Automated Recording of RNA Differential Display Patterns from Pig Granulosa Cells

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ABSTRACT

We have developed a protocol for fast, nonradioactive, mRNA differential display reverse transcription PCR (DDRT-PCR) based on a commerical automated sequencer with RNA isolated from pig granulosa cells. We sought to discover conditions that would minimize the problem of using relatively small primers labeled with large infrared dye molecule, IR41, required for the sequencer. Extended IR41-labeled primers IR41-AAGC-T_{11}-A, IR41-AAGC-T_{11}-C and IR41-AAGC-T_{11}-G gave more consistent differential display patterns than shorter anchored primers (IR41-T_{11}A, IR41-T_{11}C and IR41-T_{11}G) without the additional (AAGC) cloning site. The optimal concentration of the extended labeled (downstream) primers was 20 pmol when 13-mer arbitrary (upstream) primers were used at a concentration of 4 pmol. Background smear and the intensity of amplified bands was significantly improved by changing from conventional Taq *DNA polymerase to AmpliTaq® Gold® polymerase, which permits an improved "hot start" for the reaction. Running time (during which a digitized gel image is recorded) for a 26-cm polyacrylamide gel was 4 h, enabling us to analyze 90 reactions in an 8-h day. This protocol offers a rapid and reliable nonradioactive method for comparing gene expression patterns for various research or diagnostic purposes.*

INTRODUCTION

Normal development and differentiation as well as pathological changes in mammalian cells are predominantly driven by changes in gene expression (3,12,13,19). An effective way to identify and isolate genes that are differentially expressed in various cell types or under altered conditions has been described (9). In this original approach, termed mRNA differential display, mRNA was first reverse-transcribed into cDNA using a set of four two-base anchored, oligo(dT) primers (T_{12}MA, T_{12}MC, T_{12}MG and T_{12}MT), where M represents either G, A or C (8,9). In a subsequent polymerase chain reaction (PCR) amplification reaction, these primers were used together with arbitrary upstream primers to yield radioactively labeled products from the template cDNA to display the pattern of gene expression (1,2,10).

When the major objective of differential display reverse transcription (DDRT)-PCR is to rapidly produce a diagnostic banding pattern, and cloning of differentially expressed genes is of secondary importance, it is superior to use a nonradioactive detection method in combination with automatic analysis of the resulting gel pattern (1,2). DNA sequencers with denaturing or nondenaturing gel electrophoresis are well suited for this application (10). A major technical problem is the effect exerted by large fluorescent dye molecules on the priming efficiency of small oligonucleotides. This problem could be partly solved for the dyes TAMRA and FAM (for DDRT-PCR on a PE Applied Biosystems sequencer) by adding dye-labeled primers after an initial 20 rounds of amplification with unlabeled primers (1,10) or, for differential display with the ALF DNA Sequencer™, by using fluorescein isothiocyanate (FITC)-labeled 3′-anchored oligo(dT) (downstream) primers in both the RT reaction and the PCR (5).

Here we report experiments using infrared dye (IRD-41)-labeled, one-base anchored, 12-mer primers, with and without a 4-base extension sequence. PCR amplification conditions were optimized for these primers to obtain reproducible cDNA fingerprints, which could be used for rapid diagnostic purposes.

MATERIALS AND METHODS

Isolation of Porcine Granulosa Cells

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in ice-cold phosphate-buffered saline (PBS, pH 7.4). Small (S; 2-mm in diameter) and middle-sized (M; 4–6-mm in diameter) antral follicles with transparent, vascularized follicular walls were dissected and hemi-sectioned. With the aid of two preparation needles, large pieces of the granulosa cell layer were carefully separated from the theca, washed in ice-cold PBS, resuspended and the granulosa cell number was determined by repeated hemocytometer counting (6). The same approach was used for isolation of membrana granulosa cells

