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DNA Polymorphisms in the Controlling Region of the Human Haptoglobin Genes: A Molecular Explanation for the Haptoglobin 2-1 Modified Phenotype

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Summary

A haptoglobin 2-1 modified (Hp2-1mod) phenotype results when the amount of Hp2 polypeptide synthesized in Hp^2/Hp^1 heterozygotes is less than that of Hp1 polypeptide. Cloned Hp^2 DNA from an individual with the Hp2-1mod phenotype is here shown to have a C in place of the normal A at nucleotide position −61 in one of the interleukin-6 (IL-6) responsive elements of the haptoglobin promoter region. Direct sequencing of the haptoglobin promoter region, amplified by PCR, from DNA from unrelated American blacks showed a C at −61 in all of 10 individuals with the Hp2-1mod phenotype, in two of four with a "possible Hp2-1mod" phenotype, but in none of 15 with the Hp2-1 phenotype. Thus the −61C mutation in the $Hp^{2\text{-}61C}$ allele is strongly associated with the Hp2-1mod phenotype. Sequencing results also show that there are three other promoter sequences in the population studied; each can be associated with either Hp^2 or Hp^1. The variability seen in the Hp2-1mod phenotype, a variability which ranges from close to Hp2-1 to close to Hp1-1, can be explained, in part, by the existence of several Hp^2 alleles differing in their promoters—and possibly, in part, by differences in the promoters of the accompanying Hp^1 allele. A further part of the variability may be the consequence of differences in the way that the $Hp^{2\text{-}61C}$ and the Hp^2 alleles respond to the IL-6-dependent factor during an acute-phase response.

Introduction

Haptoglobin is the hemoglobin-binding protein of serum (for a review, see Bowman and Kurosky 1982). Although the physiological function of the protein is not completely understood, it may limit iron loss during hemolysis and during normal red cell turnover. Heritable normal variations of the haptoglobins in humans were first described by Smithies (1955), who demonstrated three common phenotypes—Hp1-1, Hp2-1, and Hp2-2—by starch-gel electrophoresis.

Haptoglobin is composed of an α-chain and a β-chain, which are generated by a posttranslational cleavage from a single polypeptide encoded by the *Hp* gene (Raugei et al. 1983; van der Straten et al. 1983; Yang et al. 1983). The Hp^2 allele contains a 1.7-kb intergenic DNA duplication that includes two of the α-chain-coding exons (Maeda et al. 1984). The hp2α polypeptide (142 amino acids) is consequently longer than the hp1α polypeptide (83 amino acids; Black and Dixon 1968). The hp2α chain also contains two free cysteine residues, compared with one in hp1α. The phenotypic differences revealed by electrophoresis of the native proteins are explained by these observations as follows: haptoglobin polypeptides in Hp^1/Hp^1 individuals form a tetramer, $(hp1\alpha,hp\beta)_2$, which migrates as a single band (Hp1-1), while the polypeptides in Hp^2/Hp^1 and Hp^2/Hp^2 individuals, respectively, form various heteropolymers—$(hp1\alpha,hp\beta)_m$ $(hp2\alpha,hp\beta)_n$—and homopolymers—$(hp2\alpha,hp\beta)_m$—that migrate as a series of slower bands (Hp2-1 and Hp2-2). There are two common variants of the Hp^1 allele—i.e., the Hp^{1F}

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and Hp^{1S}—that code for hp1α-chains having different charges.

Another phenotypic variant, haptoglobin 2-1 modified (Hp2-1mod), was first described by Connell and Smithies (1959). This is a relatively common phenotype in blacks (Giblett 1959) but is also found in other races (Harris et al. 1960). The electrophoretic band pattern of Hp2-1mod is identical to that of Hp2-1, except that the concentration of the faster-migrating (low-molecular-weight) bands is higher and the concentration of the slower-migrating (high-molecular-weight) bands is lower. During electrophoresis of the reduced protein in acid urea starch gels, the hp2α chains from Hp2-1mod migrate identically to the hp2α chains from Hp2-1, although the concentration of hp2α is noticeably reduced in relation to that of hp1α chain (Giblett 1964). These various observations have suggested that the synthesis of the haptoglobin chains coded by the Hp^{2M} allele is reduced relative to that of haptoglobin chains coded by the Hp^2 allele, and they led Parker and Bearn (1963) to suggest that the Hp2-1mod phenotype is the consequence of an allele-specific genetic alteration in the control system for haptoglobin synthesis.

