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Cloning of intestinal phospholipase A2 from intestinal epithelial RNA by differential display PCR

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Abstract. Differential display polymerase chain reaction (DD-PCR) is a powerful technique for comparing gene expression between cell types, or between stages of development or differentiation. Differentially expressed genes may be cloned and analysed further. Here we extend the use of DD-PCR to analyse differences in gene expression between two complex epithelia: that of the small intestine and of the large intestine. The aim of this study was to identify genes expressed preferentially in Paneth cells. Paneth cells are secretory epithelial cells putatively involved in host defense and regulation of crypt cell proliferation and are found at the base of the small intestinal crypts adjacent to the stem cell zone. Of 34 clones that were analysed, partial sequencing identified two clones related to known Paneth cell products: a homologue of secretory phospholipase A2 (clone B1) and a homologue of a neutrophil defensin (clone C5). B1 was strongly expressed in Paneth cells, as demonstrated by *in-situ* hybridization. B1 was also expressed at a lower level in the large intestinal epithelium. A full length B1 cDNA clone was isolated and sequenced, and shown to be highly homologous to type II secretory phospholipase A2 genes, and almost identical to the enhancing factor gene and the putative gene for the MOM-1 locus. B1 expression is limited to the intestinal tract, and we propose that it be designated intestinal phospholipase A2, or *i*-PLA2. The method we describe is well suited to the rapid identification of genes expressed exclusively or predominantly in Paneth cells.

Differential display polymerase chain reaction (DD-PCR) is a powerful technique for studying differences in gene expression between cells (Liang & Pardee 1992, Liang & Pardee 1995). The technique uses the amplification potential of PCR to produce detectable quantities of nearly randomly primed cDNA species from a complex mixture of cellular mRNA species. cDNA synthesis is primed using an oligo(dT) primer with a dinucleotide 3′ anchor sequence to increase the specificity of priming, and PCR is performed with the same anchored oligo(dT) primer, and a randomly selected 10mer oligonucleotide, calculated to bind specifically to a limited number of cDNA molecules. The resultant PCR generates a complex but finite mixture of amplified products of differing lengths which are separated and analysed by polyacrylamide gel electrophoresis. This mRNA fingerprint which, in theory, is predictable and reproducible for a given combination of anchored oligo(dT), 10mer, and mRNA source, may be compared to the mRNA fingerprint from another mRNA source.

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Specific cDNA molecules which are differentially expressed may then be extracted from the polyacrylamide gel, cloned, and analysed further.

In practice, the mRNA fingerprint pattern is found by many researchers to be variable and incompletely reproducible between experiments. For this reason DD-PCR has mainly been used to compare the regulation of genes in closely related populations of cells (Sager *et al.* 1993). To eliminate non-specific differences between the mRNA finterprints of cells being compared, cells used for DD-PCR are typically cultured under tightly controlled conditions, matching all variables apart from the one being investigated. Using this approach DD-PCR has been successfully applied in a number of studies (Blok *et al.* 1995, Chapman *et al.* 1995, Jin *et al.* 1997).

We have used DD-PCR to compare gene expression between complex mixtures of freshly isolated cells from the small intestinal epithelium and the large intestinal epithelium. Epithelial cells isolated in this way comprise all the various subtypes of cells found in the small and large intestine; including absorptive enterocytes, colonocytes, goblet cells, enteroendocrine cells, stem cells and Paneth cells (Trier & Madara 1981). Using such a complex mixture of cells necessitates abandoning the requirement that the mRNA templates for DD-PCR be as closely matched as possible. Similarly, it is not feasible to reproduce mRNA fingerprints from experiment to experiment.

The aim of this study was to identify novel genes expressed exclusively, or most abundantly, in Paneth cells. Paneth cells are found in relatively large numbers in the crypts of the small intestine, where they are located at the base of the crypt, adjacent to the stem cell zone. They have been implicated in host defence functions and in the regulation of crypt cell proliferation (Keshav *et al.* 1990). As Paneth cells are present in large numbers in the small intestine, but are absent from the large intestine, differentially expressed mRNA species restricted to Paneth cells are represented at higher levels in RNA from the small intestine compared to RNA from the large intestine.

DD-PCR applied in this way has allowed us to identify a number of differentially regulated cDNA molecules, and we have used DNA sequencing, *in-situ* hybridization and Northern blotting to identify a clone of the intestinal secretory phospholipase A2 gene (*i*-PLA2) which is expressed most abundantly in Paneth cells. This study demonstrates the potential use of DD-PCR to clone genes from complex mixtures of cells obtained directly from tissues and organs.

MATERIALS AND METHODS

Mice

Adult female PO mice were used. Mice were bred and housed at the Sir William Dunn School of Pathology animal facility. Mice were killed by CO_2 inhalation.

