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Haplotype Analysis and $^{A}\gamma$ Gene Polymorphism Associated With the Brazilian Type of Hereditary Persistence of Fetal Hemoglobin

Silvana Bordin,[1] Juliana T. Martins,[1] Marilda S. Gonçalves,[1] Mônica B. Melo,[1] Sara T.O. Saad,[1,2] and Fernando F. Costa[1,2]*

[1]Centro de Hematologia e Hemoterapia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil

[2]Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil

We have identified three unrelated individuals and three members of a family with the non-deletion form of $^{A}\gamma$–hereditary persistence of fetal hemoglobin (HPFH). Molecular analysis showed that each individual is a heterozygote for a previously described −195 $^{A}\gamma$ (C→G) mutation. The β-globin gene cluster was studied using the polymerase chain reaction and related techniques. Haplotyping using nine restriction sites identified two closely related chromosomes with the −195 $^{A}\gamma$ mutation, differing only in a single site 3′ to the β-globin gene. Further analysis of β-globin framework indicated that the HPFH allele segregates with haplotype V, according to Orkin's classification. The second haplotype probably originated by a point mutation or DNA rearrangement of a pre-existing −195$^{A}\gamma$ chromosome. We also determined the sequences from −622 to +55 bp upstream to the $^{A}\gamma$ gene and part of the $^{A}\gamma$ IVS-2. We found four polymorphisms associated to the −195$^{A}\gamma$ promoter region. All −195 $^{A}\gamma$ chromosomes had a G at positions −588 and +25 relative to the $^{A}\gamma$ gene. One individual was also homozygous for polymorphisms at −398 (G→A), and another at −369 (C→G). Cloning and sequencing of the polymorphic patterns of the 3′ region of $^{A}\gamma$ IVS-2 showed that the mutated allele is linked to β-globin chromosome B. Some correlations between chromosome characteristics and $^{A}\gamma$ point mutations were also observed. Am. J. Hematol. 58:49–54, 1998.

Key words: hereditary persistence of fetal hemoglobin (HPFH); point mutation; haplotypes

INTRODUCTION

Hereditary persistence of fetal hemoglobin (HPFH) is a clinically benign condition characterized by the continuous synthesis of fetal hemoglobin (HbF) in adult life without major related hematological changes. The non-deletion form of HPFH affects predominantly the expression pattern of one type of γ-globin gene, ranging from 3 to 20% of HbF in heterozygous individuals. Examples of the non-deletion form of HPFH include point mutations at positions −202, −175, −161, and −114 of the $^{G}\gamma$ gene, and −202, −198, −196, −195, −175, −117, and −114 of the $^{A}\gamma$ gene [see ref. 1 and references cited therein]. Although the molecular mechanisms affecting the pattern of HbF expression are not known, there is strong evidence of a critical role for the HPFH promoter in γ-globin gene expression. Thus, for example, it has been suggested that structural changes induced by base substitutions from positions −194 to −215 generate new interactions between *cis* regulatory elements [2,3]. Another example is the identification of a broad promoter region, extending at least from −202 to −50, which interacts with either ubiquitous and/or erythroid specific DNA-binding proteins [4].

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*Correspondence to: Fernando F. Costa, FCM-UNICAMP, C.P. 6111, 13081-970 Campinas, São Paulo, Brazil. E-mail: ferreira@turing.unicamp.br

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Among the non-deletion form of HPFH, an uncommon substitution at position −195 (C→G) has been described in one Brazilian family [5]. As far as we know, no other case of Brazilian type-HPFH has been reported in any population. In contrast, the relatively high frequency of other $^{A}\gamma$-HPFH mutation (e.g., −202, −198, −196, and −117) has allowed a direct correlation between the genetic background and the inherited specific substitution [6,7]. We have identified six Brazilian individuals, three unrelated and three members of a family with a non-deletional form of HPFH, in which the presence of a −195 mutation is closely associated with the high HbF phenotype. In the present study, we have characterized the chromosomes carrying the −195 $^{A}\gamma$ mutation by determining the RFLP haplotype and the polymorphic sequences within the $^{A}\gamma$-globin gene locus.

