The genes encoding for group A and B glycosyltransferases and for a non functional 0 transferase have recently been cloned and sequenced. The cDNAs encoding for group A and group B transferases both consist of 1062 base pairs that result in a protein of 353 amino acids. The nucleotide sequences of the A and B genes are highly homologous (99%); the A and B transferase genes differ in 7 base substitutions (297, 526, 657, 703, 796, 803 and 930), but only 4 of these (526, 703, 796, 803) determine an amino acid change, while the substitutions 297, 657, 930 are silent. The 0 and A transferase genes are identical except for a single cytosine deletion at position 261. The loss of this single nucleotide disrupts the normal triplet sequence in which base pairs are read and results in a frame shift of a premature stop codon, leading to a truncated, enzymatically inactive protein of 115 amino acids.

Three of the 4 nucleotide differences which cause amino acid differences create allele-specific cleavage sites for the following restriction enzymes: BssH II, Hpa II and BstN I (A allele) and Nar I, Alu I and Nla III (B allele). The single nucleotide deletion in the 0 allele also creates a Kpn I site and eliminates the BstE II site. DNA fragments amplified by PCR can be tested for the presence of these sites.

Yamamoto et al. used this approach to...
describe the molecular basis for the AB0 blood group system. Using two pairs of primers, they were able to amplify two regions of DNA including the four effective nucleotide substitutions. The PCR fragments were then digested with different restriction enzymes that revealed the differences among A, B, and 0 individuals.

Chang and Lee\textsuperscript{5,6} and O’Keefe \textit{et al.}\textsuperscript{7} corroborated the molecular genetic model proposed by Yamamoto \textit{et al.} by employing similar simplified techniques but on a limited number of individuals.

In order to assess the validity of this molecular genetic model on a larger group of serologically defined AB0 phenotypes, Grunnet \textit{et al.}\textsuperscript{8} analyzed 300 healthy Danish blood donors using a PCR-based diagnostic restriction enzyme assay of positions B261, B526 and B703. In all A, B and AB cases at least one allele of the predicted status was found, but in 0 phenotype individuals a novel allele called 0\textsuperscript{2} was discovered with a frequency of 3.7\% in their study.

Since restriction enzymes and oligonucleotide primers detect differences in only one region of genes, when unexpected variations in the A, B and 0 transferase genes exist, it may be necessary to analyze these genes in several regions. This issue was recently addressed by Stroncek \textit{et al.}\textsuperscript{9} They modified the reported methods and used a panel of selected restriction enzymes to analyze differences at nucleotides 261 and 526. This method allowed them to determine the genotypes of several individuals as well as in selected families. Group A was differentiated from group B by analysis of the polymorphism at position 526. Among the 60 individuals tested, two were identified by serology as group A but had a group B nucleotide sequence at position 526. Further studies of nucleotide 703 and 796 showed that these individuals were indeed group A. This finding, recognized previously by Yamamoto \textit{et al.},\textsuperscript{2,11} demonstrates that analysis of the AB0 genes at nucleotide 526 does not always lead to accurate gene identification.

The aim of this study was to evaluate the applicability of the rapid genotyping method described by Chang \textit{et al.}\textsuperscript{5} to the analysis of the genes at nucleotides 261 and 703, utilizing four restriction enzymes (BstE II, Kpn I, Hpa II and Alu I) instead of two. We used this method to determine the AB0 genotype of 250 Italian blood donors and compared the PCR determined genotypes with the phenotypes obtained by standard AB0 typing.

**Materials and Methods**

**Blood samples**

We collected 7 mL of EDTA blood from 100 group 0, 50 group A, 50 group B and 50 group AB healthy Italian blood donors.

**Techniques**

**PCR**

DNA was isolated from buffy coats using the salting out method described by Miller and colleagues.\textsuperscript{10} Two DNA fragments of the AB0 gene, each containing a different nucleotide polymorphism, were amplified by PCR using two pairs of primers.

Nucleotides 261 and 703 were used to distinguish A, B, 0 alleles by restriction enzyme digestion of the PCR products. The primers and restriction enzymes employed, previously described by Yamamoto \textit{et al.},\textsuperscript{2,11} are shown in Table 1.