Isolation of epithelial cells

Intestinal epithelial cells were isolated using a modification of the method of Bjerknes & Cheng (1981). The abdominal cavity was opened, and a section of small intestine (5 cm distally from the duodenojejunal flexture) or large intestine (entire length distally from the junction of caecum and colon) was mobilized. A small incision was made in the antimesenteric border at the distal end of the selected segment, a 25-g needle was inserted into the proximal end of the selected segment, and the lumen was flushed with 20 ml of phosphate buffer saline (PBS) followed by 20 ml of PBS + 5 mM EDTA. The chest was opened, and the inferior vena cava sectioned. The left ventricle was cannulated with a 25-g needle attached to

a syringe, and 50 ml of Hank's balanced salt solution (HBSS, Gibco BRL, Paisley, UK) containing 20 mM HEPES at pH 7.4, and either 10 mM EDTA for small intestinal crypts 30 mM EDTA for colonic epithelium, was perfused through the heart. All solutions were at room temperature.

The intestinal segment was removed into HBSS, everted, threaded over a 5-mm diameter glass rod, and fixed in place with cotton ties. The rod was attached to a mechanical shaker and shaken into a tube containing HBSS at 4°C. Shaking was performed in 5-s bursts. The frequency of shaking which effectively detached epithelial cells without causing excessive damage was empirically determined. Initial estimates of cell yield and damage were made visually using a dissecting microscope. For colonic epithelial cells, cells and debris released after the first three bursts of shaking were discarded. All cells released by further shaking were collected into a fresh tube of HBSS at 4°C, centrifuged, and lysed in RNA lysis solution (RNAzol, Cinna/Biotex Laboratories, Houston, TX).

Segments of small intestine were shaken in tubes of HBSS at 4°C. Cells released by this procedure were immediately examined under a dissecting microscope. Initial shaking released mainly epithelial cells from villi. Shaking was continued until no further epithelial cells were released. The segment of intestine was then placed in a tube of HBSS with 30 mM EDTA for 10 min at room temperature. Further shaking released crypt epithelial cells, with negligible contamination from villus cells. Crypt cells were centrifuged, and the pallet lysed in RNAzol.

Collection of tissues

For isolation of RNA for Northern blotting, organs were removed and frozen in liquid N_2. For *in-situ* hybridization, organs were removed and placed in formol saline fixative overnight before dehydration, embedding in wax, and cutting of 10 μM sections according to standard protocols.

RNA preparation

RNA was prepared using the RNAzol reagent and the method of Chomczyski & Sacchi (1987). RNA was phenol–chloroform extracted twice, ethanol precipitated, and stored at −70°C. RNA was quantitated by spectrophotometry, and the degree of degradation and DNA contamination assessed by agarose gel electrophoresis and ethidium bromide staining. PolyA$^+$ RNA was prepared using oligo(dT) coated magnetic beads (Dynal, Wirral, UK).

DD-PCR

DD-PCR was performed using the technique of Liang & Pardee, as modified (Liang *et al.* 1992, Liang *et al.* 1994). Aliquots of 10 μg, 2μg, 0.2 μg and 0.02 μg of total RNA were reversed transcribed using T_{12}MA (where M represents an equimolar mixture of adenine (A), cytosine (C) and guanine (G)) as the primer, in the presence of 20 μM dNTPs, and RNAase inhibitor, for 1 h at 37°C. The reverse transcription reaction was then diluted 10-fold with water, and 2.5 μl of the reaction mixture used in a 25 μl PCR, using T_{12}MA (2.5 μM) and a random 10mer (0.5 μM) as PCR primers. The 10mers used in this study were: 5′-TACAAC-GAGG-3′, 5′-TGGATTGGTC-3′, 5′-CTTTCTACCC-3′, 5′-TTTTGGCTCC-3′, 5′-GGAACCAATC-3′, 5′-TCGATACAGG-3′, 5′-GTCCTACTCT-3′. The reaction was performed in the presence of 2 μM dNTP and 3.7 kBq/μl α-^{33}P-dATP (44.4 TBq/mmol, Amersham, UK), at 40°C annealing temperature, for 40 cycles, using Taq polynmerase (Gibco BRL, Paisley, UK).

PCR products were denatured by mixing with an equal volume of gel-loading buffer containing formamide (Sequenase 2.0 DNA sequencing kit, USB, Cleveland, OH) and heating to 95°C for 5 min before rapidly cooling on ice. Aliquots of 8 μl were loaded into the wells of a 6% polyacrylamide, 8 M urea gel. PCR samples from small intestine and large intestine were loaded on adjacent lanes for ease of comparison. Vacuum-dried polyacrylamide gels were exposed to X-ray film for 24 h at room temperature. The gel and autoradiograph were then aligned, and relevant cDNA bands excised from the gel using a sharp scalpel blade.

cDNA was eluted from the gel slice by heating to 95°C for 10 min, with constant shaking, in 50μl of water, ethanol precipitated in the presence of 5 μg glycogen, and resuspended in 10 μl H_2O. Four microlitres of the eluted cDNA was used as a template in a PCR using the original DD-PCR primers (T_{12}MA and the appropriate 10mer), 20 μM dNTP, Taq polymerase and an annealing temperature of 40°C, cycling for 40 cycles. This second PCR amplified the selected cDNA fragment, which could then be easily visualized by agarose gel electrophoresis and ethidium bromide staining. These amplified PCR products were directly subcloned into the pCRII plasmid (Invitrogen, San Diego, CA).

