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Variation in the *HLA-G* Promoter Region Influences Miscarriage Rates

Carole Ober,[1,2] Carrie L. Aldrich,[1] Inna Chervoneva,[3] Christine Billstrand,[1] Fedik Rahimov,[1] Heather L. Gray,[1] and Terry Hyslop[3]

Departments of [1]Human Genetics and [2]Obstretrics and Gynecology, University of Chicago, Chicago; and [3]Biostatistics Section, Division of Clinical Pharmacology, Thomas Jefferson University, Philadelphia

The *HLA-G* gene is primarily expressed in placental cells that invade the maternal decidua during pregnancy. This gene encodes multiple isoforms that fulfill a variety of functions at the maternal-fetal interface throughout gestation. Recently, a null allele for the most abundant HLA-G isoform was associated with recurrent miscarriage in two independent studies, suggesting that reduced levels of the HLA-G1 protein may compromise successful pregnancy. We initiated the present study to determine whether other polymorphisms that could affect expression levels of *HLA-G* were associated with fetal loss in women participating in a 15-year prospective study of pregnancy outcome. We genotyped these subjects for 18 single-nucleotide polymorphisms in the 1,300 bp upstream of exon 1, 13 of which were identified as part of this study, as well as for an insertion/deletion (in/del) polymorphism in the 3′ untranslated region. The 18 SNPs defined eight unique haplotypes. One polymorphism, −725C/G, was associated with fetal loss, with an increased risk for miscarriage in couples in which both partners carried the −725G allele, compared with couples not carrying this allele (odds ratio 2.76, 95% confidence interval 1.08–7.09; $P = .035$). Further, the G at nucleotide −725 creates a CpG dinucleotide, and we demonstrate that this CpG site is methylated on −725G alleles. Overall, this study identified extraordinary levels of variation in the 5′-upstream regulatory region of *HLA-G* and provides evidence for an association between a promoter-region SNP and fetal loss rates, further attesting to the novel features and critical role of this gene in pregnancy.

Introduction

The discovery in 1990 that *HLA-G* (MIM 142871) was the nonclassical *HLA* gene expressed in fetal placental cells at the maternal-fetal interface (Ellis et al. 1990; Kovats et al. 1990) captured the attention of reproductive immunologists and biologists. The primary expression of this gene on trophoblast cells that embed deeply into the maternal tissues and come into direct contact with maternal immune cells made it an outstanding candidate for clarifying the paradox of the fetal allograft (Medawar 1953) and for shedding light on the novel mechanisms by which fetuses survive unharmed in a genetically foreign host during successful pregnancies. Much has been learned about HLA-G in the >10 years since its discovery, and it has proved to be novel in many respects. For example, the *HLA-G* transcript is alternatively spliced to produce at least six different isoforms (Ishitani and Geraghty 1992; Fujii et al. 1994). One transcript, *HLA-G1,* is nearly identical in structure to the other class I genes, with an extracellular domain that includes α1, α2, and α3 domains. The second primary transcript, *HLA-G2,* is missing exon 3, which encodes the α2 domain, and may form homodimers that more closely resemble HLA class II molecules in structure (Ishitani and Geraghty 1992). Both *HLA-G1* and *HLA-G2* are also expressed as soluble isoforms (called "sHLA-G1" or "-G5" and "sHLA-G2" or "-G6," respectively), because of the inclusion in the mature message of intron 4 sequences, which has a stop codon prior to translation of the membrane and intracellular portion of the protein. Other smaller transcripts lacking both exons 3 and 4 (*HLA-G3*) and exon 4 (*HLA-G4*) have been reported, but their translated proteins may not reach the cell surface (Bainbridge et al. 2000*b*). The full-length G1 isoform is the most abundant message in placental tissues (Ishitani and Geraghty 1992; Hiby et al. 1999) and may be the only isoform expressed at the cell surface (Bainbridge et al. 2000*b*; Mallet et al. 2000). However, both sG1 and sG2 proteins are abundant at the maternal-fetal interface (Hunt and Orr 1992) and are present in the maternal circulation throughout pregnancy (Rebmann et al. 1999; Hunt et al. 2000).

HLA-G proteins appear to serve many classical functions, including antigen presentation (Lee et al. 1995), regulation of HLA-E expression (Lee et al. 1998), and inhibition or activation of natural killer (NK) cells (King et al. 1996; Colonna et al. 1998; Lopez-Botet et al. 1999). However, HLA-G proteins have also been shown to in-

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Address for correspondence and reprints: Dr. Carole Ober, Department of Human Genetics, 920 East 58th Street, CLSC 507C, University of Chicago, Chicago, IL 60637. E-mail: c-ober@genetics.uchicago.edu

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hibit maternal T cell proliferation (Le Gal et al. 1999; Bainbridge et al. 2000*a*; Kapasi et al. 2000), induce IL-10 production in placental trophoblast cells and peripheral blood monocytes (Moreau et al. 1999), and regulate chorionic angiogenesis (Le Bouteiller et al. 1999), indicating unique roles for these molecules in pregnancy that likely include the induction or maintenance of tolerance, both locally in the uterus and in the maternal periphery. Thus, this single gene has evolved many functions, presumably to ensure the survival of the allogeneic fetus. However, the identification of an adult individual who was homozygous for a null allele (G*0105N) for all HLA-G1 isoforms demonstrated that successful gestation could occur without any G1 proteins and raised questions about the essential role of HLA-G in human pregnancy (Ober et al. 1998*a*).