Considerable variation has been observed in the electrophoretic patterns of Hp2-1mod heterozygotes (e.g., see Giblett and Steinberg 1960): it ranges from an Hp1-1-like pattern with only a single faint polymer band to a pattern resembling that of the common Hp2-1 heterozygotes (for an extensive review, see Giblett 1969). To account for this variability, various hypotheses have been advanced, including that by Sutton and Karp (1964), who proposed that in American blacks there are three Hp^2 alleles, of which two are responsible for the Hp2-1mod phenotype. The overall conclusion from these studies was concisely summarized by Giblett (1969, p. 85), who stated that "it seems likely that some kind of quantitative regulation exists, either as part of the Hp^2 gene itself, or closely linked to it."

The present work was undertaken to investigate the possibility that the Hp2-1mod phenotype is the result of some polymorphic differences, in the DNA sequences in the promoter region of the *Hp* allele, that can cause an imbalance in the relative expression of the Hp^1 and Hp^2 alleles. In the present paper, I describe three polymorphic point mutations in the promoter region of the haptoglobin gene. One of them is strongly associated with the Hp2-1mod phenotype and leads to a testable hypothesis that can explain the observed variability in the Hp2-1mod phenotype.

Material and Methods

Samples

Genomic DNA from lymphoblast cells derived from an individual with the Hp2-1mod phenotype was supplied by Dr. B. Bowman of the University of Texas at San Antonio. Peripheral blood samples were obtained from black individuals who visited the Hematology Laboratory at the University of North Carolina Hospitals at Chapel Hill. Samples from nonblack individuals with the Hp0 phenotype were provided by Dr. S. M. Pueschel of Brown University, by Dr. J. Myrick of the Department of Health and Human Service, and by Dr. E. Beautyman of St. Luke Roosevelt Hospital. Genomic DNA was prepared from peripheral blood by the method of Poncz et al. (1982).

Cloning

Genomic DNA (1 μg) was digested completely with *Bam*HI and was ligated with Charon 35 lambda phage arms (4 μg). The ligated material was packaged in vitro by using the method of Hohn (1979). The resulted phage library was screened by using a probe corresponding to the promoter region of the *Hp* gene (Maeda et al. 1986). Clones having *Bam*HI fragments of 9.2 kb, 7.5 kb, and 5.0 kb in length, which, respectively, contain the Hp^2 allele, the Hp^1 allele, and the *Hpr* gene (Maeda 1985), were purified, and their inserts were subcloned into Bluescript plasmid vectors (Stratagene, La Jolla, CA).

Haptoglobin Typing

Phenotypes were determined by mixing serum (1–4 μl) with 1 μl of a fresh lysate of red cells (approximately 3% hemoglobin concentration) followed by electrophoresis in 5% polyacrylamide gel at pH 9.5 (Davis 1964). The gels were soaked in 0.1 M potassium acetate, pH 5.0, for 15 min prior to being stained with 0.2% benzidine in 0.5% acetic acid containing 0.3% hydrogen peroxide. Hp1S and Hp1F subtypes in Hp2–1 heterozygotes were nearly always identified from the gel (see Linke 1984). Genotypes were determined by hybridization of an hpα probe to Southern blots of genomic DNA. *Hin*dIII digests were used to distinguish Hp^2 from Hp^1, and *Xba*I digests were used to distinguish Hp^{1F} from Hp^{1S} according to a method described elsewhere (Maeda et al. 1984).

PCR and Sequencing of Amplified Products

PCR reactions were performed in 50 μl of 0.05 M Tris/HCl buffer, pH 8, containing 0.02 M ammo-

nium sulfate and 1.5 mM $MgCl_2$. The reaction mixtures contained 200 ng of genomic DNA, 1 μg of each primer, 200 μM of dNTP, and 1 unit of thermostable DNA polymerase (a gift from Molecular Biology Resources, Milwaukee). The amplifications consisted of 35 cycles, each comprising denaturation at 94°C for 30 s, annealing at 55°C for 1.5 min, and extension at 63°C for 3.5 min. The primers used were 5′-ACTCTAGAACCATGAGAACCAC-3′ and 5′-CCTAAGCTTGGTTGGTCTTGC-3′; they are shown in figure 1 (*Xba*I and *Hin*dIII sites were incorporated into these primers for other purposes). The amplified fragment (650 bp) was purified from 1.5% low-melting-temperature agarose gel (Sea Plaque, FMC BioProducts, Oakland, ME), and approximately 10 ng of purified fragment was reamplified under the same conditions, except that 1 μg of the 5′ primer and 0.01 μg of the 3′ primer were used in a 100-μl reaction. The products of these second amplifications were purified by using push columns (Stratagene) according to the supplier's manual.