Following transformation of competent DH5α bacteria, recombinants were selected on agarose plates containing ampicillin and colonies containing cDNA inserts were identified using the β-galactosidase selection procedure (Sambrook, Fritsch & Maniatis 1989). White bacterial colonies were screened for insert size using a colony PCR technique using oligonucleotide primers flanking the cloning site (Sambrook *et al.* 1989). Selected colonies were expanded in liquid culture and plasmid DNA extracted using the alkaline lysis miniprep procedure (Sambrook *et al.* 1989). DNA was sequenced using the dideoxy chain termination technique, using M13 forward and reverse primer sequences flanking the cloning site of the pCRII plasmid (Sequenase 2.0 DNA sequencing kit, USB, Cleveland, OH). Complete sequence contigs were compiled by sequencing overlapping regions using appropriately designed primers.

5′-RACE

The 5′ region of the murine B1 cDNA was cloned by rapid amplification of cDNA ends (RACE), using the 5′ AmpliFINDER RACE kit (Clontech, Palo Alto, CA). Briefly, two primers complementary to the known B1 sequence were synthesized (P1, 5′-AGAGGTGTGATGGGAGACGC-3′ and P2, 5′-CAGCATTTGGGCTTCTTCCC-3′). P1 was used to prime the synthesis of single stranded cDNA using polyA^+ RNA prepared from mouse small intestine. Following first strand cDNA synthesis, the RNA template was hydrolysed with sodium hydroxide and excess primer removed by glass matrix adherence. T4 RNA ligase was used to ligate the AmpliFINDER oligonucleotide anchor (supplied by the manufacturer) to the cDNA. A nested PCR was then performed using the AmpliFINDER anchor oligonucleotide and P2 (internal to the P1 sequence) as primers, with the anchor ligated cDNA as the template. The resulting PCR product was ligated into the pCRII vector, cloned and sequenced as described above.

***In-situ* hybridization**

Slides for *in-situ* hybridization were processed using a modification of a standard method (Hogan, Costantini & Lacey 1986). Sections were dewaxed and rehydrated to distilled water through graded ethanol baths, treated with 0.2 M HCl for 20 min, rinsed with 10 mM Tris/1 mM EDTA buffer then treated with 20 μg/ml Proteinase K in the same buffer for 10 min. Proteinase K digestion was terminated by incubating the slides with 0.2% (w/v) glycine in

water for 30 s. Slides were washed three times in PBS then dehydrated through graded ehtanol baths and air dried for approximately 1 h. All steps were carried out at room temperature.

Hybridization mixture with 10^5 cpm/μl of ^{35}S-labelled riboprobe was pipetted onto sections on dry slides. The hybridization mixture consisted of 50% formamide, 10% dextran sulphate, 0.01% bovine serum albumin, 0.01% Ficoll 4000, 0.01% polyvinylpyrollidone, 1μg/ml tRNA, 10mM Tris-HCL pH 7.4, 1 mM EDTA, 1 mM sodium pyrophosphate, and 10 mM dithiothreitol. Slides were hybridized for 18 to 24 h at 42°C in shallow, airtight, plastic containers.

Hybridized slides were washed in a solution containing 0.5 M NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA (NTE buffer) for 15 min then treated with 20 μg/ml RNAase in A in NTE buffer for 30 min at 37°C. Slides were rinsed once with NTE buffer then washed in 2 × SSC (20 × SSC = 3 M NaCl, 0.3 M tri-sodium citrate) at 42°C for 1 h followed by 0.2 × SSC at 55°C for 1 h, with constant gentle agitation. Washed slides were dehydrated through graded ethanol baths containing 0.3 M ammonium acetate, air dried and processed for autoradiography.

Autoradiography

Dried slides were dipped in Amersham light microscopy emulsion (Amersham, Little Chalfont, Buckinghamshire) and exposed in the dark for 3 to 6 days at 4°C. Autoradiographs were developed for 5 min in Kodak D19 developer at 17°C and counterstained with haematoxylin and eosin.

Northern blotting

Northern blots were prepared using standard techniques with 20 μg per lane of RNA from various tissues (Sambrook *et al.* 1989). The positions of ribosomal RNA bands were established by ethidium bromide staining and ultraviolet (UV) transillumination prior to blotting. RNA was blotted onto nylon membrane and the blot was hybridized to a ^{32}P-labelled B1 cDNA probe for 18 h at 42°C. Filters were washed to a final stringency of 0.1 × SSC at 60°C and exposed to X-ray film at −70°C.

Radio-labelled probes

DD-PCR products were subcloned into the pCRII vector as described, which supports T7 and SP6 DNA dependent RNA polymerase activity, allowing α-^{35}S-rATP labelled sense and antisense riboprobes to be generated directly using a standard protocol and the Boehringer-Mannheim SP6/T7 transcription kit (Boehringer-Mannheim, Germany). α-^{32}P-dATP labelled DNA probes for Northern blots were prepared by the random oligonucleotide primer method using DNA templates obtained by restriction enzyme digests of pCRII subclones containing DD-PCR inserts (Sambrook *et al.* 1989). The sizes of cloned DD-PCR products ranged from approximately 250 bp to 500 bp.