Five \textmu L of DNA were amplified in 100 \textmu L of reaction mixture which contained a pair of oligonucleotide primers (fy 121/122 [PCR 1] or fy 29/47 [PCR 2], 20 pmol/\textmu L each), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1.2 mM MgCl\textsubscript{2} (fy 121/122) or 1.8 mM MgCl\textsubscript{2} (fy 29/47), and 2.5

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**Table 1.** Specific primers used to amplify DNA, transferases differentiated and restriction enzymes employed.

<table>
<thead>
<tr>
<th>Primers*</th>
<th>Polymorphic site (nucleotide)</th>
<th>Transferases differentiated</th>
<th>Restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR 1 fy 121-122</td>
<td>261</td>
<td>0 versus A or B</td>
<td>Kpn I, BstE II</td>
</tr>
<tr>
<td>PCR 2 fy 29-47</td>
<td>703</td>
<td>B versus A or 0</td>
<td>Hpa II, Alu I</td>
</tr>
</tbody>
</table>

*sequence of oligonucleotide primers: 
fy-121:5’-CGGAATTCCATGTGACCGCACGCCT-3’
fy-122: 5’-CGGAATTCTCTACCCTCGGCCACC-3’
fy-29:5’-CGTTCTGCTAAACCAAG-3’
fy-47:5’-TCCTGGAGGTGCGCGCCTAC-3’
units of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), using the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA).

PCR was performed as described by Yamamoto et al.2,11 but with the following modification: in PCR 1 the reaction mixture was incubated at 94°C for 5 minutes before the 45 reaction cycles in which denaturation was conducted at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds. The reaction mixture was then incubated for an additional 6 minutes and 30 seconds at 72°C. In PCR 2, there were 35 reaction cycles with denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes. After 35 cycles, the reaction mixture was incubated for an additional 6 minutes and 30 seconds at 72°C. Amplified samples were analyzed on 2% agarose gel electrophoresis (BioRad Laboratories, Hercules, CA, USA).

**PCR product analysis by restriction enzymes**

Amplified DNA was purified with phenol-chloroform:isoamyl alcohol, 24:1), ethanol-precipitated and resuspended in 20 µL of double-distilled H2O. The product (2 µL) of PCR 1, a DNA fragment of 199 or 200bp containing the polymorphic site at nucleotide 261, was digested with restriction enzymes Kpn I (10U) and BstE II (10U). If nucleotide 261 was deleted (group 0 subjects), the fragment was completely digested by Kpn I; on the other hand, if nucleotide 261 remained, the Kpn I site was not created and DNA was digested by treatment with BstE II, which digests type A or B transferases.

Moreover, the product of PCR 2, a DNA fragment (621bp) containing the polymorphic site at nucleotide 703, was digested with Hpa II (12U) and Alu I (16U) in a final volume of 20 µL. Enzyme Hpa II digests the transferase gene of individuals with type A or 0 transferase and Alu I digests the DNA of individuals with type B transferase. All enzymes were purchased from New England Biolabs, Beverly, MA, USA. Incubation temperatures were 37°C for Hpa II, Alu I and Kpn I, and 60°C for BstE II. DNA was incubated with the enzymes for 2 hours. The digested amplified DNA products were subsequently analyzed on 2% agarose gel electrophoresis or on 12% PAGE. The bands were visualized by ultraviolet light.

The strategy for analyzing AB0 alleles on genomic DNA is illustrated in Figure 1. By examining the digestion patterns of the AB0 genotypes, one of 7 patterns (AA/A02, A01, BB, B01, AB, 00’ and 00") was possible.8,11

**Results**

**AB0 genotyping by restriction enzyme analysis**

The results of AB0 genotyping of the 250 individuals studied are shown in Table 2.

In 98 of the 100 type 0 individuals the typing at nucleotide position 261 showed a homozy-
gous pattern with both alleles carrying the 0\textsubscript{261} deletion (0\textsuperscript{01}). In the remaining two 0 subjects no deletion was found in one of the 2 alleles; therefore the genotype was 0\textsuperscript{1} and 0\textsuperscript{2}.

Of the 50 subjects with serologically defined A blood, 37 were found to express both A and 0 transferase genes and 13 were homozygous for the A transferase gene.

Of the 50 subjects with serologically defined B blood, 39 had both B and 0 transferase genes, while 2 B transferase genes were detected in 11 subjects.

