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Isolation of developmentally regulated genes by differential display screening of cDNA libraries

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ABSTRACT

mRNA differential display RT–PCR has been extensively used for the isolation of genes differentially expressed between RNA populations. We have assessed its utility for the identification of developmentally regulated genes in plasmid cDNA libraries derived from individual tissues dissected from early mouse embryos. Using plasmid Southern blot hybridisation as a secondary screen, we are able to identify such genes and show by whole-mount *in situ* hybridisation that their expression pattern is that expected from the differential display profile.

The identification of developmentally regulated genes by differential display (DD) RT–PCR (1) would ideally involve the comparison of two RNA populations and the confirmation of differential expression by whole-mount *in situ* hybridisation. However, the bands obtained in a conventional DDRT–PCR experiment are too small to make good *in situ* probes. We show here that the addition of a secondary screening step that involves Southern blot hybridisation, which is relatively insensitive to probe size and quality, allows the ready confirmation of differential expression and facilitates the isolation of longer probes which can be assayed *in situ*.

Directionally cloned cDNA libraries have previously been constructed in the pSPORT1 vector using mRNA from the embryonic region of mid-gastrulation (E7.5) mouse embryos, and from dissected ectoderm, mesoderm, endoderm and primitive streak fractions (2). RNAs were generated from the mesoderm and endoderm germ layer libraries by *in vitro* transcription (Fig. 1). DDRT–PCR was performed using the primers indicated and the reaction products separated by native polyacrylamide gel electrophoresis (Fig. 2A). Bands of interest were eluted in 100 µl sterilised water at room temperature for 4 h. Five microlitres of eluate were reamplified in a 40 µl reaction containing 2 U AmpliTaq® DNA polymerase (Perkin-Elmer), 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 25 µM each dNTP and 1 µM each of primers EdT (5′-GGCAGGGAATTCGGGTTTTTTTTT) and BO6 (5′-GATTGTCGGGATCCGATCTGACAC). The reaction parameters were two cycles of 94°C for 30 s, 40°C for 60 s, 73°C for 60 s and then 34 cycles of 94°C for 20 s, 58°C for 30 s, 73°C for 30 s followed by a final extension for 5 min at 73°C. Reamplified products were cloned using the pCR™II vector (Invitrogen). Six clones were picked per band and sequence analysis performed using an ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer). Typically we isolated two to five different species which were generally identical in size.

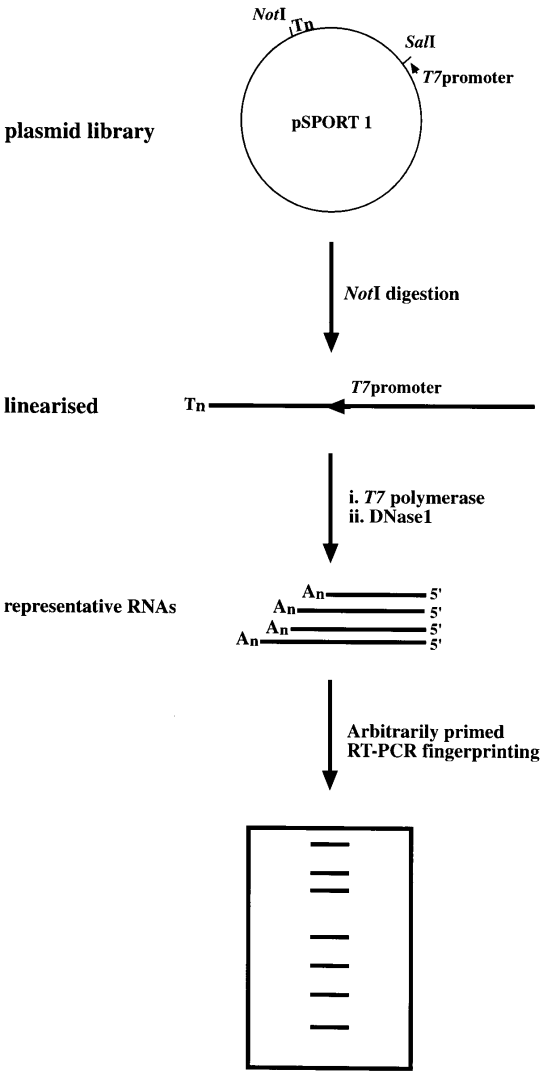

Figure 1. Each library was cloned directionally into the pSPORT1 vector (GIBCO-BRL) after reverse transcription with a *Not*I-dT_{24} primer. An aliquot of 1 µg of each library was digested for 1 h with 10 U *Not*I (Boehringer) and the linearised templates were used for the generation of representative RNAs by incubation with 10 U T7 RNA polymerase (Boehringer) for 2 h. Nascent RNAs were incubated with 5 U DNase I (Promega) for 1 h before precipitation with 4 M lithium chloride.

