Abstract—Two common alleles exist at the haptoglobin (Hp) locus, and the Hp2 allele is associated with an increased incidence of cardiovascular disease, specifically in diabetes mellitus (DM). Oxidative stress is increased in Hp2 mice and humans with DM. Oxidative modification of the apolipoprotein A-I inhibits reverse cholesterol transport. We sought to test the hypothesis that reverse cholesterol transport is impaired in Hp2 DM mice and humans. In vitro, using serum from non-DM and DM individuals, we measured cholesterol efflux from 3H-cholesterol–labeled macrophages. In vivo, we injected 3H-cholesterol–loaded macrophages intraperitoneally into non-DM and DM mice with the Hp1-1 or Hp2-2 genotype and monitored 3H-tracer levels in plasma, liver, and feces. In vitro, in DM individuals only, we observed significantly decreased cholesterol efflux from macrophages incubated with serum from Hp2-1 or Hp2-2 as compared with Hp1-1 individuals (P<0.01). The interaction between Hp type and DM was recapitulated using purified Hp and glycated Hb. In vivo, DM mice loaded with 3H-cholesterol–labeled macrophages had a 40% reduction in 3H-cholesterol in plasma, liver, and feces as compared with non-DM mice (P<0.01). The reduction in reverse cholesterol transport associated with DM was significantly greater in Hp2-2 mice as compared with Hp1-1 mice (54% versus 25% in plasma; 52% versus 27% in liver; 57% versus 32% in feces; P<0.03). reverse cholesterol transport is decreased in Hp2-2 DM. This may explain in part the increased atherosclerotic burden found in Hp2-2 DM individuals. (Circ Res. 2006;99:1419-1425.)

Key Words: diabetes mellitus ■ haptoglobin polymorphism ■ high-density lipoprotein ■ reverse cholesterol transport ■ oxidative stress

In humans, 2 common alleles (type 1 and type 2) exist at the haptoglobin (Hp) locus.1 The Hp1 and Hp2 allelic protein products are structurally distinct (reviewed extensively in the online data supplement, available at http://circres.ahajournals.org). We have previously reported in several independent, population-based longitudinal studies that individuals with diabetes mellitus (DM) who are homozygous for the Hp2 allele (Hp2-2) are at increased risk for myocardial infarction, stroke, and cardiovascular death as compared with DM individuals homozygous for the Hp1 allele (Hp1-1). An intermediate risk was found in DM individuals heterozygous for this polymorphism (Hp2-1). In the absence of DM, the risk of cardiovascular disease was not found to be Hp genotype dependent.2–7

The chief function of Hp is to bind and aid in the clearance of extracorpuscular hemoglobin (Hb), thereby preventing Hb-mediated oxidative modification of serum and cellular proteins.8–10 We and others have demonstrated marked differences between the Hp1 and Hp2 allele protein products in blocking Hb-induced lipid peroxidation in vitro.9,10 In vivo, we have found significant differences between Hp1 and Hp2 DM mice in myocardial levels of specific lipid peroxidation products measured by mass spectrometry.11 We have proposed that enhanced oxidative modification of serum lipoproteins (both low-density [LDL] and high-density [HDL] lipoprotein) in Hp2 individuals is a major factor contributing to the accelerated atherosclerosis seen in Hp2 DM individuals.12,13

Plasma levels of HDL and its major constituent protein apolipoprotein A-I (apoA-I) are inversely correlated with the incidence of atherosclerotic cardiovascular disease.14,15 The chief mechanism whereby HDL and apoA-I confer protection against atherosclerosis is through their ability to promote cholesterol efflux from macrophages in a process termed reverse cholesterol transport (RCT).16,17 The interaction of apoA-I with the ATP-binding cassette protein A1 and the activation of the enzyme lecithin:cholesterol acyltransferase (LCAT) are critical steps in the production of HDL and the
RCT process. In DM, not only is the plasma level of HDL reduced but also the atheroprotective role of the existing HDL is impaired. One possible explanation for the loss of the protective role of HDL in DM is that oxidative modification of apoA-I severely impairs cholesterol efflux from macrophages by the ATP-binding cassette protein A1 and LCAT pathways.

