Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 309-315

# A Human (3.3 kb) Haptoglobin-CAT Transgene Is Modulated in Lungs of Transgenic Mice by Inflammation

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Received January 20, 1995

Four independent lines of transgenic mice were produced carrying integrated copies of a chimeric gene composed of 3.3 kb of the human haptoglobin 5' regulatory region fused to the CAT (chloramphenicol acetyl transferase) reporter gene. Although the endogenous mouse haptoglobin (Hp) and human haptoglobin (HP) genes express mainly in liver and lung, expression of the human 3.3-kb HP-CAT transgene was not detected until after induction of inflammation and then only in lungs. The results indicated that the transgene maintained the regulatory DNA elements required for lung specific responsiveness to inflammation *in vivo* but lacked the DNA sequence required for robust expression in liver. The DNA sequence(s) responsible for the normally high level of HP expression in liver either reside outside the 3.3-kb regulatory region of the HP chimeric gene or this region contains a suppressor sequence affecting tissue specific expression in the liver. 

Haptoglobin (HP) is an $\alpha_2$-glycoprotein that binds free hemoglobin in plasma and tissues of humans and other vertebrates. Haptoglobin protein structure and function have been reviewed previously (1, 2).

Following the cloning and characterization of the human HP cDNA (3), the gene was mapped to chromosome 16q22 (4). Genomic sequencing demonstrated that HP was an evolutionary product of multiple genetic events including intragenic duplication and viral sequence insertion (5). The mouse Hp cDNA revealed 80% sequence identity with the coding region of human HP cDNA (6).

HP is a positive acute phase reactant, increasing in concentration 8.6-fold in human hepatocytes *in vitro* after treatment with recombinant IL-6, a cytokine eliciting the acute phase reaction (7). The increase of HP levels during the acute phase reaction is a result of the interactions of specific nuclear proteins that respond to IL-6 with three regions, A, B and C, in the 5' regulatory region of

0006-291X/95 $5.00

the HP gene. The three regions are located around: -157 (A), -111 (B) and -61 bp (C) (8). Reports of diverse expression caused by mutations within these regions have been reported in rodent and primate cells (9, 10, 11).

We previously reported the synthesis of several acute phase reactants in extra-hepatic tissues and proposed that a local source of proteins having protective functions could be a defense mechanism for the organism during inflammation and other stressful conditions (12, 13, 14, Yang et al., 1994, unpublished). A protective role of haptoglobin has been suggested by others who demonstrated that HP may play an important antioxidant role *in vivo* by preventing iron-catalyzed formation of oxygen radicals (15). HP also functions as an angiogenic factor that stimulates endothelial cell differentiation and vascularization (16).

The study described here demonstrates that the 3.3 kb of the regulatory region of the human HP gene directs expression of the human HP-CAT gene only after the acute phase reaction has been induced and then only in lungs of transgenic mice. The results indicate that the transgene maintained the regulatory DNA elements required for lung specific responsiveness to inflammation *in vivo* but lacked the DNA sequence required for robust expression in liver or carried a suppressor sequence affecting tissue specific expression in the liver.

## Material and Methods

### Production of human 3.3-kb HP-CAT transgenic mice

Production of transgenic mice following microinjection of DNA into fertilized mouse eggs was carried out as previously described (17). The DNA construct introduced into the mouse genome was prepared by inserting 3.3 kb of the 5' flanking sequence of the human HP gene into the HindIII site of the plasmid pSV0CAT. This plasmid contains the CAT coding sequence plus the SV40 small t intron and polyadenylation signal (18). The plasmid was transformed into *E. coli* HB 101. Before microinjection the 4.9 kb HP-CAT construct was cut out of the plasmid with Bam HI.

Identification of transgenic mice and estimation of the copy number of the transgene was carried out, respectively, by PCR using the CAT primers described (19) and by Southern analysis of DNA (20) obtained from tails of mice. The human 4.9 kb HP-CAT construct used for microinjection of mouse embryos to produce transgenic mice was used as a probe for Southerns.