In addition to the G*0105N allele, which is defined by a single-base-pair deletion in exon 3 (1597ΔC), there are four *HLA-G* alleles that differ at the protein level. Three amino acid substitutions result in differences from the most common allele (*0101): Thr→Ser at amino acid 31 defines the HLA-G*0103 allele, Leu→Iso at amino acid 110 defines the HLA-G*0104 allele, and Thr→Met at amino acid 258 defines the G*0106 allele (reviewed by Ober and Aldrich [1997] and Hviid et al. [2001]). Additional silent variation at five nucleotides (nts) in exons 2 and 3 define subtypes of the *0101 and *0104 alleles. The paucity of amino acid polymorphisms in *HLA-G* is striking, compared with the classical class I loci, which are the most polymorphic in the human genome (Diogo and Thomson 2001). However, given the location of *HLA-G* expression, it is not surprising that selection against mutations that result in highly antigenic proteins would result in low levels of polymorphism at this locus (Hunt and Orr 1992; Ober and Aldrich 1997; Le Bouteiller and Blaschitz 1999). In fact, the two nonsynonymous substitutions present in the in α1 and α2 domains, which form the peptide-binding groove of the HLA molecule, are quite conservative and do not reside in amino acids that are predicted to either bind peptide or interact with T cell receptors (Ober and Aldrich 1997). The more radical Thr258Met polymorphism is located in the α3 domain (Hviid et al. 2001), which is highly conserved in the classical genes. However, this amino acid may not contact directly with maternal immune cells.

Recently, it has been shown that some of the polymorphic variants of *HLA-G* are associated with recurrent miscarriage. Pfeiffer et al. (2001) reported an increased frequency of the G*01013 and the G*0105N alleles in German patients with recurrent miscarriage, compared with controls, and Aldrich et al. (2001) reported an increased risk for subsequent miscarriage in U.S. couples with a history of recurrent miscarriage if either partner carried the G*0104 or G*0105N alleles. The former study did not find an association with the G*0104 allele, and the latter did not find an association with the G*01013 allele, but it was striking that both studies in primarily white populations identified an association with the G*0105N allele, because this allele is generally quite rare in these populations (Aldrich et al. 2000). Nonetheless, the association with the null allele in two studies indicated that reduced levels of HLA-G1 protein might be a risk factor for spontaneous miscarriage, even though, in some cases, homozygotes for the null G*0105N allele survive to term (Ober et al. 1998*a*; Castro et al. 2000). Furthermore, the association with the G*01013 allele in the German study suggested that other variation on the G*01013 haplotype may influence HLA-G expression (Pfeiffer et al. 2001), because this allele does not differ from the other G*0101 alleles at the amino acid level. Alternatively, one or more of the three silent substitutions that define the G*01013 allele could influence mRNA splicing (Vockley et al. 2000) and could possibly affect the relative abundance of the G1 and G2 isoforms.

Linkage disequilibrium (LD) with additional variation that influences levels of expression of HLA-G could also account for the association between the G*0104 allele and miscarriage in one study but not the other. That is, the G*0104 allele in the Aldrich et al. (2000) study and the G*01013 allele in the Pfeiffer et al. (2001) study may merely have been markers for additional variation in the gene that influences expression levels. For example, the 14-bp insertion/deletion (in/del) polymorphism in the 3′ UTR (exon 8) (Humphrey et al. 1995) could influence mRNA stability (Bohjanen et al. 1991), and polymorphisms in the 5′ upstream region could influence transcriptional regulation of *HLA-G* (Hviid et al. 1999).

The purpose of this study was to genotype our patient samples for the 14-bp in/del in exon 8 (Humphrey et al. 1995) and the five promoter-region SNPs reported by Hviid et al. (1999). During the course of these studies, we identified an additional 13 polymorphisms in the promoter region of HLA-G that defined eight unique haplotypes. Here we describe the full promoter-region haplotypes that are associated with nine *HLA-G* alleles, as well as an association between variation in the promoter region and fetal loss. These data indicate that regulation of HLA-G expression is critical for successful pregnancy and that perturbation of this process results in fetal loss.

Material and Methods

Population

The Hutterites are an Anabaptist sect that originated in the Tyrolean Alps in the 1500s and settled in what is

now South Dakota in the 1800s (Steinberg et al. 1967; Hostetler 1974). The >35,000 extant Hutterites are descendants of <90 founders who lived in the early 1700s to early 1800s (Martin 1970). The Hutterites of South Dakota have participated in our studies of HLA and fertility since 1982. These individuals live on 36 communal farms (called colonies) and are descendants of only 64 of the 90 Hutterite founders (Ober et al. 1997). As a result of the small number of founding genomes, only 67 unique HLA haplotypes are present in the population (51 ancestral and 16 recent recombinant haplotypes) (Weitkamp and Ober 1999). In addition, the Hutterites' traditional proscription of contraception and a naturally high fertility rate resulted in large families (median completed sibship size was 10 in 1965) and relatively few (2%) childless couples (Sheps 1965; Ober et al. 1999).

In 1986, we initiated a prospective study to assess the relationship between parental HLA types and pregnancy outcome in the South Dakota Hutterites (Ober et al. 1992, 1998*b*). The women in this study are provided with calendar diaries and EPT pregnancy test kits (kindly provided by Warner-Lambert). They record in the diary dates of menses, changes in nursing patterns, illnesses or travel for the husband and wife, and dates of miscarriages or deliveries. In addition, they are instructed to test for pregnancy if they do not start menses exactly 1 mo after the 1st d of their previous period and to record the results of all pregnancy tests in the diaries. They are also asked to start testing for pregnancy on a monthly basis starting 6 mo after delivery, until menses resumes. Results of all pregnancy tests and outcomes of each pregnancy are recorded in the diaries, which are collected yearly, either in person or through the mail. The results reported here include data collected through 2001, representing 15 years of study.