In this study, we have sought evidence in support of the hypothesis that the increased incidence of atherosclerotic cardiovascular disease observed in Hp2 DM patients might be attributed to impaired RCT. We tested this hypothesis at multiple levels and demonstrate, in vitro, an impairment in cholesterol efflux from macrophages incubated with Hp2-2 DM serum and, in vivo, a decrease in cholesterol efflux from macrophages injected intraperitoneally in Hp2-2 DM mice.

Materials and Methods

Human Blood Products
All protocols in this study were approved by the Institutional Review Boards of participating centers. All individuals provided informed consent. Serums used in this study were obtained from outpatient clinics at the Rambam Medical Center and the Haifa and Western Galilee district of Chalit Health Services.

Chemicals and Reagents
All reagents were from Sigma Israel (Rehovot) unless otherwise indicated. Radiochemicals were purchased from Amersham. Materials for cell culture were purchased from Biological Industries (Bet Haemek). Hp was purified from healthy volunteers by antibody affinity chromatography. The Hp concentration of purified Hp was determined spectrophotometrically using the known extinction coefficients of Hp (53.9 for Hp-1 and 58.65 for Hp-2.2). The Hp molar concentration was calculated based on the monomer of each Hp type. HDL was prepared from the serum of fasted normolipidemic normal human volunteers by density gradient ultracentrifugation as previously described.

Biochemical Measurements
Serum cholesterol was assayed using commercially available enzymatic-colorimetric methods (Roche Chol CHOD-PAP). HDL in human serum was assayed after sodium phosphotungstate-Mg2+ precipitation. HDL in murine serum was assayed by ELISA (Bioclinics). Hp in human serum was measured immunonephelometrically and in murine serum by ELISA (Mercodia).

Isolation and Glycation of Hb
Native Hb was isolated from fresh human blood. Hb concentrations were calculated using the Bradford reagent. Hb was glycated in vitro using glycolaldehyde.

Measurement of HDL-Associated Lipid Peroxides
Glycosylated or nonglycosylated Hb (1 μmol/L) was incubated with 100 μg of HDL and 20 μmol/L ascorbic acid in PBS with or without Hp1-1 or Hp2-2 (equimolar to Hb) for 3 hours at 37°C. Lipid peroxides were measured as previously described.

Determination of the Hp Genotype
The Hp genotype of participants in this study was determined by nondenaturating gel electrophoresis and peroxidase staining, using a modification of a previously described method.

Cell Culture
J774 A.1 murine macrophage cells were purchased from the American Type Culture Collection (Manassas, Va) and grown in DMEM supplemented with 5% FBS.

Cholesterol Efflux From Macrophages
Murine J774 cells (1 × 10^6/mL) were plated in 24-well plates for 48 hours, then washed and incubated in DMEM without serum containing 3H-cholesterol (2 μCi/mL) for 1 hour. Cells were washed to remove unincorporated label and then incubated in 1 mL of DMEM supplemented with: (1) nothing (negative control); (2) purified HDL (100 μg/mL protein) (positive control); or (3) 30 μL of serum from individuals with or without DM with the different Hp genotypes. In studies using purified Hp and Hb rather than serum, the cells were incubated with purified HDL (50 μL/mL protein) with different combinations of native Hb, glycated Hb, Hp1-1, and Hp2-2 (all at 0.8 μmol/L). After a 3-hour incubation at 37°C to permit efflux of 3H-cholesterol from the cells into the medium, 500 μL of the medium was collected, the cells washed with PBS, and 0.1 N NaOH added to the cells. Cellular and medium 3H-cholesterol were determined by liquid scintillation counting (LSC). The percentage of cholesterol efflux was calculated as the ratio of total counts per minute in the medium divided by the total counts per minute in the medium and in the cells. HDL-mediated cholesterol efflux (resulting from purified HDL or HDL found in the serum) was calculated after subtraction of the nonspecific efflux obtained in cells incubated in the absence of purified HDL or serum. Results reported for efflux elicited by serum samples are normalized for the serum HDL concentration derived as (measured efflux)/(measured HDL in mg/dL)/50.

