The Duffy blood group system is of clinical and biological significance. Antibodies to Duffy antigens are responsible for some cases of transfusion incompatibility and newborn hemolytic disease. The Duffy protein is a receptor for the Plasmodium vivax erythrocyte-binding protein and is also a receptor for various chemokines (thus renamed Duffy Antigen Receptor for Chemokines (DARC)). The two Duffy polymorphic antigens, Fya and Fyb (coded by the FY*A and FY*B alleles), are present on erythrocyte membranes. The Fya(b−) phenotype is the predominant one in populations of black people and also occurs in other populations, including some non-Ashkenazi Jewish groups. The Fya(b−) phenotype has been associated with a mutation in the FY*B promoter at the GATA box that abolishes the expression of erythrocyte Duffy protein. We describe here a novel mutation, present in the FY*B coding sequence (271C → T), that is associated with some Fyb(b−) phenotypes among non-Ashkenazi Jews and among Brazilian blacks. The mutation is present in Fyb(b−) individuals, who have wild-type FY*B GATA and carry the previously described 304G → A substitution. The 271C → T and 304G → A can be identified by restriction enzyme–generated restriction fragment length polymorphisms. The 271C → T substitution represents a considerable change in chemical nature (Arg91 → Cys), one which may affect the antigenic determinants of DARC, and thus be of clinical significance. The mutation may have implications for some physiological roles of DARC and be of interest in malaria research and in studies of population genetics.

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sample was found to be FY*B/FY*B as determined by Ban I RFLP, and only heterozygous for the mutation at the GATA box, as identified by Sty I RFLP.11 DNA sequencing showed two mutations in the coding sequence, a novel mutation at nucleotide (nt) 271 from C to T (271C → T), and the previously reported mutation at nt 304 from G to A (304 G → A).8,13 Subsequently, polymerase chain reaction (PCR)-RFLP assays were established to screen for these mutations. As described here, the simultaneous presence of these two mutations resulted in the silencing of the Fyb antigen in erythrocytes. This phenomenon is of clinical significance and may have implications for physiological roles of DARC in tissues other than erythrocytes, and it may be of interest in studies of population genetics.

MATERIALS AND METHODS

Phenotyping of erythrocyte Duffy antigens. Blood samples were from donors whose identity was unknown (unlinked). The Fyb(−) samples were selected based on routine phenotyping of washed erythrocytes with anti-Fya and anti-Fyb used according to the manufacturer’s instructions. Erythrocytes from the non-Ashkenazi Jews in Israel were tested with antisera from Gamma Biologicals Inc (Houston, TX). Erythrocytes from Brazilian blacks were tested with antisera from three companies (Gamma Biologicals Inc; Biotest-São Paulo, Brazil; and Diamed, Cressier sur Morat, Switzerland). It should be noted that the serological testing used here does not distinguish between Fya(−) and Fya(b−weak) erythrocytes, often type as Fya(−) if only the usual anti-Fya are used by routine methods. Further testing with a variety of anti-Fyb reagents as well as a quantitative adsorption and elution analysis have to be performed on erythrocytes identified as Fyb(−) by standard agglutination assays, to characterize such samples. DNA was prepared at the time of Fyb testing and by the time that analysis of DNA showed the mutations described here, erythrocytes were not available for further testing.

DNA preparation. White blood cells (WBCs) from whole blood were obtained after erythrocyte lysis with a solution containing 155 mmol/L NH4Cl, 10 mmol/L KHCO3, and 1.0 mmol/L Na2-EDTA. The washed pellets were suspended in buffer containing 10 mmol/L Tris-HCl, pH 7.5, 75 mmol/L NaCl, 24 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and 150 µg proteinase K/mL, and kept for 4 hours at 55°C. Proteins were precipitated by salting out, using saturated NaCl solution, vigorous mixing, and centrifugation.14 The supernatants were mixed with cold ethanol. The precipitated DNA was solubilized in 10 mmol/L Tris-HCl, pH 7.5, 1.0 mmol/L EDTA. Alternatively, WBC DNA was extracted using DNAzol Kit (GIBCO-BRL, Gaithersburg, MD), according to the manufacturer’s recommendations. The DNA solutions were analyzed for quality by agarose gel electrophoresis and for quantity by optical density measurements at 260 nm.

