nancies with one male fetus. Conversely, normative reference values for twin-bearing pregnancies with two or more male fetuses should be assessed on a sample population displaying the same number of male fetuses.

We found no statistically significant association between the concentration of fetal DNA in the maternal circulation and chorionicity in MM pregnancies, which does not help in elucidating the origin of cell-free fetal DNA in maternal plasma. Nevertheless, the number of monochorionic twin pregnancies was too small (reflecting the lower frequency of spontaneous monochorionic vs bichorionic twin pregnancies) to allow reliable conclusions. The placental weight data available to us seem to be similar between mono- and bichorionic pregnancies. Data obtained for placental weight may vary considerably; depending on how the placenta is prepared, the weights may differ by nearly 50% *(11)*. Thus, because of the small proportion of twin pregnancies with respect to singleton ones, collecting multicentric data could be useful to reach an adequate sample size to further elucidate the origin of cell-free fetal DNA in maternal plasma.

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Characterization of the *Bcl*I Polymorphism in the Glucocorticoid Receptor Gene, *Isabelle Fleury,[1] Patrick Beaulieu,[1] Melanie Primeau,[1] Damian Labuda,[1,2] Daniel Sinnett,[1,2] and Maja Krajinovic[1,2*]* ([1] Service d'Hématologie-Oncologie, Centre de Recherche, Hôpital Sainte-Justine, Montréal (Québec), H3T 1C5 Canada; [2] Département de Pédiatrie, Université de Montréal, Montreal (Quebec), H3T 1C5 Canada; [*] address correspondence to this author at: Centre de Recherche, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine, Montréal (Québec), H3T 1C5 Canada; fax 514-345-4731, e-mail maja.krajinovic@umontreal.ca)

Glucocorticoids (GCs) have a major antiproliferative effect, which has led to the use of their synthetic homologs for immunosuppression, treatment of inflammation, and induction of cytotoxicity *(1, 2)*. GCs exert their effect by binding to an intracellular GC receptor (GR), forming a complex that translocates to the nucleus, where GCs then regulate the expression of target genes interacting with promoter GC-responsive elements *(1)*. The different GR forms, resulting from *GR* gene variability, can affect the regulation of many biological functions, such as hypothalamic-pituitary-adrenal axis regulation and GC responsiveness, thereby underlying susceptibility to many diseases. Indeed, *GR* mutations have been associated with altered cardiovascular function, metabolic disturbances, and hematologic malignancies *(3–5)*. Likewise, functional GR variability might affect the therapeutic response to corticosteroid drugs *(5)*. Identification of different *GR* gene variants may thus be helpful in assessing the role of the *GR* gene in disease susceptibility or in adjudging predisposition to corticosteroid-associated adverse drug reactions.

Several polymorphisms of the *GR* gene, which might have an impact on GC sensitivity, have been reported *(6–8)*. Among these, the *Bcl*I polymorphism was identified by Southern blotting using human *GR* cDNA-specific probes *(9)* that identified two alleles with fragment lengths of 4.5 and 2.3 kb. Several clinical investigations have subsequently suggested that this *GR* polymorphism is linked to altered GR function *(6, 10–15)*. An association between the *Bcl*I polymorphism and changes in tissue-specific corticosteroid sensitivity, as well as with poor feedback regulation of the hypothalamic-pituitary-adrenal axis, has been reported *(6, 10)*. This was further documented by association of the *Bcl*I polymorphism with abdominal obesity *(11, 12)*, insulin resistance *(6, 13)*, and development of an atherogenic profile *(6, 14)*. Similarly, the larger allele of *Bcl*I is more frequent in a group of individuals genetically predisposed to develop hypertension *(15)*. The molecular identity of this polymorphism, however, is still unclear, and its analysis has been based on the laborious and time-consuming Southern blot approach, which requires large quantities of DNA and is difficult to apply to large-scale genotyping *(6, 10–15)*.

Here we present the characterization of the *Bcl*I polymorphism, as well as the application of two simple genotyping assays for its detection: PCR with restriction fragment length polymorphism (RFLP) analysis and al-

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lele-specific oligonucleotide (ASO) hybridization. Using these approaches, we assessed the frequency of this polymorphism in populations of different origin.