MATERIALS AND METHODS

Subjects

Three cases (S.R., J.E., and S.G.) had been identified during the routine screening of blood donors at the Center of Hematology and Hemotherapy at the State University of Campinas (UNICAMP). S.R. and J.E. were, respectively, 32- and 38-year-old white males. S.G., a 12-year-old black male, was the son of a blood donor. The family (family D) was identified through V.D., a 30-year-old mulatto female outpatient who had attended the University Hospital to treat neurocysticercosis. Eight members of family D were available for the family study.

Hematological Tests and DNA Analysis

Hb analyses were carried out by electrophoresis on both cellulose acetate [8] and urea-Triton-acrylamide gels [9], followed by quantification of the γ chain by densitometry. Hb A_2 was quantified spectrophotometrically [10]; HbF was measured by resistance to alkali denaturation [11].

DNA was isolated from peripheral blood leukocytes according to standard procedure. RFLP-haplotyping was performed by amplification of nine β-cluster segments containing the following restriction site polymorphisms [12]: XmnI in the $^{G}\gamma$ 5′-flanking region, Hind III in IVS-2 of $^{G}\gamma$ and $^{A}\gamma$, Hinc II in Ψβ and 3′ to it, Hinf I and Rsa I 5′ to β, Ava II in β and Hpa I 3′ to the β gene. Primers and buffer conditions were as previously described [13,14]. The $^{A}\gamma$-globin promoter spanning position −622 to + 55 was amplified and completely sequenced employing the primers described in Figure 1. Amplification and sequencing of the β-globin framework was performed using previously reported primers [15]. Sequence analysis of polymorphic (TG) repeats within $^{A}\gamma$ IVS-2 was carried out on both isolated alleles. For this, the PCR product (2 μl) used for RFLP-haplotyping (for Hind III digestion of $^{A}\gamma$-globin IVS-2) was subcloned using a pMOSblue T-vector kit (Amersham Life Science, Buckinghamshire, UK) as recommended by the supplier. The positive clones were isolated and sequenced using plasmid primers.

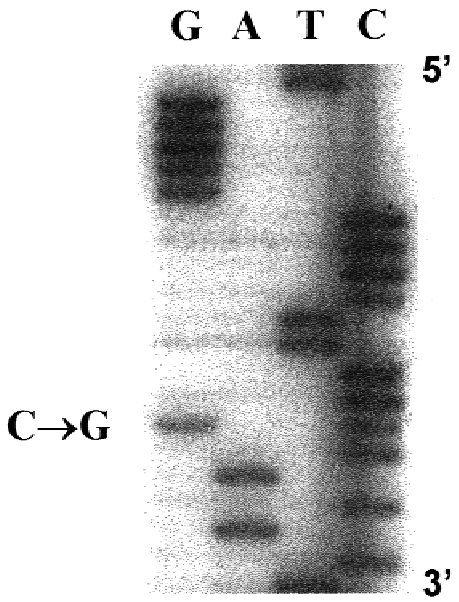


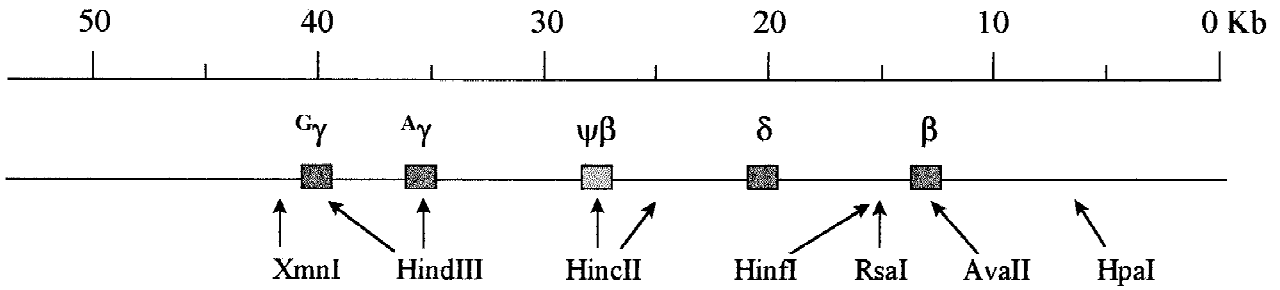
Fig. 1. Sequencing gel showing the −195 C→G mutation in the promoter of the $^{A}\gamma$ gene. Sequences of PCR and sequencing primers are shown below. (PCR primers: TGAAACTGTGGTCTTTATGAAAATTG and GGCGTCTGGACTAGGAGCTTATTG; sequencing primers: AGAAATTAAGAGATAATGG, GAAGTTCCTGGTATCCTC and TGAAAACGGTCCCTGGCTAA).

