Nonisotopic, PCR-Based Method for 5¢ Insulin Gene VNTR Allele Class Assignment

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The identification of genes that contribute to the susceptibility to polygenic human diseases is a laborious task that requires the analysis of multiple polymorphic markers in search of variants putatively associated with the disease phenotype. Besides, it is often necessary to study large cohorts of affected and unaffected individuals to achieve acceptable levels of significance. Once preliminary evidence of association has been obtained, a confirmation of results in independent samples from different populations is also required. If this type of research is to be cost effective, genotyping methods must be straightforward, easily scalable to large numbers of samples and results must be obtained in a short time.

A clear example of a polygenic disease is insulin-dependent (type 1) diabetes mellitus, an autoimmune disorder in which T-cell-mediated destruction of the insulin-producing pancreatic beta cell occurs, and is currently the object of intense genetic investigation in the search for susceptibility genes. During the last few years, several groups have focused their attention towards the allelic variation at the polymorphic region situated 5¢ upstream of the human insulin gene (INS) on chromosome 11p15.5, which arises from a variable number of tandem repetitions (VNTR) of a 14–15 bp oligonucleotide with the consensus sequence 5¢-ACAGGGGT(G/C)(T/C)-GGGG-3¢. Based on Southern blot analysis (2), these VNTR alleles fall into three distinctive size classes according to the number of repeats of the oligonucleotide (classes I, II and III average 570, 1200 and 2200 bp, respectively).

It has been shown that in Caucasians, class I alleles are positively associated with type I diabetes, class II alleles are rare and class III alleles are dominantly protective. More recently, a PCR-based method has been used for class I allele subtyping, and allele-specific effects on disease susceptibility have been described (3,8). However, all reported methods for PCR amplification of the INS-VNTR polymorphism are limited to the analysis of class I alleles and fail to amplify either class II or class III alleles (3,5,6) probably because of (i) the increased length of these alleles, (ii) the strong secondary structure derived from the high GC-rich content of the template and (iii) the high frequency of class I/class III heterozygous in which the amplification of the short class I alleles is strongly favored over the long class III alleles. In fact, class I alleles may only be detected by traditional Southern blot hybridization with an internal radioactive-specific probe, and this is a laborious, time-consuming technique unsuitable for large population studies requiring large amounts of purified, high molecular weight DNA, which is not always available.

The analysis of diallelic markers in the 5¢ region of the insulin gene, especially the -23/HphI RFLP, has proven useful as an alternative to Southern blot typing of the INS-VNTR, at least in populations of European descent, in which the VNTR locus may be considered diallelic (class I/class III) and tight linkage disequilibrium between -23/ HphI and VNTR alleles has been demonstrated (4). However, it might be necessary to investigate both the frequency of class II alleles and the degree of linkage disequilibrium between the proposed marker and the INS-VNTR locus when a non-Caucasian population is investigated for the first time. We report a set of PCR conditions that allow efficient amplification of INS-VNTR alleles of all classes, offering a nonradioactive and rapid alternative to the conventional, tedious Southern blot technique for reliable VNTR typing in large samples from any population.

Genomic DNA was isolated from blood samples using NucleoSpin® Blood kit (Macherey-Nagel, Düren, Germany). DNA (150 ng) was subjected to PCR amplification with flanking primers (5) (5FP1: 5¢-CACCTTGGCC-CATCCATGGCCGCATC-3¢ and 5FP2: 5¢-CTCCAGGAGAGCAAAG CCCTCACCTG-3¢, which anneal 21 and 64 bp beyond the 5¢ and 3¢ ends of the VNTR, respectively). The downstream primer is labeled with fluorochrome Cy5 on its 5¢ end, so the pair of primers is routinely used for INS-VNTR class I subtyping on an ALFexpress® automatic DNA sequencing apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR amplifications were performed in 100-µL reactions containing NH4-based PCR buffer (16 mM ammonium sulfate, 67 mM Tris-HCl, pH 8.8, and 0.01% Tween® 20), 2 mM MgCl₂, 10% glycerol, 0.4 mM
each dNTP, 0.47 mM each primer and 5 U BIoTaq DNA polymerase (Bioline, London, UK). Thermal cycling was carried out in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) as follows: initial denaturation at 94°C for 6 min, 18 cycles of 1 min at 94°C and 10 min at 72°C, followed by 12 cycles in which the 10-min extension time is increased by 15 s each cycle, and a final extension step of 10 min at 72°C. Reactions were ethanol-precipitated and resuspended in 8 mL double-distilled water. Samples were loaded onto 0.75 mm thick, 10 cm long, 3.5% denaturing polyacrylamide gels. Electrophoretic separation was performed at room temperature and 8 V/cm constant setting until the xylene-cyanol dye ran off the bottom of the gel (4.5–5 h). Bands were detected by standard silver staining of the gel attached to one of the casting glasses (1).

