day after treatment. At 24 hours after infection, cultures were fixed and stained for  $\beta$ -gal activity. Control cultures show no  $\beta$ -gal activity in internal hepatocytes (A), whereas cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days display an increased percentage of both internal and peripheral cells positive for  $\beta$ -gal activity (B). C: Time-course analysis indicating the percentage of internal cells positive for  $\beta$ -gal activity at 24 hours after infection with 400 pfu of CMV-lacZ baculovirus/cells plotted against time of treatment with 20  $\mu\text{mol/L}$  of TMH-ferrocene. Each marker represents the mean percentage with SD of  $\beta$ -gal-positive internal primary rat hepatocytes at the indicated time after treatment. Original magnifications,  $\times 100$ .

Having recently demonstrated that calcium depletion appears to enhance baculovirus entry into internal hepatocytes by disrupting paracellular junction complexes,<sup>41,42</sup> we wanted to determine whether iron loading of primary rat hepatocytes alters paracellular junction integrity.

#### Decreased Expression of Paracellular Junction Complex Gene mRNA Levels in Iron-Loaded Hepatocytes

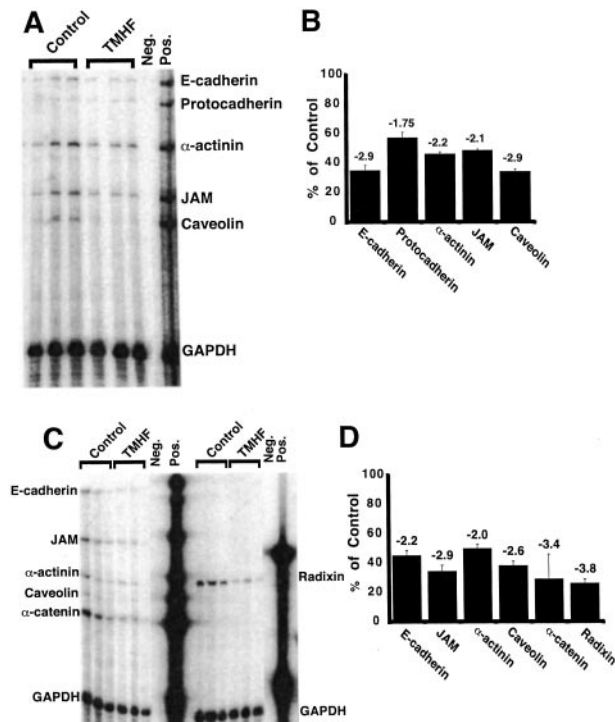
Gene array analysis was performed on RNA isolated from control cultures and cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days. The expression of numerous genes, including several paracellular junction genes, was altered in iron-loaded primary rat hepatocyte cultures compared to control cultures. The data revealed that the number of transcripts whose levels were increased was 258, whereas the number of transcripts whose levels were decreased was 627. A change of 1.7 or greater was considered significantly different in this analysis. The levels of E-cadherin, caveolin, JAM, protocadherin, and  $\alpha$ -catenin mRNA expression were reduced by 2.1-

3.6-fold in iron-loaded compared to control cultures of primary rat hepatocytes (Table 1). RPAs were also performed on RNA from control and TMH-ferrocene-treated hepatocyte cultures (Figure 2). In these studies, the expression of E-cadherin, protocadherin,  $\alpha$ -actinin, JAM, and caveolin mRNAs was reduced by 2.9-, 1.75-, 2.2-, 2.1-, and 2.9-fold in iron-loaded compared to control cultures of primary rat hepatocytes, respectively (Figure

**Table 1.** Gene Array Analysis of Iron-Related Gene Expression in Long-Term Iron-Loaded Primary Rat Hepatocytes

Gene	Fold expression
E-cadherin	-3.6
Caveolin	-3.2
JAM	-2.9
Protocadherin	-2.1
$\alpha$ -catenin	-2.1

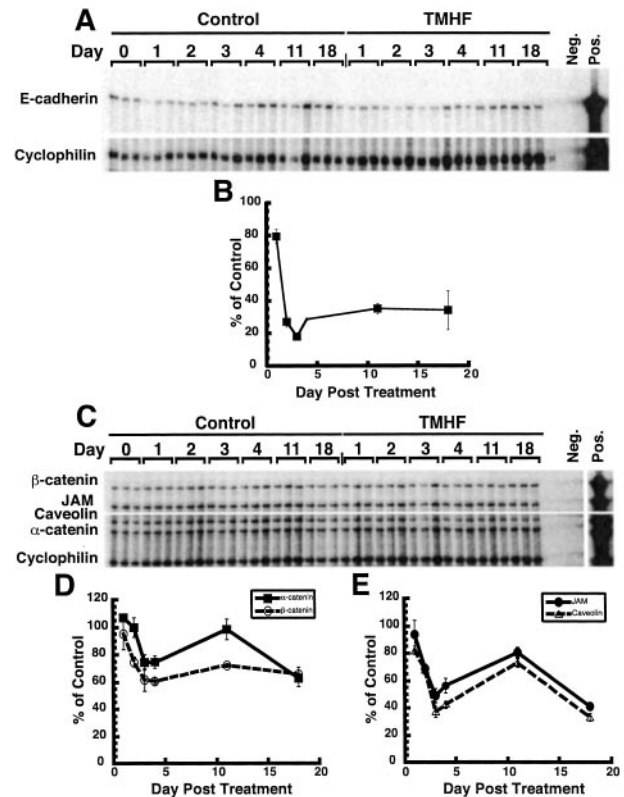
Primary rat hepatocytes were either untreated or treated with 20  $\mu\text{mol/L}$  TMHF for 21 days. Total RNA was harvested and gene expression was analyzed using gene array analysis. Decreases in transcript levels are expressed as the negative inverse of the ratio of treated normalized to control.