Determination of LCAT Cholesterol Esterification Rate in Serum
LCAT cholesterol esterification rate in serum was measured using the method of Ohta et al. Briefly, 0.25 μCi of 3H-free cholesterol (3H-FC) was added to a 1:5 dilution of serum (500 μL of total volume) and incubated at 37°C for 90 minutes. The enzyme reaction catalyzing the esterification of FC was stopped by immersing the sample tubes in an ice bath. Lipids were extracted with n-hexane:isopropanol 3:2 (vol/vol), dried under nitrogen and resuspended in chloroform. Lipid extract was spotted on thin-layer chromatography plates and developed in n-hexane:diethyl ether:acetic acid:methanol (85:20:1:1) (vol/vol). Spots corresponding to FC and cholesterol ester were cut out from the plates and the radioactivity was determined by LSC. The fractional esterification rate (FER) was expressed as the difference between the percentage of radioactive cholesterol esterified before and after incubation at 37°C and the molar esterification rate was calculated based on the specific activity (counts per minute per nanomole of FC) of each sample. Results reported for FER in the serum samples are normalized for the serum HDL concentration derived as (measured FER)/(measured HDL in mg/dL)/50.

In Vivo Studies
Mice
Mice were housed and procedures approved according to the guidelines of the Animal Care and Use Committee of the Technion. All mice used in this study had a C57Bl/6 genetic background. The Hp2 allele exists only in humans. The C57Bl/6 wild-type murine Hp gene is a class 1 allele with more than 90% homology to the human class 1 Hp allele. A murine Hp2 allele was created by molecular engineering of the murine Hp1 allele as described in the online data supplement. The murine Hp2 allele was targeted for insertion at the murine Hp locus by homologous recombination resulting in a replacement of the wild-type Hp1 allele with a murine Hp2 allele. The generation of Hp2-2 mice after this targeted insertion is described in the online data supplement. Characterization of haptoglobin in Hp2-2 mice by gel electrophoresis demonstrated that the distribution of Hp polymers in Hp2-2 mice was similar to that in Hp2-2 humans.

DM was induced by intraperitoneal injection of streptozotocin (200 mg/kg) dissolved in 50 mmol/L citrate buffer (pH 4.5) at 6 weeks of age. Glucose levels were monitored with a glucometer and Hba1c was measured using a diagnostic kit from Sigma. Mice were...
fed a standard chow diet (Teklad-Harlan, Certified Global 18% Protein Rodent Diet; catalog no. 2018SC+F). DM and non-DM littermates followed in parallel were used for these studies.

Measurement of RCT
We used a recently described method for measuring RCT in mice. Male C57BL/6 mice at the age of 9 weeks (DM duration of 3 weeks) were used for this study. Each animal was caged separately with unlimited access to food and water. J774 cells were cultured in DMEM supplemented with 5% FBS, 5 μg/mL H-cholesterol, and 30 μg/mL acetylated LDL for 48 hours. Cells were washed twice and cellular associated radioactivity determined. The ratio of radiolabeled FC and radiolabeled cholesterol ester in these cells was assessed by thin-layer chromatography, more than 70% of the 3H-cholesterol incorporated into J774 foam cells was esterified. 3H-Cholesterol–labeled and cholesterol-loaded J774 foam cells were injected intraperitoneally into Hp1-1 or Hp2-2 mice with or without DM (4×10^6 cells containing 4.5×10^6 cpm in 0.5 mL medium for each mouse). Mice were bled at 24 hours (from the retroorbital plexus) and at 48 hours (from the inferior vena cava). Blood was used for LSC and for lipid analysis. At 48 hours, mice were euthanized and liver tissue stored at −20°C until lipid extraction was performed. Feces were collected continuously more than the study interval and were stored at 4°C until cholesterol and bile acid extraction were performed.

Tissue Lipid Extraction
Tissue lipids from 100 mg of homogenized liver tissue were extracted twice with n-hexane and isopropanol 3:2 (vol/vol), evaporated under nitrogen, dissolved in chloroform, and counted by LSC. The distribution of radioactive FC and cholesterol ester in liver tissue was assessed by thin-layer chromatography.

Fecal Cholesterol and Bile Acid Extraction
Fecal cholesterol and bile acids were extracted from the feces as previously described. Briefly, the total feces collected over the 48-hour study period were soaked in water for 16 hours (1 mL per 100 mg of feces). An equal volume of ethanol was then added and the mixture homogenized. Total 3H-stereols was determined by taking 400 μL of the homogenized feces and counting in LSC. To extract the 3H-cholesterol from homogenized feces, 2 mL of the homogenized feces was mixed with an equal volume of ethanol followed by the addition of 500 μL of 1 mol/L NaOH and the samples saponified at 95°C for 2 hours. This homogenate was then extracted 3 times with hexane, evaporated under nitrogen, and resuspended with chloroform, and the 3H-cholesterol was counted in LSC. To measure 3H–bile acids, the feces solution was acidified with concentrated HCl, extracted 3 times with ethyl acetate, evaporated under nitrogen, resuspended in ethyl acetate and counted by LSC.