Nucleotide Sequence Determination

Nucleotide sequences were determined by using a Sequenase kit (United States Biochemical, Cleveland) and the protocol recommended by the supplier. Thirty nanograms of one of the two PCR primers was used to sequence a quarter of the DNA fragments purified after the second PCR amplification. To determine the sequence close to the primer, a reaction buffer containing Mn^{2+} ions was used (Tabor and Richardson 1989).

Results

Promoter Sequences of the Hp Alleles from an Hp2-1mod Individual

Southern blots of DNA isolated from an individual selected as having a typical Hp2-1mod phenotype after digestion with various restriction enzymes were indistinguishable from those of DNA from Hp2-1F individuals (data not shown). They showed that the

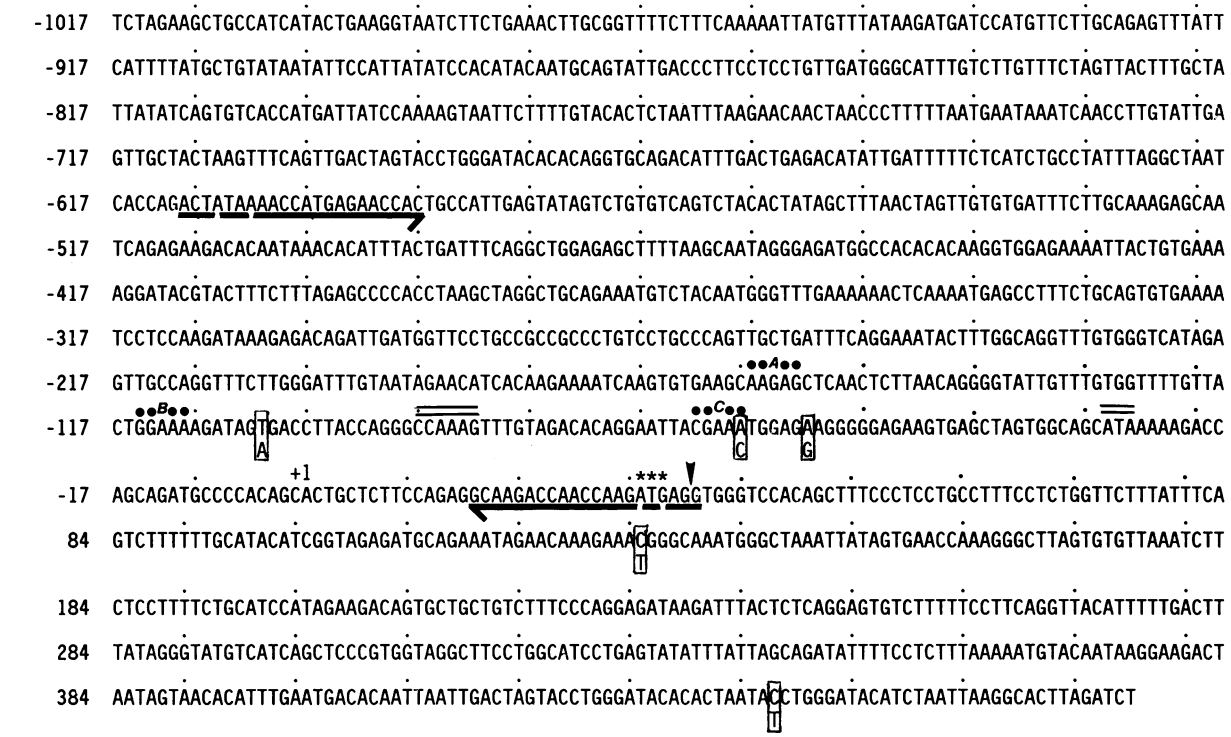

Figure 1 Nucleotide sequence differences in haptoglobin gene promoter region. The nucleotide sequence of the promoter region of four haptoglobin alleles are compared. The continuous sequence from −1017 to 475 is a corrected version of the previously published sequence shared by Hp^2 and Hp^{1S} (Maeda 1985). The nucleotide shown below the sequence at position −61 was found in the $Hp^{2\text{-}61C}$ allele; those at −104, −55, +131, and +443 were found in the $Hp^{1F\text{-}104A\text{-}55G}$ allele (see text). These sequences have been submitted to GenBank. The 5′ and 3′ primers used for PCR are indicated by horizontal arrows, with breaks indicating the nucleotide mismatches introduced into the primers. The splicing donor site for intron 1 is indicated by a vertical arrowhead. The starting codon, ATG, is indicated by three asterisks. The CAT box and the TATA box are indicated by double overlines, and three IL-6 responsive elements—*A*, *B*, and *C*—(identified by Oliviero and Cortese 1989) are indicated by the sets of large black dots.

individual is a heterozygote having a genotype indistinguishable, at this level of discrimination, from Hp^2/Hp^{1F}.