**Figure 2.** RPA analysis demonstrated decreased expression of paracellular junction genes in primary rat hepatocytes after treatment with TMH-ferrocene. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. After 21 days of treatment, control and iron-loaded cultures were harvested for total RNA. RPA analyses for the selected paracellular junction genes indicated in each figure (A and C) were performed on RNA from control (control) and iron-loaded (TMHF) cultures. Quantitative analyses of expression of selected paracellular junction genes indicated in each figure (B and D) were performed for RPA analyses for RNA from control (control) and iron-loaded (TMHF) cultures. As a control for sample processing and gel loading, the data were normalized to GAPDH expression. Each bar represents the mean percentage with SD of mRNA expression in iron-loaded cultures compared to control cultures. The fold-decrease in mRNA expression in iron-loaded cultures relative to control cultures is displayed above each bar.

2, A and B). The results closely matched the gene array analysis. We next extended the RPA analysis to two additional paracellular junction genes,  $\alpha$ -catenin and radixin. Decreased expression of  $\alpha$ -catenin was detected in the gene array analysis and radixin gene expression was not measured in the gene array analysis (Table 1). The same concentration of TMH-ferrocene (20  $\mu\text{mol/L}$ ) and time point of treatment (21 days) were selected for these analyses. In addition, the effects on expression of E-cadherin, JAM, caveolin, and  $\alpha$ -actinin were re-evaluated. We conclude from these studies, that  $\alpha$ -catenin and radixin mRNA levels were decreased by 3.4- and 3.8-fold, respectively, in iron-loaded compared to control hepatocyte cultures (Figure 2, C and D). In addition, the previously observed decreases in E-cadherin, JAM, caveolin, and  $\alpha$ -actinin mRNA expression were reproduced.

The effect of iron loading throughout time on the expression of numerous paracellular junction genes was also analyzed. At 1, 2, 3, 4, 12, and 18 days of treatment with TMH-ferrocene, cultures were harvested for total RNA. Time-course analyses demonstrated that by day 3 after treatment with TMH-ferrocene, the expression of



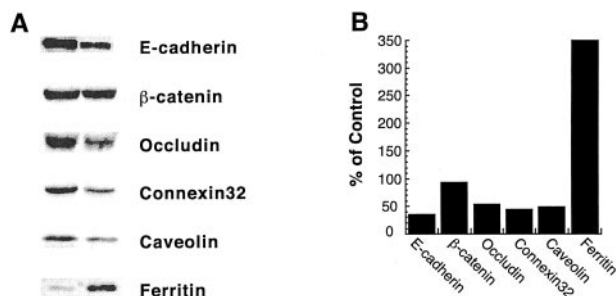
**Figure 3.** Time-course analysis of paracellular junction gene mRNA expression in primary rat hepatocyte cultures during iron loading by TMH-ferrocene. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. At 1, 2, 3, 4, 12, and 18 days of treatment with TMH-ferrocene, cultures were harvested for total RNA. RPA analysis probing for E-cadherin (A) and  $\alpha$ -catenin,  $\beta$ -catenin, JAM, and caveolin (C) mRNA expression throughout time after TMH-ferrocene treatment was performed on RNA from control (control) and iron-loaded (TMHF) cultures. Quantitative analyses of the expression of E-cadherin (B),  $\alpha$ - and  $\beta$ -catenin (D), and JAM and caveolin (E) mRNA in iron-loaded primary rat hepatocyte cultures compared to control cultures. As a control for sample processing and gel loading, the data were normalized to cyclophilin expression in iron-loaded cultures compared to the control cultures. Where no error bar is observed, the error falls within the size of the marker.

E-cadherin mRNA levels decreased to  $\sim 20\%$  of the control level and remained between 20% and 40% of the control level through 18 days of treatment (Figure 3, A and B). Additionally, time-course analysis demonstrated that by day 3 after treatment with TMH-ferrocene, the mRNA expression of  $\alpha$ -catenin,  $\beta$ -catenin, JAM, and caveolin was  $\sim 75\%$ , 60%, 50%, and 40% of control, respectively (Figure 3; C, D, and E). At day 12 after treatment with 20  $\mu\text{mol/L}$  of TMH-ferrocene, mRNA expression increased slightly over day 3 levels; however, by day 18 after treatment, the expression of  $\alpha$ -catenin,  $\beta$ -catenin, JAM, and caveolin was  $\sim 60\%$ , 60%, 35%, and 40% of control cultures, respectively (Figure 3; C, D, and E).

### Decreased Expression of Paracellular Junction Complex Proteins in Iron-Loaded Hepatocytes

To determine whether decreased mRNA expression of paracellular junction genes translated into a decrease in

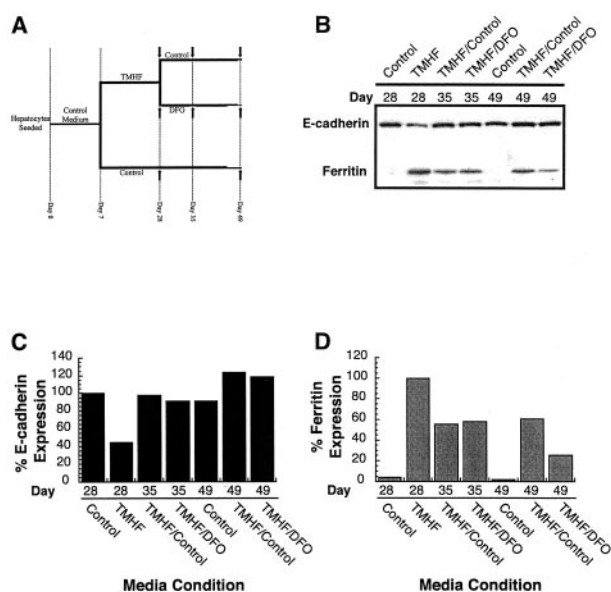




**Figure 4.** Protein expression of numerous paracellular junction genes was decreased in iron-loaded primary rat hepatocytes. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. After 21 days of treatment, control and TMH-ferrocene-treated cultures were harvested for protein and subjected to immunoblot analysis. **A:** Blots were analyzed for E-cadherin,  $\beta$ -catenin, occludin, connexin32, caveolin, and ferritin expression. **B:** The percent expression of each paracellular junction protein analyzed in TMH-ferrocene-treated (TMHF) cultures compared to control cultures.

expression of paracellular junction proteins, immunoblot analyses were performed on cellular lysate from primary rat hepatocyte cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days. E-cadherin protein levels in primary rat hepatocyte cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 20 days were  $\sim 35\%$  of control cultures (Figure 4, A and B), which corresponded to the decrease observed in the mRNA levels. Decreases in protein expression were also observed for the tight junction protein, occludin (54%); the gap junction protein, connexin32 (45%); and caveolin (50%), which was just recently demonstrated to associate at paracellular junctions (Figure 4, A and B).<sup>43</sup> Conversely, no significant change in  $\beta$ -catenin protein expression was observed in iron-loaded cultures compared to control cultures. The protein expression of ferritin increased to  $\sim 350\%$  of the level in control cultures, indicating that iron-loading by TMH-ferrocene induced a known iron-responsive gene (Figure 4, A and B).