Statistical Analysis
Results are reported as the mean±SEM. Pairwise comparisons between groups was performed using Student’s t test, with a probability value of <0.05 considered statistically significant.

Results

Impaired Cholesterol Efflux From Macrophages Elicited by Serum From Hp2 DM Individuals
We sought to determine whether there were differences in cholesterol efflux from macrophages incubated with serum from 90 DM and 72 non-DM individuals segregated by Hp genotype. Patients included in this analysis were randomly selected from a larger cohort of individuals from whom stored sera were available to ensure an equal distribution of the three Hp genotypes. Consistent with previous reports, we found that the serum Hp concentration was Hp-type dependent, with significantly mean higher values in Hp1-1 and lower mean values in Hp2-2. The serum Hp concentration segregated by Hp genotype in the DM cohort was 1.78±0.34 mg/mL for Hp1-1 individuals, 1.92±0.11 mg/mL for Hp2-1 individuals, and 1.25±0.08 mg/mL for Hp2-2 individuals. In the non-DM cohort, the Hp concentration was 1.75±0.12 mg/mL for Hp1-1 individuals, 1.47±0.09 mg/mL for Hp2-1 individuals, and 1.16±0.12 mg/mL for Hp2-2 individuals. There were no significant differences between the Hp types in demographic characteristics (ie, age, gender), comorbid conditions, or lipid parameters (total cholesterol, HDL).

We found that there were no significant differences in cholesterol efflux from J774 cells incubated with serum from non-DM individuals with the Hp1-1 (n=22), Hp2-1 (n=26), or Hp2-2 (n=24) genotypes. Incubation of J774 cells with serum from DM individuals resulted in a significant reduction in the cholesterol efflux compared with cells incubated with serum from non-DM individuals (14.84±1.85% versus 8.1±1.12% for non-DM versus DM individuals; P<0.001). The reduction in cholesterol efflux associated with DM serum was Hp-type dependent. Efflux elicited with serum from DM Hp1-1 (n=30) individuals was significantly higher as compared with efflux elicited with serum from DM Hp2-1 (n=30) or Hp2-2 (n=30) individuals (P<0.01) (Figure 1).

LCAT Cholesterol Esterification Rate Is Markedly Reduced in Diabetic Patients With the Hp2 Allele
We sought to determine whether there were any differences in the LCAT cholesterol esterification rate in the diabetic state and whether LCAT cholesterol esterification rate was associated with the Hp type. We measured LCAT cholesterol esterification rate in the serum of 84 DM and 62 non-DM individuals with Hp1-1, Hp2-1, and Hp2-2 (the same patients in whom cholesterol efflux was measured). We found a pattern similar to what was observed for cholesterol efflux. In non-DM individuals there were no differences in LCAT cholesterol esterification rate according to the Hp type,
whereas in the serum of DM individuals, there was a significant reduction in the LCAT cholesterol esterification rate in only individuals with the Hp2-1 and Hp2-2 genotypes ($P<0.01$) (Figure 2). In DM individuals, we found that the highest LCAT cholesterol esterification rate was observed in Hp1-1 individuals, the lowest in Hp2-2 individuals, and an intermediate level in Hp2-1 individuals. In the Hp1-1 group, there was no significant difference in LCAT cholesterol esterification rate between DM and non-DM individuals.

**Decreased Cholesterol Efflux From Macrophages Incubated With Glycated Hb and Hp-2**

We sought to examine whether the reduction in the cholesterol efflux from cells elicited by serum from DM individuals with the Hp2 allele could be recapitulated using purified Hp and native Hb. We found that the addition of Hp1-1, Hp2-1, or Hp2-2 had no effect on efflux. However, glycated Hb significantly reduced this efflux (34% reduction, $P<0.001$). The reduction in cholesterol efflux by glycated Hb was blocked to a significantly greater degree with Hp1-1 as compared with Hp2-2 (80% vs 30%, $P<0.001$).