The sample included 474 pregnancies that were either miscarried or followed beyond 20 wk of gestation in 191 women. The fetal loss rate in this sample was 15.6% (74 losses in 474 pregnancies), which is nearly identical to estimates of clinically recognized miscarriage rates in outbred populations (Wilcox et al. 1988). All women with fetal losses also had successful pregnancies, and none would be considered to have recurrent miscarriage (Aldrich et al. 2001; Pfeiffer et al. 2001). Of these 191 women, genetic data were available for both partners in 162 couples, providing 403 pregnancies.

Haplotype Assignments and Polymorphism Detection in the Hutterites

HLA haplotypes were initially characterized in 1,045 Hutterites by the direct observation of alleles segregating in families at five serologically-typed loci: *HLA-A, HLA-B, HLA-C, HLA-DR,* and *HLA-DQ,* (Kostyu et al. 1989). HLA haplotypes were inferred in an additional 216 individuals on the basis of the haplotypes present in their children, spouse, siblings, and/or parents. The subsequent molecular and biochemical genotyping of 21 HLA region loci, including *HLA-G* (Ober et al. 1996), has been conducted in a sample of ~85 Hutterites selected to represent all of the 51 ancestral haplotypes present in the Hutterites of South Dakota (Weitkamp and Ober 1999). In these ~80 individuals, all haplotypes were represented by at least three Hutterites who were selected from distant branches of the pedigree, with the exception of eight rare haplotypes that were represented by two ($N = 7$) or one ($N = 1$) individual. Using this approach, we determined the alleles on each haplotype at 21 loci and then assigned these alleles to all Hutterites with that particular haplotype, as described by Weitkamp and Ober (1999). More recently, HLA haplotypes were assigned to an additional 730 Hutterites by molecular genotyping at informative loci and inferring haplotypes from family data. Among the 324 spouses (162 couples) in this study, haplotypes were determined by serology in 217 and by molecular genotyping and inference in 107.

For our studies of polymorphisms in the *HLA-G* promoter region, we selected a sample of 42 Hutterites who carried 47 of the 51 ancestral HLA haplotypes and all eight *HLA-G* alleles present in the population. These 42 individuals were not selected on the basis of reproductive history. Alleles could not be assigned to four ancestral haplotypes, because there were insufficient amounts of DNA from individuals representing these rare haplotypes. In addition, we included in our studies DNA from a women who was homozygous for the null G*0105N allele (Ober et al. 1998*a*), because this allele is not present in the Hutterites (Ober et al. 1996). Studies of the exon 8 14-bp in/del alleles in the Hutterites were described elsewhere (Ober et al. 1996). For surveying the 5′-upstream promoter region, DNA from all individuals was PCR-amplified using primers that generated a 1,752-bp fragment from −1477 bp (5′) to +208 (3′) relative to the transcriptional start site: 5′-ACATTCTAGAAGCTTCACAAGAATG and 3′-TGGGCCTTGGTGTTCCGTG. The PCR product was sequenced in both directions by Big Dye Terminator v.3 (Applied Biosystems) on an ABI 3100 Automated Sequencer (Applied Biosystems), using the PCR primers as sequencing primers as well as six internal primers: G-908-TTCACCTCACAGTTGTAAGTGTTC, G-830F-CACACGGAAACTTAGGGCTACG, G-1123F-GCCTCGCTGGGTGTTCTTTGC, G-304R-GCCAAGCGTTCTGTCTCAGTGT, GPR-247-CTCAAGCGTGGCTCTCAGGGTC, and GIN1-98-GTTTCCCTCCTGACCCCGCACT.

In addition, the sequence of the promoter haplotype was determined in cloned DNA from 17 heterozygous individuals. For these experiments, PCR-amplified DNA

was cloned into pCR 4-TOPO vector (Invitrogen) and was sequenced using the same protocols described above. DNA from two chimpanzees (*Pan troglodytes*) and two gorillas (*Gorilla gorilla*) was also sequenced, to determine the ancestral allele at each polymorphic site. The human BAC clone AP000521.1 was used as the reference sequence.

Alleles at each of the polymorphic sites were assigned to 47 Hutterite haplotypes, as described above. Allele frequencies were determined from the haplotype frequencies, which were estimated from a sample of 962 married Hutterites representing all four lines of colony descent (Ober et al. 1997), as described elsewhere (Ober et al. 1996; Weitkamp and Ober 1999).

Methylation Studies

To determine whether the −725C/G SNP has different methylation patterns associated with each allele, we sequenced bisulfite-treated DNA from individuals with different genotypes at this SNP. Genomic DNA was treated with bisulfite, following published protocols (Olek et al. 1996; Warnecke et al. 2002) with minor modifications. In brief, 100 ng of DNA was pretreated with proteinase K (1 mg/ml; 37°C for 18 h) prior to treatment with 1 ml of bisulfite solution (5.7 g of sodium metabisulphite, 165 mg of hydroquinone, 2.25 ml of 2 M NaOH, and 9 ml of ddH_2O). Tubes with 500 ml of mineral oil were kept at −20°C for 10–20 min prior to adding agarose beads (Olek et al. 1996); after adding the beads, they were kept on ice for an additional 10–20 min. PCR primers were designed following the suggestions of Warnecke et al. (2002) and were as follows: forward, AGGAGATGTTTTGGATTTAT; and reverse, AAATAACATAACCTTAATAACC. These same primers were used for sequencing, as described above.