To determine whether removal of iron from cultures maintained in medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene restored E-cadherin protein levels to that of control cultures, intracellular iron was chelated by treatment with 500  $\mu\text{mol/L}$  of DFO. As displayed in Figure 5A, primary rat hepatocytes were maintained in control medium for 7 days after seeding (day 7), after which, cultures were either maintained in control medium or in control medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days (day 28). At day 28, cultures on control medium were maintained in control medium; however, iron-loaded cultures were now either further maintained in control medium or control medium supplemented with 500  $\mu\text{mol/L}$  of DFO for an additional 21 days (day 49). At days 28, 35, and 49 of the experiment, protein was isolated and immunoblot analyses were performed for E-cadherin and ferritin. At day 28 (corresponding to 21 days of treatment with 20  $\mu\text{mol/L}$  of TMH-ferrocene), E-cadherin protein levels were  $\sim 40\%$  of control cultures (Figure 5, B and C) and ferritin protein expression was  $\sim 20$ -fold higher than in control cultures (Figure 5, B and D). The effects of placing cells in me-



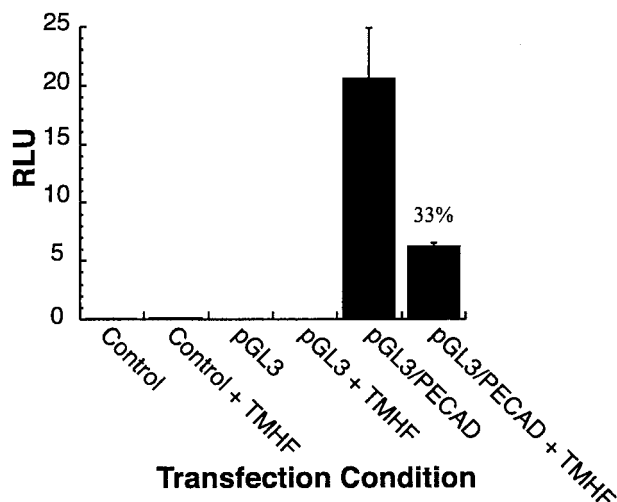
**Figure 5.** E-cadherin protein expression after release from iron-loading and addition the iron chelator DFO. **A:** Experimental schematic for iron loading and removal of iron from primary rat hepatocyte cultures. Isolated primary rat hepatocytes were plated onto collagen-coated tissue culture dishes and fed control medium for a period of 7 days (days 0 through 7). Seven days after plating, cultures were either re-fed in control medium (control) or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene (TMHF). Cultures were re-fed three times per week for 21 days (days 7 through 28), after which, cultures were either re-fed three times per week with either control medium (control or TMHF/control) or medium supplemented with 500  $\mu\text{mol/L}$  of DFO (TMHF/DFO). Protein samples were harvested at days 28, 35, and 49 after removal of TMH-ferrocene from the media or chelation using medium supplemented with DFO (arrows). **B:** Immunoblot analysis for E-cadherin and ferritin protein levels in primary rat hepatocyte cultures after removal of iron and addition of DFO. Quantitative analyses of immunoblots for E-cadherin (**C**) and ferritin (**D**) were performed by densitometry.

dium free of TMH-ferrocene or supplemented with the chelator DFO were as expected. Specifically, placing cells in control media reduced the level of ferritin expression. When cells were treated with DFO, ferritin levels were reduced and with time (day 49) to lower levels than when cells were simply cultured in iron-free medium. After removal of iron from the medium for 7 days (day 35) by either feeding with control or DFO-supplemented medium, E-cadherin protein levels returned to that of control cultures consistently maintained in control medium throughout the experiment. Twenty-one days after the removal of iron from the cultures (day 49), E-cadherin levels exceeded that of the control cultures. The results of this experiment demonstrate that the effects of iron on E-cadherin are reversible, further indicating a direct regulatory role of iron on E-cadherin expression.

#### *E-Cadherin Promoter Activity Was Decreased in Iron-Loaded Primary Rat Hepatocytes*

Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. After 12 days of treatment, control and TMH-ferrocene-treated cultures were trans-





**Figure 6.** E-cadherin promoter activity was decreased in iron-loaded primary rat hepatocyte cultures. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene. After 12 days of treatment, control and TMH-ferrocene-treated (+TMHF) cultures were transfected with either: CMV-renilla plasmid alone (control); CMV-renilla and pGL3 plasmids (pGL3); or CMV-renilla and pGL3/PECAD plasmids. At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/renilla) luciferase activity (RLU).

ected with either: 1) CMV-renilla plasmid alone (control); 2) CMV-renilla and pGL3 plasmids (pGL3); or 3) CMV-renilla and pGL3/PECAD (containing the firefly luciferase gene under control of the human E-cadherin promoter) plasmids. At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/renilla) luciferase activity (Figure 6). E-cadherin promoter activity in iron-loaded primary rat hepatocyte cultures (+TMHF) was ~33% of that observed in control cultures. No luciferase activity was observed in control or pGL3 transfections. Therefore, iron regulation of the E-cadherin promoter may be wholly or partially responsible for the decreased E-cadherin mRNA and protein levels observed in iron-loaded primary rat hepatocytes.