We have speculated that these observations can be explained by differences in the oxidation of proteins or lipids involved in cholesterol efflux. To demonstrate that glycosylated Hb can oxidatively modify molecules involved in the efflux process within the time frame of this experiment (3 hours), we assessed the ability of glycosylated and nonglycosylated Hb to oxidize HDL-associated lipids. We found a marked increase (mean of 142.3 nmol of lipid peroxide per milligram of HDL in 2 independent experiments) in lipid peroxides when HDL was incubated with glycated Hb for 3 hours, whereas no increase in HDL-associated lipid peroxides was found over this interval when using nonglycated Hb. Furthermore, Hp1-1 nearly completely blocked the ability of glycated Hb to induce HDL-associated lipid peroxides (mean inhibition of 94% in 2 independent experiments), whereas Hp2-2 had only a partial inhibitory activity (50% in 2 independent experiments).

**RCT Is Dramatically Decreased In Vivo in Diabetic Mice in a Hp-Dependent Manner**

We injected $^{3}$H-cholesterol-labeled J774 macrophages into the peritoneum of 16 DM and non-DM Hp1-1 or Hp2-2 mice (n=4 for each subgroup). The lipid profile and diabetes characteristics of these mice is provided in the Table. There was no significant difference in either the total or HDL cholesterol among any of the 4 subgroups. Glucose and HbA1c were not significantly different between DM mice with the Hp1-1 and Hp2-2 genotypes (Table). Furthermore, we found no difference in the serum Hp concentration among the different groups.

### Lipid Profile and DM Characteristics of Mice

<table>
<thead>
<tr>
<th>Hp Type</th>
<th>n</th>
<th>DM Age (wk)</th>
<th>Hp (mg/mL)</th>
<th>Glu (mg/dL)</th>
<th>HbA1c</th>
<th>Total Cholesterol (mg/dL)</th>
<th>HDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp1-1</td>
<td>4</td>
<td>9</td>
<td>1.4±0.5</td>
<td>482±53</td>
<td>11.2±1.1</td>
<td>214±48</td>
<td>56.5±5.5</td>
</tr>
<tr>
<td>Hp2-2</td>
<td>4</td>
<td>9</td>
<td>1.4±0.6</td>
<td>487±94</td>
<td>12.3±1.3</td>
<td>194±36</td>
<td>49.6±3.1</td>
</tr>
<tr>
<td>Hp1-1</td>
<td>4</td>
<td>9</td>
<td>1.6±0.5</td>
<td>487±94</td>
<td>12.3±1.3</td>
<td>196±39</td>
<td>60.2±3.5</td>
</tr>
<tr>
<td>Hp2-2</td>
<td>4</td>
<td>9</td>
<td>1.2±0.4</td>
<td>123±9</td>
<td>191±22</td>
<td>62.6±5.8</td>
<td></td>
</tr>
</tbody>
</table>

Hp, glucose (Glu), HbA1c, total cholesterol, and HDL are presented as mean±SEM. No significant differences in total cholesterol or in HDL levels were found among the different groups.
between Hp1-1 and Hp2-2 mice in the presence or absence of DM (Table).

There were no significant differences in plasma, liver, or fecal $^3$H-cholesterol between the non-DM mice with the different Hp types ($P=0.2$). In DM mice as compared with non-DM mice, we found a $38 \pm 10\%$ reduction in the appearance of $^3$H-cholesterol in plasma as compared with non-DM mice at 24 hours and a $41 \pm 11\%$ reduction at 48 hours after injection of the J774 cells ($P=0.012$) (Figure 4A). We found striking Hp-type differences in the amount of $^3$H-cholesterol in the plasma, liver, and feces in DM mice (Figure 4). The reduction in $^3$H-cholesterol associated with DM was significantly greater in Hp2-2 mice as compared with Hp1-1 mice ($54 \pm 9\%$ versus $25 \pm 13\%$ in plasma; $52 \pm 10\%$ versus $27 \pm 14\%$ in liver; $57 \pm 10\%$ versus $32 \pm 10\%$ in feces; $P<0.03$). $^3$H-bile acids levels were not significantly different among the groups.

**Discussion**

In this study, we present experimental data in both in vitro and in vivo paradigms supporting the concept that the Hp genotype can regulate the process of RCT specifically in DM. These data thereby provide a possible mechanism to account for Hp genotype-dependent differences in atherosclerotic cardiovascular disease burden in DM.