Statistical Analysis

The relationship between polymorphisms in the *HLA-G* gene and fetal loss was assessed in Hutterite couples participating in the prospective study, through use of a generalized estimating equation logistic regression model (chapter 8, section 8.2 of Diggle et al. 1994). This model adjusts for the clustering of pregnancies within couples (Hauck and Ober 1991). The models were fit using STATA software (STATA Corporation).

To minimize the number of comparisons, we selected promoter-region SNPs for the analysis of fetal loss that both captured the patterns of LD and identified the major haplotype groups. To do this, we identified the smallest number of SNPs that, together with the exon 8 in/del, differentiated the four haplotypes with frequencies >0.10. We first selected one SNP from each of the two haplotype blocks described in the "Results" section below: −1306G/A and −689A/G. These two SNPs, along with the in/del, identified all haplotypes with frequencies >0.10 except one, which was differentiated from the others by the −725C/G/T SNP. Taken together, these four variants defined six haplotype groups and were the smallest number of variants required to identify all haplotypes with frequency >0.10. Given the large number of possible combinations of haplotypes in the husbands and wives in our sample, we classified couples as to the number of spouses per couple (on a scale of 0–2) that carried a −1306A/−689G (AG) haplotype, the −725G allele, or an insertion (ins) allele. This selection and categorization process was performed prior to examining the effects of these polymorphisms on fetal loss rates.

In the multivariable logistic model, we examined the effects of the −1306A/−689G (AG) haplotypes, the −725G allele, and 14-bp ins allele. In all analyses, we controlled for variables that are known to affect fetal loss in the Hutterites. These include maternal age and HLA-B matching (Ober et al. 1998*b*); we also included maternal inbreeding, because it provided more precise estimates for the covariates. We previously showed that neither inbreeding in the father nor relatedness between the parents influences fertility in this population (Ober et al. 1998*b*, 1999). The final model was reduced to include only significant genetic variables.

Results

Patterns of Variation in the Promoter Region

Eighteen polymorphisms were present in the 1,300 bp upstream from the transcriptional start site of HLA-G in the Hutterites (fig. 1). Five SNPs (−201A/G, −964A/G, −1140T/A, −1179G/A, and −1306A/G) were previously identified (Hviid et al. 1999); the remaining 13 SNPs were discovered in the present study. Thirteen of the polymorphisms are within or very close to known transcription factor binding sites or regulatory elements. Nearly all others are either in complete LD with these sites (such as the −964A/G SNP with the −1306A/G SNP) or are in other putative binding sites. For example, the −666T allele disrupts a potential binding site for the GATA2/GATA3 transcription factors.

The frequencies of the alleles at each polymorphic site are shown in table 1. Most are common polymorphisms, with minor allele frequencies >0.10 for 15 and >0.30 for 12 of the 18 variants. The more common allele corresponds to the chimpanzee sequence for 9 of the 18 variants and to the gorilla sequence for 8 of the variants (fig. 2). Interestingly, one site (−369C/A) has an unusual pattern, in that different primate species have one or the other allele present in humans. Thus, for 8 variants, the more common allele is the ancestral allele (present in the chimpanzee and gorilla); for 10 variants, the more common allele is the derived allele (absent in the chimpanzee and gorilla); and for 1 variant (−369C/A), the ancestral state cannot be determined with certainty, be-

cause each allele is present in either the chimpanzee or the gorilla.

Overall, there is very strong LD between variants in this 1,300-bp region. Three variants that span the regulatory region, at nts −1306, −964, −201, are in complete LD in this population, with either G-G-G or A-A-A occurring on each haplotype. The former corresponds to the derived and the latter to the ancestral alleles at each of these polymorphic sites. Similarly, a set of six variants clustered in the middle of the regulatory region, at nts −762, −716, −689, −666, −633, and −486, are in complete LD. These occur as either T-G-G-T-A-G or C-T-A-G-G-A. In this case, however, the haplotypes contain a mix of ancestral and derived alleles.

These 18 polymorphisms defined eight unique promoter haplotypes in the Hutterites (fig. 2). In some cases, different *HLA-G* alleles, which are defined by variation in the coding region, share the same promoter haplotype: G*01011, G*01014, and G*01018 share one sequence, and G*01012, G*0105N, and G*01061 share another sequence. These likely represent the evolutionary relationships between alleles: the G*0105N and G*01061 alleles arose on a G*01012 haplotype background (Suarez et al. 1997; Hviid et al. 2001), and molecular studies indicated that G*0105N is a relatively young allele (Aldrich et al. 2002). On the other hand, some *HLA-G* alleles

Table 1

Frequencies of the Minor Alleles at 18 Polymorphic Sites in the 5′-Upstream Regulatory Region of *HLA-G*

Position[a]	Polymorphism	Minor Allele	Frequency
−1306	G/A	A	.38
−1179	A/G	G	.41
−1155	G/A	A	.13
−1140	A/T	T	.25
−1138	A/G	G	.011
−1121	C/T	T	.035
−964	G/A	A	.38
−762	C/T	T	.36
−725	C/G	G	.16
−716	T/G	G	.36
−689	G/T	T	.36
−666	G/T	T	.36
−633	G/A	A	.36
−486	A/C	C	.36
−477	G/C	C	.35
−369	C/A	A	.35
−201	G/A	A	.38
−56	C/T	T	.036

[a] Nucleotide position relative to transcriptional start site.