In order to study the promoter region of the Hp^2 allele, DNA from the same individual was completely digested with *Bam*HI and was ligated into lambda phage. From the resulting phage library, the promoter region and the 1.7-kb internal duplication of the α-coding region of the Hp^2 allele were isolated in a single 9.2-kb fragment. (Comparable sequences of the Hp^1 allele were isolated in a 7.5-kb fragment). The nucleotide sequence of a 1.5-kb *Xba*I-*Bgl*II fragment that contains the promoter region was then determined by using an M13 phage system. The resulting data (fig. 1) show that the Hp^2 allele from the Hp2-1mod individual has the same sequence in this 1.5-kb region as do the Hp^2 and Hp^{1S} alleles previously studied (Maeda 1985), except for one position: at nucleotide position −61, a C occurs in this Hp^2 allele whereas an A occurs in the other two alleles, which are identical to each other over the whole 1.5-kb region. I will refer to the newly sequenced allele as "$Hp^{2\text{-}61C}$." Note that the position of difference lies between a CAT box at −90 and a TAT box at −28. The remainder of the sequenced region (−1010 to +470) is identical in all three alleles. The −61C mutation in the $Hp^{2\text{-}61C}$ allele is therefore an excellent candidate for a change that can alter Hp2-1 to Hp2-1mod.

The sequence of the Hp^{1F} allele from this individual differs from those of previously sequenced Hp^2 and Hp^{1S} alleles—at two positions 5′ to the start of transcription, i.e., positions −104 and −55, and at two positions in the first intron, i.e., positions +131 and +443. The nucleotides in this Hp^{1F} allele are, respectively, A, G, T, and T—instead of the T, A, C, and C in previously sequenced alleles. I will refer to this allele as "$Hp^{1F\text{-}104A\text{-}55G}$." Another Hp^{1F} gene has been previously sequenced by Bensi et al. (1985). It has T at −104 and G at −55 (the first intron was not sequenced). These polymorphic sites are shown in figure 1.

Other Individuals with Hp2-1mod Phenotype

To determine whether the substitution at −61 found in the $Hp^{2\text{-}61C}$ allele in the individual with the Hp2-1mod phenotype is regularly found associated with this phenotype, DNA from other individuals was studied. Sera from 96 unrelated American black individuals were obtained and subjected to PAGE at pH 9.5, in order to determine their haptoglobin phenotypes. Examples of the electrophoretic patterns observed are shown in figure 2. Among these samples, 18 were typed as Hp2-2 homozygotes (see fig. 2, lane 1), 25 were typed as Hp1-1 homozygotes (see fig. 2, lane 9), three had undetectable haptoglobin proteins, and 50 were heterozygotes (see fig. 2, lanes 2–8). Of these 50 heterozygotes, 36 were categorized as Hp2-1 (exemplified in fig. 2, lanes 3, 5, and 7), and 10 had the phenotype Hp2-1mod (exemplified in fig. 2, lanes 4, 6, and 8). Four of the heterozygous individuals (exemplified in fig. 2, lane 2) had electrophoretic patterns very similar to those of a standard Hp2-1 individual, except that the first heteropolymer band is heavier than the second (see fig. 2, arrows 2 and 3), which is a characteristic seen in Hp2-1mod. These four individuals were, therefore, classified as "possible Hp2-1mod." I also observed in the Hp2-1mod samples that, the higher the total level of haptoglobin protein is in serum, the closer the electrophoretic pattern of Hp2-1mod is to that of Hp2-1.

Promoter Sequence Determination after PCR Amplification

DNA was isolated from blood samples from 32 of the typed individuals, and a 650-bp fragment from the promoter regions of their haptoglobin genes was