Both A and B alleles were found in all 50 AB individuals.

**Discussion**

The molecular basis of red cell AB\textsubscript{0} group antigens was recently determined.\textsuperscript{1-3} The A and B genes are similar; in fact, they only differ in a few single-base substitutions, changing four amino acid residues that may cause differences in A and B transferase specificity. The 0 gene contains a single base deletion which causes a reading frame shift. The nucleotide sequences of the coding regions of different AB\textsubscript{0} alleles have led to the identification of variations that determine a change in amino acid sequences which are responsible for the different specificities in the AB\textsubscript{0} blood group system.

Traditional blood group serology, which is based on immunoreactivity with the carbohy-

Table 2. AB\textsubscript{0} blood group genotyping of 250 Italian blood donors.*

<table>
<thead>
<tr>
<th>Phenotype</th>
<th># of cases</th>
<th>Nucleotide 261</th>
<th>Nucleotide 703</th>
<th>Inferred AB\textsubscript{0} genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13</td>
<td>+/–</td>
<td>+/-</td>
<td>A/A0\textsuperscript{01}</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>+/+</td>
<td>+/-</td>
<td>A0\textsuperscript{1}</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>+/-</td>
<td>–/+</td>
<td>BB</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>+/-</td>
<td>+/+</td>
<td>B0\textsuperscript{1}</td>
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<tr>
<td>AB</td>
<td>50</td>
<td>+/-</td>
<td>+/-</td>
<td>AB</td>
</tr>
<tr>
<td>0</td>
<td>98</td>
<td>–/+</td>
<td>+/-</td>
<td>0\textsuperscript{01}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+/-</td>
<td>+/-</td>
<td>0\textsuperscript{02}</td>
</tr>
</tbody>
</table>

*The symbols minus (–) and plus (+) indicate a negative and positive cutting for each allele, respectively.

In this study we employed a rapid molecular method to determine the AB\textsubscript{0} genotype of 250 healthy blood donors, using four restriction enzymes at two nucleotide positions (0\textsubscript{261} and A/B\textsubscript{703}).

A similar approach had previously been reported by Yamamoto et al.,\textsuperscript{2,11} who employed 8 different endonucleases at nucleotide positions 261, 526, 703 and 796. This molecular method was recently applied by Grunnet et al.\textsuperscript{4} to a larger group of individuals and by Stroncek et al.\textsuperscript{9} to selected families.

This study demonstrates that using molecular genetic techniques for AB\textsubscript{0} genotyping at nucleotide positions 261 and 703 makes it possible to distinguish A, B and 0 alleles. Complete agreement with the proposed molecular genetic model for AB\textsubscript{0} blood group polymorphism was found for the A, B and AB individuals. An A or B allele was always identified when appropriate, and in the case of heterozygous A0 or B0 individuals an 0\textsuperscript{1} allele with the 0\textsubscript{261} deletion was found. In the 100 type 0 individuals, we found 2 subjects with unexpected 0 alleles that did not contain the usual deletion at position 261 and were typed as A at position 703. These subjects were defined as 0\textsuperscript{1}0\textsuperscript{2}. The fact that none of the A or B individuals were found to carry the 0\textsuperscript{2} allele may be due to the relatively small number of cases examined. However, in 150 Danish blood donors with 0 type Grunnet et al.\textsuperscript{4} detected 11 individuals with genotype 0\textsuperscript{01}, while the frequency of the alleles A and B was similar to that found in our Italian blood donor population. Both studies seem to confirm the relative advantage for heterozygous individuals (well known from other genetic studies), as appears from the frequency of A0\textsuperscript{1} and B0\textsuperscript{1} in the subjects with type A and B RBCs.

In all cases, analysis of the AB\textsubscript{0} genes at nucleotide 703 led to accurate type identification.\textsuperscript{9}

Such low-frequency variants may remain important for DNA analysis in forensic medi-
cine. Moreover, similar potential applications exist for the DNA typing of the A2, A3 and cis-AB genes.12,13

The methods used in this study, in addition to other molecular methods previously described that allow the identification of unusual genotypes, represent a useful tool for AB0 typing in difficult situations and for the detection of weakly expressed AB0 antigens when serology fails.

References