#### *Immunolocalization of Paracellular Junction Proteins Was Altered in Iron-Loaded Cultures of Primary Rat Hepatocytes*

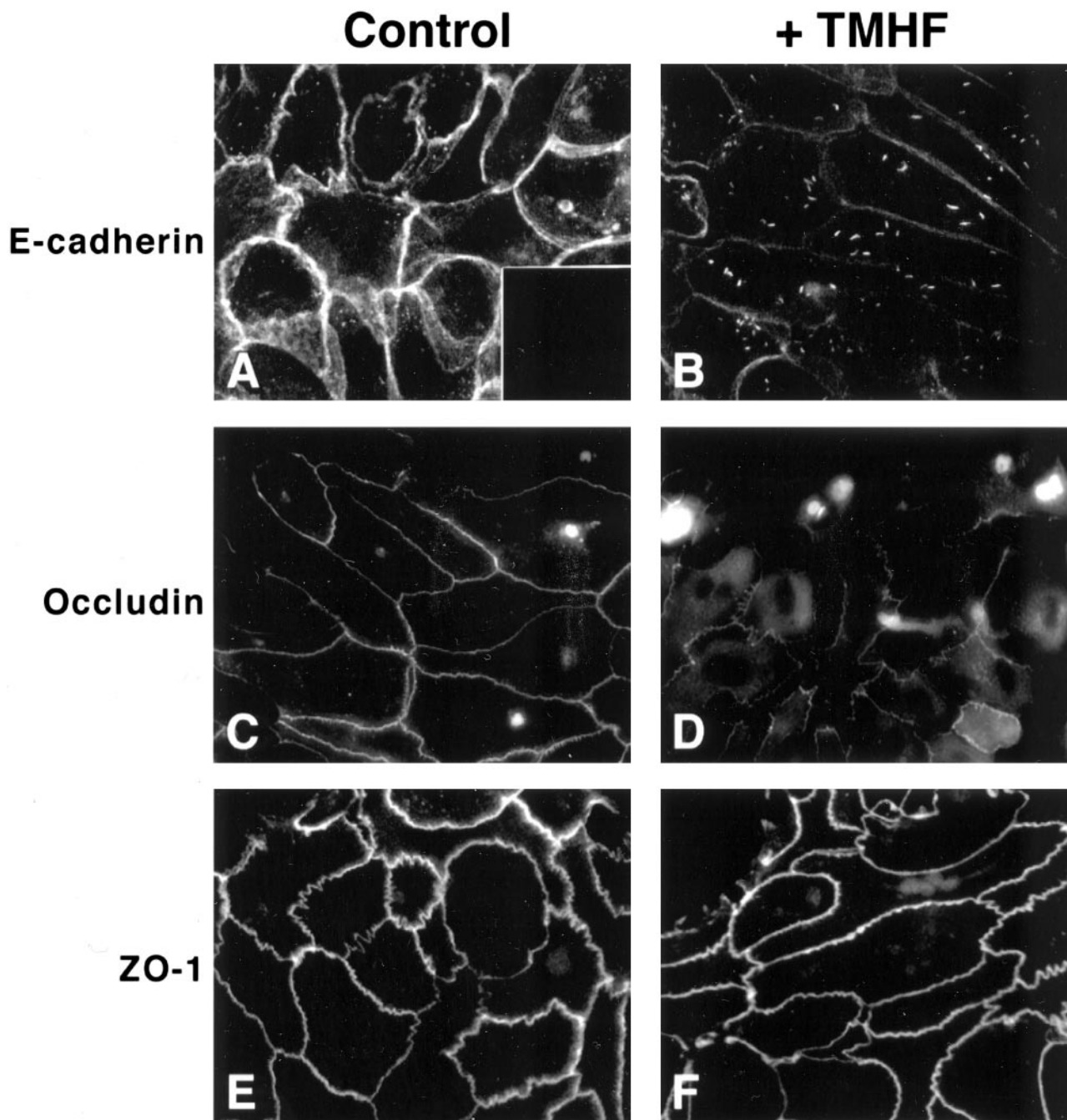
As previously described, E-cadherin mRNA and protein expression were reduced after iron loading of primary rat hepatocytes. To determine whether the localization of E-cadherin was affected by iron loading of primary rat hepatocytes, cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days were examined for E-cadherin immunolocalization. In control cultures, E-cadherin localized to the membranes of adjoining cells (Figure 7A), whereas, in agreement with the immunoblot analysis, immunolocalization of E-cadherin to the membranes of adjoining cells was reduced in iron-loaded cultures (Figure 7B). Interestingly, iron-loaded cultures displayed a localization of E-cadherin into distinct intracellular punctate regions.

To determine whether treatment of primary rat hepatocyte cultures with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days affected paracellular tight junctions, control and iron-loaded primary rat hepatocyte cultures were examined for immunolocalization of occludin and the tight junction protein, ZO-1. In control cultures, occludin localized to the membranes of adjoining cells (Figure 7C), whereas, in agreement with the immunoblot analysis, immunolocalization of occludin to the membranes of adjoining cells was reduced in iron-loaded cultures (Figure 7D). Occludin also localized intracellularly after iron loading, which was not observed in control cultures. In contrast, ZO-1 immunolocalization was not altered by iron loading (Figure 7, E and F), indicating that the effects of TMH-ferrocene treatment on paracellular protein localization were limited to certain paracellular junction proteins. It should be noted that this finding was in contrast to what we observed for transient calcium depletion, in which EGTA-treatment caused ZO-1 internalization.<sup>42</sup>

Iron loading of primary rat hepatocytes with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days also altered the localization of the gap junction protein, connexin32 (Figure 8, A and B). Whereas in both control and iron-loaded cultures, connexin32 was observed diffusely throughout the cytoplasm, punctate staining was observed in control cultures, indicative of connexin32 oligomerization into hemichannels, and subsequently gap junctions, at the membranes of adjoining cells. This decrease in gap junction formation translated into a decrease in GJIC as measured by the scrape-loading and dye-transfer technique. Diffusion of Lucifer yellow (green) dye was decreased in iron-loaded cultures, diffusing into only one cell layer (Figure 8D) beyond the scratch line indicated by rhodamine dextran (red). Conversely, Lucifer yellow diffused through several cell layers beyond the rhodamine dextran scratch line in control cultures (Figure 8C).