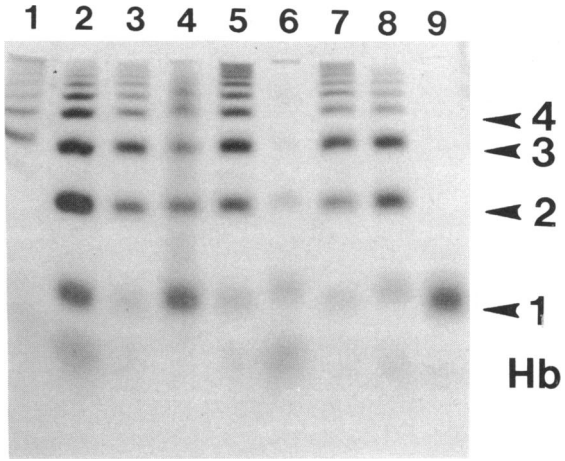

Figure 2 Haptoglobin phenotyping of sera from American black individuals. Lane 1, Hp2-2. Lane 2, possible Hp2-1mod. Lanes 3, 5, and 7, Hp2-1. Lanes 4, 6, and 8, Hp2-1mod. Lane 9, Hp1-1. Hb = free hemoglobin; arrowhead 1 indicates $(Hp1\alpha,\beta)_2$ homopolymer; arrowheads 2–4 indicate $(Hp1\alpha,\beta)_m(Hp2\alpha,\beta)_n$ heteropolymers. Note that in Hp2-1mod individuals the higher heteropolymer bands are faint and that the first heteropolymer band (arrowhead 2) is slightly heavier than the second heteropolymer band (arrowhead 3)—and vice versa in Hp2-1 individuals. In some cases (such as lanes 2 and 8) the distinction between Hp2-1, possible Hp2-1mod, and Hp2-1mod could not be made from a single electrophoresis run. In these cases, the electrophoresis was repeated, and the amount of serum loaded was varied.

amplified by using the PCR as described above. Examples of sequencing gels obtained from the noncoding strand of these amplified fragments are presented in figure 3. At nucleotide position −61, two bands indicated by asterisks and corresponding to G and T are observed in individual II. This individual is phenotypically Hp2-1mod. A single band corresponding to T is seen in individuals I and III, who are phenotypically Hp2-1. This result shows that there is a C on the coding strand at nucleotide position −61 in one of the *Hp* genes in sample II, while the other allele has an A on the coding strand at this position. Both alleles in individuals I and III have an A. The $Hp^{1F\text{-}104A\text{-}55G}$ allele seen in the cloned sample was detected frequently in the PCR-amplified samples. Individual I in figure 3 shows an example of double bands at these two positions. (Note double bands of T and A at nucleotide position −104 and double bands of C and T at position −55 in this $Hp^{1F\text{-}104A\text{-}55G}/Hp^{2}$ individual.)

Individual III has a double band (T and C) at position −55 but has single bands at all other positions. Thus a promoter with −55G also occurs in this population. It was elsewhere reported as present in the Hp^{1F} allele sequenced by Bensi et al. (1985). In all, the nucleotide sequences of the haptoglobin promoter region from 33 blacks and six nonblack individuals were determined as presented in table 1.

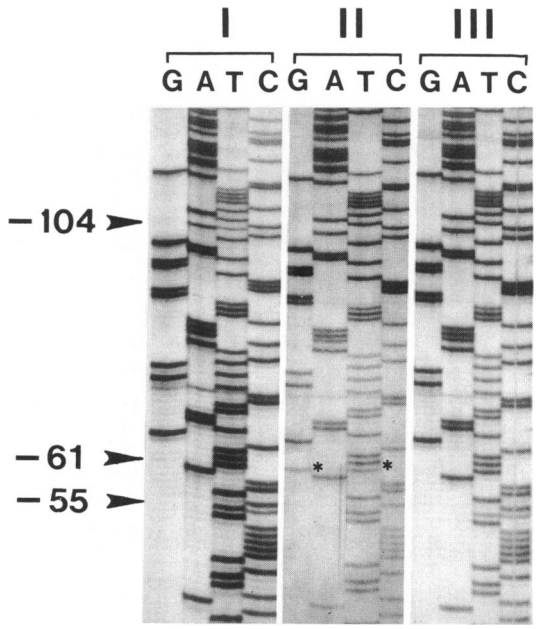

Figure 3 Polymorphism in haptoglobin promoter regions. The 3′ primer (see fig. 1) was used to sequence a fragment obtained after PCR amplification. The sequence shown is from the noncoding strand. Three positions where double bands occur are indicated by arrowheads. The double bands at position −61 are marked with asterisks in the G and T lanes of individual II.

Association of the C at −61 and the Hp2-1 mod Phenotype

The tentative identification of the −61C mutation as being sufficient to change an Hp2-1 phenotype to Hp2-1mod phenotype predicts that all Hp^{2}/Hp^{1} heterozygotes with the Hp2-1mod phenotype will have this substitution. The data presented in table 1 show that this prediction is fulfilled; all 10 heterozygous individuals with the Hp2-1mod phenotype have the −61C mutation on one allele. Two of four individuals with the possible Hp2-1mod phenotype also have the −61C mutation. None of 15 individuals who have an unequivocal Hp2-1 phenotype have this mutation.