DNA amplification. PCR was performed using 100 to 200 ng of DNA, 3 pmol of each primer, 2 nmol of each dNTP, 1.0 U Taq polymerase and buffer (Perkin Elmer, Norwalk, CT), in a total volume of 40 µL. The primers used for PCR amplification, FY3, 5′-CCCTCTTGTTCCCTCCCTT, located at nt 276 → 256, and FY4, 5′-CAGAGTCGGAGTGCTACCTA, located at 385 → 365, were designed to encompass the coding region containing nt 131 (site for FY*A/FY*B polymorphism4,5), nt 271 (site of novel mutation described here), and nt 304 (site of mutation previously described).13 Reactions were performed in an automated thermal cycler (PTC 100 MJ Research, Watertown, MA), with denaturation at 94°C for 4 minutes, followed by 30 cycles of amplification (94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute) and a final extension at 72°C for 10 minutes. A second PCR amplification of a DNA segment containing the GATA mutation site (nt −46) was performed using the published conditions and primers F38 and F3911 (here named FY1 and FY2).

RFLP analysis of PCR products. The restriction enzymes, buffers, and details for their use were supplied by New England Biolabs (Beverly, MA). For the identification of FY*A and FY*B, 15 µL of the PCR product (DNA amplified by the FY3 and FY4 primers) was digested with Ban I. The restriction fragments were resolved by electrophoresis on 1% agarose gel. For the identification of the GATA mutation, 25 µL of the PCR product (DNA amplified by the FY1 and FY2 primers) was digested with Sty I.12 Followed by electrophoresis on 12% acrylamide gel. For the identification of the 271C → T mutation, 10 µL of the PCR product (DNA amplified by the FY3 and FY4 primers) was digested with Aci I, and for the identification of the 304G → A mutation, 10 µL of the same PCR product was digested with Mwo I. The restriction fragments were resolved on 1% agarose gel.

Nucleotide sequence analysis. The PCR-amplified fragments were sequenced on both strands by thermocycling sequencing with automatic 377 DNA sequencer (Perkin Elmer). For the initial sample that showed the discrepancy between the phenotype and genotype determined by Ban I and Sty I [phenotype Fya(−)b−], and genotype FY*B/FY*B (Fig 1). As described above, we analyzed the DNA samples of unrelated individuals having Fya(−) and Fya(a+b−) phenotypes, followed by sequencing other samples were sequenced using FY3 for the PCR products generated by FY3 and FY4.

RESULTS

Alleles FY*A and FY*B in Fyb(−) phenotypes among non-Ashkenazi Jews. Although the phenotype Fya(a−b−) is known to be present in about 20% of Jews from Yemen and has also been observed among other non-Ashkenazi Jews,19,20 there is no published information on the genotypes among these ethnic groups. Using the Ban I RFLP for the identification of the FY*A and FY*B alleles,5,7 we analyzed the DNA samples of unrelated individuals having Fya(−) and Fya(a+b−) phenotypes (Fig 1). Among the Fya(a−b−) phenotypes, we found FY*A/FY*A (lanes 4, 6, and 7) and FY*A/FY*B (lanes 3 and 9); the Fya(a−b−) phenotypes were found to be FY*B/FY*B (lanes 5, 8, and 10). The Ban I restriction patterns of the PCR products indicate that the FY*B allele is the silent one in the Fya(a−b−) samples from non-Ashkenazi Jews, as is the case for the Fya(a−b−) phenotypes in the black populations.8,11

