placenta) in which the ADAM mRNA was extremely rare (Figure 2D). This is probably the origin of the observed high mutation rate and the artifactual amplification of hybrid products (Pitfalls 3 and 5). The correct tissue distribution of the cDNA under study was obscured because of Pitfall 4. A clear lesson from this work is that full-length cDNA cloning must involve library screenings for as much as possible of the coding sequence and that RACE or RT-PCR protocols, if used at all, should be limited to obtain sufficient sequence for hybridizations or for the elucidation of the less critical untranslated regions.

It is well-known that library cDNA clones also often contain extraneous sequences, mostly in the form of unspliced exons, but these are nearly always derived from the same gene. Usually, only a single clone is found per aberrant construct.

Subcloned, gel-purified DNA fragments are usually contaminated with the plasmid DNA from which they had been excised. When used as a probe, these contaminations often manifest themselves by their strong hybridization to the size markers. These contaminations are usually harmless in Northern blot hybridizations or library screenings (except when plasmid libraries are screened). However, RT-PCR-generated contaminations such as those described in Pitfall 4 are far more insidious and should be avoided at all times.

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Use of the Restriction Enzyme *AvaI* and *Exo⁻Bst* Polymerase in Strand Displacement Amplification

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Strand displacement amplification (SDA) is an isothermal in vitro method of amplifying DNA (10-12). This technique is based on the ability of a restriction enzyme to nick one strand of a hemiphosphorothioated form of its double-stranded recognition site and the ability of a polymerase to initiate synthesis at the nick and displace the downstream DNA strand during replication. The method consists of two parts: (i) a target generation process that makes copies of the target sequence flanked by nickable restriction sites and (ii) the exponential amplification of these modified target sequences by repeated nicking, strand displacement and priming of displaced strands, as depicted in Figure 1. The first SDA system we developed used the restriction enzyme *HincII* and the 3'→5' exonuclease-deficient Klenow fragment of *E. coli* polymerase I (exo⁻ Klenow). This system achieves 10⁸-fold amplification in 2 h at 37°-40°C (10-13). More recently, we have developed a thermophilic SDA system that operates at 60°C and uses a restriction enzyme from *Bacillus stearothermophilus* (*BsoBI*) and a 3'→5' exonuclease-deficient Klenow fragment of a DNA polymerase from *B. caldotenax* (exo⁻ *Bca*) (7). This system is faster and more powerful, achieving a 10¹⁰-fold amplification in 15 min. It is also more specific, with a significant reduction in background amplification.

The restriction enzyme *AvaI* from the blue-green algae *Anabaena variabilis* (3) is an isoschizomer of *BsoBI*. Previously, we had preliminarily demonstrated SDA at 37°C (12) using *AvaI* and the 3'→5' exonuclease-deficient Klenow-type fragment of a DNA polymerase from *B. stearothermophilus* (exo⁻ *Bst*) (1). In the present study, we recombined *AvaI* and exo⁻ *Bst* at 50°-55°C, testing their ability to amplify a target sequence in the IS6110 insertion-like

element from *Mycobacterium tuberculosis*, which contains approximately 10 IS6110 copies per genome (8).

SDA reactions were performed in a 50- μ L volume with final concentrations of 6 mM magnesium acetate, 1.4 mM dCTP α S, 0.5 mM dUTP, 0.2 mM dGTP, 0.2 mM dATP, 0.1 mg/mL acetylated bovine serum albumin, 10 ng/ μ L human placental DNA, 25 mM K₂HPO₄ (pH 7.6), 0.5 μ M primers S1 and S2, 0.05 μ M primers B1 and B2, 10.5% glycerol (vol/vol), 3 U/ μ L *Ava*I (50 U/ μ L stock; New England Biolabs, Beverly, MA, USA), 0.36 U/ μ L *exo*-*Bst* (120 U/ μ L stock; New England Biolabs) and varying amounts of *M. tuberculosis* genomic target DNA. SDA primers S1 (5'-ACCGCATCGAATGCATGTCTCGGGTAAGGCGTACTC-

GACC-3'), S2 (5'-CGATTCCGCTCCAGACTTCTCGGGTGTACTGAGATCCCCCT-3'), B1 (5'-CGATCGAGCAAGCCA-3') and B2 (5'-CGAGCCGCTCGCTGA-3') were previously described for the *Bso*BI/*exo*-*Bca* system (7). Human DNA is included to mimic DNA present in clinical specimens, while dUTP is used as a substitute for dTTP to enable control of carryover contamination of SDA products using uracil DNA glycosylase (2,7,9,13). For each reaction, 45- μ L sub-samples were assembled with all components except enzymes. These 45- μ L sub-samples contained only 5.5% glycerol; subsequent addition of the enzyme stock solutions raised the final glycerol concentration to 10.5%. Target DNA was denatured in a boiling water bath for 3