***In-situ* hybridization with digoxigenin-labelled probes**

Digoxigenin-labelled riboprobes were generated using digoxigenin-labelled dUTP as a substrate for the T7 or SP6 transcription reaction, using the Boehringer-Mannheim DIG RNA labelling kit (Boehringer-Mannheim, Germany). *In-situ* hybridization and washing were carried out as for ^{35}S-labelled probes, exept that slides were not dehydrated after the final washes. Hybridized slides were incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer-Mannheim DIG Nucleic Acid Detection kit). Hybridiza-

tion was subsequently detected by reacting antibody treated slides with NBT colour reagent and x-phosphate substrate (Boehringer-Mannheim DIG Nucleic Acid Detection kit) for 6 to 18 h at room temperature, after which slides were mounted under glass coverslips and examined directly.

RESULTS

Differential display PCR using RNA derived from small and large intestinal epithelium produced results that were comparable to those obtained from cell lines. As can be seen in Figure 1, there were a number of clear differences between the small intestinal and large intestinal samples. A minor modification of the DD-PCR protocol was introduced in an attempt to reduce the complexity of the resulting DD-PCR band pattern. This consisted of reducing the amount of RNA used in the initial reverse transcription reaction. The amount of input mRNA could be reduced by 50-fold (from 10 μg to 0.2 μg) before the intensity of bands was comprised (results not shown). The band pattern was simplified to some extent by reducing the amount of input RNA (Figure 1), but this modification was ultimately not required as individual bands could easily be distinguished using the standard protocol.

Four separate experiments were performed, each using different random 10mers. A total of 34 DD-PCR clones were isolated for further analysis, care being taken to select prominent bands which appeared to be differentially expressed, and which had a size greater than or equal to approximately 200 bp. Seven different 10mers were used. Fifteen of the 34 clones were not sequenced or yielded incomplete sequence information. Eight clones showed homology to genes of doubtful significance, and two clones were clearly artefactual. Three clones showed homology to genes of potential interest in Paneth cell biology, and were selected for further study (see below). Six clones showed no homology to known genes, and some of these clones were used for *in-situ* hybridization. Characteristics of the isolated clones are summarized in Table 1.

The initial sequence from clone B1 showed homology to rat platelet phospholipase A2. Phospholipase activity and immunoreactivity had previously been localized to Paneth cells (Lechene-de-la-Porte, Lafont of Lombardo 1986, Kiyohara *et al.* 1992).

Sequence analysis demonstrated that clone C5 was homologous to the murine homologue of the APC (adenomatous polyposis coli) gene, which is thought to be involved in regulation of the mitotic and migratory behaviour of intestinal epithelial cells (MacPhee *et al.* 1995). Clone D3 was homologous to a human neutrophil defensin gene. A number of defensin homologues (known as cryptdins) have recently been identified as Paneth cell products (Lehrer, Lichtenstein & Tanz 1993, Huttner, Selsted & Ouellette 1994). These clones were therefore analysed further by *in-situ* hybridization. However only B1 mRNA transcripts were specifically detected in Paneth cells and this clone was selected for further study.

The localization of B1 transcripts in the small intestinal epithelium, by *in-situ* hybridization, is shown in Figure 2. B1 riboprobes hybridized strongly to cells at the bases of the crypts, but not to other cells of the lining epithelium. Using digoxigenin labelled riboprobes, it is evident that the B1 antisense probe hybridized only to Paneth cells, characterized by prominent apical granules which can be seen at higher magnification (Figure 2c). This hybridization is specific as shown by the absence of reactivity in control intestinal sections hybridized to sense strand riboprobes (Figures 2b & d).

Unexpectedly, B1 riboprobes also hybridized to cells within the epithelium of the large intestine (Figure 3). This hybridization signal was of a lower intensity, and was localized uniformly to epithelial cells at the bases of the colonic crypts, throughout the large intestine.