TABLE I. Hematological Data and Hb Analysis of the-195 $^{A}\gamma$ HPFH Heterozygotes

Subjects	RBC (10^{12}/l)	Hb (g/dl)	Ht (%)	MCV (fl)	MHC (pg)	HbA_2 (%)	HbF (%)
J.E.	5.16	16.2	44.2	85.7	31.4	2.7	5.5
S.R.	4.70	15.0	42.0	89.0	31.0	1.7	8.5
S.G.	5.35	13.6	42.7	79.8	25.4	2.3	6.8
Family D							
V.D. (II.1)	4.52	12.7	36.9	81.7	21.0	1.5	7.5
Mother (I.1)	4.88	15.5	44.3	90.7	31.9	2.5	0.8
Sister (II.2)	5.29	13.4	42.8	81.0	25.3	1.7	5.9
Sister (II.3)	4.35	12.3	36.9	84.7	28.3	1.7	5.6
Sister (II.4)	4.50	13.8	41.1	91.4	30.7	2.2	0.9
Son (III.1)	4.37	11.5	36	82.6	26.5	2.0	0.7
Niece (III.2)	4.03	10.5	32.6	81.0	26.1	2.4	0.8
Niece (III.3)	4.70	11.8	36.7	78.0	25.1	2.2	0.7

RESULTS

Table I shows the hematological and hemoglobin analyses of three blood donors (S.G., J.E., and S.R.) and of the family members of patient V.D.. All of the hematological parameters were within the normal range, except for the increased HbF levels. The hemoglobin study



Subjects									
J.E.	+/-	+/-	-/-	+/-	+/-	+/-	+/+	+/+	+/+
S.G.	-/-	+/-	+/-	-/-	+/-	+/-	+/-	+/-	+/+
S.R.	-/-	-/-	-/-	-/-	-/-	+/-	+/+	+/+	+/+
V.D.	-	-	-	-	-	-	+	+	-
	-	-	-	-	-	-	+	+	+/-

Fig. 2. β-Globin cluster haplotype analysis of the four subjects carrying the −195 $^{A}\gamma$ mutation. The arrows indicate the polymorphic restriction sites examined.

showed that the moderate increase in HbF consisted mainly of the $^{A}\gamma$ chain (73 to 100%), suggesting the presence of a non-deletion form of HPFH.

The association between the substitution C→G at position −195 in the $^{A}\gamma$ gene and an increased production of HbF was confirmed. Direct sequencing of the $^{A}\gamma$ promoter revealed the presence of a $-195^{A}\gamma$ mutation in three out of eight members of family D (proband and two sisters), as well as in three other unrelated individuals (Fig.1). All individuals carrying the $-195^{A}\gamma$ mutation exhibited a moderate elevation in HbF levels (range 5.5 to 8.5%), as previously described [5].

Haplotypes of the chromosomes carrying −195 $^{A}\gamma$ mutations were determined in eight members of the family and in the three other cases. Nine polymorphic sites were evaluated and a summary of the data is provided in Figure 2. No major alteration in the β-globin gene cluster was detected. In the family study, the haplotype of the chromosome carrying the mutation was [− − − − − − + + −], which differs in one site from the haplotype [− − − − − − + + +] observed in the three other −195 alleles (Fig. 3).