Table 1. Upstream and Downstream Primers

(1) PolyA2 (PCR product size 252 bp)	
	5′-GTTTCCTCGGTGGTGTTTCCTGGGCTATGC-3′
IRD-41 +	5′-TGGAGTTCTGTTGTGGGTATGCTGGTGTAA-3′
(2) Cx43 (PCR product size 516 bp; Reference 16)	
	5′-GGGAAAGAGCCATCCTTACCACACTACCAC-3′
IRD-41 +	5′-CCACCTCCACCAATGAAATGAACACCTA-3′
(3) Ubiquitin (PCR product size 215 bp)	
	5′-GGAGCCCAGCGACACCATCGAGAAC-3′
IRD-41 +	5′-TGAAATTTGTTGAAAGCTTAAAAGGGAGAA-3′
(4) GAPDH (PCR product size 764 bp)	
	5′-GTTCCAGTATGATTCCACCCACGGCAAGTT-3′
IRD-41 +	5′-TGCCAGCCCCAGCATCAAAGGTAGAAGAGT-3′

from large (L) preovulatory follicles excised from the ovaries of miniature gilts that were stimulated at day 16 with 500 IU pregnant mare's serum gonadotrophin (PMSG; Sergon, Bioveta, Czech Republic) and slaughtered 72 h after.

RNA Isolation and Reverse Transcription

Granulosa cell samples ($10 \times$ S, $10 \times$ M, $6 \times$ L) were centrifuged at 4°C for 5 min (500× *g*), supernatants were discarded and 350 µL of lysis buffer (RNeasy® Total RNA Kit; Qiagen, Hilden, Germany) were added to each 10×10^6 cell pellet. DNA was sheared by passing the lysates through a 21-gauge needle attached to a sterile plastic syringe. This procedure was completed with two elution steps, and RNA concentrations were measured with a GeneQuant™ RNA/DNA Calculator (Pharmacia Biotech, Cambridge, England, UK). Each RNA sample (5 µg) was run on a formaldehyde/4-morpholinepropanesulfonic acid (MOPS) agarose gel and showed sharp bands for 18S and 28S rRNA. To remove residual contaminating DNAs, the samples were treated with DNase I (RNase-free; Boehringer Mannheim, Mannheim, Germany) in the presence of RNasin® (Promega, Madison, WI, USA). RNA samples were stored at concentrations of 2–3 µg/µL at -70°C.

A GenHunter RNAimage™ Kit 1 (GenHunter, Nashville, TN, USA) was used for RT of the mRNAs. S, M and L RNA samples were diluted to 0.1 µg/µL, and 2 µL were used in a 20-µL reaction (buffer: 25 mM Tris-HCl, pH 8.3, 38 mM KCl, 1.5 mM $MgCl_2$ and 5 mM dithiothreitol [DTT]; primer: 0.2 µM H-T_{11}M; nucleotides: dNTP 20 µM) and transcribed for 50 min with 1 µL Moloney murine leukemia virus (MMLV) reverse transcriptase provided in the kit. RT reactions for S, M and L granulosa cell RNAs were primed with the three different one-base anchored H-T11M primers (where M are G, A or C and H is AAGC). After 5-min inactivation at 75°C, tubes were placed immediately on ice, the cDNA products of these RT reactions were divided into 2-µL aliquots and stored at -20°C. To test the effect of dimethyl sulfoxide (DMSO) on the efficiency of the RT reaction, DMSO (Merck KGaA, Darmstadt, Germany) was added to the RT buffer at a final concentration 10% in five experimental replicates.

Nonradioactive DD-PCR

For PCR amplification, a 20-µL reaction was prepared with a buffer consisting of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$ and 0.001% gelatin. Nucleotides were added from a 200 µM dNTP mixture (Amersham International, Little Chalfont, Bucks, England, UK) to give a reaction concentration of 20 µM. The reactions were performed in the tubes in which the 2-µL cDNA samples had been stored. The PCR was primed with arbitrary upstream primers H-AP1 (5′-AAGCTTGATTGCC-3′), H-AP2 (5′-AAGCTTCGACTGT-3′) and H-AP3 (5′-AAGCTTTGGTCAG-3′) from the RNAimage Kit 1. Additional H-AP2, used for optimization experiments, was synthesized and HPLC-purified by MWG-Biotech GmbH (Ebersberg, Germany). The following concentrations of arbitrary upstream primers were tested: 4, 40 and 400 pmol. The anchored oligo(dT) downstream primers were labeled with infrared dye IRD-41 (MWG-Biotech GmbH). In the Model 4000L DNA Sequencer (LI-COR, Lincoln, NE, USA), this dye is excited by an infrared laser diode emitting at 785 nm. The absorbance maximum of IRD-41 is at 795 nm, and emission maximum is at 833 nm. The following IRD-41-labeled primers were tested: IR41-5′-T11A, C and G; IR41-5′-AAGCT11A, C and G at concentrations of 4, 20 and 40 pmol.

