Repulsive Elements Amplified during Differential Display


The differential display (DD) technique (5) was used to compare gene expression between undifferentiated embryonic stem (ES) cells and ES cells induced to differentiate by withdrawal of the growth factor leukemia inhibitory factor (LIF). Two out of eleven isolated cDNA fragments contained a repetitive B1 element. The B1 elements were amplified because of binding of the oligo(dT) primer to the poly(A)^+ stretch of internal B1 elements located in the corresponding mRNA species. We show that the highest number of PCR products comprising an interfering B1 repeat was produced by the oligo(dT) T12VG primer.

Murine B1 elements, human Alu repeats and rat identifer (ID) sequences belong to the family of short interspersed elements (SINEs) and occur in a copy number of approximately 10^9 per genome (2,9,10). Structural features include a length of 80–240 nucleotides (nt) and a poly(A)^+ terminus comprising approximately 40 bases. The terminus is preceded by an AC-rich region in the case of Alu and B1 elements. The AC-rich region following the poly(A)^+ stretch of internal B1 elements located in the corresponding mRNA species. We show that the highest number of PCR products comprising an interfering B1 repeat was produced by the oligo(dT) T12VG primer.

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ES1 murine embryonic stem cells were cultured as previously described (3). Differentiation of ES cells was induced by withdrawal of LIF, and gene expression was compared with undifferentiated ES cells. Total RNA was prepared by the isothiocyanate-guanidine cesium chloride method (7). For DD analysis, the method of Liang and Pardee (5) was performed essentially as published with certain modifications. Total RNA was reverse-transcribed at 37°C using 25 µM of the primers T12VA, T12VG, T12VC or T12VT and 200 U of Moloney murine leukemia virus (M-MLV) Reverse Transcriptase (Life Technologies, Eggenstein, Germany) in a total volume of 20 µL. The cDNAs in 2 µL of the reverse transcription reaction were amplified by polymerase chain reaction (PCR) in the presence of 1 mM MgCl₂, 4 µM of each dNTP, 5 µM 5’ primer, 2.5 µM arbitrary primer (AP) (1), 0.5 µM [α-32P]dATP (1200 Ci/mmol; Amersham Pharmacia Biotech, Braunschweig, Germany) and 1.5 U Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA). Annealing temperature was 40°C.

A final set of 11 cDNA fragments was obtained and further analyzed by Northern blot studies and sequencing (I. Steinebrunner and G.M. Hocke, unpublished results). Two of these cDNA fragments, termed uno and variant, contained a B1 element and were assigned European Molecular Biology Laboratory (EMBL) Accession Nos. Y14822 and Y14821, respectively.

The repetitive sequence within the cDNA fragments isolated by DD strongly impaired the detection of specific signals in Northern blot studies as demonstrated in Figure 1. No specific hybridization signal was obtained when the whole 321-bp DD uno fragment was used as a probe in Northern blot analysis (Figure 1A). However, deletion of the B1 element at nucleotide position 128–321 from the uno cDNA fragment clearly led to the detection of a single 4.7-kb transcript (Figure 1B).

Instead of deleting repetitive elements after amplification, a better approach is to prevent the amplification of cDNA fragments containing repeats from the start. The variant and uno cDNAs were amplified using the oligo(dT) primer T12VG. The last G residue offers an excellent binding partner for the cytidines located in the AC-rich region following the poly(A)^+ stretch of the B1 element. Therefore, the number of amplified cDNA fragments containing B1 elements was compared with respect to the oligo(dT) primer used.

DD reactions (30 µL each) without labeled dATP were precipitated and dissolved in 5 µL of water each. The concentrated nonradioactive PCR products were electrophoresed in parallel with radioactive DD reactions on 6% denaturing polyacrylamide sequencing gels containing 1× TBE buffer (8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA, pH 8.3) for 4 h at 1700 V. Three cDNA fragments of 150 bp, 320 bp and 1.1 kb containing B1 elements served as size markers for the unlabeled PCRs. For the labeled DD reactions, a sequencing reaction was chosen as molecular weight standard. The nonradioactive part of the gel was electroblotted on an uncharged nylon membrane (Hybond-N; Amersham Pharmacia Biotech) using 0.5× TBE as transfer buffer. Transfer was achieved by applying 125 mA for 2 h. Thereafter, the DNA was cross-linked to the nylon membrane by UV irradiation. Pre-hybridization and hybridization were performed as described for Northern blot analysis (7). A 150-bp cDNA fragment comprising a B1 element was used as labeled probe.

The number of resulting PCR products was determined from the autoradiograph of the DD gel by counting distinct bands between the size markers 1.1 kb and 150 bp (Figure 2A). The number amounted to 150 ± 10 for both oligo(dT) primers T12VG and T12VA. To identify which of the PCR products contain B1 elements, the DD fragments hybridizing with the B1 probe were counted (Figure 2B). Using the T12VG oligo(dT) primer, the number of PCR products containing a B1 element was enormously increased compared with the T12VA primer (20 ± 3 vs. 9 ± 2). Means and standard deviations were calculated from three independent experiments. No hybridization signals were observed.
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stemmed from contaminating genomic DNA (Figure 2B).