### Analyses of CAT activity

Levels of CAT enzyme activity in tissue extracts from transgenic mice 54-90 days old were determined by the procedure of Gorman et al. (18). Tissue extracts from liver, lung, adipose, heart, kidneys, and brain were heated for 10 min at 65°C prior to the assay. Radioactivity in free and acetylated forms of [$^{14}$C] chloramphenicol was determined by scintillation counting of corresponding regions of thin layer chromatography plates. Protein concentration of the soluble extracts was determined (21).

### Induction of acute phase reaction with LPS in transgenic mice

Transgenic mice 54-90 days old were given intraperitoneal injections of pyrogen-free saline or of 4.5 μg of lipopolysaccharide (Sigma, *E. coli* 0111:B4) per gram of body weight. LPS was dissolved in sterile pyrogen-free saline solution. Animals were sacrificed and tissues were examined 30 hrs. after intraperitoneal injections. To confirm the inflammatory state in the transgenic mice, rocket immunoelectrophoresis was carried out according to the procedure of Laurell (22). The antibody preparation against mouse SAP (serum amyloid protein) was obtained from Calbiochem Corporation (San Diego, CA). SAP is a positive acute phase reactant in mouse plasma. Plasma SAP levels were analyzed by measuring the peak height of the rocket immunoprecipitate of each sample from saline and LPS treated transgenic mice.

## Results

### Production of human 3.3-kb HP-CAT transgenic mice

A schematic diagram of the construct introduced into the mouse genome is shown in Figure 1. The 3.3 kb of the 5' regulatory region of human HP directed the CAT reporter gene. The sequence of the 5' 3.3 kb of the transgene had been determined by Martinez et al. (1994, unpublished). Four founder lines of transgenic mice were identified by PCR with the CAT primers as previously described (19). PCR analysis is shown in Figure 2. Transgene DNA mixed with nontransgenic DNA is shown in lane 1 as the positive control. TSHβ is an endogenous gene present in both transgenic and nontransgenic mice. It is the negative control seen in lane 2. Transgenic mice DNAs are in lanes 3, 5, 6, 7, 11, and 13. The remaining lanes have DNA from nontransgenic siblings of transgenic mice.

Copy number of the transgene in each of the founder lines was estimated by Southern analysis shown in Figure 3. The DNAs were cut with XbaI. DNA was hybridized with the 4.9 kb HP-CAT construct used to inject the embryos. The four independent lines of transgenic mice obtained are shown in lanes 1-4, and compared to a nontransgenic sibling of one of the transgenic litters (lane 5) and a mouse from a non-transgenic litter (lane 6). DNA representing 1, 5 and 10 copies

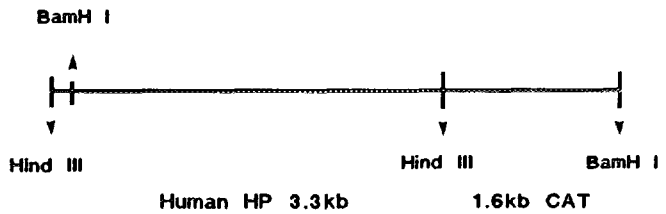


**Figure 1. The human HP 3.3-kb CAT transgene.**
3.3-kb CAT of the 5' regulatory region of the human HP gene (Ref. 10) was fused to the plasmid SV0CAT which contains the CAT coding sequence plus the SV40 small t intron and polyadenylation signal (Ref. 18). HP-CAT was cut out of plasmid with BamHI.

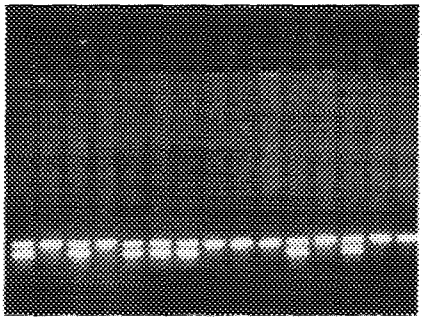


**Figure 2. PCR identification of transgenic mice.**
PCR analysis was carried out following the method of Walter et al. (Ref. 19). Two bands seen in the transgenic mice DNA are TSH (top) and CAT (lower). Lane 1, positive control contains both bands. Lane 2, the negative control contains only TSH. Transgenic mice DNAs are in lanes 3, 5, 6, 7, 11, 13. Nontransgenic sibling DNAs are in lanes 4, 8, 9, 10, 12, 14, 15.

of the construct are shown in lanes 7, 8, and 9, respectively. It was estimated that the transgenic mouse DNA in lane 1 had approximately 10 copies of the transgene, in lane 2 the DNA from the transgenic mouse had greater than 10 copies and the DNA from the transgenic mouse in lane 3 had about 1 copy, while the DNA from transgenic mouse in lane 4 had about 2 copies.