Two DNA polymerases were tested in DDRT-PCRs: *(i)* Taq DNA polymerase (Life Technologies, Eggenstein, Germany) at a concentration of 1 U/reaction and *(ii)* AmpliTaq® Gold™ (Perkin-Elmer, Norwalk, CT, USA) at a concentration of 0.5 U/reaction. All PCRs were carried out in thin-walled, 0.2-mL reactions tubes in a MultiCycler® PTC-200 Thermal Cycler with heated lid (MJ Research, Watertown, MA, USA) without mineral oil. The amplification program was 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles; a final extension of 72°C for 5 min and a final holding temperature of 4°C. In the experiments with *Taq* DNA polymerase, "hot start" was accomplished by pausing the program after the initial denaturing step, adding the enzyme and then continuing the cycling program. In experiments with AmpliTaq DNA polymerase, which must be activated by heat, the first step was at 95°C for 9 min, but the cycling program that followed was identical.

PCR

Two additional thermal polymerases were compared for IRD-41 compatibility in PCRs with IRD-41-labeled primers for poly-AAA polymerase (PolyA2), connexin 43 (Cx43), ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *(i)* PrimeZyme™ DNA Polymerase (Biometra,

Göttingen, Germany) was used with the buffer, nucleotides and enzyme (2 U/100 µL reaction) provided with the kit. *(ii)* Taq DNA Polymerase (Qiagen) was used with the buffer, nucleotides and enzyme (2 U/100 µL reaction) provided with the kit. In both cases, the thermal cycler program was 94°C for 1 min, followed by 34 cycles of: 94°C for 15 s; 57°, 58° or 62°C (according to the annealing temperature of each primer pair) for 15 s; 72°C for 15 s. The last cycle was followed by a final extension at 72°C for 5 min and a holding temperature of 4°C. Table 1 describes the upstream and downstream primers used.

The products of successful reactions were subsequently used as molecular weight markers to calibrate differential display gels on the LI-COR Model 4000L DNA Sequencer.

Gel Electrophoresis, Collection and Image Analysis

Stop/loading solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue) was added to PCR products (4 µL + 4 µL), and samples were incubated at 95°C for 3 min before loading. Only 1 µL per lane was applied on 5% Sequagel™ XR (0.25 mm; Biozym, Hess.-Oldendorf, Germany), which had been prerun for 40 min with 1× TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0 at 50°C). The automated sequencer was a Model 4000L (LI-COR). Both 41- and 26-cm long gels were used under with an electric potential of 1500 and 800 V, respectively. Band patterns were acquired and analyzed with Base ImageIR™ software (LI-COR).

RESULTS

Protocols for IRD41-Primed DDRT-PCR

As we started this project to adapt the GenHunter RNAimage Kit for use with the Model 4000L Automated Sequencer, we chose to do the RT step without labeled primers in order to benefit from the optimization of cDNA production already carried out by the manufacturer. Using standard conditions, we obtained only very weak bands on the image from the Model 4000L after PCR amplification reaction with several different primer pair combinations. Since there are reports (11, 15) that the addition of DMSO can increase the efficiency of RT, we then performed five further RT reactions with each of the three downstream primers (LHT11A, C and G) with the addition of 10% DMSO to the original RT buffer supplied with the RNAimage Kit. Using these cDNA samples as templates for subsequent PCR, we obtained stronger display patterns containing more bands with all primer combinations (see Figure 4, A and B).

We also attempted to optimize the PCR amplification step. PCR products must incorporate the IRD41 dye label to be detectable in the Model 4000L sequencer. In our first experiments, we tested three simple IRD41-labeled, 12-mer downstream primers (5′ IRD41-TTT TTT TTT TTA 3′; 5′ IRD41-TTT TTT TTT TTC 3′; and 5′ IRD41-TTT TTT TTT TTG 3′). These labeled primers were used in concentrations of 4, 20 or 40 pmol in combination with two upstream primers (AP1 and AP2) each at concentrations of 4 and 40 pmol. All six combinations were tested several times but produced only a weak banding patterns in the low molecular weight region below 200 bp.