The GATA mutation in Fyb(−). FY*B non-Ashkenazi Jews. To determine whether the GATA mutation, identified in the Fya(a−b−) black population,11 was associated with the Fya(b−) phenotype among the non-Ashkenazi Jews, Sty I RFLP11 were performed on PCR-amplified genomic DNA from samples of Fya(a−b−) FY*B/FY*B, Fya(a+b−) FY*A/FY*A, and Fya(a+b−) FY*A/FY*B (genotypes as identified by Ban I). As can be seen in Fig 2, the Sty I RFLP identifies samples that are homozygous and heterozygous for the mutation, with several samples that exhibit discrepancy between their phenotypes and genotypes, as determined by Ban I and Sty I RFLPs (genotypes of samples shown in lanes 1, 2, 4, and 7 correspond to their phenotypes; genotypes of samples shown in lanes 3, 5, and 6 did not correspond to their phenotypes). As shown in Table 1, among 16 Fyb(−) individuals, the genotype corresponded to the phenotype in 12; 6 individuals were Fya(a−b−), FY*B/FY*B by Ban I and homozygous for the GATA mutation; 4 individuals were Fya(a+b−), FY*A/FY*B by Ban I and heterozygous for the GATA mutation; and 2 were Fya(a+b−),
FY*A/FY*A by Ban I and homozygous for the wild-type promoter. In contrast, 4 individuals showed a discrepancy between the phenotype and genotype: 2 individuals, who were Fy(a+b), FY*B/FY*B by Ban I, were only heterozygous for the GA TA mutation, and two individuals, who were Fy(a+b), FY*A/FY*B by Ban I, were homozygous for wild-type promoter, i.e., lacked the GATA mutation. These results indicate that in some of the Fy(b−) FY*B individuals among the non-Ashkenazi Jews, some mutation(s) other than the GATA mutation is responsible for the erythrocyte “silent” FY*B.

Identification of mutations at nucleotides 271 and 304.

DNA from the first discordant sample, identified as Fy(a+b), FY*B/FY*B and heterozygous for the GA TA mutation, was sequenced and found to have two mutations in the coding.

Fig 1. Ban I RFLP for the identification of FY*A and FY*B alleles in non-Ashkenazi Jews. DNA was amplified using FY3 and FY4 primers for the amplification of a DARC fragment containing the 131G → A substitution, responsible for FY*A and FY*B, respectively. Restriction fragments were separated on 1% agarose gel. (A) Schematic diagram of fragments generated by the Ban I digestion of FY*A and FY*B DNA. (B) RFLP patterns of DNA from samples with the indicated phenotypes, identified by antisera (the 52- and 44-bp fragments are not detected in this gel). Lanes: 1, 100-bp ladder; 2, uncut; 3, 4, 6, 7, and 9, Fy(a+b−); 5, 8, and 10, Fy(a−b−).

Fig 2. Sty I RFLP for the identification of the GATA mutation (−46 T→C). DNA was amplified using FY1 and FY2 primers for the amplification of a DARC fragment encompassing nt −46. The restriction fragments were separated on 12% acrylamide gel. (A) Schematic diagram of fragments generated by Sty I digestion of the DARC fragment encompassing nt −46 FY*B, GATA mutation. (B) RFLP patterns of DNA from samples with the indicated phenotypes, identified by antisera, and genotypes, as determined by Ban I (the 12-bp fragment is not detected in this gel). Lanes: 1, 2, and 4, Fy(a+b−)FY*A/FY*A; 3 and 7, Fy(a−b−)FY*B/FY*B; 5 and 6, Fy(a+b−)FY*A/FY*B.
sequence, as compared with the sequence of the wild FY*B allele.\(^8,10\) The first one was a novel mutation of C → T at nucleotide 271 (271C → T) and the second one was a previously described mutation of G → A at nucleotide 304 (304G → A).\(^5,13\) Based on these mutations, PCR-RFLP were developed for the identification of the mutations. \(\text{Aci I}\) RFLP for 271C → T (Fig 3) and \(\text{Mwo I}\) RFLP for 304G → A (Fig 4). As can be seen in Table 1, all four individuals, whose GATA genotypes did not correspond to their phenotypes were found to be heterozygous for both mutations. The mutations detected by RFLP using \(\text{Aci I}\) and \(\text{Mwo I}\) were further confirmed by sequencing the PCR-amplified DNA of these samples. The simultaneous presence of the 271C → T and 304G → A in the discordant cases implies that these mutations are responsible for some cases of Fy(b–), wild-type GATA erythrocytes among Fy(b–) non-Ashkenazi Jews.

