their phosphorylation state that can occur during sample preparation in the conventional manner. Figure 2 demonstrates a recent application of the adsorption/desorption procedure described above for the analysis of ser/arg-rich proteins associated with nuclear ribonucleoprotein particles that had been fractionated in a sucrose gradient (7). In this experiment, the entire volume of 20 sucrose-containing gradient fractions (550 µL each) was loaded on the gel. Proteins were then revealed by the standard Western blot procedure (8).

In summary, the advantages of our method for the effective concentration of extremely dilute protein samples for PAGE are as follows: *(i)* a wide range of proteins can be easily analyzed; *(ii)* the concentration procedure is fast and, in most cases, quantitative; *(iii)* large volumes of dilute protein solutions can be loaded on SDS polyacrylamide gels without affecting the antigenicity of the proteins, particularly when they contain posttranslational modifications; and *(iv)* a large number of samples (e.g., fractions of sucrose gradients) can be handled simultaneously in a single step.

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Minimizing False Positives in Differential Display

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Differential display was first described by Liang and Pardee in 1992 (11) as an alternative to conventional methods like subtractive hybridization for analyzing changes in gene expression. The technique hinges on the use of anchored oligo(dT) primers and defined arbitrary primers to generate by polymerase chain reaction (PCR) sets of amplified cDNAs unique to individual cell types. These fingerprints can be resolved side by side on large polyacrylamide gels. Bands appearing in one sample but not another presumably represent differentially expressed genes. These candidate bands are eluted from the dried gel, re-amplified and used as probes for subsequent verification by Northern blot. Although bolstered by relative flexibility, ease of use and exquisite sensitivity, differential display nonetheless suffers several drawbacks, most notably a high incidence of false positives. These are candidate bands that, upon further analysis by Northern blot, are either nondifferentially expressed or fail to detect a transcript. More than half of candidate bands isolated by differential display are consistently reported as false positives (1,9,15).

Vagaries of PCR are an obvious potential source for at least some of these false positives. This was recognized early in the development of differential display and has been dealt with by routinely performing multiple reactions and choosing only reproducible bands for further study. This typically involves performing duplicate or triplicate reverse transcription (RT)-PCRs from a single RNA preparation (9,17). In our application of differential display to the study of human B-cell development, we have identified cDNA fragments that are reproducibly differentially expressed in one set of RNA preparations but expressed at equivalent levels in RNA preparations derived from independent cultures of identical cell lines (Figure 1). Mohr et al. (12)

made similar observations. These fragments likely represent genes that are differentially expressed, but as a result of slight variations in culture conditions or cell density at the time of RNA isolation rather than innate physiological differences between cell types. We eliminated this problem simply by performing all RT-PCRs for differential display in triplicate using three independent RNA preparations and only then choosing reproducible bands for further study.

A significant proportion of candidate differential display bands fail to detect a transcript on Northern blots (9,15). In part, this probably reflects the very low level of expression of the majority of eukaryotic mRNA species, most of which are present at fewer than 15 copies per cell (2,3). However, differential display bands that fail to detect a transcript by Northern blot might not always be derived from exceptionally rare species of mRNA. Our data indicate that they can also be derived from the unforeseen source of immature RNA transcripts. At least one of the five fragments we verified to be differentially expressed by RT-PCR was later determined to be derived from an intron (Figure 2). The possibility of amplification from chromosomal DNA was excluded by treating all RNA samples with DNase, performing RT-PCR controls and doing all RT-PCRs for differential display in triplicate from independent RNA preparations.

The amplification of an intron sequence requires that the primers used for differential display anneal to sites far removed from the poly(A) tail. Degenerate annealing of primers is inherent to differential display (11). Consistent with previous reports, the decamer AP-13 (5′-AGTTAGGCAA-3′; GenHunter, Brookline, MA, USA) used in our reaction annealed with one mismatch at the 5′ end. Furthermore, the anchored oligo(dT) primer (T_{12}MC; GenHunter) did not always anneal at the poly(A) tail. In our case, it annealed instead to a site containing 9/12 A residues that was within an intron of an unspliced RNA. Annealing of anchored oligo(dT) primers to sites far removed from the 3′ UT region has been demonstrated by others as well (11,13). Sometimes, though, it has been assumed that these primers have annealed to the 3′ UT region when fragments contain neither an open reading frame (ORF) nor a poly(A) addition site (7). Because of the intrinsically AT-rich nature of 3′ UT regions, these fragments have been explained as being derived from the 3′ UT region of mature transcripts but upstream from the poly(A) addition site. In other reports, degenerate poly(A) addition sites like ACTAAA (1,8) have been presented as evidence that anchored oligo(dT) primers have annealed at the poly(A) tail. However, 90% of all confirmed poly(A) addition sites perfectly match the consensus sequence AATAAA (16). Only one variant, ATTAAA, occurs with any significant frequency; i.e., in about 10% of transcripts. This variant has been shown to have about 80% of the activity of the wild-type sequence. No other variant has any significant activity or occurs at any considerable frequency. The sequence ACTAAA, for instance, has only about 10% of the activity of the wild-type sequence. Therefore, this is at best an extremely unlikely candi-