#### *Paracellular Junction Integrity Was Compromised and Membrane Permeability Was Increased in Iron-Loaded Primary Rat Hepatocytes*

To examine if decreases in paracellular junction gene expression after iron loading of primary rat hepatocytes altered paracellular junction integrity and membrane permeability, FDA staining was performed on control cultures and cultures treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days. FDA, a nonpolar nonfluorescent fatty acid ester, freely enters living cells and is hydrolyzed by cellular esterases to produce fluorescein. In cells with intact cell membranes, the polar fluorescein accumulates intracellularly, a phenomenon known as fluorochromasia.<sup>44,45</sup> The accumulation or release of fluorescein after hydrolysis of FDA is dependent on the cell membrane permeability and integrity.<sup>45-48</sup> When membrane permeability is increased, fluorescein is able to passively diffuse out of the cells into intercellular clefts. The fluores-

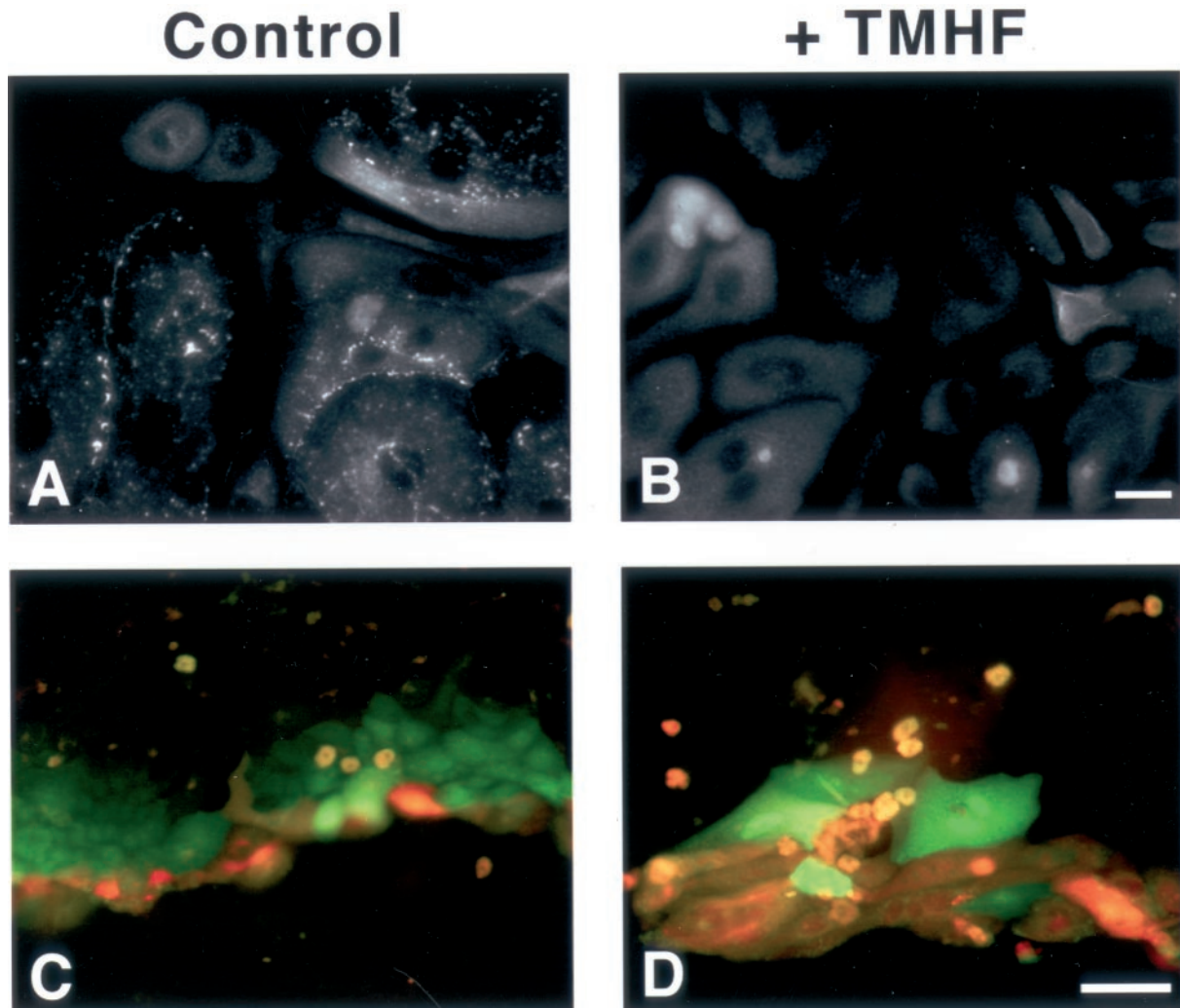


**Figure 7.** Immunolocalization of paracellular junction proteins after iron loading of primary rat hepatocytes. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days. After 21 days of iron loading, cultures were fixed and immunofluorescence was performed for E-cadherin (**A** and **B**), occludin (**C** and **D**), and ZO-1 (**E** and **F**) on control (control) and iron-loaded (+TMHF) cultures. (**A**, inset) No immunofluorescence was observed when cultures were incubated in control mouse IgG antibody rather than primary antibody. Original magnifications,  $\times 600$ .

cein is not retained intercellularly when paracellular junctions are compromised and consequently, the fluorescein diffuses into the medium. The level of fluorescein retention within the hepatocytes decreased after iron loading (Figure 9, A and B), suggesting that iron loading increases membrane permeability and intercellular leakage of fluorescein resulting from loss of paracellular junction integrity.