The *Bcl*I site was suggested to be situated in either the first or second intron of the *GR* gene *(6)*. To search for *Bcl*I sites in these gene segments, we used the genomic sequence derived from human chromosome 5 contig (GenBank accession no. NT_029289), on which the exon/intron boundaries were positioned according to the *GR* mRNA sequence information (GenBank accession no. NM_000176.1) and BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). The genomic sequence corresponding to the first two introns was analyzed for *Bcl*I restriction sites using WebCutter 2.0 software (http://www.firstmarket.com/cutter/cut2.html). We found three *Bcl*I sites, one in intron 1 and two in intron 2, whose relative positions to the exon 2/intron 2 boundary are indicated in Fig. 1A. Only the polymorphic *Bcl*I site in intron 2, 647 bp from exon/intron junction, produced the fragment sizes corresponding to those obtained by Southern blot exper-

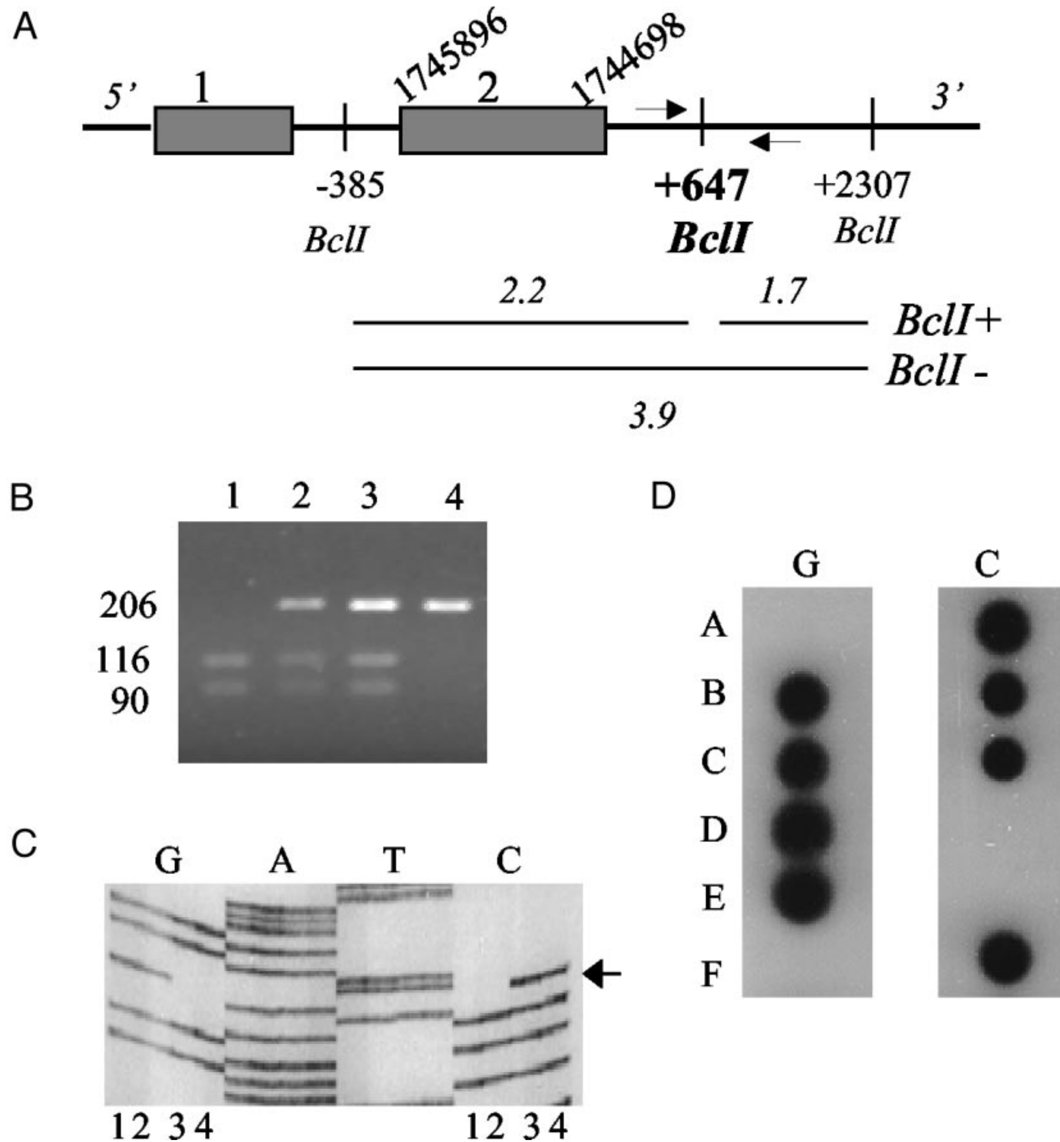

Fig. 1. Molecular characterization and genotyping of the *Bcl*I polymorphism in the *GR* gene.