Effects That Various Promoter Sequences Have on the Phenotype in Heterozygotes

The results given in table 1 also show that 10 of the 11 individuals who have the unequivocal Hp2-1mod phenotype are genotypically Hp^{2}/Hp^{1F}; the eleventh individual had the Hp^{2}/Hp^{1S} genotype. In addition, these 10 individuals all have double bands at positions −104 and −55. Thus it is most likely that they carry the same two alleles, $Hp^{2\text{-}61C}$ and $Hp^{1F\text{-}104A\text{-}55G}$, as does the individual from whom the promoter regions were cloned and sequenced. The 10:1 Hp^{1F}:Hp^{1S} ratio in the 11 Hp2-1mod individuals contrasts with an 8:7 ratio in the 15 unequivocal Hp2-1 individuals I have studied. Since the reported gene frequencies of Hp^{1F} (.26) and Hp^{1S} (.29) in American blacks are nearly equal (Giblett and Brooks 1963), this high occurrence of the Hp^{1F} allele in the Hp2–1mod individuals is particularly noteworthy. Possibly the $Hp^{1F\text{-}104A\text{-}55G}$ promoter is stronger than the Hp^{1S} promoter. Earlier population studies (Nance 1967; Giblett 1969), however, have shown no bias in the occurrence of Hp^{1F} and Hp^{1S} in Hp2-1mod individuals.

Because the cloned Hp^{1F} allele has an A at −104 and a G at −55, as described above, and because the double bands at these positions are only seen in the DNA from individuals who carry an Hp^{1F} allele, it is most likely that the −104A-55G promoter sequence is linked to the Hp^{1F} allele. Three Hp^{1F} alleles from two Hp^{1}/Hp^{1} individuals (individuals 32 and 33) also have these promoters, a finding which is consistent

Table 1

Individual Differences in Haptoglobin Promoter Sequences

Genotype[a]	Nucleotide Positions			Probable Promoter Genotype
	−104	−61	−55	
Individuals who are phenotypically Hp2-1mod:				
1.[b] 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
2. (2/1F).....	A/T	C/A	G/A	2-61C/1F-104A-55G
3. 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
4. (2/1F).....	A/T	C/A	G/A	2-61C/1F-104A-55G
5. 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
6. (2/1F).....	A/T	C/A	G/A	2-61C/1F-104A-55G
7. 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
8. (2/1F).....	A/T	C/A	G/A	2-61C/1F-104A-55G
9. 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
10. 2/1F.....	A/T	C/A	G/A	2-61C/1F-104A-55G
11. 2/1S.....	T/T	C/A	A/A	2-61C/1S
Individuals who are possibly Hp2-1mod:				
12. 2/1S.....	T/T	C/A	A/A	2-61C/1S
13. 2/1S.....	T/T	C/A	A/A	2-61C/1S
14. 2/1F.....	A/T	A/A	G/G	2-55G/1F-104A-55G
15. 2/1S.....	T/T	A/A	G/A	2-55G/1S
Individuals who are phenotypically Hp2-1:				
16. 2/1S.....	T/T	A/A	A/A	2/1S
17. 2/1F.....	T/T	A/A	G/A	2-55G/1F or 2/1F-55G
18. 2/1S.....	T/T	A/A	A/A	2-1S
19. 2/1F.....	A/T	A/A	G/A	2/1F-104A-55G
20. 2/1F.....	A/T	A/A	G/A	2/1F-104A-55G
21. 2/1F.....	A/T	A/A	G/A	2/1F-104A-55G
22. 2/1F.....	A/A	A/A	G/G	2-104A-55G/1F-104A-55G
23. 2/1S.....	T/T	A/A	A/A	2/1S
24. 2/1S.....	T/T	A/A	A/A	2/1S
25. 2/1F.....	A/T	A/A	G/A	2/1F-104A-55G
26. 2/1F.....	T/T	A/A	G/A	2/1F-55G or 2-55G/1F
27. 2/1S.....	T/T	A/A	A/A	2/1S
28. 2/1S.....	T/T	A/A	A/A	2/1S
29. 2/1F.....	A/T	A/A	G/A	2/1F-104A-55G
30. 2/1S.....	T/T	A/A	A/A	2/1S
Individuals with undetectable haptoglobins:				
31. 2/2.....	T/T	C/A	A/A	2-61C/2
32. 1F/1S	A/T	A/A	G/A	1F-104A-55G/1S
33. 1F/1F	A/A	A/A	G/G	1F-104A-55G/1F-104A-55G
Nonblack individuals:				
34. 1F/1S	T/T	A/A	G/A	1F-55G/1S
35. 2/2.....	T/T	A/A	A/A	2/2
36. 2/2(Hp0).....	T/T	A/A	G/G	2-55G/2-55G
37. 2/2(Hp0).....	T/T	A/A	A/A	2/2
38. 2/2.....	T/T	A/A	G/A	2-55G/2
39. 2/2.....	T/T	A/A	A/A	2/2