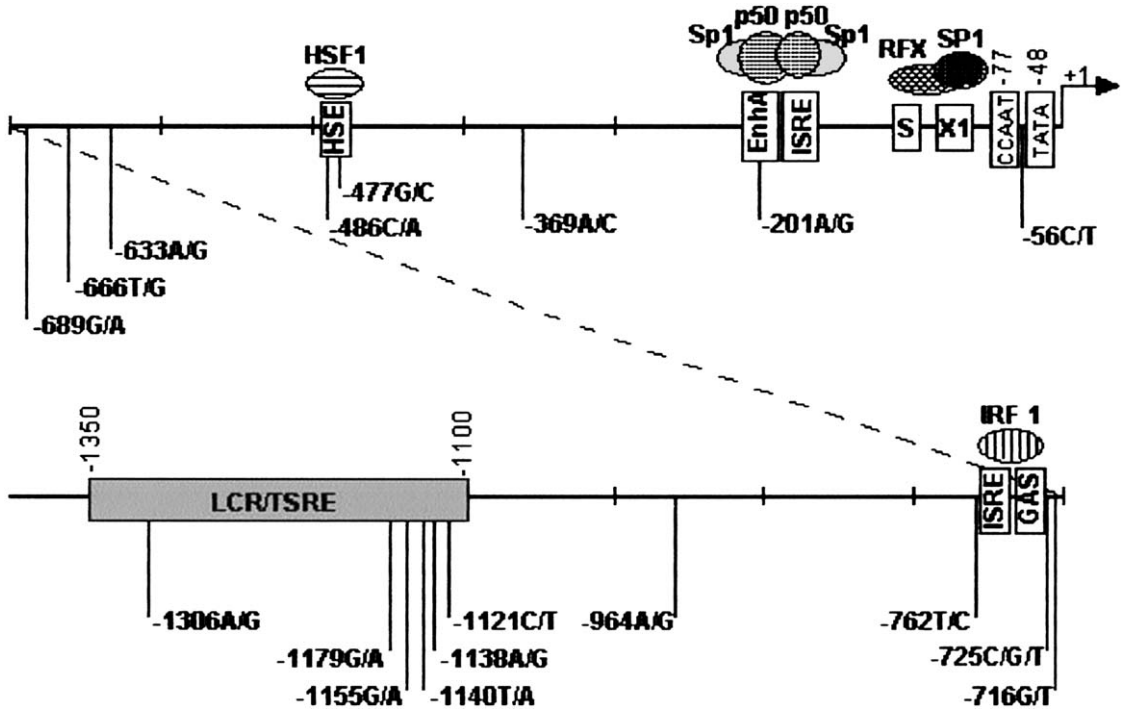

Figure 1 *HLA-G 5′*-upstream regulatory region (modified from Solier et al. 2001). "+1" designates the transcriptional start site. Cis-acting regulatory elements are shown: TATA = TATA box; CCAAT = CAAT box; S/X1 = *Pan* HLA regulatory elements; ISRE = interferon-specific regulatory element; EnhA = enhancer A; HSE = heat shock protein element; GAS = gamma (interferon) activated site; LCR = locus control region (also known as "TSRE" [tissue-specific regulatory element]). The LCR (or TSRE) contains the regulatory elements that direct trophoblast-specific expression (Schmidt and Orr 1995).

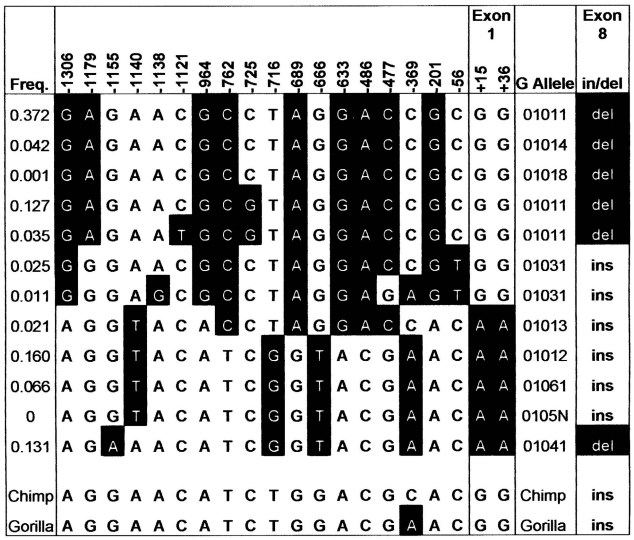

Figure 2 Haplotypes comprised of 18 polymorphisms in the 5′-upstream regulatory region, in exons 1 and 8, and their associated HLA-G allele. Frequencies of haplotypes in the Hutterites are shown. Alleles shown as black letters on a white background correspond to the chimp sequence and are presumed to be ancestral; alleles shown as white letters on a black background are presumed to be derived alleles. Alleles at two polymorphic sites in exon 1 are shown for comparison with earlier studies (Hviid et al. 1999).

are associated with more than one promoter haplotype. Two variants (−1121T and −725G) define two additional G*01011 promoter haplotypes, which are associated with G*01011 alleles that are present only on HLA-A3 haplotypes. Three variants, −1138G, −477G, and −369A, define a second G*01031 promoter haplotype and are present on the only HLA-A34 haplotype in the Hutterites. On the basis of these results, we resequenced exons 2 and 3 of the variant *01011 and *01031 alleles but did not find any additional variation. Six promoter variants, all with frequencies <0.20, were found exclusively with single *HLA-G* alleles: the −56T and −1138G alleles occur only with G*01031, the −725G and −1121T alleles occur only with G*01011, and the −1155A allele occurs only with G*01041. The remaining 13 variants were associated with more than one *HLA-G* allele.