(*A*), schematic representation of the *Bcl*I polymorphic locus. The predicted positions of *Bcl*I sites in the first and second introns are given relative to exon 2, whose boundary positions on the chromosome 5 contig (GenBank accession no. NT_029289) is also indicated. The predicted polymorphic *Bcl*I site is indicated by *bold font*. Exons 1 and 2 are represented as *gray boxes*. The *arrows* indicate the positions of the PCR amplimers. The expected restriction fragment lengths, obtained using Southern blot, are shown *below* the schematic. (*B*), PCR-RFLP analysis of the *Bcl*I polymorphism. The pattern of PCR product digestion in *lane 1* indicates a homozygous individual for the smaller allele. *Lanes 2* and *3* contain samples from heterozygous individuals, and *lane 4* contains a sample from an individual homozygous for the larger allele. (*C*), nature of the underlying DNA variant. Four individuals were sequenced: two homozygous for the smaller allele (*lanes 1* and *2*) and two homozygous for the larger allele (*lanes 3* and *4*). The G-to-C substitution in intron 2 at position +646 is indicated by the *arrow*. (*D*), PCR-ASO hybridization assay of the *Bcl*I polymorphism. PCR products containing the *Bcl*I polymorphic site were dot-blotted in duplicate. Membranes were hybridized with ASO probes for the G (*left*) or C (*right*) variant. Hybridization signals in *rows D* and *E* indicate homozygous GG individuals, signals in *rows B* and *C* indicate heterozygotes, and those in *rows A* and *F* indicate individuals with the CC genotype.

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iments (i.e., fragments of 2.2 kb generated in the presence and 3.9 kb in the absence of the *Bcl*I site; Fig. 1A). Although the size of the larger allele would be slightly different from that reported previously *(9)*, no other *Bcl*I site produced a fragment of similar size.

Using this information, we designed primers flanking the *Bcl*I site at position +647 (forward, 5′-AAATT-GAAGCTTAACAATTTTGGC-3′; reverse, 5′-GCAGT-GAACAGTGTACCAGACC-3′) and amplified genomic DNA samples from the Institutional DNA bank of healthy volunteers recruited for the study of human genomic sequences variability. (The study was approved by the Institutional Ethical Committee, and informed consent was obtained from all participating individuals.) The PCR 206-bp product was amplified in 20 μL containing 20 ng of genomic DNA, 0.5 μM each amplimer, 100 μM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 U of *Taq* polymerase (Platinum; Invitrogen) with the following conditions: initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 45 s; followed by a final extension at 72 °C for 7 min.

To confirm the presence of the *Bcl*I polymorphism, we digested 10 μL of the PCR products with 4 U of *Bcl*I (New England BioLabs) for 6 h at 50 °C and separated the resulting digested fragments on a 3% agarose gel. Digestion of the PCR product gave the following predicted fragment sizes: 90 and 116 bp in the case of homozygotes for the smaller allele, an additional band of 206 bp for heterozygous individuals, and a single band of 206 bp for larger allele homozygotes (Fig. 1B). To characterize the underlying base substitution of the *Bcl*I polymorphism, we directly sequenced the PCR product using the Thermo Sequenase™ Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation) according to the manufacturer's instructions. We found a G-to-C transition in the second intron, 646 bp from the exon 2/intron 2 junction (Fig. 1C), that abolished the *Bcl*I restriction site (T$\underline{G}$ATCA to T$\underline{C}$ATCA, where the underlined bases indicate the transition), thus producing the larger allele. Similar efforts to replace Southern blot analysis by PCR-based methods in analysis of different polymorphisms have been reported in the literature *(16, 17)*.

The identification of the underlying base substitution allowed us to apply a PCR-ASO genotyping assay that, in addition to PCR-RFLP, has been widely used to analyze gene mutations and variations in numerous genes *(18)*. We previously reported the successful use of this approach for large-scale genotyping *(19)*. For this method, PCR products were denatured in 0.33 mol/L NaOH and 16.5 mmol/L EDTA in a total volume of 200 μL and subsequently transferred, in duplicate, to a HYBOND-n+ membrane (Amersham Pharmacia Biotech) and cross-linked to the membrane by use of ultraviolet light. Blots were prehybridized for 30 min at 37 °C in 10 mL of a solution containing 1× saline–sodium phosphate–EDTA (SSPE; 150 mmol/L NaCl, 10 mmol/L NaH_2PO_4, 1.1 mmol/L EDTA, pH 7.4), 0.75 mol/L NaCl, 70 mmol/L Tris-HCl, pH 7.4), 10 g/L sodium dodecyl sulfate, and 200 mg/L heparin. ASO probes (50 pmoles) were 5′-labeled using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 kinase (Life Technologies) to a specific activity of 1–3 × 10^6 cpm/pmol. Hybridization with the 5 pmol of ASO-specific probe was carried out for 30 min at 42 °C in an excess (10×) of the nonlabeled probe for the other variant allele of the same polymorphism. The membranes were then washed with 2× SSPE containing 1 g/L sodium dodecyl sulfate for 10 min at room temperature and exposed overnight at −80 °C with intensifying screens. Identical twin membranes were hybridized with the allelic probes specific for the G (gag att Gat cag cag) or C (gag att Cat cag cag) variant of the *Bcl*I polymorphism and read in parallel. DNA samples of known genotypes served as controls. Representative examples of genotypes obtained by the ASO approach are presented in Fig. 1D.