RCT is thought to be influenced in large part by the quantity and quality of HDL. We have not observed Hp genotype dependent differences in HDL concentration either in the diabetic human cohort or in our transgenic mice. However, we have shown that the HDL-mediated cholesterol efflux elicited with serum from DM individuals with the different Hp types is markedly different.

The in vivo model of RCT initially described by Rader and colleagues has its limitations. This model is far removed from the process of RCT occurring within an atherosclerotic plaque in the vessel wall. Moreover, the efflux of label from IP-injected macrophage may not be mediated by the same efflux pathway that occurs in the plaque. To rule out the possibility that $^3$H-tracer levels in the liver simply represent the migration of injected macrophages from the peritoneum to the liver, we have examined the distribution of $^3$H-FC and $^3$H-cholesterol in labeled macrophages before injection and in the mouse liver 48 hours after injection by thin-layer chromatography. We found that the majority of labeled cholesterol was esterified ($\approx 75\%$), whereas the majority of labeled cholesterol in the liver was free (less than 10% was esterified), indicating that the labeled cholesterol seen in the liver was not attributable to cellular migration. Moreover in plasma we found that more than 70% of the labeled cholesterol was esterified, suggesting that this labeled cholesterol was transported through an HDL-mediated efflux from macrophages.

The observed Hp-genotype dependence of cholesterol efflux may reflect in part differences in LCAT cholesterol esterification rate. Abrescia and colleagues have recently demonstrated that Hp can bind to a site on helix 6 of apolipoprotein A-1, which overlaps with the binding site of LCAT. The displacement of LCAT from apolipoprotein...
A-1 has been shown to result in an inhibition of LCAT cholesterol esterification rate in vitro and in a reduction of RCT in human ovarian follicular fluid in vivo.\textsuperscript{35,36} Moreover, decoy peptides corresponding to this region of apolipoprotein A-1 (Leu141-Ala169) have been shown to block the ability of Hp to reduce LCAT cholesterol esterification rate in vitro.\textsuperscript{36} However, we have not found any specific binding of Hp to HDL or to purified apoA-1 using either plasmon resonance spectroscopy (BiaCore apparatus) or by ELISA with \textsuperscript{125}I-labeled Hp. Furthermore, we have not seen any relationship between Hp concentration and LCAT cholesterol esterification rate indicating that the Hp-dependent differences in LCAT cholesterol esterification rate cannot be accounted for by differences in Hp concentration.

Although the mechanism responsible for Hp type-dependent differences in RCT remains to be fully elucidated, an attractive hypothesis is that these differences are reflective of the degree of oxidative modification of proteins involved in the RCT process. Several components of HDL that are critical for RCT are known to be inactivated by oxidative mechanisms including LCAT and apolipoprotein A-1.\textsuperscript{1,2,22–25} In addition, oxidative mechanisms may impair the activity of other components of the RCT process such as the ATP-binding cassette protein A1 transporter.\textsuperscript{37,38} We have identified the primary culprit oxidant mediating Hp-type dependent differences in oxidative stress as non–transferrin-bound iron.\textsuperscript{13} It would be of considerable interest to determine whether chelation of the non–transferrin-bound iron component in Hp2 DM humans and mice could improve RCT in our experimental paradigms.

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**Disclosures**

None.

**References**


Haptoglobin Genotype Is a Regulator of Reverse Cholesterol Transport in Diabetes In Vitro and In Vivo

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Methods.

Construction of a murine Hp 2 allele.

The human genomic locus as well as cDNAs encoding the Hp gene, both for the Hp 1 and Hp 2 alleles have been cloned and sequenced (1). The Hp 1 allele contains 5 exons and 4 introns. The Hp 2 allele contains 7 exons and 6 introns (Figure 1a). The only difference between the two alleles is that the third and fourth exons of the Hp 1 allele have been duplicated in Hp 2 to give rise to exons 5 and 6 as well. Exon 5 in Hp 1 allele and exon 7 in the Hp 2 allele are identical. The reading frame of the duplicated region (exon 3 and 4) is maintained so the primary amino acid sequence produced by this duplicated region is a direct repeat of exons 3 and 4. Furthermore the translated in-frame amino acid sequence of exon 7 is the same as exon 5.