[a] Determined by Southern blot analysis. Genotypes given in parentheses were obtained from PAGE.
[b] Promoter sequences of this individual were determined on the basis of cloned DNA.

with $Hp^{1F\text{-}104A\text{-}55G}$ being the most frequently occurring Hp^{1F} allele in the sampled population. Accordingly, of the eight Hp2-1 individuals who have an Hp^{1F} allele, five most likely have the genotype $Hp^{2}/Hp^{1F\text{-}104\text{-}55G}$. Thus any possible effects that the −104A-55G promoter might have on the level of expression of the Hp^{1F} gene are not sufficient to produce an Hp2-1mod phenotype without the presence of a down-regulated Hp^{2} gene. The −104A-55G promoter is also linked to an Hp^{2} gene in one individual (individual 22 in table 1), who gives only single bands at positions −104 and −55 but is genotypically Hp^{2}/Hp^{1F}.

Two of the possible Hp2-1mod individuals (individuals 12 and 13) have the probable genotype $Hp^{2\text{-}61C}/Hp^{1S}$ as described above. One (individual 14) of the other two possible Hp2-1mod individuals has only G at position −55 but has A and T at position −104. This establishes that a −55G promoter can be linked to an Hp^{2} allele. The other possible Hp2-1mod individual (individual 15) also carried the −55G promoter and has the probable genotype $Hp^{2\text{-}55G}/Hp^{1S}$. This suggests that a G at position −55 can negatively affect expression. However, my sample size is too small for this possibility to be explored further at this time. Two individuals (individuals 17 and 26) who are phenotypically Hp2-1 and genotypically Hp^{2}/Hp^{1F} have both G and A at −55 but have only T at −104; the linkage of the −55 promoter in these individuals has not yet been established. Both $Hp^{2\text{-}55G}$ and $Hp^{1F\text{-}55G}$ were found among a small number of samples from nonblacks (see table 1). Elsewhere, Benzi et al. (1985) have reported the same −55G promoter sequence on an Hp^{1F} allele from an individual of unspecified racial background, as indicated above.

Promoter Sequences in Hp0 Individuals

Three of my samples from blacks had haptoglobin concentrations too low for detection by simple electrophoretic tests. Southern blot analysis and direct sequencing of the promoter region of DNA from these three individuals revealed that one of them was genotypically $Hp^{2}/Hp^{2\text{-}61C}$; the second individual was genotypically $Hp^{1F\text{-}}/Hp^{1F\text{-}104A\text{-}55G}$; and the third was $Hp^{1F\text{-}104A\text{-}55G}/Hp^{1S}$. Thus none of these three persons with an undetectable amount of haptoglobins is homozygous for −61C. Two samples from nonblack individuals with the Hp0 phenotype were also analyzed. Genotypically one is $Hp^{2\text{-}55G}/Hp^{2\text{-}55G}$ and one is Hp^{2}/Hp^{2}. Therefore, within the limitation of my sample size, there is no obvious association between any particular promoter sequence and the Hp0 phenotype.

Discussion

My data establish a strong association both (*a*) between the presence of C at position −61 of the *Hp* gene and the Hp2-1mod phenotype and (*b*) between the presence of A at −61 and the Hp2-1 phenotype. Although further experiments will be needed to prove that this difference is the direct cause of the reduced level of expression associated with the $Hp^{2\text{-}61C}$ allele, the following argument supports the validity of this inference.

Serum haptoglobin levels increase during inflammation (Javid 1978), as do the levels of other acute-phase reactants, and IL-6 is one of the most important mediators of the liver acute-phase response (Gauldie et al. 1987). Oliviero and Cortese (1989) have identified three regions in the human *Hp* gene promoter region that are important for IL-6 response, as judged by a transient-transfection assay in Hep3B hepatoma cells. The three regions are located around and including nucleotide positions −157, −111, and −61 (for the *cis*-acting elements A–C, see fig. 1). These authors found that a new protein is produced in the cell after induction with IL-6. This protein replaces the proteins previously bound to elements A and C in uninduced cells, and this in turn is associated with the formation of a fully transcribable complex. There were no detectable changes in the complexes formed with the B element after IL-6 induction. The authors also reported that DNA with mutations introduced into a localized region that included position −61 did not form any complexes with nuclear extracts from either uninduced or IL-6-induced Hep3B cells.