We then tested IRD41-labeled primers that included the addition of an AAGC sequence, which adds a *Hin*dIII site that facilitates subsequent cloning but also increases the affinity of the primer for its recognition sequence. In an initial screening, these extended primers (5′ IRD41-AAG CTT TTT TTT TTT A 3′; 5′ IRD41-AAG CTT TTT TTT TTT C 3′; and 5′ IRD41-AAG CTT TTT TTT TTT G 3′) were tested at concentrations 4 and 40 pmol in combination with the above two upstream primers (AP1 and AP2) at a concentration of 4 pmol. These primer

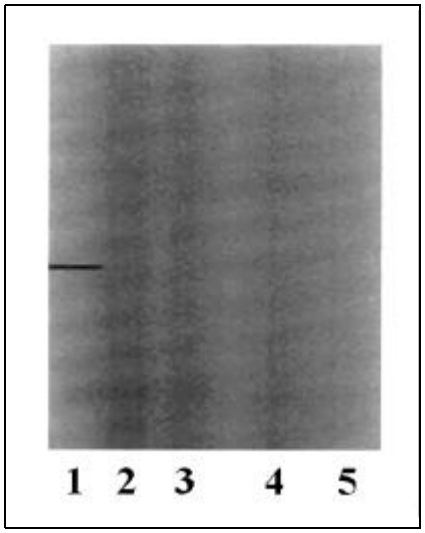

Figure 1. DDRT-PCR primed with IR41-5′-AAGCT$_{11}$A. When *Taq* DNA polymerase was used without hot start; primers AP1 (lanes 2 and 3) and AP2 (lanes 4 and 5) in combination with IR41-5′-AAGCT$_{11}$A at concentrations of 40 pmol (lanes 2 and 4) and 4 pmol (lanes 3 and 5) gave a continuous smear only. Lane 1 shows the 252-bp polyA2 product for molecular weight calibration.

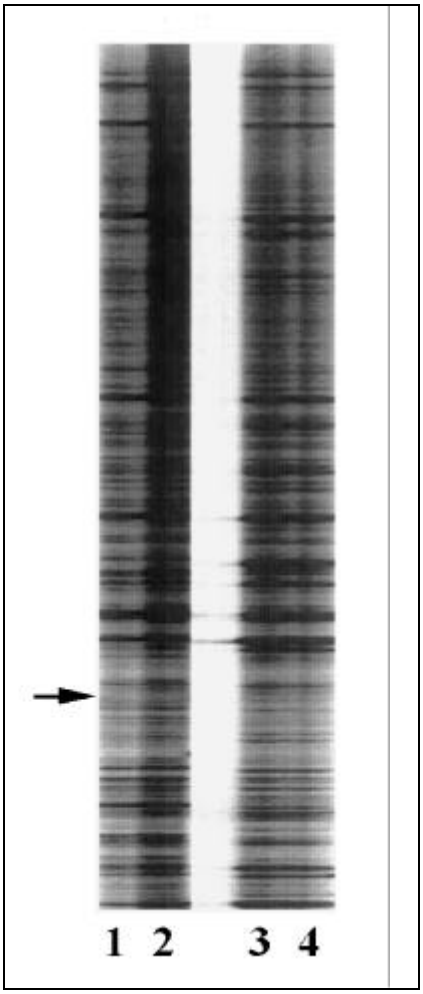

Figure 2. DDRT-PCR banding after hot-start PCR. Addition of *Taq* DNA polymerase after the denaturation step improved DNA banding. AP1 (lanes 1 and 2) and AP2 (lanes 3 and 4) primers in combination with IR41-5′-AAGCT$_{11}$G at concentrations of 40 pmol (lanes 1 and 3) and 4 pmol (lanes 2 and 4) and gave considerable background smear above 300 bp. Molecular weight of 252 (polyA2 product) is arrowed.

combinations were tested in amplification reactions with conventional *Taq* DNA polymerase and produced a dramatic improvement by extending the length of products up to 800 bp. However, under these conditions, nonspecific priming caused a continuous smear of products over the entire range (Figure 1). This smearing was substantially reduced when the same primer combinations were used, but a "hot start" was performed by adding the *Taq* DNA polymerase immediately after the initial denaturing step. However, this form of "hot start" did not completely remove the background smear, particularly in the important range of PCR products above 300 bp (Figure 2).