#### *Iron Treatment of Human Hepatic Cells with the FeNTA Iron Donor Decreased E-Cadherin Protein Expression*

To determine whether the decreased E-cadherin expression observed in iron-loaded primary rat hepatocytes could be duplicated in a human hepatic culture system, the Huh7 human hepatoma cell line was treated with 100



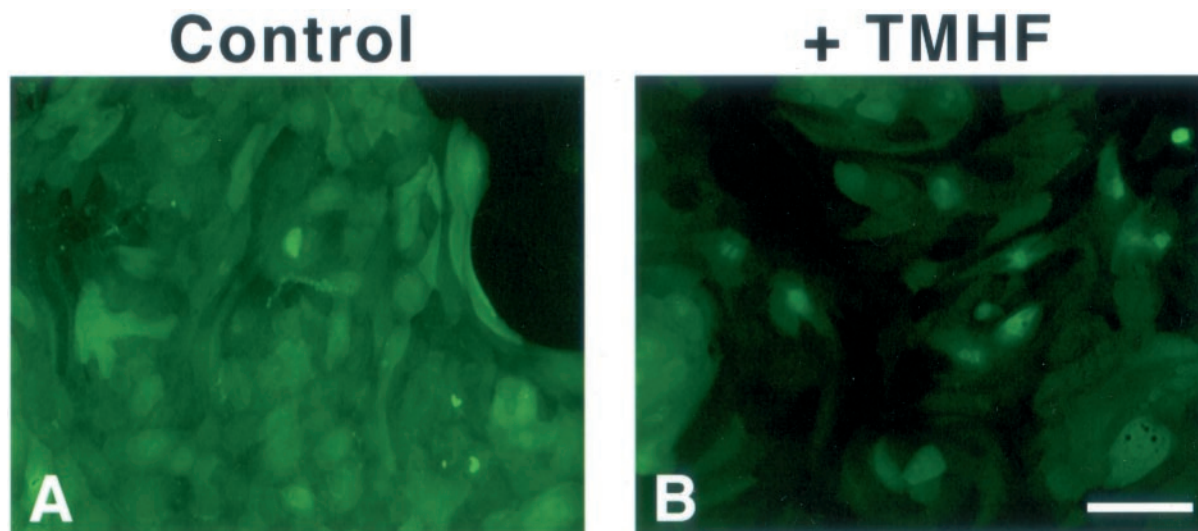
**Figure 8.** Immunolocalization of gap junction proteins and decreased GJIC in iron-loaded primary rat hepatocytes. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene for 21 days. After 21 days of iron loading, cultures were fixed and immunofluorescence was performed for connexin32 (**A** and **B**) on control (control) and iron-loaded (+TMHF) cultures. GJIC was analyzed by the scrape-loading and dye-transfer technique on control (control) and iron-loaded (+TMHF) cultures after 21 days of iron loading (**C** and **D**). Note that in control cultures (**C**), Lucifer yellow (green) diffused into multiple layers of cells, demonstrating functional GJIC, whereas in iron-loaded cultures (**D**), Lucifer yellow only diffused into a single layer of cells, demonstrating inhibition of GJIC. Original magnifications:  $\times 400$  (**A**, **B**);  $\times 200$  (**C**, **D**). Scale bars: 20  $\mu\text{m}$  (**A**, **B**); 100  $\mu\text{m}$  (**C**, **D**).

$\mu\text{mol/L}$  of FeNTA for 72 or 96 hours. Twenty-four hours after plating, Huh7 cells were treated, in the absence of fetal bovine serum, with control medium or control medium supplemented with 100  $\mu\text{mol/L}$  of FeNTA (Figure 10A). Cultures were either not refed (72 hours or 96 hours) or refed every 24 hours (72 hours  $\times$  3 or 96 hours  $\times$  4) with the appropriate medium. After 72 or 96 hours, cultures were harvested for protein and analyzed by immunoblotting for E-cadherin or ferritin protein expression. All treatments demonstrated a significant decrease in E-cadherin protein expression over control cultures, with the greatest decrease occurring in the 96 hours  $\times$  4 treatment. Ferritin expression was significantly increased over controls for each FeNTA treatment condition. This study demonstrates that iron regulation of E-cadherin and ferritin expression occurs in human as well as rat cells of hepatic origin.

#### *E-Cadherin Promoter Activity Was Decreased in FeNTA-Treated Human Hepatic Cells*

To determine whether the E-cadherin promoter activity was altered by iron treatment in a human cell culture system, Huh7 cells were transfected in serum-free medium in the absence or presence of 100  $\mu\text{mol/L}$  of FeNTA with either: 1) CMV-renilla plasmid alone (control); 2) CMV-renilla and pGL3 plasmids (pGL3); or 3) CMV-renilla and pGL3/PECAD plasmids. At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/renilla) luciferase activity (Figure 10B). At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/renilla) luciferase activity. E-cadherin promoter activity was  $\sim 13\%$  in FeNTA-treated (+FeNTA) compared to untreated cultures. No luciferase activity





**Figure 9.** Paracellular junction integrity and membrane permeability in iron-loaded primary rat hepatocyte cultures. Primary rat hepatocytes were maintained for 7 days in control medium, after which, cultures were maintained in either control medium (control, **A**) or medium supplemented with 20  $\mu\text{mol/L}$  of TMH-ferrocene (+TMHF, **B**). After 21 days of treatment, control and TMH-ferrocene-treated cultures were stained with 2 ng/ml of FDA for 45 minutes at 37°C. Cultures were then rinsed five times with PBS and observed by fluorescence microscopy. Original magnifications,  $\times 200$ . Scale bar, 100  $\mu\text{m}$ .

was observed in control or pGL3 transfections. This study further demonstrates iron-dependent regulation of the E-cadherin promoter and suggests the existence of a novel transcriptional regulatory mechanism present in both human and rat hepatic cell culture systems.