It is of considerable interest that the position of the A-to-C difference at −61 in the $Hp^{2\text{-}61C}$ allele coincides with one of the IL-6-responsive cis-elements, and I therefore suggest that this single nucleotide substitution causes the Hp2-1mod phenotype via its effects on the binding ability of the protein factors in both uninduced and IL-6-induced cells. Experiments to test the predicted negative effects that the A-to-C substitution at nucleotide position −61 has on both promoter function and IL-6 responsiveness are currently underway.

The wide range of Hp2-1mod patterns noted in the early studies of Hp2-1mod phenotype is likely to be explained in part by the existence of several Hp^{2M} al-

leles, each responsible for a different level of expression of the Hp2 protein, as was proposed by Sutton and Karp in 1964. Thus I have observed a less extreme, possible Hp2-1mod form in two individuals heterozygous for an $Hp^{2\text{-}55G}$ allele and an $Hp^{1F\text{-}104\text{-}55G}$ or Hp^{1S} allele. This suggests that the −55G promoter can also affect gene expression negatively and that, accordingly, $Hp^{2\text{-}55G}$ may be a second allele able to change the Hp2-1 phenotype to the Hp2-1mod phenotype.

Since the Hp2-1mod phenotype can only be detected in heterozygotes, the promoter function of the Hp^1 alleles may also contribute to the range of the phenotype. My data show that there are three different promoters associated with the Hp^1 alleles. Especially notable is the fact that 10 of 11 individuals with the phenotype have an $Hp^{1F\text{-}104A\text{-}55G}$ allele together with an $Hp^{2\text{-}61C}$ allele. This suggests that the −104A-55G promoter may have a *positive* effect on the level of expression of the Hp^{1F} allele, although it is not sufficient by itself to produce an Hp2-1mod phenotype.

Although the majority of the Hp2–1mod individuals in the samples I have studied are genotypically $Hp^{2\text{-}61C}/Hp^{1F\text{-}104A\text{-}55G}$, the Hp2-1mod phenotype shows variation among these samples, as illustrated in figure 2. Consequently, there must be an additional mechanism that leads to the variation of the Hp2-1mod phenotype in these individuals. Possibly the −61C promoter leads to a substantially reduced expression of its linked allele in an individual in good health but, during an acute-phase response when IL-6 is induced, the $Hp^{2\text{-}61C}$ allele responds to the IL-6-dependent factor to the extent that its phenotype becomes almost indistinguishable from that associated with the Hp^2 allele. This possibility can be tested, for it leads to the prediction that the electrophoretic pattern in any individual with an $Hp^{2\text{-}61C}/Hp^1$ genotype will change from close to Hp1-1 in a healthy state to almost indistinguishable from Hp2-1 in the presence of inflammation or infection. A longitudinal study of individuals with the $Hp^{2\text{-}61C}$ allele is planned to test this prediction. (Samples for the current study were collected without regard to the state of health of the individuals.)

The Hp0 phenotype has been studied extensively in various populations, and a high correlation of the Hp0 phenotype with the Hp2-1mod phenotype has been shown in previous family studies (Giblett and Steinberg 1960; Nance 1967; Azevedo et al. 1969). The pattern of inheritance of this phenotype is, however, not simple and has not yet been fully explained. Furthermore, ascertainment of the Hp0 phenotype is difficult both because haptoglobin can be depleted in plasma in some conditions, such as internal bleeding or hemolysis, and because haptoglobin is undetectable in the serum of most newborns. In the present small sample, no changes in the haptoglobin gene clusters of five Hp0 individuals (three blacks and two nonblacks) were detected by the Southern blotting. Similar observations have been reported by Hill et al. (1986) in a population survey of Melanesians. In addition, my current failure to observe any association of the promoter polymorphisms with the Hp0 phenotype suggests that null expression of haptoglobins is more likely to be related to the expression of the trans-acting factors necessary for the Hp expression than to cis-acting promoter sequences.

In conclusion, I have demonstrated that a polymorphic single-basepair difference in one of the IL-6-responsive elements in the haptoglobin promoter is strongly associated with the differences between the Hp2-1 and Hp2-1mod phenotypes. A proposed hypothesis is that the variability of the Hp2-1mod phenotype is in part the consequence the fact that, compared with other promoter sequences, this promoter sequence has a different response to the acute-phase signals mediated by IL-6. This hypothesis should be testable by observing the protein phenotypes of Hp2-1mod individuals over time.

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