Surprisingly, the promoter haplotype associated with the G*01012, G*0105N, and G*01061 alleles is the closest to the nonhuman primate sequence, differing from the chimpanzee at only four sites (nts −1140, −716, −666, and −369) and from the gorilla at three sites (nts −1140, −716, and −666). The *01041 allele differs from the *01012 allele at only a single site in the promoter region (the derived allele −1155A). In contrast, the common *01011 allele differs from the chimpanzee at 8–10 sites and from the gorilla at 9–11 sites and is therefore the most divergent sequence, despite being the most common allele. The relatively rare *01013 allele is more similar to the ancestral sequence than are the *01011 alleles to the ancestral sequence, with only five differences from the chimpanzee and six from the gorilla. The promoter haplotypes associated with the *01031 allele are more similar overall to the *01011 alleles than to the *01012 alleles and differ from the chimpanzee at 9–11 sites and from the gorilla at 9 or 10 sites. Apart from these polymorphic sites, the human sequence differs from the chimpanzee sequence at 10 fixed sites and from the gorilla sequence at 11 fixed sites, or just under 1% of the sites surveyed (data not shown), which is consistent with divergence rates between humans and chimpanzees/gorillas at other genes (Cavalcanti et al. 2002).

Analysis of Fetal Loss

On the basis of the patterns of LD among promoter SNPs and on promoter haplotype frequencies in the Hutterites, we selected three SNPs, −689T/G, −725C/G, and −1306G/A, a priori, for analyses of associations with fetal loss rates. These three SNPs, together with the 3′ UTR in/del, defined five distinct groups of alleles (see the "Material and Methods" section for additional details). Because HLA-G is primarily expressed in fetal placental tissues and because we did not collect tissues from aborted fetuses, we examined *HLA-G* genotypes in both the husbands and the wives, as in our previous study (Aldrich et al. 2001). We hypothesized that transmission of a high-risk allele from either parent to the fetus would be associated with fetal loss.

The parental genotype combinations for the −1306A/−689G promoter haplotype, the −725C/G alleles, and the 14-bp in/del polymorphism are shown in table 2. Fetal loss rates were highest among couples in which neither spouse carried a −1306A/−689G haplotype, in which both partners carried a −725G allele, and in which neither spouse carried an insertion allele (table 2). For the logistic models, we considered as the baseline couples in which one or both spouses carried a −1306A/−689G haplotype, neither spouse carried a −725G allele, and one or both spouses carried an insertion allele. The other groups were compared against these baselines.

In the multivariable models controlling for mother's age, mother's inbreeding, and HLA-B matching, the presence of the −725G allele in both the husband and the wife was the only *HLA-G* polymorphism that was significantly associated with fetal loss rates (odds ratio [OR] 2.72; 95% CI 1.08–6.87; $P = .034$) (table 3). Neither the −1306A/−689G SNPs (OR 1.63; 95% CI 0.79–3.38; $P = .186$) nor the ins allele (OR 1.50; 95% CI 0.81–2.78; $P = .200$) was significantly associated with risk for miscarriage, when each was considered in separate logistic models controlling for mother's age,

Table 2

Parental Genotype Combinations for the −1306G/A and −689A/G Haplotypes, the −725C/G Polymorphism, and the 14-bp in/del Polymorphism, in Hutterite Couples

No. of Spouses per Couple	No. of Pregnancies	Genotype Combinations in Couples	Fetal Loss Rate
With −1306A/−689G haplotype:			
0	68	nonAG/nonAG × nonAG/nonAG	.22
1	182	AG/nonAG × nonAG/nonAG or AG/AG × nonAG/nonAG	.13
2	153	AG/nonAG × AG/nonAG, AG/AG × AG/nonAG or AG/AG × AG/AG	.13
With −725G allele:			
0	186	CC × CC	.10
1	188	CC × CG or CC × GG	.17
2	29	CG × CG or CG × GG	.28
With exon 14 ins allele:			
0	101	del/del × del/del	.21
1	190	ins/del × del/del or ins/ins × del/del	.11
2	112	ins/del × ins/del, ins/ins × ins/del, or ins/ins × ins/ins	.16

mother's inbreeding, and HLA-B matching. The multivariate logistic regression model was fit including all three genetic variables and controlling for mother's age, mother's inbreeding, and HLA-B matching. After excluding nonsignificant ($P > .05$) genetic variables, it was reduced to the final model with only the number of spouses carrying a −725G allele remaining (table 3).

In the final model, the effects of matching for *HLA-B* alleles and of mother's age on fetal loss rate were reduced and no longer significant ($P > .05$). This may indicate that the presence of the −725G allele in both spouses is a more significant risk factor for fetal loss in the Hutterites than either maternal age or HLA-B matching. We did not find evidence of association between maternal age and the −725G allele. However, there could be confounding between the −725G allele and *HLA-B* matching, because these genetic factors are associated in this sample. Also, the 95% CIs are fairly large, because of a small subgroup with the −725G allele. Further, it is not possible to determine from these data whether the risk for miscarriage is increased only in fetuses that are homozygous for the −725G allele or if heterozygous fetuses are also at risk. However, the fact that fetal loss rates are higher in couples in which only one spouse carries the −725G allele compared with those in couples with no allele (0.17 vs. 0.10, respectively) suggests that heterozygous fetuses may also be at risk, even though these differences were not significantly different in our sample (table 3). Further, consistent with the modest risk associated with this allele in the Hutterites (OR 2.72; 95% CI 1.08–6.87) (table 3), the frequencies of −725G heterozygotes and homozygotes were in Hardy-Weinberg proportions in the population (data not shown).