To further improve the clarity of the DDRT-PCR banding pattern, we tested AmpliTaq Gold DNA Polymerase, which is described as a modified form of AmpliTaq DNA Polymerase (Perkin-Elmer). It is provided in an inactive form and is activated by a 9–12-min, pre-PCR step at 93°–95°C. In our hands, 95°C was significantly better than the lower temperature of 93°C when the activation period was 9 min. AmpliTaq Gold produced a smaller number of strong sharp bands with a very low background, while conventional *Taq* with manual "hot start" produced many weaker bands against a higher background smear.

Working further with AmpliTaq Gold, we optimized primer concentrations for IRD41-labeled PCR and found that the optimal combination was 4 pmol for the arbitrary primer (AP2) and 20 pmol for the labeled downstream primer (Figure 3A). Equal concentrations of labeled (downstream) primer and nonlabeled arbitrary (upstream) primer did not produce a satisfactory differential display pattern with any of the three labeled downstream primers tested (Figure 3B).

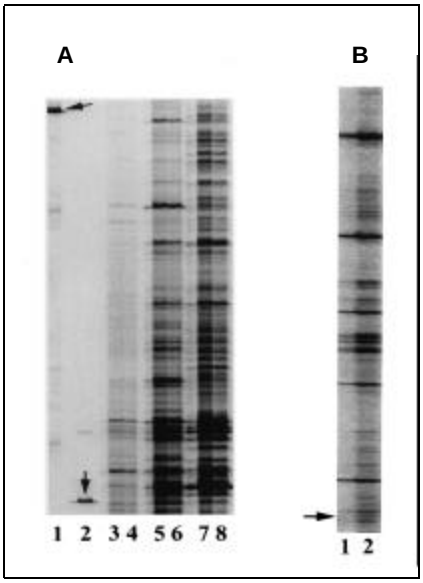

Figure 3. Effect of 5′ and 3′ primer concentration on DDRT-PCR banding. (A) Upstream AP2 primer concentrations of 400 (lanes 3 and 4), 40 (lanes 5 and 6) and 4 pmol (lanes 7 and 8) were tested in combination with downstream IR41-5′-AAGCT$_{11}$G primer at a concentration of 20 pmol. Lanes 3, 5 and 7 were from one RT reaction, and lanes 4, 6 and 8 were done with a second RT reaction from small (S) antral follicles. The lowest AP2 concentration (4 pmol) gave the best results. Lanes 1 and 2 show connexin43 (516 bp) and ubiquitin (215 bp) products (arrows) as mol wt calibration. (B) IR41-5′-AAGCT$_{11}$C primer concentrations of 4 pmol (lane 1) and 40 pmol (lane 2) in combination with AP2 at a concentration of 4 pmol. The tenfold higher (40 pmol) concentration of IR41-labeled primer was optimal for the DDRT-PCR. Molecular weight of 215 bp (ubiquitin product) is arrowed.

Sensitivity of IRD41-Primed PCR

RT reactions were done with granulosa cell RNA from S, M and L follicles using all three downstream (anchored) primers (LH-T11A, LH-T11C and LH-T11G) supplied with the GenHunter RNAimage kit. All nine RT reaction products were then tested as templates in PCRs with specific primers designed for PolyA2, Cx43, ubiquitin and GAPDH. The downstream primer was always labeled with IRD41. The specific primer pairs for PolyA2 and ubiquitin produced the correct diagnostic bands (252 and 215 bp, respectively) only from RT reactions with the LH-T11G primer (Figures 1, 3A and 4A). Molecular weight calibration was performed with IRD-40-labeled fragments of pBR322 cleaved by *Hin*fI. Interest-