Figure 4 summarizes the chromosomal sequence variations assessed by $^{A}\gamma$ gene sequencing. Two other substitutions (G at positions −588 and +25) were identified in all individuals with the HPFH phenotype. However, one normal family member also presented these polymorphisms (see II.4 in Fig. 3). Patients J.E. and S.R. were also homozygous for polymorphisms −398 (G→A) and −369 (C→G), respectively. We also sequenced part of the IVS-2 of the mutated $^{A}\gamma$ gene because this is a region rich in differences between β-globin chromosomes A and B. For this, we cloned and separately sequenced the two alleles of the $^{A}\gamma$ IVS-2 polymorphic sequence from the four subjects and from a normal relative (mother of subject V.D.). All of the subjects heterozygous for the $-195^{A}\gamma$ mutation carried both $(TG)_9(CG)_5(TG)_8$ and $(TG)_{13}$ configurations. In contrast, all clones sequenced from the normal relative showed only the $(TG)_{13}$ configuration. The presence of two allelic forms in the HPFH subjects and one allelic form in the normal individual was confirmed by SSCP analysis (not shown).

We further analyzed the β-globin polymorphic sequences of CD2 and IVS-2 at positions 16, 74, 81, and 666, in order to determine the β-gene framework. Figure 4 shows that all subjects were found to have a CAC-C-T-C-T framework (CD2, 16, 74, 81, and 666, respectively), which corresponds to framework 2.

DISCUSSION

During the routine screening for hemoglobinopathies carried out in the University Hospital at UNICAMP, four unrelated individuals bearing the $^{A}\gamma$-HPFH phenotype were found. Direct sequencing of the upstream region of the $^{A}\gamma$ gene identified all of them as heterozygous for the Brazilian type of HPFH. This study analyzed the RFLP-haplotypes and sequence variations in the promoter region and IVS-2 of the $^{A}\gamma$ gene from these four −195 $^{A}\gamma$ chromosomes. The observation of the same rare mutation in four unrelated individuals raises the question about a single or multiple origin of the mutation. Several mutations in the β-gene cluster have been reported to have independent origins [16]. In this respect, RFLP-haplotypes have been used as a powerful marker for de-