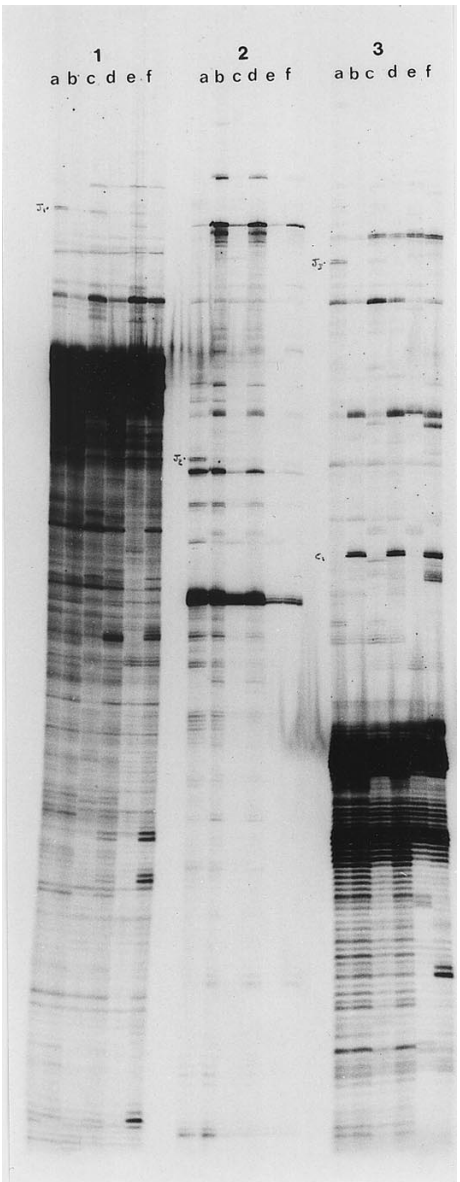

Figure 1. Representative DD-PCR of RNA isolated from small intestinal epithelium and large intestinal epithelium. RNA samples from small intestinal epithelium and large intestinal epithelium were reverse transcribed and subjected to DD-PCR as described in Materials and Methods. DD-PCR was carried out using trace amounts of α-^{33}P-dATP and the autoradiograph of the polyacrylamide gel is shown. Aliquots of the DD-PCR of small intestinal epithelium and large intestinal epithelium were loaded side by side. The lanes are: (a) small intestine, 10 μg RNA, (b) large intestine, 10 μg RNA, (c) small intestine, 2μg RNA, (d) large intestine, 2 μg RNA, (e) small intestine, 0.2 μg RNA and (f) large intestine, 0.2 μg RNA. The 10mers used were: (1) 5′-TACAACGAGG-3′, (2) 5′-TGGATTGGC-3′, and (3) 5′-GTCCTACTCT-3′. Despite the cellular complexity of the source from which cDNA samples were prepared, the number of bands obtained by DD-PCR was comparable to that obtained using single cell lines and most higher molecular weight bands could be easily distinguished. The cDNA marked J1 (top band in lane 1a) migrated with the 300 bp marker on agarose gel electrophoresis.

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Table 1. Summary of analysis of 34 DD-PCR clones

No homology to sequences in database*	6 clones
Homologous to ubiquitously expressed (housekeeping) genes	3
Homology of doubtful significance	5
Homology to intestine specific genes, or other potentially interesting genes*	1 (APC‡ gene homologue)
Homology to known Paneth cell products*	2 (secretory phospholipase A2, and human neutrophil defensin)
Cloning artefacts or contaminants	2
Not sequenced, or incomplete sequence obtained	15
Total	34

Thirty-four differentially expressed cDNA bands from four separate DD-PCR experiments were subcloned into the pCRII plasmid and partially sequenced as described in Materials and Methods. Partial sequences were compared to sequences in the EMBL and Genbank cDNA sequence databases. Some clones, marked *, were selected for *in-situ* hybridization screening; of these only clone B1 hybridized specifically to Paneth cells. ‡Adenomatous polyposis coli.

Paneth cells are absent from the large intestine and the cells to which the B1 probe hybridized were not morphologically similar to Paneth cells. The point along the longitudinal axis of the crypt at which B1 transcripts disappear from colonic crypt epithelial cells is not morphologically distinct from the rest of the crypt epithelium.

Hybridization of B1 riboprobes to large intestine was confirmed by Northern blot analysis which showed that an approximately 1 kb transcript was expressed in colon as well as small intestine, although the colonic expression was less intense (Figure 4). Northern blot hybridization also confirmed, *in-situ* hybridization experiments (results not shown) which showed that B1 mRNA was not expressed in normal liver or spleen sections.

The B1 cDNA clone was further analysed by complete sequencing of the clone obtained by DD-PCR and by cloning and sequencing of the 5′ upstream region obtained using the 5′-RACE (rapid amplification of cDNA ends) technique. The full length sequence of the B1 cDNA is shown in Figure 5. The sequence shows almost complete identity to that of the enhancing factor cDNA described by Mulherkar *et al.* 1993a). Furthermore the B1 sequence is homologous to other secretory phospholipase A2 cDNA species. We therefore propose that B1 be designated the murine intestinal secretory phospholipase A2 cDNA (murine *i*-PLA2).

DISCUSSION

The successful cloning of an intestinal secretory phospholipase by DD-PCR, using whole epithelial preparations as a source of RNA is a significant advance in the application of DD-PCR technology. Given the complexity of the RNA mixture upon which DD-PCR was performed, it may have been anticipated that the density of bands on DD-PCR gels would be too great to allow individual differences between small intestinal and large intestinal RNA samples to be detected. This, however, was not the case. Nonetheless, it was also possible to reduce the density of bands by using lower amounts of RNA template in the cDNA synthesis step prior to the DD-PCR reaction. This modification did not adversely affect the quality of DD-PCR, although it did reduce the intensity of all bands and alter the pattern of bands (Figure 1). Reducing the amount of RNA used in the DD-PCR reaction was not required for

