BsaXI/RFLP analysis of initial or selectively reamplified PCR product is unreliable in detecting the V617F mutation in JAK2

G. C. SHEPARD*, H. L. LAWSON*, G. A. HAWKINS†, J. OWEN*

INTRODUCTION

The discovery that a common point mutation in the JAK2 gene is closely associated with myeloproliferative disease (MPD) has changed our view of MPD diagnosis (Kralovics et al., 2005; Tefferi, Skoda & Vardiman, 2009). The V617F mutation is found in some 90% of patients with polycythemia vera and as many as 30% of patients with essential thrombocytosis (Jones et al., 2005). A number of methods have
been proposed for the detection of this mutation, ranging from simple RFLP of PCR product to more sophisticated quantitative methods (Cankovic et al., 2009; Le et al., 2010; Ochsenreither et al., 2010; Paradis, Simard & Gaudet, 2010; Shammaa et al., 2010; Trifa, Cucuiianu & Popp, 2010; Ugo et al., 2010). The RFLP approach depends on BsaXI as the only known restriction enzyme capable of distinguishing the mutation from the ancestral sequence. This simple and rapid method can easily detect the V617F mutation in patients with advanced disease where the fraction of mutation is often well in excess of 20%. We were interested in whether the method could be modified to detect small fractions of V617F mutated DNA.

BsaXI apparently is not a very efficient enzyme when digesting a low concentration of PCR substrate. In practice, this translates to incomplete digestion, with some five to ten percent of normal V617-coding PCR product remaining intact after as much as 24 h of digestion. This places an unacceptable limit on the use of PCR-RFLP for the detection of the V617F mutation when present as a small fraction of total JAK2 DNA. To circumvent this limitation, we chose to examine PCR reamplification after BsaXI digestion of the ancestral sequence. This was expected to significantly enrich the fraction of mutant sequence allowing unequivocal detection of the presence of V617F when initially present as a small fraction of the total JAK2 DNA. A detailed analysis of this method and comparison with quantitative PCR are presented here.

**MATERIALS AND METHODS**

Patients were recruited from the Wake Forest University Baptist Medical Center hematology clinics and inpatient hospital. All patients provided informed consent for blood specimen analysis and record review and signed the consent document approved by the Institutional Review Board. Clinical data were collected retrospectively at the time of sample collection and prospectively from July 2005 to December 2007.

DNA was isolated from whole blood samples using the QiAmp DNA Minikit (Qiagen, Valencia, CA, USA). PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) was performed with AG-CAAGCTTTTCTCAACAGCA and CTGACACCTAGCTGACATGCTCGT-GATCCTG primers and 125 ng of template DNA in 25 μl total volume. This amplified a 155-bp sequence of genomic DNA spanning the V617F mutation site. Complete cleavage of the ancestral PCR product by BsaXI in NEB Buffer #4 (NEB, Ipswich, MA, USA) is predicted to produce three fragments, 96, 29, and 30 bp, which would be easily visualized by 10% PAGE and staining with Sybr Gold (Invitrogen, Eugene, OR, USA).

The purified DNA from a patient with <5% wild-type allele was mixed with DNA obtained from a normal individual to create a series of samples with decreasing fraction of the V617F mutation in a background of normal sequence. These samples were subjected to the same PCR amplification and BsaXI enzyme digestion protocol to determine sensitivity.

**Selective enrichment**

Initial PCR was performed as aforementioned, and BsaXI digestion of the PCR product was carried out overnight at 37 °C. The digested product was diluted 1:1000, and a second round of PCR amplification carried out using the same primers. This product was subjected to BsaXI cleavage overnight at 37 °C, and the cleavage pattern determined by PAGE and Sybr Gold staining. Digital photographs of the Sybr Gold-stained gels were converted to gray scale images using Photoshop (Adobe, San Jose, CA, USA) then ‘scanned’ using Image J software (NIH, Bethesda, MD, USA), and the intensity of each band quantitated using the Fityk software program (http://www.unipress.waw.pl/fityk/).

**Characterization of noncleavable products from selective enrichment**

After the second round of PCR and BsaXI cleavage, uncleaved PCR product from the normal donor was cloned into the pCR® 2.1 vector (Invitrogen) using the TA cloning kit also from Invitrogen. The vector was transfected into NovaBlue competent cells (Novagen, San Diego, CA, USA), and individual colonies with inserts were selected. The plasmids were isolated, and the inserts sequenced using M13 primers matching the plasmid. The resulting sequences were examined using Sequencher software (Gene Codes, Ann Arbor, MI, USA) and classified as either having ancestral sequence at the BsaXI recognition site or a mutation that destroyed the BsaXI recognition sequence.
Quantitative PCR for V617F

Real-time quantitative PCR was performed according to the method of Kroger et al. (2007) To generate the standard curve for quantification, we used isolated DNA from a patient with <5% wild-type allele by BsaXI digestion mixed at defined percentages into isolated DNA from a healthy donor. Percent JAK2 V617F in patient samples was determined from the resulting standard curve.

RESULTS

The qPCR result was taken as a reference point. By this method, all patients could be unequivocally classified either as having a significant fraction (>5%) of V617F or as having less than one mutation in 10,000 copies. Using these results, we found 23 of the 67 patients positive for the presence of the V617F mutation in whole blood DNA.

