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Differential display using one-base anchored oligo-dT primers

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Differential display was developed as a method to identify and analyze altered gene expression at the mRNA level in any eukaryotic cell (1). A similar method utilizing a single arbitrary primer to produce RNA finger-printing was developed independently and used for the same purpose as differential display (2). Despite the wide use of differential display in the analysis of altered gene expression, two major obstacles remained with the method. One is the redundancy and under-representation of certain mRNA species and the other is a rather high number of 'false positives' that cannot be confirmed by Northern blot analysis. To this end, various efforts have been made to optimize and streamline the method (3–5). While the problem could, to some extent, be attributed to the extrinsic factors such as pipetting errors, the integrity of RNA samples, contamination of RNA with chromosomal DNA or even bad PCR tubes (6), the intrinsic factors with the current scheme of the method have not yet been critically addressed.

This work describes the use, in differential display, of three one-base anchored oligo-dT primers which provide excellent selectivity in subdividing mRNA into three populations. In comparison with two-base anchored oligo-dT primers, the use of one base-anchored oligo-dT primers further cuts down the number of reverse transcription reactions needed for each RNA sample, minimizes the redundancy and under-representation of certain RNA species due to the degeneracy of the primers. The introduction of a restriction site at the 5′ ends on both the anchored and arbitrary primers makes the primers longer and more efficient in cDNA amplification while allowing the cloned cDNA to be more readily manipulated.

METHODS

To demonstrate the selectivity of one-base anchored primer, H-T_{11}C (5′-AAGCTTTTTTTTTTTC-3′) was used in combination with H-AP1 (5′-AAGCTTGATTGCC-3′), a 13mer derived from the Ltk3 primer located 310 bp upstream of the poly(A) tail of TK mRNA (1) to amplify a cloned mouse thymidine kinase (TK) cDNA template (7). The design of a *Hin*dIII restriction site (5′-AAGCTT-3′) at the 5′ ends of both primers was intended for both the easier manipulation of the amplified cDNAs after cloning into the pCR-TRAP cloning vector and more efficient amplification of the cDNA due to the longer primers used. The *Hin*dIII site is the least expensive restriction enzyme with 2 T's at the 3′ of its recognition site so the minimum number of non-T bases would be introduced to the anchored primers. Furthermore, since the first 7 to 8 bases at the 3′ end of the arbitrary primers are crucial for the initial hybridization with the first strand cDNAs (8), a series of arbitrary 13mers with the same 5′ *Hin*dIII restriction site but with 7 changing 3′ bases would maximize the selectivity of the arbitrary primers. The 6 bases of the *Hin*dIII site at the 5′ ends of the arbitrary primers statistically provides an additional 1.5 base match for any arbitrary sequence. These primers hybridize as 8mers, which were mostly found in the initial priming during the PCR reaction. Figure 1 shows the excellent selectivity of the one-base anchored oligo-dT primer, since only H-T_{11}C, with its last 3′ base being able to pair with the G located upstream of the TK poly(A) tail, was able to amplify the expected 310 bp TK cDNA in combination with the upstream primer H-AP1. Importantly, the longer H-T_{11}C and H-AP1

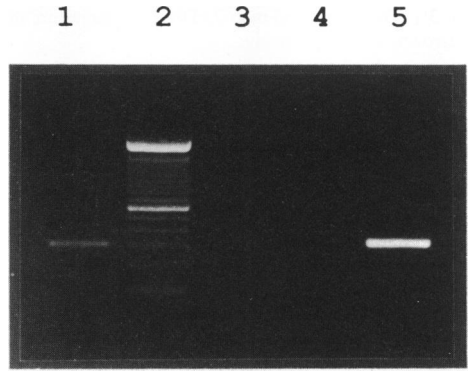

Figure 1. Selectivity of one-base anchored oligo-dT primers. Two-base anchored primer T_{12}VA (GenHunter) (lane 1) or three one-base anchored primers H-T_{11}G (5′-AAGCTTTTTTTTTTTG- 3′), H-T_{11}A (5′-AAGCTTTTTTTTTTTA- 3′), H-T_{11}C (5′-AAGCTTTTTTTTTTTC- 3′) (lanes 3, 4 and 5, respectively) were used in combination with either Ltk3 (5′-CTTGATTGCC- 3′), a TK specific decamer (lane 1) or H-AP1 (5′-AAGCTTGATTGCC-3′), a TK specific 13mer with 3 mismatches at the 5′ end (lanes 3 to 5) to amplify a 310 bp TK cDNA fragment using 5 ng of TK cDNA plasmid, pAMTK (1). Lane 2 is the 100 bp ladder (BRL) as a size marker.