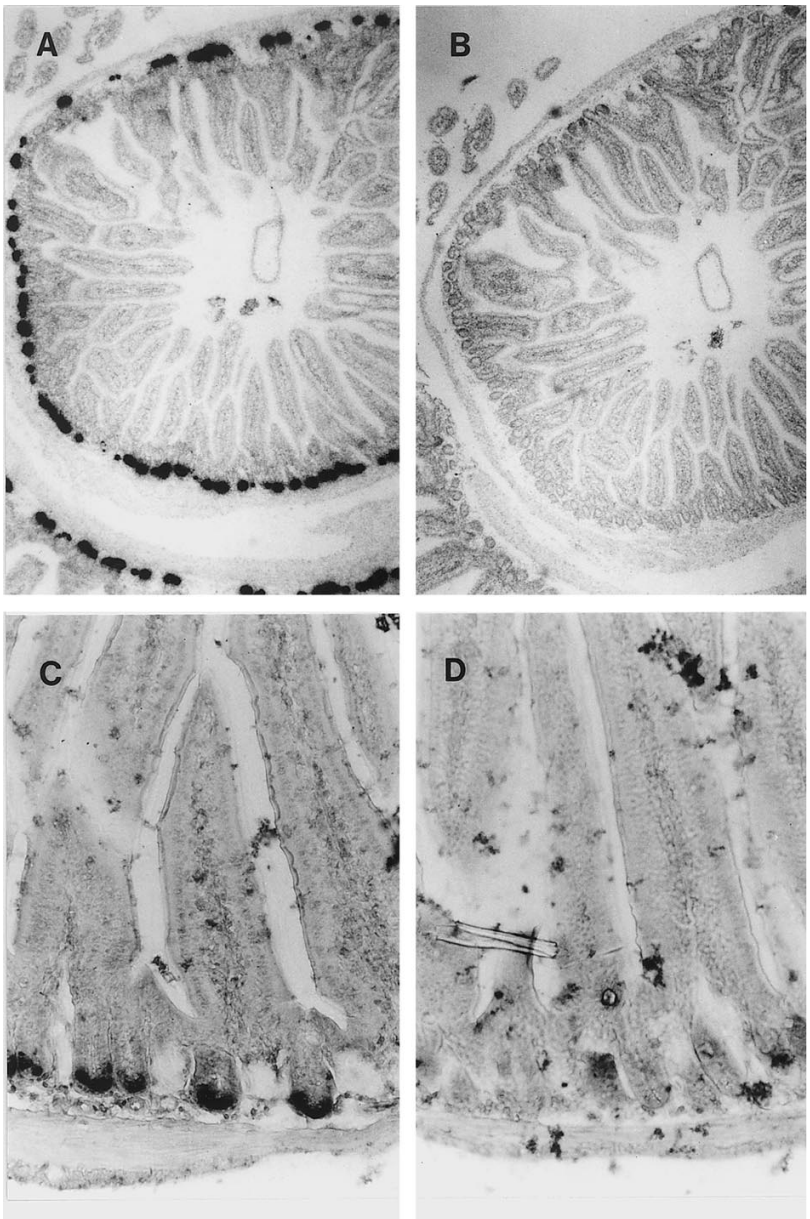

Figure 2. The B1 DD-PCR cDNA clone hybridizes specifically to Paneth cells of the small intestinal crypt epithelium. Sections of mouse small intestine were hybridized to sense and antisense riboprobes derived from the B1 DD-PCR clone, as described in Materials and Methods. (A) There is a high density of autoradiographic grains over cells at the bases of the small intestinal crypts after hybridization to a ^{35}S-labelled antisense riboprobe derived from B1. (B) Using a control ^{35}S-labelled sense strand riboprobe derived from B1, no specific hybridization is seen. (C) Digoxigenin labelled antisense B1 riboprobe hybridizes to cells at the bases of the small intestinal crypts which contain a basal nucleus and promine apical granules. This position in the crypt and these microscopical appearances are typical of Paneth cells. (D) Hybridization of sections to control B1 sense strand digoxigenin labelled riboprobes results in no detectable signal. A and B are photomicrographs photographed through a $\times 10$ objective, and C and D were photographed through a $\times 20$ objective.

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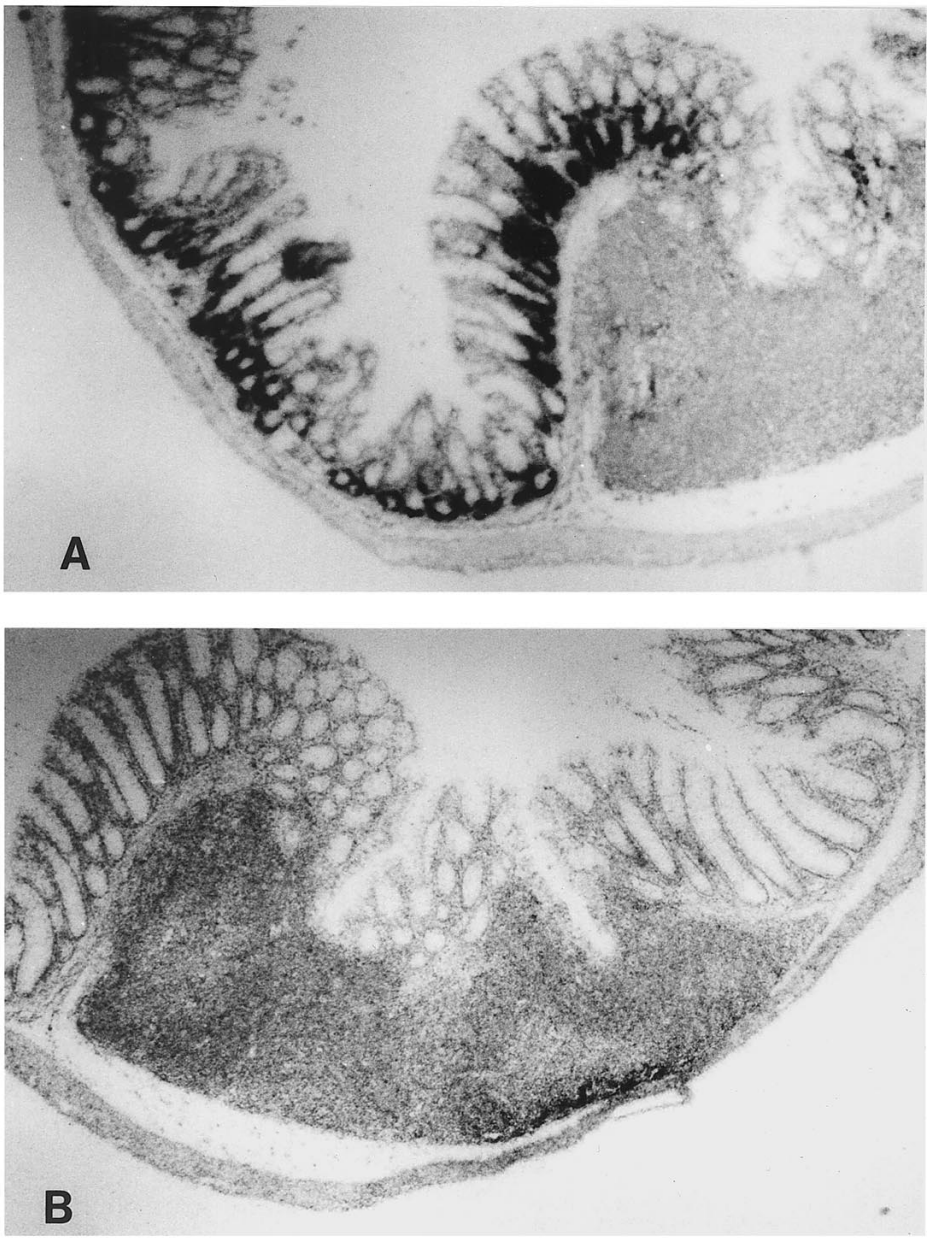