**Table 1. Duffy Phenotypes and Genotypes in Fy(b–)**

<table>
<thead>
<tr>
<th>Samples (n = 36)</th>
<th>Phenotype [Anti-Fy(a)]</th>
<th>Genotype FY<em>A/FY</em>B FY/FY-</th>
<th>Genotype FY, FY+</th>
<th>Genotype 271 (C → T)</th>
<th>Genotype 304 (G → A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Fy(a–b–)</td>
<td>FY<em>B/FY</em>B FY-FY-</td>
<td>C/C</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fy(a–b–)</td>
<td>FY<em>B/FY</em>B FY/FY-</td>
<td>T/C</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fy(a+b–)</td>
<td>FY<em>A/FY</em>B FY/FY-</td>
<td>C/C</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fy(a+b–)</td>
<td>FY<em>A/FY</em>A FY/FY</td>
<td>T/C</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fy(a+b–)</td>
<td>FY<em>A/FY</em>A FY/FY</td>
<td>C/C</td>
<td>G/G</td>
<td></td>
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</table>

†[ ], Identified by antisera, by restriction enzymes. ‡FY, wild-type GATA; FY-, GATA mutation.

Identification of the 271C → T and 304G → A mutations among Brazilian black Fy(b–) individuals. Thirty-four Fy(a–b–) and 15 Fy(a+b–) samples from Brazilian black people were analyzed for FY*A and FY*B alleles, using the \(\text{Ban I}\) RFLP. As shown in Table 2, all Fy(a–b–) phenotypes were homozygous for the FY*B allele. Among the Fy(a+b–), 3 were homozygous for FY*A and 12 were heterozygous, FY*A/FY*B. These results correspond to those observed in other studies on black populations,\(^11,12\) in which homozygosity for the FY*B allele was found in Fy(a–b–), and homozygosity for FY*A or heterozygosity for FY*A/FY*B was shown in Fy(a+b–), as determined by \(\text{Ban I}\) RFLP. Analysis of the 49 samples with \(\text{Sty I}\) showed that 33 Fy(a+b–)FY*B/ FY*B were homozygous for the GATA mutation; thus, their phenotype can be accounted for by the GATA mutation. One Fy(a–b–)FY*B/ FY*B individual was heterozygous for the GATA mutation, thus showing a discrepancy between his phenotype and genotype. Among the 15 Fy(a+b–) individuals, the FY and GATA genotypes corresponded to their phenotypes in 12; as expected, the three Fy(a+b–)FY*A/FY*A had the wild-type GATA and 9 Fy(a+b–)FY*A/FY*B were heterozygous for the GATA mutation. In contrast, in three individuals, who were Fy(a+b–)FY*A/FY*B, wild-type GATA was found. Thus, in four individuals of the 49 analyzed, their Duffy phenotypes and genotypes could not be explained by FY and GATA genotyping. RFLP analysis by \(\text{Aci I}\) and \(\text{Mwo I}\) showed that all four individuals were heterozygous for both the 271C → T mutation and the 304G → A mutation. These two mutations were not found in the other 45 Fy(b–) individuals, in whom the genotype

\[\text{A}\]

\[\text{271 wild type}\]

\[\begin{array}{c|c|c|c|c}
\text{Aci I} & \text{164 bp} & \text{139 bp} & \text{242 bp} & \text{116 bp}
\end{array}\]

\[\text{271 mutant (C→T)}\]

\[\begin{array}{c|c|c}
\text{Aci I} & \text{164 bp} & \text{139 bp}
\end{array}\]

\[\text{358 bp}\]

\[\text{B}\]

\[\begin{array}{c|c|c|c|c|c|c|c|c|c|c|c}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12
\end{array}\]

\[358 \text{ bp}\]

\[242 \text{ bp}\]
corresponded to the phenotype according to the FY and GATA analysis (Table 2). These results show that, as in non-Ashkenazi Jews, an FY*B mutation different from the common GATA mutation in black populations is also associated with some Fy(b−) phenotypes among the Brazilian blacks. The findings indicate that the presence of both mutations result in an Fy(b−) phenotype.