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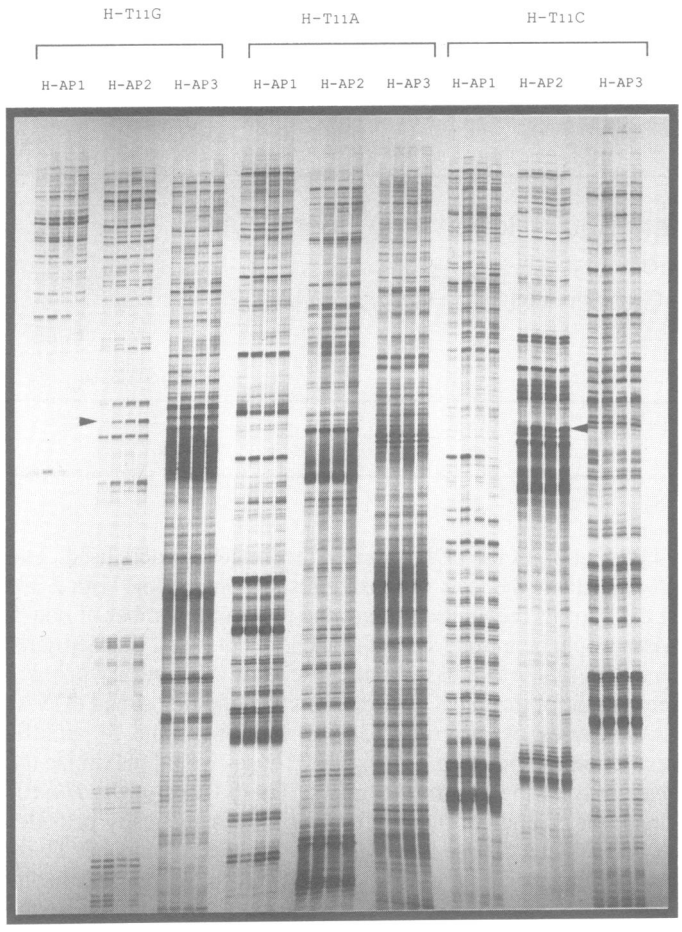

Figure 2. Differential display using one-base anchored oligo-dT primers. Four RNA samples from non-transformed cell line Rat 1 and H-ras transformed cell lines rat 1 (ras), T101-4 and A1-5 (11) (lanes from left to right, respectively) were compared by differential display using three one-base anchored oligo-dT primers, H-T_{11}G, H-T_{11}A and H-T_{11}C in combinations with three arbitrary 13mers, H-AP1, H-AP2 (5′-AAGCTTCGACTGT- 3′) and H-AP3 (5′-AAGC-TTTGGTCAG- 3′). The mob-1 and mob-7 cDNA fragments are marked by the right and left arrowheads, respectively.

primers appeared to be much more efficient than the shorter conventional primers T_{12}VA and Ltk3 in amplifying the TK cDNA as based on the intensity of the amplified PCR product.

To evaluate the utility of these one-base anchored oligo-dT primers in an actual differential display, total RNAs from rat embryo fibroblasts before and after transformation with H-ras oncogene and mutant p53 tumor suppressor gene were compared by differential display using three different one-base anchored oligo-dT primers in combination with three *Hin*dIII restriction site-containing arbitrary 13mers. Total RNAs from rat embryo fibroblasts were isolated using RNAzol B (Biotecx). After removing the chromosomal DNA contamination in the RNA samples using the MessageClean kit (GenHunter), the reverse transcription, PCR reactions, a 6% denaturing polyacrylamide gel electrophoresis, subsequent retrieval of the cDNA fragments of interest and Northern Blot analysis were carried out essentially as described (3), except 1 μCi of α-[^{33}P]dATP (2000 Ci/mmole, NEN) was used instead of 5 μCi of α-[^{35}S]dATP. Highly comparable patterns of amplified cDNAs from the four relevant RNA samples being compared were obtained (Figure 2). These showed excellent primer selectivity from both directions (as indicated by the change of cDNA patterns with changing either one-base anchored primers or arbitrary 13mers). As a positive control, H-AP2 primer (5′-AAGCTTCGACTGT-3′) which contains a *Hin*dIII site at its 5′ end and with its first 7 bases at the 3′ end matching the mob-1 cDNA (9) was designed to see if this previously identified gene differentially expressed only in the transformed cells could be detected with the corresponding one-base anchored primer H-T_{11}C. Indeed a cDNA band with the expected size (247 bp) was expressed mainly in the transformed cells (Figure 2, indicated by the right arrowhead). Cloning into the pCR-TRAP cloning vector (GenHunter) and sequencing using the Sequenase kit (USB), confirmed that this cDNA indeed corresponded to the 3′ tail of the mob-1 mRNA as expected (10). The 247 bp mob-1 cDNA cloned into pCR-TRAP vector could be either readily amplified with the primers flanking the cloning site or excised by *Hin*dIII digestion and used as a probe for Northern blot analysis (10). Among the initial five cDNA probes analyzed from the experiment, four including mob-1 and Mob-7 (Figure 2, marked by the left arrowhead) were confirmed by Northern blot analysis to be differentially expressed only in the transformed cells; the remaining one being a false positive (10). It should be noted that cautions taken to minimize the false positives in using two-base anchored primers (3) should still apply to the application of differential display using one-base anchored primers.

In conclusion, the successful use of one-base anchored oligo-dT primers and rationally designed arbitrary 13mers in differential display further cuts down the redundancy of two-base anchored primers, avoids the use of degenerate two-base anchored primers which tend to under-represent certain sub-class of mRNA since primers with an 'A' as the penultimate base were rarely used (10) and eliminates the problem of frequent oligo-dT contamination in T_{12}VT during its synthesis, which has been the major cause of 'smearing' in differential display using two-base anchored primers with T being the 3′ base. As a result, more reproducible and discrete patterns of differential display are obtained that allow the probes for differentially expressed genes to be more readily identified and manipulated.

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