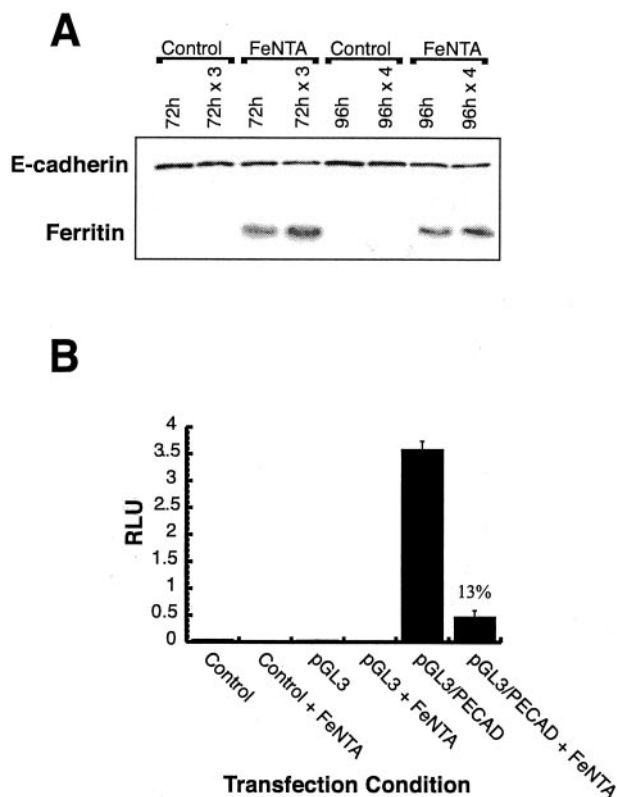
### Discussion

Based on the results presented in this article, we conclude the following: 1) paracellular junction complex junction integrity was compromised and membrane permeability was increased in iron-loaded primary rat hepatocytes. 2) Expression of numerous paracellular junction genes was decreased in a time-dependent manner in primary rat hepatocytes treated with 20  $\mu\text{mol/L}$  of TMH-ferrocene throughout 21 days. 3) The expression of numerous paracellular junction proteins was also decreased in iron-loaded hepatocytes. 4) The effects of iron on E-cadherin were reversible as indicated by the findings that protein expression of E-cadherin returned to levels comparable to control cultures after TMH-ferrocene was removed from the media and/or iron was chelated from the cells by exposure to DFO. 5) Localization of the adherens junction protein, E-cadherin, the tight junction protein, occludin, and the gap junction protein, connexin32, was altered in iron-loaded primary rat hepatocytes. 6) Gap junction communication was decreased in primary rat hepatocytes after iron loading. 7) E-cadherin protein expression was decreased in FeNTA-treated human hepatic cells. 8) E-cadherin promoter activity was decreased in iron-loaded primary rat hepatocytes and FeNTA-treated human hepatic cells. 9) Baculoviruses infected a greater proportion of internal hepatocytes in iron-loaded compared to control long-term nondividing hepatocyte cultures most likely because iron loading reduces expression of paracellular

junction complex genes and hence compromises paracellular junction complex integrity.

Hepatocytes in long-term DMSO culture are highly differentiated and an excellent *in vitro* model for studying growth, gene expression, and pathological changes in adult hepatocytes. The long-term DMSO hepatocyte culture system has several notable advantages that have made it possible for our laboratory and other laboratories to use the system to study differentiated hepatocyte function.<sup>49–54</sup> Some of these characteristics are of particular importance to the experiments reported in this study. It was possible to examine the effect of iron loading on paracellular junction complexes because hepatocytes in long-term DMSO culture have intercellular junctions and express paracellular junction complex genes. In addition, the way in which the hepatocytes are maintained in this system made it possible to use techniques such as FDA staining to measure paracellular junction integrity and membrane permeability, gap junction communication, and immunofluorescence staining for paracellular junction proteins. Specifically, the cells are homogeneous because DMSO essentially eliminates contaminating nonparenchymal liver cells normally present in primary rat hepatocyte cultures and there is no need to add an exogenous source of contaminating cells for co-culture. The hepatocytes are plated on rat-tail collagen-surfaced plates and therefore, the morphology of the cells is easily visualized because the cells are not in a matrix or co-cultured. Another advantage of the long-term DMSO culture system that made these studies possible is that enough viable cells can be obtained ( $5 \times 10^5$  to  $1 \times 10^6$  cells per 60-mm dish) to perform most molecular studies including RPA and gene array analyses.

It is important to note that the studies in this article have also added to our knowledge regarding the properties of primary rat hepatocytes in long-term DMSO culture. Spe-



**Figure 10.** E-cadherin expression and promoter activity in iron-loaded human hepatic cell cultures. **A:** E-cadherin protein expression was decreased in Huh7 cells treated with FeNTA. The Huh7 human hepatoma cell line was treated with 100  $\mu\text{mol/L}$  of FeNTA for 72 or 96 hours. Twenty-four hours after plating, Huh7 cells were treated, in the absence of fetal bovine serum, with control medium or medium supplemented with 100  $\mu\text{mol/L}$  of FeNTA. Cultures were either not refed (72 hours or 96 hours) or refed every 24 hours (72 hours  $\times$  3 or 96 hours  $\times$  4) with the appropriate medium. After 72 hours or 96 hours, cultures were harvested for protein and analyzed for E-cadherin or ferritin protein expression. **B:** E-cadherin promoter activity was decreased in FeNTA-treated human hepatic cells. Huh7 cells were transfected with either CMV-renilla plasmid alone (control), CMV-renilla and pGL3 plasmids (pGL3), or CMV-renilla and pGL3/PECAD (pGL3/PECAD) plasmids in serum-free medium in the absence or presence of 100  $\mu\text{mol/L}$  of FeNTA (+FeNTA). At 24 hours after transfection, cultures were harvested and analyzed for relative (firefly/renilla) luciferase activity (RLU).

cifically, we report here for the first time that these cells retain the ability to express paracellular junction genes including: E-cadherin, protocadherin,  $\alpha$ -actinin, JAM, caveolin,  $\alpha$ -catenin,  $\beta$ -catenin, radixin, occludin, connexin32, and ZO-1. In addition, we have demonstrated that this culture system can be used to study gap junction intercellular communication.