Figure 1 shows an example of efficient, simultaneous amplification of class I, II and III alleles using the PCR conditions described. We have modified the PCR conditions initially used for class I amplification and use a two-step cycle with high denaturation and annealing/extension temperatures. We also include 10% glycerol in the reaction mixture to help destabilize the complex DNA in solution and to overcome the secondary structures derived from the GC-rich content (>70%) of the template, which may further hinder the amplification of long alleles (7). Moreover, we have observed that careful adjustment of the concentration of dNTPs (and consequently of MgCl$_2$), together with a gradual extension (15 s/cycle) of the polymerization step to more than 10 min, favors amplification of the long alleles, which is especially necessary in heterozygous subjects. To evaluate the consistency of the PCR method, we performed a comparative study with 50 samples previously typed using the conventional technique (16 were homozygous for class I, 8 were homozygous for class III and 26 were class I/III heterozygous), and the results were absolutely concordant with those obtained with the Southern blot method (Figure 2).

In brief, we have developed a PCR technique that is capable of simultaneously amplifying INS-VNTR alleles of different sizes (600–2500 bp) and can be used for the analysis of this and other polymorphic markers of similar structure. Although electrophoresis might limit the genotyping throughput, this method is easy and less time consuming, so it can replace more time-consuming methods that cannot be used in large-scale population studies. Furthermore, the method is based on modifications of the physical and chemical parameters of PCR, without the use of special and costly enzymes or additives, and makes use of highly sensitive DNA staining techniques.

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Integration-Proficient Pseudomonas aeruginosa Vectors for Isolation of Single-Copy Chromosomal lacZ and lux Gene Fusions

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Transcriptional analyses of many genes of unknown function identified in whole genome sequencing projects or genes whose products are difficult to assay are facilitated by gene fusion technologies (10,12). Although many plasmid-based systems are available for constructing such gene fusions (3,10), they suffer from inherent problems that have been discussed before (3). To facilitate gene integration at a defined neutral site, various phage systems have been designed to integrate exogenous gene sequences at chromosomal phage attachment sites. In E. coli, l-based systems have been developed for single-copy chromosomal integration of gene fusions and other gene cassettes (4,11). We recently developed an integration-proficient fCTX-based system for Pseudomonas aeruginosa (3), modeled after an approach that was previously described for mycobacterial integration vectors (13). This approach included (i) inclusion of the fCTX int gene on the same vector containing attP and (ii) inclusion of a multiple cloning site (MCS) for facilitated cloning of DNA fragments. We further improved our system by the engineering of FRT target sites, which allow subsequent in vivo removal of unwanted plasmid sequences from the genome (2). In this communication, we describe two new mini-CTX vectors, mini-CTX-lacZ and mini-CTX-lux, that

Figure 1. Maps of the integration vectors mini-CTX-lacZ and mini-CTX-lux (A) and illustration of integrase-mediated chromosomal integration of a lasB lacZ fusion (B). Plasmids are drawn to scale. The locations of genes and their transcriptional orientations are shown, including the T4 transcription terminators (W). The R in mini-CTX-lacZ marks the location of an RNase III processing site that is located between the MCS and the luxZ ribosome binding site. Other abbreviations: atb, chromosomal fCTX attachment site; attP, fCTX attachment site; FRT, Flp recombinase target sites; int, fCTX integrase encoding gene; lacZ, E. coli b-gal structural gene; ori, ColE1 origin of replication; oriT, origin of transfer; PlasB elastase structural gene promoter; and tet, tetracycline resistance encoding gene. Only important restriction enzyme cleavage sites are shown. The nucleotide sequences of mini-CTX-lacZ and mini-CTX-lux were deposited in GenBank (5) and assigned the accession nos. AF140579 and AF251497, respectively.