**DISCUSSION**

We describe here a novel mutation in the FY*B allele of the Duffy chemokine receptor gene. This mutation, together with a previously described mutation, results in erythrocyte Fy(b−) phenotype as identified by standard agglutination assays (see Materials and Methods). The phenotype Fy(a−b−), similarly identified by standard reagents, is present in about 70% of both American blacks and Brazilian blacks, and is also found in the FY*B allele among the Brazilian blacks and is also present in about 70% of both American blacks and Brazilian blacks, and is also present in the FY*B allele among the Brazilian blacks. The 304G → A mutation, on the other hand, converts the residue 91 (amino acid residue according to Chaudhuri et al6; residue 89 according to Iwamoto et al7), assumed to be in the first cytoplasmic loop, to Cys. This substitution represents a considerable change in the chemical nature of the local region and may affect the behavior of DARC and its extracellular antigenic sites.

The finding that a combination of two mutations within the

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**Table 2. Duffy Phenotypes and Genotypes in Fy(b−) Brazilian Blacks**

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>33</td>
<td>Fya (a−b−)</td>
<td>FY<em>B/FY</em>B</td>
<td>C/C</td>
<td>G/G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Fya (a−b−)</td>
<td>FY<em>B/FY</em>B</td>
<td>T/C</td>
<td>A/G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Fya (a−b−)</td>
<td>FY<em>A/FY</em>B</td>
<td>C/C</td>
<td>G/G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fya (a−b−)</td>
<td>FY<em>A/FY</em>B</td>
<td>T/C</td>
<td>A/G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fya (a−b−)</td>
<td>FY<em>A/FY</em>A</td>
<td>C/C</td>
<td>G/G</td>
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</tbody>
</table>

†[ ] Identified by antisera, by restriction enzymes.

‡FY, wild-type GATA; FY−, GATA mutation.
coding sequence may result in an apparent erythrocyte Fy(b−)
phenotype raises several important questions. The promoter
GATA substitution, which impairs the binding site of the
erythroid transcription factor and results in a silent erythroid
FY*B allele and lack of erythrocyte Duffy receptor, does not
affect the expression of the gene in other cells. It is not
known at present whether the DARC Arg91→Cys, Ala102→Thr
mutant protein is present in the erythrocyte membranes.
The point mutations leading to amino acid substitutions
would be expected to allow the expression of the protein, albeit in
a possibly altered conformation and altered ligand-binding
properties. However, it cannot be excluded that such mutations
result in a deficiency or absence of the protein (eg, due to failure
of being incorporated into the cell membrane, or being suscepti-
table to degradation). It would be of interest to study whether
this DARC mutant is fully or partially expressed in or absent
from erythrocytes and from other cells. In addition, because the
spliced transcript may normally be the predominant one, it may
be relevant to find out whether there is any preferential effect on
the expression of one of the two transcripts in the mutant
cells. In any case, the overall phenotype of the mutant described
here is expected to be different from the GATA mutation,
because both the erythrocytes and other DARC expressing cells
would be affected by mutations in the coding sequence that alter
the expression and/or ligand-binding properties of the protein.
Additional studies on the binding of a variety of FyH,
including quantitative titrations of antibody binding, are neces-
sary to determine whether the mutant erythrocytes described
here behave as a Fy(bweak) variant. It may be important to define
the properties of the mutant erythrocytes and other DARC-
expressing cells for binding malarial parasites and chemokines.
It should also be pointed out that chemokine binding to DARC
has characteristics different from those of antibody binding, and
that differences exist among various chemokines in their
interaction with DARC. Thus, DARC mutant erythrocytes
that do not bind anti-FyH may nevertheless react with chemo-
kines. Although the precise roles of DARC in various tissues are
not known at present, the properties of a mutant such as the one
described here may be of physiological significance.

In view of the importance of Duffy blood group system in
clinical medicine, eg, in cases of transfusion incompatibility
and hemolytic disease of the newborn, forensic medicine,
clinical medicine, eg, in cases of transfusion incompatibility
described here may be of physiological significance.

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A Novel Mutation in the Coding Sequence of the FY*B Allele of the Duffy Chemokine Receptor Gene Is Associated With an Altered Erythrocyte Phenotype

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