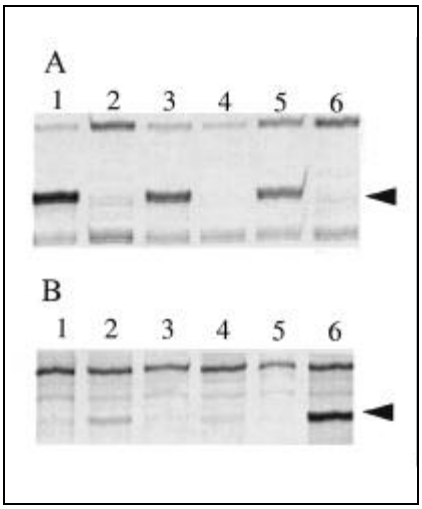

Figure 1. Reproducibility of differential display. Differential display was performed as previously described (9) on a human B-cell line (lanes 1, 3, 5) and a clonally related pre-B-cell line (lanes 2, 4, 6) using three independent RNA preparations. (A) A reproducible B-cell-specific band. (B) A nonreproducible band.

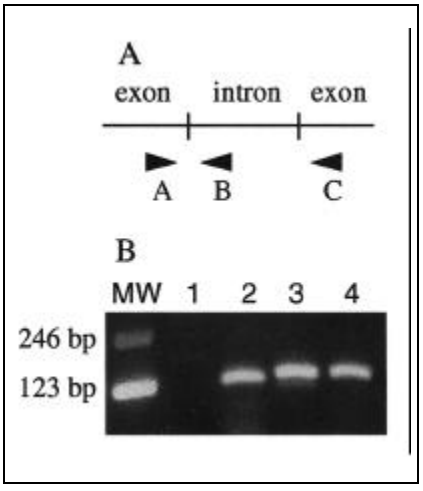

Figure 2. PCR confirmation of intronic sequence. PCR assays from genomic DNA (lanes 1 and 3) or total RNA isolated with TRI Reagent (lanes 2 and 4) using primers A and C (lanes 1 and 2) or A and B (lanes 3 and 4). Primers A and C yielded a 124-bp product only from a spliced RNA transcript. These primers yielded several much larger bands from genomic DNA, but we did not determine which of these, if any, contained the full-length intron. Primers A and B yielded a 132-bp product from genomic DNA and unspliced RNA.

date for a poly(A) addition site. Consistent with these reports is the fact that many of our candidate fragments contained neither a continuous ORF nor a consensus poly(A) addition site. We propose that a notable proportion of these might be derived from intron sequences rather than 3′ UT regions. These intron-derived fragments might account for some of the differential display probes that give no signal on Northern blots.

Intron-derived sequences can be eliminated using a method of RNA isolation that leaves the nuclei intact (6). These methods use the detergent Nonidet® P-40 (NP40), which lyses only the plasma membrane. The method most commonly used to isolate RNA for differential display is acid guanidinium thiocyanate-phenol-chloroform extraction (5). We used TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA), a commercially available mixture that is prepared according to this procedure. Both of these methods disrupt nuclei, accounting for the presence of immature transcripts. Use of poly(A) RNA might not be a viable alternative because methods of poly(A)$^+$ RNA isolation often leave the preparations contaminated with oligo(dT) fragments. In subsequent RT reactions, these fragments serve as unanchored primers that anneal at various points along the poly(A) tail and result in a smeared differential display gel (10). To the best of our knowledge, there have been only two reports that used cytoplasmic RNA rather than total RNA for differential display (4,14). Our isolation of an intronic sequence from differential display suggests that cytoplasmic RNA should be used whenever possible. By eliminating the possibility of intron-derived sequences and by performing multiple reactions from independent RNA preparations, many of the common problems associated with differential display might be alleviated.

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