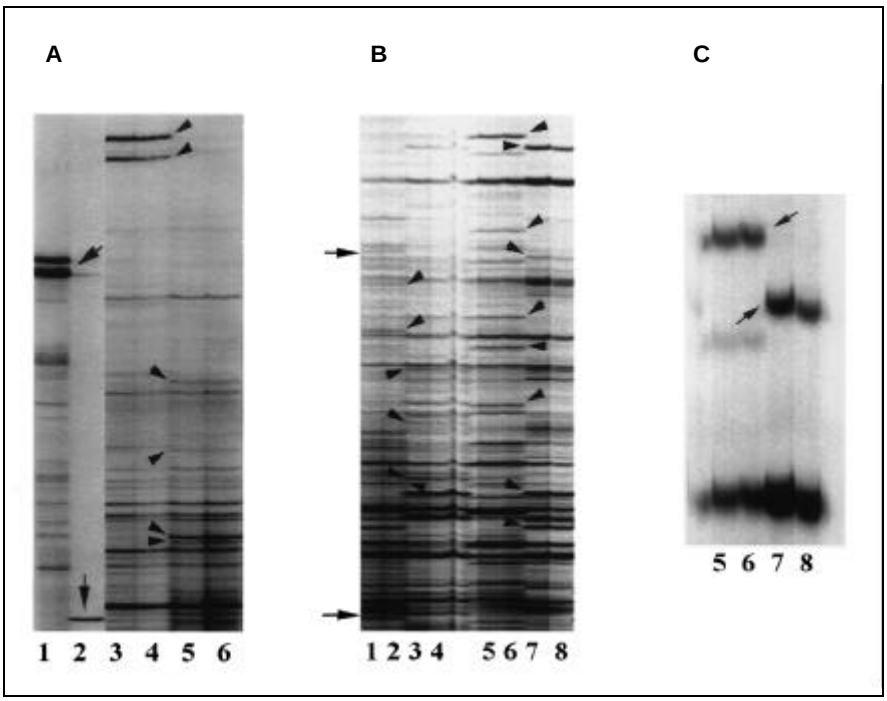

Figure 4. Differential display band pattern derived from pig granulosa cell RNA. (A) Two RNA samples from small follicles (lanes 3 and 4) and two samples from large follicles (lanes 5 and 6) were compared in DDRT-PCRs primed with AP1 and IR41-5′-AAGCT$_{11}$A. Differentially expressed bands are indicated (arrowheads). Lanes 1 and 2 show the positions of the connexin43 (516 bp) and poly(A) (252 bp) products (arrowed) for mol wt calibration. (B) RNA samples from small follicles (lanes 1, 2, 5 and 6) and large follicles (lanes 3, 4, 7 and 8) were compared in DDRT-PCRs primed with AP2 and IR41-5′-AAGCT$_{11}$G (lanes 1, 2, 3 and 4) and with AP3 and IR41-5′-AAGCT$_{11}$G (lanes 5, 6, 7 and 8). Differentially expressed bands are marked with arrowheads. Molecular weights of 252 bp (polyA2 product) and 516 bp (connexin43 product) are arrowed. (C) Original image showing high resolution of the differential display pattern obtained by Model 4000L DNA Sequencer. The region shown corresponds to the uppermost bands in lanes 5–8 of Figure 4B.

ingly, while a strong Cx43 product (512 bp) was produced only in cDNA samples reverse-transcribed with LH-T11A, a faint band could be observed with cDNA samples produced with LH-T11G (Figures 3A and 4A). A strong GAPDH product (764 bp) was consistently produced from cDNA samples obtained with LH-T11C only with the QIAGEN® *Taq* DNA Polymerase Kit (Qiagen) containing an additive to "change the melting behavior of DNA" but not with a conventional *Taq* DNA polymerase. In addition, these specific primer pairs provided positive controls and molecular weight calibration for the differential display gels (see Figures 1, 3A and 4A). PCR with specific primers pairs could be performed with as little as 1/10 the amount of cDNA necessary for good DDRT-PCR patterns.

Automatic Recording of Granulosa Cell RNA Display Patterns

Nine distinct PCRs covered all possible combinations of the three anchored (downstream) and the three arbitrary (upstream) primers for each granulosa cell cDNA sample. Reaction products for small (S), medium (M) and large (L) samples were loaded side by side to permit accurate, direct comparison between these three different populations of granulosa cells. With each of the nine primer combinations, approximately 20 distinct bands were present in the size range from 200–600 bp in all samples tested (see Figure 4, A and B). Only a few distinct, reproducible cDNA differences between S and M follicles were identified. On the other hand, the comparison of S and L follicles revealed 2–6 distinct differentially expressed bands with 100% reproducibility with each of the nine primer combinations. Additional differences among the weaker bands were less reproducible. Examples of the differential display images obtained with three different upstream primers (AP1, AP2 and AP3) are portrayed in Figure 4, A, B and C. Figure 4A shows the pattern obtained with the combination of AP1 and IR41-5′-AAGCT$_{11}$A. Figure 4B shows the pattern obtained with the combination of AP2 and AP3 with downstream primer IR41-5′-AAGCT$_{11}$G. Figure 4C shows the original resolution of the Model 4000L sequencer, while Figure 4, A and B are vertically compressed by a factor of five using standard image-processing accessories.