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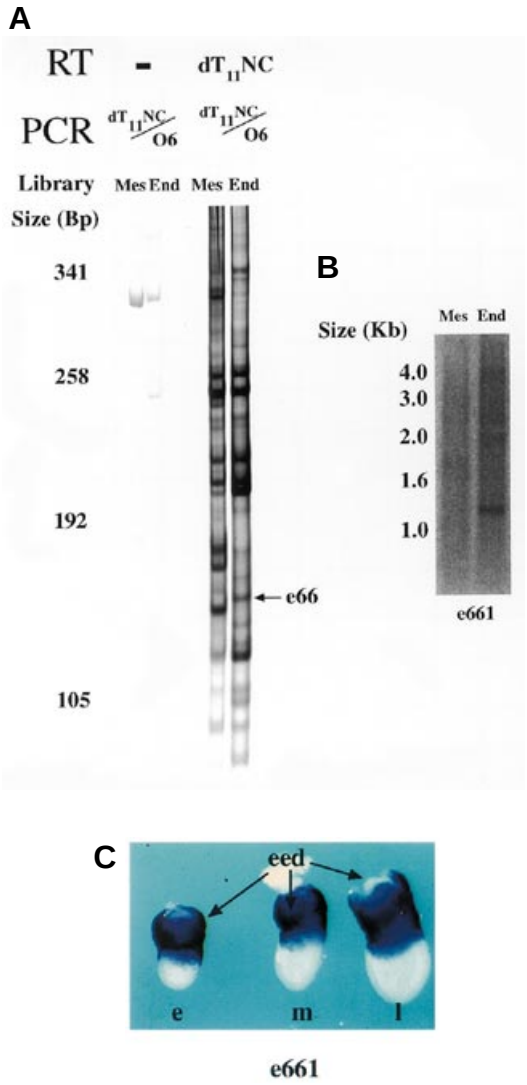

Figure 2. (**A**) Primary screen (differential display). An aliquot of 1 µg RNA from each library was reverse transcribed with 2.5 µM $dT_{11}NC$ prior to PCR with 2.5 µM $dT_{11}NC$ and 0.5 µM O6 (5′-GATCTGACAC) as described previously (5). The PCR was radiolabelled with 2.5 mCi [α-^{33}P]dATP and the products separated by electrophoresis through a 6% (w/v) native polyacrylamide gel. The products from the mesoderm (mes) and endoderm (end) libraries are shown. The products of the non-reverse transcribed control PCR are also shown. (**B**) Secondary screen (Southern blot hybridisation). Aliquots of 500 ng plasmid DNA from each library were digested with 10 U each of *Not*I and *Sal*I and the reaction products separated by electrophoresis through a 1% (w/v) agarose gel. After Southern blotting filters were hybridised with a DNA probe generated from the clone e661. This cDNA is represented only in the endoderm library. (**C**) Tertiary screen (*in situ* hybridisation). A clone corresponding to e661 which contains the entire ORF was isolated from the endoderm library and an antisense digoxygenin-labelled riboprobe was generated for *in situ* hybridisation analysis of early-, mid- and late-primitive streak stage mouse embryos (6). Strong expression is seen only in the extra-embryonic endoderm (eed).

A representative of each sequence type was hybridised to Southern blot filters of the entire libraries (Fig. 2B). In each of 17 cases at least one of the multiple species present in each band was differentially represented between the libraries by this assay, demonstrating that this secondary screen effectively identifies differentially expressed genes. Each of these differentially represented species was used to isolate longer clones for use in *in situ* hybridisation studies.

The clone e661 was derived from the endoderm specific band e66 (Fig. 2A). In the secondary screen it was highly enriched in, if not specific to, the endoderm library (Fig. 2B), and was used to isolate a 1750 bp clone which when used for *in situ* hybridisation analysis of mouse embryos of the stage at which the libraries were made, showed intense expression only in the extra-embryonic endoderm (Fig. 2C). Northern blotting shows that this clone is an approximately full-length copy of the mRNA present in the embryo and sequence analysis (DDBJ/EMBL/GenBank accession no. AJ010388) identifies a 1263 bp open reading frame (ORF) which is entirely composed of CUB repeats (3), a motif thought to mediate interactions with other proteins, carbohydrates and lipids. This sequence is derived from the same transcription unit as cubilin, the receptor for the intrinsic factor–vitamin B_{12} complex (4). Although extra-embryonic endoderm is a transporting epithelium, the e661 transcript is much smaller than that which encodes cubilin and does not appear to encode the signal sequence or the EGF repeats found in cubilin; it appears to result from the use of an alternative promoter and differential splicing.

Eight long clones were analysed in this way. One was a false positive, being expressed in all three germ layers of the embryo, two gave an expression pattern entirely consistent with the inital screen while the majority (five out of eight) of clones tested gave no *in situ* signal. It has previously been suggested (5) that DDRT–PCR readily identifies rare transcripts and our data show that this is true. However, although many clones correspond to transcripts below the detection sensitivity of the preferred screening technique, other analytical procedures could be employed in favourable cases. More importantly, given the ease of the procedure then even if the overall success rate, as judged by *in situ* hybridisation, is 25%, one worker can easily identify a large number of genes that are differentially expressed in any given developmental situation.

These data show that DDRT–PCR can be used to identify developmentally regulated genes in situations where high quality cDNA libraries are available to enable an effective secondary screen. We are presently seeking to optimise the DDRT–PCR protocol so that probes suitable for whole mount *in situ* hybridisation can be obtained directly.

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