In this study, the effects of iron loading on different genes that play a role in paracellular junction integrity were measured. Paracellular junction complexes of hepatocytes include the tight and adherens junctions, desmosomes, and gap junctions.<sup>55–60</sup> Tight junctions, partially composed of occludin and ZO-1 and ZO-2, form continuous contacts and limit paracellular movement of molecules.<sup>11–13</sup> The tight junction complex is stabilized by the binding of JAM to ZO-1.<sup>61</sup> We showed in this study that iron-loading did not alter ZO-1 immunolocalization, which may be because of the interaction of ZO-1 and cytoskeletal elements. However, we know that alteration of ZO-1 immunolocalization can be achieved in hepato-

cytes in long-term DMSO culture; we recently demonstrated that calcium depletion induces a rapid alteration in the cellular localization of ZO-1 and that this effect can be readily reversed when calcium is returned to the hepatocyte cultures.<sup>42</sup> Although iron loading did not alter ZO-1 immunolocalization, iron loading did lead to reduced expression of JAM mRNA and this reduction was detected in both the gene array and RPA analyses.

Adherens junctions, composed of cadherins or non-classical cadherins known as protocadherins, are adhesive contacts located below the tight junctions. Epithelial intercellular contact at the adherens junction is mediated by calcium-dependent intercellular binding of interdigitated E-cadherin dimers that form a continuous zipper-like structure.<sup>14,62–64</sup> The binding of cadherins to cytoplasmic  $\alpha$ - and  $\beta$ -catenins, in association with the actin cytoskeleton through proteins such as  $\alpha$ -actinin and radixin, couples cell-cell adhesion to tissue morphology and signal transduction.<sup>14,65–70</sup> The results of the gene array analysis comparing RNA from iron-loaded and control cultures of hepatocytes indicated that the expression of E-cadherin, protocadherin, and  $\alpha$ -catenin mRNAs were all significantly reduced in iron-loaded hepatocytes. These three genes were also found to be significantly reduced when RNA from iron-loaded compared to control hepatocytes was subjected to RPA analysis. One strength of this study is that the data obtained initially by gene array analysis was verified by a second technique, RPA. The complementarity of the data are of interest because not all changes detected by gene array analysis can be validated by a second procedure. It is also noteworthy that the data obtained by RPA was highly reproducible using hepatocytes isolated from different animals and cultured independently. Other laboratories have reported in various different experimental conditions that decreased E-cadherin expression can be caused by transcriptional down-regulation or mutations in the E-cadherin gene.<sup>71–77</sup> In this study, gene array, RPA analyses, and E-cadherin promoter reporter assays indicate that transcriptional down-regulation of the E-cadherin gene may be occurring in both iron-loaded primary rat hepatocytes and FeNTA-treated human hepatic cells in culture.

Additional RPA analysis demonstrated that  $\beta$ -catenin,  $\alpha$ -actinin, and radixin mRNA levels were also decreased in iron-loaded cultures; however,  $\beta$ -catenin protein levels were not altered in iron-loaded primary rat hepatocytes. One possible explanation for this may be found in the characteristics of  $\beta$ -catenin degradation. Degradation of  $\beta$ -catenin is regulated by a multisubunit complex composed of constitutively active GSK3 $\beta$ , axin, and adenomatous polyposis coli.<sup>78–80</sup> This complex regulates the level of  $\beta$ -catenin protein accumulation in a cell to maintain homeostasis of  $\beta$ -catenin protein levels and prevent subsequent tumorigenesis that can result from overaccumulation of  $\beta$ -catenin.<sup>67,81–83</sup>  $\beta$ -Catenin protein expression in iron-loaded primary rat hepatocytes may be similar to control cultures in that the degradation of  $\beta$ -catenin may be inhibited to maintain the appropriate levels of  $\beta$ -catenin in the hepatocytes.

Recently, the roles of E-cadherin and  $\alpha$ - and  $\beta$ -catenin in signal transduction were demonstrated to be dependent on the lipid-rich scaffolding caveolae membranes.<sup>84</sup> Caveolae are composed of caveolin family members (caveolin-1, -2, and -3), cholesterol, sphingolipids, and signaling molecules, which enable them to function in signal transduction.<sup>84</sup> We conclude from both the gene array and RPA analyses that iron loading reduced expression of caveolin mRNA levels. Direct cell-cell communication is mediated through gap junctions, which are channels composed of oligomerized connexins that connect neighboring cells. Molecular movement through these channels is relatively nonspecific and occurs by passive diffusion.<sup>85</sup> Gap junction communication was also decreased, consistent with decreases observed in connexin32 protein levels and gap junction formation, in iron-loaded primary rat hepatocyte cultures. The disruption of paracellular junctions after iron loading also resulted in the increased intercellular leakage and membrane permeability of fluorescein and subsequent loss of fluorescein accumulation within the hepatocytes.

Iron overload is associated with liver pathogenicity in several human diseases including genetic HH and thalassemia.<sup>86-94</sup> Iron deposition in hepatocytes of those afflicted with genetic HH begins early in life and progresses at a constant rate.<sup>7,9</sup> As iron concentrations increase in the liver, fibrosis begins to expand the portal tracts. At later stages, iron loading is found throughout the liver acinus. Cirrhosis begins to develop as the normal architecture of the liver is destroyed, marking a decrease in life expectancy and an increased risk of development of hepatocellular carcinoma.<sup>95-98</sup> Increased development and severity of hepatocellular carcinoma has also been associated with decreased expression of the invasion-suppressor gene, E-cadherin.<sup>76,99</sup> The relationship between membranous E-cadherin levels and the tumor grade and patient prognosis is well established for carcinomas of the liver, as well as the esophagus and colon.<sup>100-108</sup> Although much evidence is available that relates iron loading of hepatocytes or decreased E-cadherin expression with the incidence and severity of hepatocellular carcinoma, the relationship between iron loading and decreased paracellular junction gene expression has not been previously investigated. The results presented in this report describe the down-regulation of paracellular junction mRNA expression and specifically, decreased E-cadherin expression at the mRNA and protein levels, in iron-loaded rat and human hepatic cells *in vitro*. These studies suggest that in HH and other iron-loaded patients, iron loading of hepatocytes may decrease paracellular junction gene expression, leading to increased risk for developing hepatocellular carcinoma. Our experiments in rat and human hepatic cells demonstrate that iron loading decreased the E-cadherin promoter activity and expression of numerous paracellular junction genes and altered cellular localization of several paracellular junction proteins, increasing membrane permeability and inhibiting intercellular communication, potentially predisposing hepatocytes to carcinogenesis by additional yet undefined mechanisms.

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