The −1121T allele resides on a single G*01011 allele that also includes the −725G allele. Therefore, to rule out the possibility that the effect of the −725G on miscarriage is due to LD with the −1121T allele, we examined the effects of this allele on fetal loss. Because the −1121T allele is relatively infrequent in the Hutterites (frequency 0.035) and there were no couples in which both partners carried this allele, we compared miscarriage rates in couples with and without this allele. The miscarriage rates did not differ between these two groups (0.14 in couples without a −1121T allele and 0.19 in couples with a −1121T allele; $P = .56$) or when the −725G allele was included in the model ($P = .97$).

Further Studies of the −725G Allele

The transposition of a C to a G at position −725 creates a CpG dinucleotide at nts −726 and −725. Therefore, we questioned whether −726C was methylated when associated with a G at nt −725 and perhaps influenced the binding of interferon response factor-1 (IRF-1) at the neighboring interferon-specific regulatory element binding site (fig. 1) (Lefebvre et al. 1999). To investigate the former question, we sequenced bisulfite-treated DNA derived from peripheral blood cells from two individuals with the −725 CC genotype and three individuals with the −725 GG genotype (all samples are CC at nt −726). Bisulfite converts cytosines (C) to thymines (T) in nonmethylated DNA but does not alter cytosines that are methylated (C^M) (Frommer et al. 1992). After bisulfite treatment, the samples with −725

Table 3

Analysis of Fetal Loss in the Hutterites

Variable	*P*	OR	95% CI
Mother's age	.061	1.07	.99–1.15
Mother's inbreeding	.751	.96	.80–1.18
HLA-B matching	.056	1.85	.98–3.34
−725G in one spouse	.116	1.71	.88–3.34
−725G in both spouses	.034	2.72	1.08–6.87

CC and −726 CC genotypes were homozygous TT at both −725 and −726, as expected in unmethylated DNA (fig. 3*A*). However, the C at −726 was unconverted in samples with the −725 GG and −726 CC genotypes (fig. 3*B*), although all other non-CpG cytosines in the sequence were converted to thymines (complete sequence not shown). Therefore, the −726C is methylated on −725G alleles in the majority of cells. A small T peak at this site (fig. 3*B*) indicates that methylation in DNA derived from peripheral blood cells is incomplete. Nonetheless, these studies demonstrate that the allele that is associated with miscarriage in the Hutterites has a C^M at nt −726.

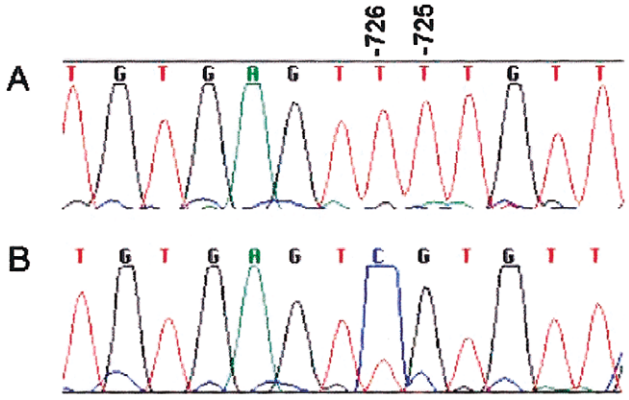

Figure 3 Sequence flanking the −725C/G polymorphism in bisulfite-treated DNA. *A*, Genotype −725 CC. *B*, Genotype −725 GG.

Discussion

Miscarriage is the most common gestational disorder, affecting ~15% of pregnancies. Although the majority of sporadic losses and a lesser proportion of recurrent pregnancy losses are due to chromosomal abnormalities in the fetus (Warburton and Fraser 1964; Stephenson et al. 2002), a significant proportion of both sporadic and recurrent fetal losses remain unexplained. Recently, two studies implicated a null allele in *HLA-G*, *0105N, in the etiology of recurrent miscarriage, suggesting that reduced levels of HLA-G1 protein may adversely affect reproductive outcome (Aldrich et al. 2001; Pfeiffer et al. 2001). Two additional alleles, *01013 in the German study (Pfeiffer et al. 2001) and *01041 in the U.S. study (Aldrich et al. 2001), were also associated with miscarriage. We undertook this study to determine whether additional variation that could affect levels of HLA-G protein was associated with miscarriage. We examined the promoter region and 3′ UTR of the *HLA-G* gene to test the hypothesis that variation in these regions could directly affect transcription rates or mRNA stability, respectively, and indirectly affect levels of protein expression and pregnancy success. In contrast to the previous studies, we conducted this study in healthy women who were unselected with respect to reproductive histories but who were participants in a 15-year prospective study of pregnancy outcome. We report a significant association between a variant in the upstream regulatory region of *HLA-G*, −725G, which is present on a subset of G*01011 alleles and is located ~10 bp 3′ to an IRF-1 binding motif (Lefebvre et al. 1999). Further, the transposition from a C to a G creates a CpG dinucleotide, at nts −726 and −725, that is methylated on −725G alleles.