Figure 1. SDA cycle. SDA starts with a target generation step during which target fragments flanked by nickable *Ava*I sites are generated from the genomic target sequence. This is immediately followed by the exponential SDA cycle in a single and concerted reaction. This figure depicts the SDA cycle but omits the target generation step, which is described elsewhere (7,10,11). Present in excess during the SDA cycle are two SDA primers (S1 and S2). The 3' end of S1 binds to the 3' end of the displaced target strand T1, forming a duplex with 5' overhangs. Likewise, S2 binds to T2. The 5' overhangs of S1 and S2 contain recognition sites for the particular restriction enzyme being used for SDA. In the present experiments, the recognition sequence (5'-CTCGGG-3') is for *Ava*I. *Exo*-*Bst* extends the 3' ends of the duplexes using dGTP, dUTP, dATP and dCTP α S, which produces hemiphosphorothioate recognition sites on S1-T1 and S2-T2. *Ava*I nicks the unmodified primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. *Exo*-*Bst* extends the 3' end at the nick on S1-T1 and displaces the downstream strand that is equivalent to T2. Likewise, extension at the nick on S2-T2 results in displacement of T1. Nicking and polymerization/displacement steps cycle continuously on S1-T1 and S2-T2 because extension at a nick regenerates a nickable *Ava*I recognition site. Target amplification is exponential because strands displaced from S1-T1 serve as target for S2 primers, while strands displaced from S2-T2 serve as target for S1 primers. Sense and antisense DNA strands are differentiated by thin and thick lines. Intact and nicked *Ava*I recognition sequences are depicted by $\blacksquare$ and $\blacksquare$, respectively. The partial *Ava*I recognition sequence 5'-TCGGG-3' and its complement 5'-CCCGA-3' are present at the 5' and 3' ends of displaced strands as represented by $\blacksquare$ and $\blacksquare$.

Benchmarks

Figure 2. Detection of *M. tuberculosis* DNA by SDA. SDA was performed for 15 min at 52.5°C. Products were detected by extension of a ³²P-labeled detector probe and analyzed by denaturing PAGE followed by autoradiography as previously described (7). Initial *M. tuberculosis* genomes in each SDA sample are indicated at the top. Extension of ³²P-detector probe (15-mer) yields two detection products (40- and 59-mer) corresponding to extension of the probe on nicked and un-nicked SDA products. Amplification factors and the total amount of SDA products (per 50-μL reaction) are shown at the bottom of the figure (after multiplication by 10⁻⁹ and 10⁻¹¹, respectively). Molecular weight markers are indicated.

min, after which the samples were transferred to a 52.5°C heat block and equilibrated for 4 min to allow primers to anneal. A 5-μL aliquot of an enzyme mixture containing *Ava*I (30 U/μL) and *exo*⁻ *Bst* (3.6 U/μL) diluted in 50% (vol/vol) glycerol was added, and samples were quickly vortex-mixed and returned to 52.5°C for 15 min. Amplification was terminated by heating for 5 min in a boiling water bath. SDA products were detected by extension of a ³²P-labeled detector probe (5'-CGT-TATCCACCATAC-3') and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) followed by autoradiography, as previously described (7). As shown in Figure 2, this system achieves 5 × 10⁹-fold amplification and can detect one genome.

These results are very similar to those obtained with the *Bso*BI/*exo*⁻ *Bca* system (7). It is not surprising that *exo*⁻ *Bst* performs similarly to *exo*⁻ *Bca*,

Benchmarks

because both polymerases come from thermophilic bacilli (phylogenetically closely related organisms) and are 83.3% identical at the nucleotide level and 90.1% identical at the amino acid level (1). On the other hand, it would seem surprising that *Ava*I and *Bso*BI perform similarly in SDA because they come from less closely related organisms; *A. variabilis* belongs to the cyanobacteria phylum, and *B. stearothermophilus* belongs to the Gram-positive phylum (4,14). Furthermore, *A. variabilis* is a mesophile, whereas *B. stearothermophilus* is a thermophile. But a recent report of a 74% similarity (55% identity) between *Bso*BI and *Ava*I

at the amino acid level (6) explains their comparable performance in SDA.

It is somewhat unexpected that, despite *Ava*I being mesophilic, the optimal temperature for this *Ava*I/*exo*⁻ *Bst* system is 52°–55°C, with very little SDA observed at 37°C or above 55°C (data not shown). Previous studies indicate an optimum temperature of 50°C for *Ava*I restriction activity (5).

Interestingly, we also found that *Ava*I shows very little star activity when compared to *Bso*BI under a wide variety of conditions (data not shown). This higher specificity of *Ava*I could prove valuable in reduction of nonspecific amplification, especially in the presence of large amounts of non-target DNA, as in the case of human DNA in clinical samples for infectious disease diagnosis.

*Ava*I and *exo*⁻ *Bst* can now be added to the pool of enzymes proven to work especially well in SDA, making a total of three polymerases, *exo*⁻ Klenow (10–13), *exo*⁻ *Bca* (7) and *exo*⁻ *Bst*, along with four restriction enzymes, *Hinc*II (10–13), *Bso*BI (7), *Ava*I and *Bsr*I (unpublished results). Consequently, SDA systems can be tailored to the needs of a particular problem and/or availability of reagents.

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