The genomic and cDNA sequence of mouse Hp is known (accession # M96827 C57BL/6J f) (2). The genomic structure of wild type murine Hp is remarkably similar to that of the human Hp 1 allele (Figure 1a). There exist 5 exons and 4 introns in murine Hp. The nucleotide sequences at the intron-exon boundaries in mouse Hp and the human Hp 1 allele are 100% conserved. The overall amino acid homology between the murine and human Hp 1 alleles is over 80% (1). Because the nucleotide sequence at the intron-exon boundaries of the murine Hp 1 allele are conserved, it was possible to create a murine Hp 2 allele by duplicating murine exons 3 and 4. This duplication does not change the reading frame of sequences that come 3’ to the duplicated region allowing the sequence of the final exon (exon 7) to be read in frame unchanged from what occurs in the murine Hp 1 allele.

Genomic mouse Hp DNA from the strain 129Sv obtained from a 129SvJ genomic library was kindly provided by Dr Sai-Kiang Lim and Dr Heinz Baumann. Our strategy to create a duplication of murine exons 3 and 4 was to modify murine exon 3 to become exon 343. In this strategy in the genomic murine Hp 2 allele there is no intron between the extra copy of exon 4 and the extra copy of exon 3. The intron normally occurring after exon 3 in the endogenous murine Hp 1 allele occurs after the 343 exon. The genomic structure of the murine Hp 2 allele is exon 1- intron 1- exon 2- intron 2 -exon3exon 4exon 3- intron 3 -exon 4- intron 4- exon 5 (see Figure 1a, murine Hp 2). The genomic structure of the murine Hp 2 allele is different
from the human Hp 2 allele in that there is no intron between the duplicated exons 3 and 4. However, in the mature mRNA (i.e., after the RNA has been spliced and intronic sequences removed) there will be no difference in the genetic organization of the murine and human Hp 2 alleles. The logic we used to generate a duplication and direct repeat of exons 3 and 4 in the murine Hp 1 allele can be explained as follows. Suppose exon 3 has sequence ABCDE and exon 4 has sequence FGHIJ. We cloned into the middle of exon 3 (at a restriction endonuclease site between AB and CDE) the sequence CDEFGHIJAB (i.e. 2nd half of exon 3, all of exon 4 and the 1st half of exon 3) thereby transforming exon 3 (ABCDE) into exon 343 (ABCDEFGHIJABCDE). Using this logic we generated a DNA fragment by RT-PCR of Hp mRNA isolated from the human HepG2 hepatoma cell line with oligonucleotides 343sense (CGGGATCCATGACAGCTGCCAAGCCCCAGAGA) and 343 antisense (CGGAATTCCAGCTGTCATCTGCCTCACATTCGGGGAGTTTCTC). After digesting the fragment with PvuII we cloned it into the PvuII site of exon 3 of the murine Hp 1 allele to create a modified exon 3 with the sequence of exon3exon4exon3.