Although the Hutterites are an isolated population, their HLA haplotypes are similar to those found in other European populations (Weitkamp and Ober 1999) and all but five rare *HLA-G* alleles (G*01015, G*01016, G*01042, G*01043, and G*0105N) are present in the Hutterites at frequencies similar to those in other populations (Ober et al. 1996; Ober 1997). We assume, therefore, that the promoter-region haplotypes described in this study will also be found in other populations and associated with the same *HLA-G* alleles, although it is possible that additional haplotypes and even additional variation are present in outbred groups

The two *HLA-G* alleles that were associated with miscarriage in previous studies, *01013 and *01041, have different promoter haplotypes, and neither carries the −725G allele. This is consistent with the fact that neither the *01013 nor the *01041 (nor the newly described *01061) allele was associated with miscarriage in the Hutterites (data not shown). However, it is possible that the −725G allele is present on *01013 and *0104 haplotypes in outbred populations or that additional functional variation, which is not present in the Hutterites, is present in the promoter region of these alleles in outbred patients with recurrent miscarriage.

The extraordinary amount of variation in the 5′-upstream regulatory region of *HLA-G* (18 polymorphisms in 1,350 bp) was unexpected, given the relatively low levels of polymorphism in the coding region of this gene (Ober and Aldrich 1997). In particular, many of the polymorphic sites described in this study coincide with upstream regulatory elements that are unique to *HLA-G*. For example, two polymorphisms (−486C/A and −477G/C) are located within a heat shock element, three polymorphisms (−762T/C, −725C/G, and −716G/T) closely flank an IRF-1 binding motif, and six polymorphisms (−1306A/G, −1179G/A, −1155G/A, −1140T/A, −1138A/G, and −1121C/T) reside within the tissue-specific regulatory element, a region critical for trophoblast-specific expression of HLA-G in transgenic mice (Schmidt and Orr 1993). Thus, many of these polymorphisms, either alone or in combination, could influence transcriptional rates of HLA-G proteins in a tissue-specific manner. Although we do not have func-

tional data showing differences in transcriptional properties of the −725G and −725C alleles, the creation of a methylated CpG close to an important regulatory element on −725G alleles (Lefebvre et al. 1999) and the association between −725G and miscarriage in the Hutterites suggest that differences likely exist. A proposed functional role for the −725G allele is further supported by a recent study demonstrating that *HLA-G* transcription is inhibited by DNA methylation (Moreau et al. 2003). Thus, the introduction of an additional methylated cytosine on −725C alleles may downregulate transcription of *HLA-G*.

Alternatively, the −725G allele may be in LD with another variant that confers risk to miscarriage. It is unlikely that this variant is within the coding region of *HLA-G,* because the variation in the exons is identical on all *01011 alleles in the Hutterites. However, we cannot rule out the presence of intronic variation that could affect splicing, or even variation in a nearby gene that is in LD with the −725G allele. In fact, there is very significant LD between *HLA-G* and *HLA-A* alleles in all populations studied, and, in the Hutterites, the −725G allele is found exclusively on six different haplotypes that carry an HLA-A3 allele (Ober et al. 1996). Although the A3 allele per se has not been associated with miscarriage (reviewed by Ober and van der Ven 1998), Christiansen et al. (1989) have proposed that some extended HLA haplotypes carry abortion-susceptibility alleles. Thus, it is possible that the −725G allele resides on such a haplotype or even that the −725G allele is the susceptibility allele on these extended haplotypes. Additional studies of the −725G allele in other populations and functional studies of the −725C/G polymorphism are required to differentiate between these hypotheses.

The pattern of variation in the *HLA-G* promoter region is also remarkable. The two most common alleles, *01011 and *01012, which differ at only two silent sites in the coding region of the gene, have the most divergent promoter sequences and define two major groups of promoter haplotypes (fig. 2). The *01012 allele is most similar to the ancestral (chimpanzee) haplotype and is identical to the promoter sequence of the *0105N and *01061 alleles, both of which arose on a *01012 background in the relatively recent past (Suarez et al. 1997; Hviid et al. 2001; Aldrich et al. 2002). The promoter region of the *01041 allele differs from the *01012 group of alleles by only a single nucleotide (−1155A). The *01011 allele, the most common allele in all populations studied, is the most divergent from the ancestral sequence and shares a common promoter sequence with the closely related *01014 and *01018 alleles. The promoter region of the more common of the two *01031 alleles differs from the more common of the three *01011 haplotype at two sites (−1179G and −56T), and two additional differences (at −1138G and −369A) define the second *01031 allele. The 5′-upstream region of the relatively rare *01013 allele is more similar to the ancestral sequence than is the *01011 allele and is more similar to the *01012 group of alleles at its 5′ end (from nts −1306 to −964) than to the *01011 group of alleles. This unusual pattern may be consistent with gene conversion events reshuffling blocks of variation among haplotypes, similar to what has been observed in HLA class II genes (Zangenberg et al. 1995).

In summary, the *HLA-G* gene continues to reveal features that distinguish it from the other classical and nonclassical class I HLA genes. We report here extraordinary polymorphism in the upstream regulatory region of this gene and an association between miscarriage and a polymorphic variant that occurs at a frequency of 0.16 in the study population. This variant flanks a binding site for the transcription factor IRF-1 and creates a methylated CpG dinucleotide, which could alter the conformation of DNA and affect IRF-1 binding. It is not yet known if this variant allele influences the transcriptional properties of *HLA-G* or which of the HLA-G isoforms might be affected. Nonetheless, the association with miscarriage suggests that the −725G allele may downregulate transcription of *HLA-G* and, like the *0105N null allele, result in reduced protein expression. Thus, the likelihood of a successful pregnancy may be determined, in part, by either the absolute levels of HLA-G protein or the relative levels of the different protein isoforms, further suggesting a critical role for this unusual gene throughout pregnancy.

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Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for HLA-G)

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