Figure 3. B1 DD-PCR cDNA clone hybridizes to cells in the basal region of the colonic crypt epithelium. Sections of normal mouse large intestine were hybridized to ^{35}S-labelled riboprobes derived from clone B1, as described in Materials and Methods. (A) Antisense B1 riboprobes hybridized to cells in the basal one-third to one-half of the colonic crypt epithelium and not to lumenal epithelium cells or to lamina propria cells. There is no clear morphological difference between cells in the basal portion of the crypt, which hybridize to the B1 probe, and other epithelial cells closer to the colonic lumen, which do not hybridize. (B) Using control B1 sense strand probes, there is no hybridization to sections. Photographs of sections wre taken through a $\times 10$ objective.

Figure 4. B1 mRNA transcripts are detected in small intestine and large intestine, but not in liver or spleen. Total RNA from whole small intestine, whole large intestine, liver and spleen of normal PO mice was subject to Northern blot hybridization as described in Materials and Methods. Twenty micrograms of RNA was loaded in each lane, electrophoresed through a 1.2% agarose gel containing formaldehyde and ethidium bromide, and blotted onto nylon membrane. UV illumination was used to determine the position of the 28S and 18S ribosomal RNA bands. The blot was hybridized to a ^{32}P-labelled B1 cDNA probe, washed to a final stringency of $\times 0.1$ SSC at 60°C, and exposed to X-ray film at -70°C for 72 h. There is a single RNA species of approximately 1 kb detected in small intestine (SI) and large intestine (LI). The intensity of hybridization is approximately five times greater in the RNA from small intestine. No signal is detected in spleen (S) or liver (L) RNA samples.