**CAT enzyme activity in lungs and livers of transgenic mice**

The levels of human 3.3 kb HP-CAT were analyzed in extracts of tissues from transgenic mice by determining enzymatic activity of CAT driven by the HP 5' flanking DNA. The expression was consistently absent until the mice had been induced into the inflammatory state by injections of LPS; 30 hours after injection of LPS, the extracts were prepared. Of the four transgenic lines tested the greatest CAT activity was shown in the same line whose Southern pattern is shown in lane 1 of Figure 3. The CAT assay results from four lines of transgenic mice are shown in Figure 4. The expression of HP-CAT even after inflammation was low. Unlike the mouse endogenous Hp gene, expression of the transgene did not appear consistently in liver, never in adipose, brain, heart or adrenals. However, in all four lines tested after inflammation was induced, expression of CAT was clearly observed in lung.

Confirmation that the transgenic mice treated with LPS were in the inflammatory state was made by noting a relative increase of SAP in those mice treated with LPS. SAP is a positive acute phase

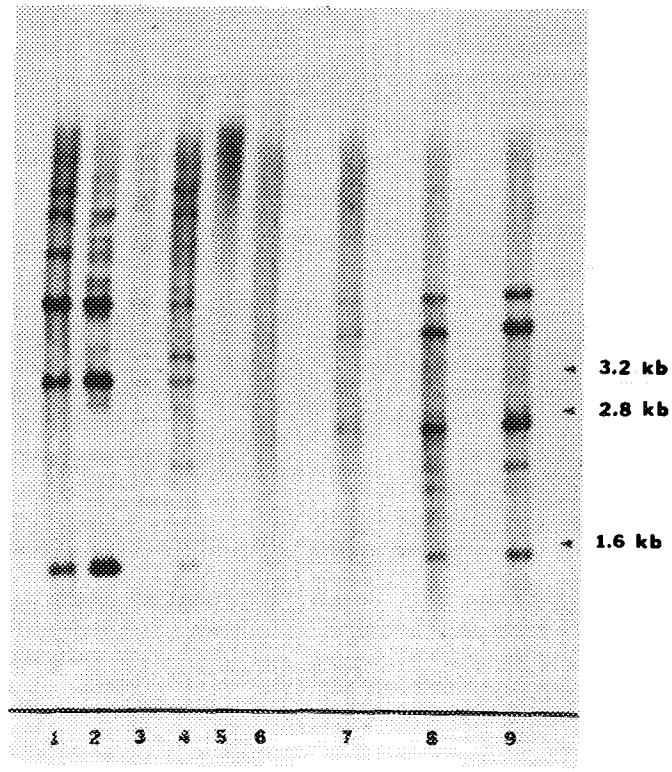


**Figure 3. Southern Blot analysis of DNA to detect and approximate 3.3-kb HP-CAT copy number.**
Southern analysis after digestion of DNA with XbaI demonstrated four transgenic mice shown in lanes 1-4. Lane 5 shows the DNA from a nontransgenic sibling. Lane 5 is the DNA from a mouse from a nontransgenic litter. The last 3 lanes (7, 8 and 9) demonstrate controls with 1, 5 and 10 copy numbers of 3.3-kb HP-CAT. It was approximated that transgenic mice in lanes 1, 2, 3, 4 had 10, 15, 1 and 1 copy of the transgene, respectively.

reactant that significantly increases with the acute phase reaction. The SAP was compared by rocket electrophoresis in mice that had been treated by saline or LPS.