Once we replaced the murine 3 exon with a 343 exon, we proceeded to generate a targeting vector for transfection into embryonic stem (ES) cells. In designing targeting vectors for homologous recombination, it is critical that there is at least 2 kb of 100% homology sequences (regions identical between targeting vector and targeted gene) 5’ and 3’ to the targeted region. In our case the targeted region was exon 3 and the homology regions were murine genomic sequences located 5’ (5.6kb) or 3’ (3.4 kb) to exon 3. A second feature of the targeting vector is a selectable marker, which can subsequently be removed. We used the neomycin antibiotic resistance gene (conferring resistance to G418) flanked by two lox P sites (allowing removal of the neo gene with the cre recombinase) for this purpose. We placed a cytosine deaminase (CD) gene casetted and a neo cassette in the intron between exon 2 and exon 343 bounded by 2 lox P sites using the cloning vector pTKLNCL (Thymidine kinase-LoxP-CD-Neo-LoxP) GB 135 (3) (see Figure 1b, for schematic picture of this construct after its successful integration showing the relationship between the wild type murine Hp 1 allele, and the targeting DNA after its integration both before (middle panel) and after (bottom panel) removal of the CD and Neo cassettes).
The targeting vector was linearized with Not I, transfected into 129O1a ES cells by electroporation (800 V, 3 uF) and individual clones selected with G418 (150ug/ml). G418 resistant clones undergoing homologous recombination for the transfected sequences were identified by southern blot analysis of BamH1 digested DNA isolated from each clone using as a probe a 300 bp Bgl II-Bam H1 fragment located outside (5') of the 5' homology region of the targeting vector. Southern blot using this probe yields a band of 5.8 kb in wild type mouse DNA (i.e. wild type murine Hp 1 allele) and 11 kb if the targeted Hp gene has undergone homologous recombination with the targeting vector. Successfully targeted ES clones were then subjected to karyotype analysis and injected into 3.5d post-coitum (dpc) C57BL/6J females to generate several chimeras. The chimeras were mated with C57BL/6J females to produce heterozygous Hp 2 mice that were then intercrossed to produce mice homozygous for the murine 2 allele. The neo gene was deleted by crossing with EIIaCre mice overexpressing the cre recombinase in all tissues (provided by Heiner Westphal, National Institutes of Health). After the neo gene was deleted the only difference between the wild type murine Hp 1 allele and the murine Hp 2 allele which we created, other than exon 3, was in the intron between exons 2 and exons 3. In the murine Hp 1 allele the intron is 250 bp. In the murine Hp 2 gene a Pvu-Bgl fragment (100 bp) in the middle of this intron was deleted and additional sequences were inserted (vector sequences from pTKLNCL consisting of the Xho-LoxP and LoxP-Bam) thereby creating an intron between exons 2 and 343 in the murine Hp 2 allele of different length than the intron between exons 2 and 3 in the murine Hp 1 allele. These differences in the size of intron 2 have been exploited for Hp genotyping of the mice by PCR using oligonucleotides that bracket this intron. These oligonucleotides are: exon 2s AGCCCTGGGAGCTGTTGTCAC (located in the coding sequence for exon 2) and 3r (located at the 3’ end of the intron between exon 2 and exon 3) TGGGTGCTCCGATGGCTCTCTG. Oligonucleotides 2s and 3r yield a PCR product of 306 bp for the murine Hp 1 allele (83 bp from exon 2 and 223 from the intron) and 406 bp for the murine Hp 2 allele (83 bp from exon 2 and 323 from the intron). Mice having both bands are heterozygotes (haptoglobin 2-1).
References


Online Figure 1. Construction of a murine Hp 2 allele.

A. Genomic organization of the Hp locus.

The human Hp 1 and Hp 2 alleles are located at chromosomal coordinates 16q22. The murine wild type Hp is a Hp 1 allele and is found on chromosome 8. A murine Hp 2 allele was created as described in this manuscript and inserted by homologous recombination at the wild type Hp locus replacing the murine Hp 1 allele. In the human Hp 2 allele, exons 5 and 6 represent a duplication of exons 3 and 4. The mouse Hp 1 allele has the identical intron-exon boundaries as the human Hp 1 allele and is 90% homologous at the amino acid. The murine Hp 2 allele, constructed as described in the text, is similar to the human Hp 2 allele in that it has a direct repeat of exons 3 and 4. The exonic organization of the human and murine Hp 2 alleles are identical after RNA splicing has occurred.
B. Fine map of the murine Hp locus before and after gene targeting.

**Top.** Genomic organization of the murine Hp 1 allele. B, Bam H1; Bg, Bgl II; E, EcoR1; P, PvuII.

**Middle.** Genomic organization of the murine Hp 2 allele after successful gene targeting by homologous recombination. A targeting vector was constructed using the pTKLNCL GB 135 vector as a backbone. TKLNCL contains lox P sites (large arrow) bracketing a neomycin resistance gene. A 5.8 kb E-P fragment of the murine Hp 1 allele was cloned in the Kpn 1-Xho 1 site of TKLNCL 5' to the neo cassette (5' homology region) and a 3.4 kb BglII fragment of the murine Hp 1 allele was cloned in the Bam H1 site of TKLNCL 3' to the neo cassette (3' homology region). Exon 3 of the murine Hp 1 was reconstructed to be exon 343 as described in methods. The vector was linearized with Not 1 prior to transfection. Identification of G418 resistant ES clones which integrated the targeting vector at the Hp locus by homologous recombination was achieved by southern blot analysis of Bam H1 digested DNA from these clones using a 300 bp BamH1-BglIII fragment (in blue) as probe. This probe hybridizes with a 5.8 kb Bam H1 fragment in wild type DNA (Hp 1) and with a 11 kb Bam H1 fragment in successfully targeted clones (Hp 2).

**Bottom.** Genomic organization of the murine Hp 2 allele after removal of the neo cassette with cre recombinase.

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**Scale:**
- 0.5 kb

- Exonic sequence
- Lox P site
- Restriction site