RFLP analysis

Figure 1 shows the Sybr Gold–stained polyacrylamide gel with the fragments obtained by BsaXI cleavage of the initial PCR amplification. Two major bands are evident at 155 and 96 bp together with smaller fragments at 29 and 30 bp. Also clearly seen are bands at 126 and 59 bp. These latter bands are produced by the failure of BsaXI completely to cleave the PCR product at the scission sites 3′ and 5′, respectively, to the BsaXI cognate sequence. The 59-bp fragment is more prominent than the 126-bp fragment in all lanes, suggesting greater resistance of the 5′ site compared to the 3′ site. In all experiments, we noted that the normal control sample showed a small amount of apparently uncleaved PCR product identical in appearance to that expected from a patient with a small fraction of V617F mutation. Prolonged incubation and/or increased concentration of BsaXI had minimal if any effect on the intensity of the ‘uncleaved’ band in normal samples.

The 67 patients were classified as positive or negative for the presence of the V617F mutation by visual inspection of the intensity of the residual 155-bp band. By this method, 23 of the 67 patients were thought to carry the V617F mutation, but great uncertainty accompanied the classification of patients showing only a weak band of uncleaved PCR product.

Comparison of qPCR and RFLP

Classification was first carried out by RFLP and subsequently by qPCR. Sample order was randomized before qPCR to minimize bias. The qPCR analysis was taken as the reference because samples could be unequivocally categorized for the presence or absence of the V617F mutation. Both methods classified 23 of 67 samples as containing the V617F mutation. However, 10 samples gave discordant results, which we felt was because of the difficulty in assessing band intensity by eye.

RFLP after selective enrichment

Figure 2 shows the Sybr Gold–stained PAGE of the fragments obtained by a second round of PCR amplification and BsaXI digestion. This gel can be directly compared with the initial digest shown in Figure 1. All samples containing V617F mutation show an increased intensity of the 155-bp band, as expected from this selective amplification protocol. Unexpectedly, we saw a marked increase in the intensity of the 155-bp band in the normal control sample. Rather than rely on visual comparison, band intensities of both gels were assessed quantitatively. The normal control showed about 8% uncleaved product in the initial round, rising to about 35% in the second reamplification.
Sequencing of the BsaXI-resistant 155-bp band

Complete sequence data was obtained for 34 inserts derived from the uncleaved band after the second round of amplification and BsaXI cleavage. Of the 34 inserts, 22 showed only ancestral (normal) sequence with one sequence having a single misincorporation well outside the BsaXI cognate sequence. There were base misincorporations in the cognate sequence in 11 inserts, and the remaining insert had a single base deletion destroying the cognate sequence. In the 12 BsaXI-resistant inserts, there were no misincorporations outside the extended cognate sequence.

**DISCUSSION**

BsaXI is a member of the unusual Type IIb class of restriction enzymes (Marshall & Halford, 2010). Its cognate sequence is GGAGnnnnnGT giving six opportunities for destroying the cognate sequence by base misincorporation. The string of five nonspecific bases can also be altered by insertion or deletion to destroy the cognate sequence. Twelve distinct ‘mutations’ were found in 34 sequenced inserts, all different and none corresponding to V617F. A single base change was found outside the extended recognition sequence, strongly supporting the original proposal that selective amplification would enrich for mutant sequences. Unfortunately, selective reamplification does not distinguish between V617F in the original sample and BsaXI-resistant sequences introduced by PCR. In this setting, the direct misincorporation rate can be estimated from the frequency of misincorporations, 1 in 4896, found outside the extended cognate sequence. This figure is broadly within those published for Taq-based PCR (Tindall & Kunkel, 1988; Keohavong & Thilly, 1989; Hengen, 1995). Within the extended cognate sequence, we identified 12 misincorporations in 374 possible sites for a rate of 1 in 31. Comparing the two misincorporation rates shows that selective reamplification does indeed work, but does not distinguish real (V617F) from spurious mutations.

Of the 34 inserts sequenced, 22 showed no alteration at or around the cognate sequence, so some other mechanism must be in play. The finding of a significant fraction of normal, BsaXI-resistant, inserts is consistent with the observation of a persistent uncleaved band in the initial BsaXI digestion of primary PCR amplified product. While no clear explanation is at hand, the work of Gowers, Bellamy & Halford, (2004) suggests the possibility of single-chain cleaved product being resistant to further cleavage. This could lead to full-length product with single-chain nicks, some of which would have one intact strand capable of acting as a template for further PCR amplification.

BsaXI leaving apparently intact PCR product does not depend on either duration of digestion or concentration of enzyme. This is consistent with the interpretation that initial single-chain cleavage and resistance of nicked DNA to complete cleavage are properties intrinsic to the BsaXI. Furthermore, it is unlikely that the manipulation of conditions of cleavage would lead to more complete cleavage. This interpretation is supported by the observation of bands at 126 and 59 bp, presumably also containing nicks at the cleavage sites and which are resistant to full degradation by BsaXI.

Our conclusion from these studies is that PCR and RFLP using BsaXI is not suitable for detecting V617F, either in a one-step or in a two-step process. However, combining reamplification with a specific direct V617F detection method might well prove efficacious.

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REFERENCES