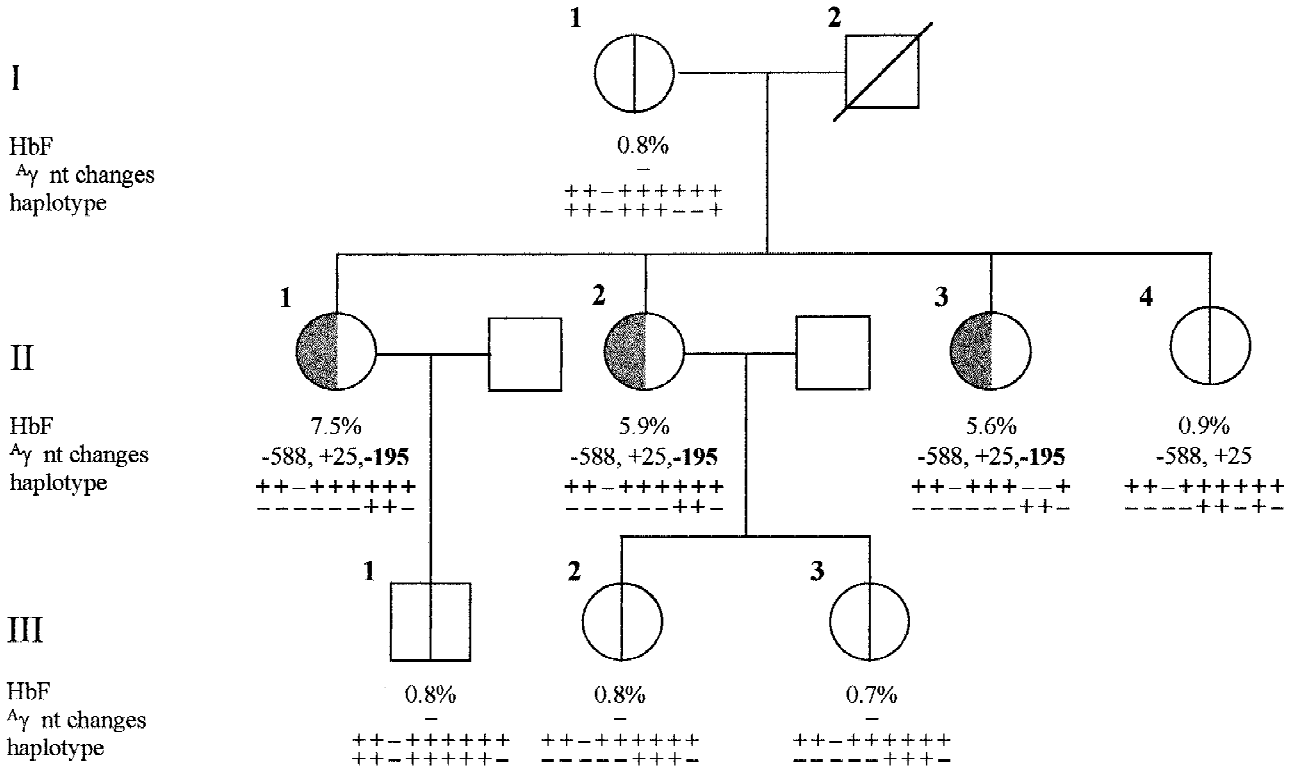


Fig. 3. Pedigree of the family with the $^{A}\gamma$-HPFH phenotype. Haplotypes and sequence substitutions in the $^{A}\gamma$ gene promoter region were determined as described in the text. Half-filled symbols indicate HPFH phenotype; slashed symbol indicates deceased; open symbol indicates not typed.

termining the genetic background inherited with the abnormal gene in hemoglobin disorders such as HbSS and β-thalassemia, thus providing valuable insights in anthropological studies.

In our investigation, two closely related haplotypes ([——+++] and [——++–]) were identified for chromosomes bearing the −195 $^{A}\gamma$ mutation. Except for the absence of the Hpa I site in one of the four subjects, RFLP analysis showed that the $^{A}\gamma$ −195 (C→G) substitution could be associated with haplotype [——+++]. However, since the HpaI site lies within an extensive LINE-1 sequence, there is a large probability of DNA rearrangement within this site [17,18]. The presence of these two haplotypes in the same HPFH mutation led us to examine in greater detail the 3′ region of the β-globin cluster. Analysis of the β-globin framework (Fig. 4) showed that intragenic variations in CD2 and nucleotides 16, 74, 81, and 666 within β-IVS2 were associated with framework 2. Based on the dimorphisms (RFLP and intragenic variations) observed in all of the subjects, we believe that the −195$^{A}\gamma$ allele most probably segregates with haplotype V [19, 20]. It is likely that these two different 3′ subhaplotypes have originated from DNA rearrangement through sequence instability within the Hpa I site rather than by multiple origin of the −195 $^{A}\gamma$ mutation.

The β 5′-subhaplotype [− − − − −] comprising five RFLP markers surrounding the γ globin gene region cosegregated with the high HbF determinant in the family members (Fig. 3), and most probably in the three other individuals as well (Fig. 2). It is noteworthy that the 5′-subhaplotype [− − − − −] is the most common in all of the populations studied so far around the world, and is usually found in association with various normal and mutated chromosomes [16]. As the subhaplotype [− − − − −] is characterized by the absence of the classical polymorphic restriction sites, which result from several single nucleotide variations, one would expect a high degree of heterogeneity for this subhaplotype at the level of the nucleotide sequence.

It has been observed that the same point mutation related to the HPFH phenotype can occur in different haplotypes or even in different γ genes. In order to verify whether the four cases of a −195 mutation described here were associated with different chromosomes, we have compared polymorphisms not amenable to RFLP analysis, particularly within the $^{A}\gamma$ gene [19]. Sequence variations in the 5′ flanking region and in the simple sequence (TG) repeats within IVS-2 of the $^{A}\gamma$ gene revealed that the −195 allele probably exists in a nonrandom association with the substitution A→G at positions −588 and +25 to the Cap site and the configuration $(TG)_9(CG)_5(TG)_8$. These two neutral substitutions in the promoter