In summary, use of the DD technique led to the amplification of cDNA fragments containing B1 elements. B1 repeats contained in the cDNA fragments variant and uno were amplified because of binding of the T12VG oligo(dT) primer to the poly(A)+ stretch of the B1 element and its following C-rich region. The T12VG primer amplified approximately twice as many DD fragments containing B1 elements as the T12VA, T12VC and T12VT primers (Figure 2 and I. Steinebrunner and G.M. Hocke, unpublished results). Therefore, the use of the T12VG oligo(dT) primer for DD should be avoided or at least monitored with care.

In this study, the method of blotting a DD gel and subsequent hybridization with a specific probe was applied to identify cDNA fragments containing B1 elements. Alternatively, the method can be used to pre-select the amplified products for any other purpose, e.g., for cDNAs containing a certain conserved domain such as SH2 domains or zinc finger motifs. Even if oligonucleotides that are already sequence-specific are used as arbitrary primers (11), the described approach allows a fast pre-screening of the obtained DD products to identify the desired cDNA products.

REFERENCES


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Use of *Staphylococcus aureus* Protein-A Subdomains as a Tag for the Sensitive Detection of Recombinant Fusion Proteins


The use of translationally fused peptide tags has greatly facilitated the detection and purification of recombinant proteins. The first tags included narrowly defined epitopes (e.g., c-myc, hemagglutinin and FLAG®) that could be detected by a corresponding monoclonal antibody (1,6). More recently, tags have been developed that also facilitate purification of the corresponding fusion with an appropriate affinity matrix. Examples of this type include poly-histidine (11), glutathione S-transferase (10), maltose-binding protein (3) and cellulose-binding peptide (9). However, each of these tags requires a specific antibody to detect the tagged molecule.

One tag that eliminates the need to prepare specific antibodies/antisera has been derived from *Staphylococcus aureus* Protein A (SPA). SPA has a natural affinity for a wide range of immunoglobulins (Ig) due to the presence of an Ig-binding motif repeated five times within the polypeptide [domains D, A, B, C and E (7,8)]. This affinity has been widely exploited for the purification of recombinant proteins on nonspecific Ig matrices. More recently, we and others have noted that the Ig-binding domains of SPA are remarkably stable and retain their Ig-binding activity even after the fused protein is subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot transfer (2,5). This stability suggested that SPA also could be used for immunoblot detection of tagged proteins and for immunopurification.

We show that SPA tags can provide a highly sensitive method to detect recombinant proteins subjected to immunoblot analysis. In fact, the limits of detection using nonspecific IgGs to recognize the SPA tag were comparable to those obtained with antisera specific for the rest of the polypeptide. Moreover, if a nonspecific secondary antibody conjugated to a reporter molecule was used alone, a less sensitive but easy one-step immunoblot procedure was possible. Thus, SPA tags can be convenient and affordable for use in recombinant protein detection.

We originally isolated the coding regions for the Ig-binding domains D and DABC from SPA by polymerase chain reaction (PCR) amplification of *S. aureus* genomic DNA as described in Gosink and Vierstra (2). The SPA<sub>D</sub> domain added 64 amino acids (aa) (7.1 kDa) to the target, whereas the SPA<sub>DABC</sub> added 238 aa (27 kDa). These coding regions were cloned into a pET Vector (Novagen, Madison, WI, USA) harboring the wheat ubiquitin conjugating enzyme (UBC4) gene to create the pET-UBC4-Protein A<sub>D</sub> (UBC4-SPA<sub>D</sub>) and pET-UBC4-Protein A<sub>DABC</sub> (UBC4-SPA<sub>DABC</sub>) vectors (2). To construct the SPA<sub>D</sub>- and SPA<sub>DABC</sub>-β-galactosidase (SPA<sub>D</sub>-LACZ and SPA<sub>DABC</sub>-LACZ, respectively) expression vectors, the Ig-binding domains, D and DABC, were PCR-amplified at low stringency from the pET-UBC4-SPA<sub>DABC</sub> using the primers, 5′-CTTACGATACCATAATGGCTCCAAAAGCTGATGCGCAAC-3′ and 5′-CTTTGTTGAATTAGATCTTTTGGTGCTTGAGC-3′. The primers added an NdeI site and an ATG codon before codon 90 and added a BglII site after either codon 153 or 327 of SPA. The products encompassing the D and DABC domains were gel-purified, digested with NdeI/BglII and ligated into NdeI/BglII-digested pET29b to create the vectors pET-SPA<sub>D</sub> and pET-SPA<sub>DABC</sub>, respect-