Using these two genotyping assays, we analyzed DNA samples of individuals from several populations, including Africans, Asians (both Southeast and East Asia), Amerindians, Middle Easterners, and Europeans. Both methods generated concordant results, thus validating the usefulness of both assays. The number of individuals with different genotypes, as well as the frequency of allele C in the tested populations, is given in Table 1; the highest frequency of allele C was observed in Asians [mean (SD), 32.8 (8.7)%], and the lowest was in Amerindians [15.2 (6.2)%]. The frequency observed here for Europeans was similar to the one reported for the larger allele in Sweden *(6)*.

We conclude that characterization of the *Bcl*I polymorphism, together with the availability of PCR-based genotyping approaches, could allow fast screening of this

Table 1. Allele and genotype frequencies of the *BclI* polymorphism in the *GR* gene among different populations.[a]

	African	Amerindian	European	Asian	Middle Eastern
No. of individuals	42	33	38	29	36
Genotype, n (%)					
GG	27 (64%)	23 (70%)	18 (47%)	12 (41%)	24 (67%)
GC	13 (31%)	10 (30%)	18 (47%)	15 (52%)	12 (33%)
CC	2 (5%)	0 (0%)	2 (5%)	2 (7%)	0 (0%)
Mean (SD) frequency of allele C, %	20.2 (6.2)	15.2 (6.2)	28.9 (7.4)	32.8 (8.7)	16.7 (6.2)

[a] Genotyping was performed using both the PCR-RFLP and PCR-ASO assays.

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polymorphism for monitoring of both disease susceptibility and therapeutic response variability. Further studies clarifying the reason for an association of this polymorphism with altered GR function could provide additional insight into the variability of the *GR* locus and the role of the *Bcl*I polymorphism.

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Free Thyroxine Measured by Equilibrium Dialysis and Nine Immunoassays in Sera with Various Serum Thyroxine-binding Capacities, *Rémy Sapin*[1*] *and Michèle d'Herbomez*[2] [[1] Laboratoire Universitaire de Biophysique, Unité d'Analyses Endocriniennes, Université Louis Pasteur (ULP)/Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche (UMR) 7004, Faculté de Médecine, 67085 Strasbourg Cedex, France; [2] Service Central de Médecine Nucléaire, Hôpital Salengro, Centre Hospitalo-Universitaire Regional (CHRU), 59037 Lille Cedex, France; * address correspondence to this author at: Institut de Physique Biologique, Faculté de Médecine, F-67085 Strasbourg Cedex, France; fax 33-3-90-24-40-57, e-mail sapin@ipb.u-strasbg.fr]

Despite the predominant role of thyrotropin measurements in the assessment of thyroid status, free thyroxine (FT_4) measurements remain useful either when thyrotropin determination is not conclusive or when a diagnosis of thyroid disease must be confirmed *(1)*. Because it represents only a minute fraction (0.02%) of total T_4 (TT_4), FT_4 is more difficult to measure *(2)*. Direct equilibrium dialysis (ED) methods are considered analytically accurate *(3)* and are the methods against which others are compared *(4)*. Compared with ED, other FT_4 immunoassays may show significant biases related to protein-bound T_4 or to the serum T_4-binding capacity (sBC: concentration $\times$ affinity of binding proteins) *(4–6)*. We assume assays are calibrated to have roughly the same euthyroid range in samples with normal sBC, and we expect that markedly negative biases may be observed in samples with low sBC and that smaller positive biases may be observed in samples with high sBC *(7)*. The aim of our study was to determine, in clinical samples from euthyroid patients classified into three groups as a function of their low, normal, or high sBC, the bias between FT_4 measured with ED and that measured with nine frequently used immunoassays. We also studied the specificity of each assay method and the concordance of immunoassays with ED.

FT_4 was determined with the Nichols ED/RIA assay (Nichols Institute Diagnostics) and the following nine immunoassays: Elecsys (EL) from Roche Diagnostics, VIDAS (VD) from bioMérieux, Vitros ECi (VT) from Ortho-Clinical Diagnostics, GammaCoat 2-step RIA (GC) from DiaSorin, Immulite (IM) from Diagnostic Products Corporation (DPC), Nichols Advantage (AD), AxSYM (AX) from Abbott Diagnostic, ACS (AC) from Bayer Diagnostics, and AIA (AI) from Tosoh Bioscience. All assays were performed in compliance with the manufacturers' instructions. The sBC was calculated by dividing the TT_4 concentration determined with the EL assay by the FT_4

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