## Discussion

The work described here furnishes evidence that 3.3 kb of the regulatory region of the human haptoglobin gene is not adequate *in vivo* to express in the liver, lung or any of the other tissues where HP is usually expressed in humans or other vertebrates. The induction of the acute phase reaction by administering an experimental inflammatory agent, LPS, boosts the level of expression somewhat, but mainly in lung. This finding is distinct when compared to results from *in vitro*

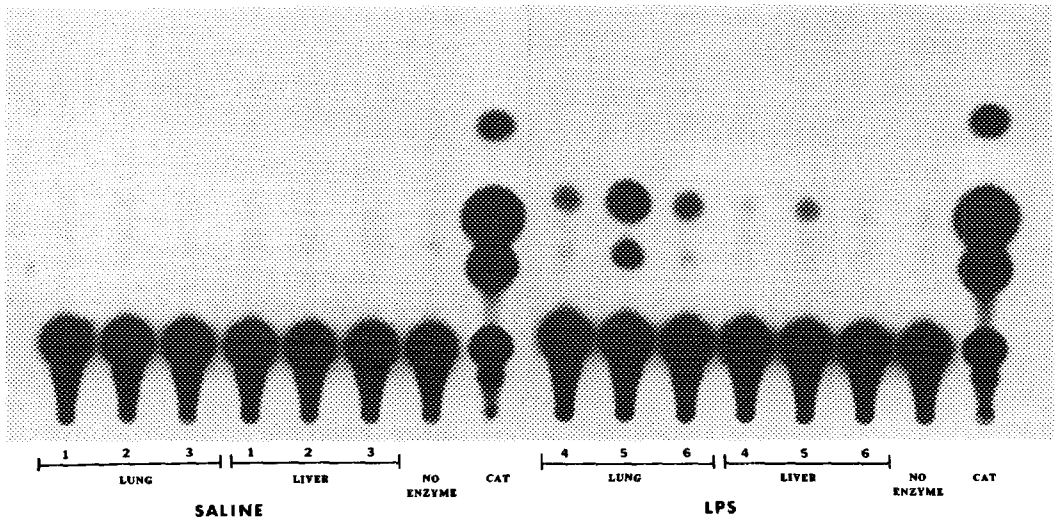


**Figure 4. CAT assay of livers and lungs of 3.3-kb HP-CAT transgenic mice after treatment with saline or LPS.**
After intraperitoneal injection with saline no trace of HP driven CAT activity was detected in 2 transgenic mouse lines carrying the 3.3-kb HP-CAT transgene. Expression of HP driven CAT activity was detected after intraperitoneal injection with LPS in three transgenic lines in lung. CAT activity in liver was present in only one line after LPS injection (lane 5).

expression in transfected cells with specific HP sequences (8), and it points out that the requirements for expression and tissue specific regulation in the whole organism is more complicated than within established cells lines.

In previous studies we demonstrated that high levels of endogenous haptoglobin mRNA were transcribed in lungs of mice (14) and baboons *in vivo* (Yang et al., 1994, unpublished). *In situ* hybridization analysis established that expression of haptoglobin in lung tissues was confined to epithelial linings of the lung airways in mouse and baboon. In mice, Hp mRNA synthesis by alveolar epithelial cells was strongly induced by inflammation (Yang et al., 1994, unpublished).

There was a 1.1 kb sequence identified in the 5' region of the regulatory region of the human HP gene that can direct synthesis of a heterologous gene in lung. A report by D'Amiento et al. (23) demonstrated that utilizing an insert containing approximately 1.1 kb of the human HP gene regulatory region to direct expression of a heterologous collagenase gene in transgenic mice assured synthesis of the enzyme in lung and the development of emphysema by transgenic mice. Significant expression of the transgene was not detected in other tissues. It was not reported whether the 1.1 kb HP region responded to inflammatory signals (23). Thus, comparison of our results with theirs offers the possibility that expression regulated by human 1.1 kb Hp 5' regulatory region is stronger in lung than the 3.3 kb HP, and that the longer construct contains a suppressor sequence not present in the 1.1 kb HP sequence.

**Acknowledgments**

We thank Dr. Nobuyo Maeda for a gift of the 3.3-kb 5' regulatory region of human HP.

This research was supported by National Institutes of Health grants AG06872 and AG00165.

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