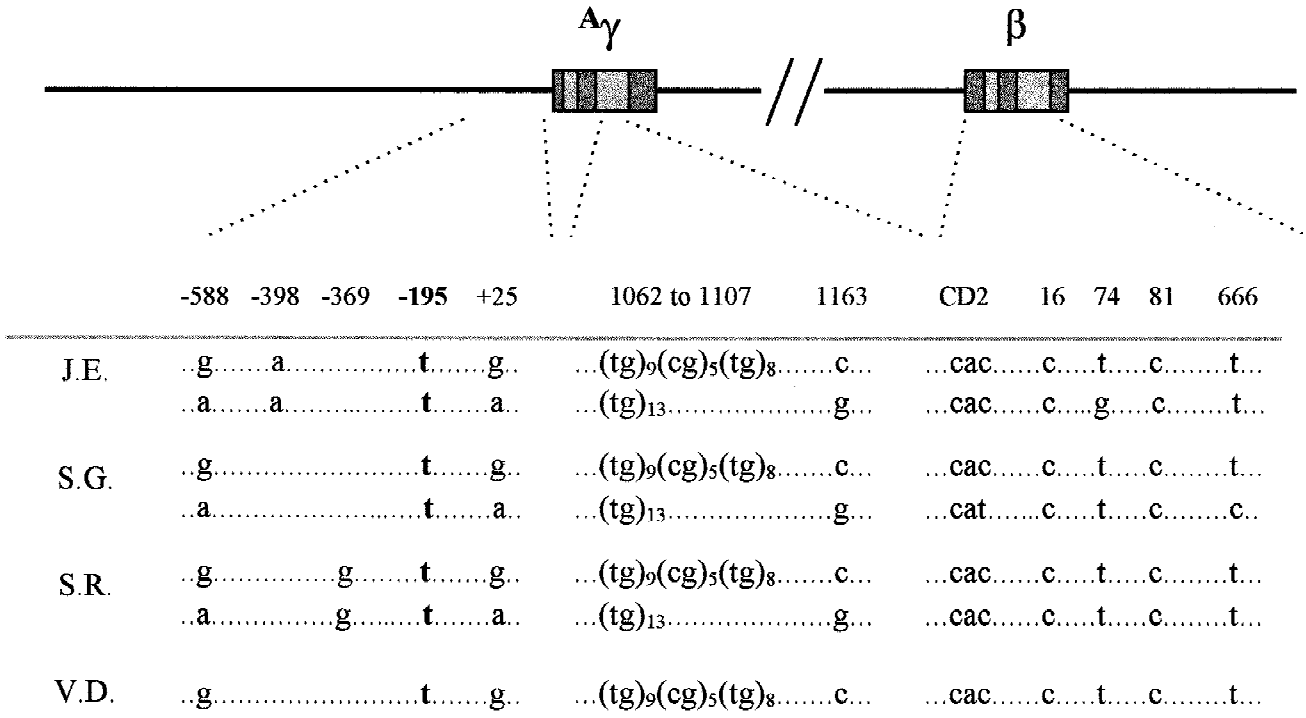


Fig. 4. Sequence comparison between the individuals carrying the Brazilian type of HPFH chromosomes. The $^{A}\gamma$ IVS-2 simple sequence (TG) repeats observed correspond to the normal type-B chromosome [24]. The polymorphic nucleotides in the β-globin gene correspond to framework 2.

region, as well as the (TG) repeat sequence, are part of the chromosomal type-B β-globin gene described by Slightom et al. [21]. Normal A and B chromosomes are the products of gene conversion and have different evolutionary histories. Both have a frequent occurrence in hemoglobinopathies and normal individuals, regardless of the ethnic origin [22,23].

The Brazilian population shows a very high degree of racial admixture as a result of intense immigration and miscegenation. In the region where this study was conducted (São Paulo State), there is a predominance of descendants from African and Italian immigrants who settled in the southeastern Brazil. The influence of these two ethnic groups on the genetic background of this region has already been demonstrated [24]. Although we can not precisely determine the ethnic background of the patients studied here, two of them are unequivocally of African origin.

Taken together, extended haplotype analysis of the β-globin gene cluster shows that all HPFH chromosomes characterized in this study share a common background, which indicates a possible single origin for the Brazilian type of HPFH. It is interesting to note that the Italian type of $^{A}\gamma$-HPFH (−196 C→T) is also associated with haplotype V within chromosome B [25]. In addition, eight out of eleven point mutations previously described in the $^{A}\gamma$ promoter have the subhaplotype [− − − −], whereas all $^{G}\gamma$ mutations have a high diversification at the same sites [4, 26]. Although there is not enough data to delineate a pattern, it is possible that some of the point mutations in the $^{A}\gamma$ promoter that led to the HPFH phenotype arose from a particular chromosomal background.

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