In general, these results show that under optimized reaction conditions, nonradioactive DDRT-PCR using IRD41-labeled downstream primers displays a characteristic and reproducible spectrum of bands, which are characteristic for distinct stages of granulosa cell differentiation.

DISCUSSION

Growth of porcine follicles is accompanied by substantial changes in the expression of mRNAs for growth factors, gonadotropin receptors and steroidogenic enzymes (14,17). The process of follicular differentiation includes expression (IGF1, IGF-2, LH receptor and aromatase) or disappearance (FSH receptor) of specific mRNAs in granulosa cells (4,18). These changes in gene expression in granulosa cells during follicular growth can be monitored by mRNA differential display. For that reason, pig membrana granulosa cells from three follicular categories provide an excellent model for such studies.

Sidhu et al. (15) have shown that amplification is enhanced and is more specific when DMSO was included in the RT reaction mixture, while inclusion of DMSO in the PCR did not produce an additional improvement. It was suggested that the DMSO effect is due to destabilization of intramolecular secondary structure of the template, which facilitates annealing of the primers (11,15). In our protocol, we added 10% DMSO only to the RT reaction, and since 1/10 of the subsequent PCR represents undiluted cDNA, our PCR contained 1% DMSO. We observed stronger bands in DDRT-PCR using buffer supplemented with 10% DMSO, suggesting that the annealing efficiency of the short downstream anchor primers was a limiting factor.

Fluorescent dye molecules are necessary for the detection of migrating DNA bands in automated sequencers. Dyes for the PE Applied Biosystems sequencer (Foster City, CA, USA), such as TAMRA, JOE and ROX, have molecular weights (mol wt) of 430, 505 and 535, respectively. FITC (mol wt 389) is used with the ALF DNA Sequencer (Pharmacia Biotech). The molecular weight of IRD40 used by the Model 4000L Sequencer is above 900. By comparison the mol wt of 11T-G is less than 4000. It is clear that dye labeling of short primers could affect reaction kinetics and annealing (10).

The present results show that priming efficiency was unsatisfactory with IRD41-labeled 12-mer (T11M) primers even under low stringency conditions. Extension of the 5′ end of one-base anchored primers by four additional nucleotides (AAGC) significantly improved priming efficiency after IRD41 labeling. The molar concentration ratio between labeled and unlabeled primers was shown to affect the differential display pattern (5). We found that a ratio of 5:1 in combination with AmpliTaq Gold DNA Polymerase (to eliminate smearing) gave optimal cDNA fingerprints in the range of 200–600 bp without background smearing. In DDRT-PCR where the upstream primer was labeled with FAM or JOE, the optimal ratio was also reported to be 5:1 (10).

The band display changed with different combinations of the primers, which indicates that each pair has its own priming specificity. Each primer combination gives a specific pattern and number of cDNAs, ranging in size from 100–700 bp. We compared different granulosa cell cDNAs between 200–500 bp. The gel region containing bands shorter than 200 bp was not used because it was overcrowded and difficult to interpret. The identical patterns obtained with RNA samples isolated from granulosa cells of the same follicular category indicate that the process of RNA preparation was not a source of variability. Some slight changes in the displays were always observed in a set of weak and very weak bands. Although RNA samples were treated with DNase I, some undigested chromosomal DNA fragments could survive (18) and produce a lower degree of reproducibility among weaker bands on nonradioactive DDRT-PCR records. Thus DNA contamination should be kept as low as possible.

The current protocol identified a highly reproducible pattern of cDNAs from the granulosa cells of S, M and L follicles as well as cDNAs present only in S or L follicles. A sample of as little as 20 ng of RNA was sufficient for the identification of positive and negative changes in gene transcription during granulosa cell differentiation. Our results show that the reproducibility of nonradioactive DDRT-PCR was improved using IR-41-labeled downstream primers. Owing to the availability of automated DNA sequencers in many laboratories and hospitals, this method of nonradioactive DDRT-PCR using IRD41-labeled primers may prove a useful protocol for a quick and reliable comparison of cell populations for general diagnostic purposes. The biological implications of the different bands obtained from granulosa cells derived from different size porcine follicles await further investigation. Cloning and sequencing of reproducible difference bands cut from parallel radiolabeled gels using the same primer combinations is currently being undertaken.

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