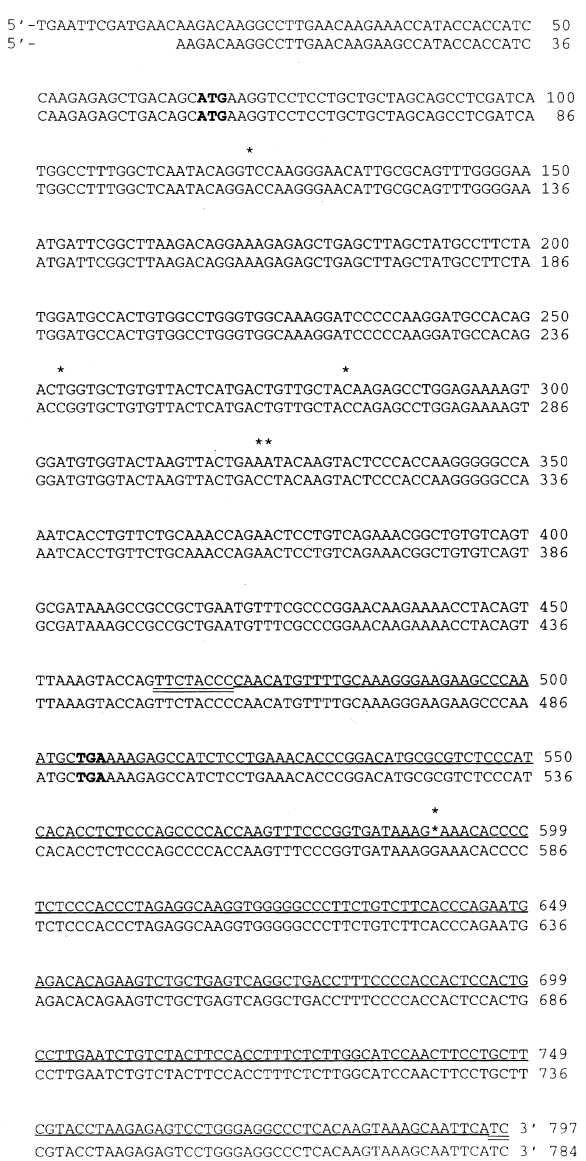

Figure 5. The nucleotide sequence of B1 cDNA clone is highly homologous to murine enhancing factor cDNA. The nucleotide sequence of the B1 cDNA clone isolated by DD-PCR is shown underlined, with the additional sequence derived from the 5′-RACE cloning shown in normal typeface. The published sequence of murine enhancing factor is shown below the B1 sequence. The positions of the DD-PCR 10mer and the T_{12}MA primer are shown by double underlining of the B1 sequence. Eight residues from the 3′ end of the 10mer match the B1 sequence. The complete B1 sequence contains an additional 14 nucleotides within the 5′ untranslated region of the cDNA. There is a single nucleotide (G) insertion in the 3′ untranslated region of the enhancing factor sequence at position 576. Base mismatches between the sequences are shown by * above the B1 sequence. These nucleotide differences would lead to four amino acid differences between thew B1 and enhancing factor protein sequences. The putative translation initiation and termination codons are shown in bold typeface. Both sequences are highly homologous to rat and human secretory phospholipase A2 and to secretory type II phospholipase A2 proteins in general.

successful cloning; for example, the B1 clone encoding the *i*-PLA2 cDNA was isolated using the conventional DD-PCR protocol.

DD-PCR has been extensively used to examine differences in gene expression between isolated cell lines maintained in tissue culture. However, more recently workers have used whole tissues comprising more than one major cell type in order to study the differential expression of genes in such tissues. One such example has been the cloning of a gene involved in mediating the obese phenotype in ob/ob mutant mice (Qu *et al.* 1996). This study is similar to the one we have undertaken insofar as whole tissue extracts were used as a source of RNA. However, in that particular study identical organs were used for comparison and a single genetic mutation had been selected for prior to the DD-PCR analysis. Thus one may expect that the complexity of mRNA differences between cell populations in that study would be much less than that between whole small intestinal epithelium and large intestinal epithelium.

The function of Paneth cells in the small intestine is still largely unknown. Recent advances in our understanding of these cells has come from experiments in which novel gene products have been localized to these cells, implicating the cells in processes related to the function of these gene products. Thus Paneth cells are thought to be involved in antibacterial defence through the localization of lysozyme and defensins, in regulation of intestinal chloride secretion through the localization of guanylin, and in the maintenance of a stem cell micro-environment through the localization of tumour necrosis factor alpha (TNFα) and epidermal growth factor (EGF) (Chung, Keshav & Gordon 1988, Keshav *et al.* 1990, de-Sauvage *et al.* 1992, Poulsen, KrygerBaggesen & Nexo 1996). Phospholipase A2 is known to have antibacterial activity, however, there is some evidence that the Paneth cell derived phospholipase A2 may enhance the effect of EGF, hence the term enhancing factor (Mulherkar *et al.* 1993b, Harwig *et al.* 1995). Furthermore, the *i*-PLA2 gene has recently been identified as the MOM-1 locus gene, responsible for modifying the pro-neoplastic effect of the APC gene (MacPhee *et al.* 1995). Thus the exact function of *i*-PLA2 derived from Paneth cells is not clear.

Expression of PLA2 has been noted before in the large intestine, but has only recently been shown to localize at the mRNA and protein level to cells other than Paneth cells (this report, and Haapamaki *et al.* 1997). Previous studies had demonstrated PLA2 expression only in Paneth cells (Nevalainen, Gronroos & Kallajoki 1995). The uniform expression of *i*-PLA2 mRNA in the normal mouse large intestine, in the basal part of the crypt epithelium, is unique. As there is no morphologically distinct population of cells which expresses *i*-PLA2 in the colonic crypt epithelium, it appears that a gradient of *i*-PLA2 gene expression is maintained along the crypt axis. This observation also suggests that Paneth cell functions may be performed by other cells in the epithelium which are morphologically distinct from Paneth cells. Metaplastic Paneth cells, which appear in the epithelium of the large intestine under inflammatory conditions, may therefore differentiate from precursors which are capable of some Paneth cell-like functions (Schumacher 1993).

The aim of the experimental strategy outlined here was to increase our understanding of Paneth cell biology by identifying novel genes which are highly expressed or exclusively expressed in Paneth cells as opposed to other epithelial cells. Although *i*-PLA2 is highly expressed in Paneth cells, it is not restricted to these cells. Thus the DD-PCR technique used here was capable of distinguishing quantitative differences in expression of *i*-PLA2 between small and large intestinal epithelium.

The DD-PCR technique may be used for further analysis of cell-specific gene expression in complex tissues such as the intestinal epithelium. Although the number of potentially

interesting clones in this study was relatively small compared to the total number of clones analysd, many clones can be rapidly screened by partial DNA sequencing and *in-situ* hybridization. Given the low number of Paneth cells relative to the total number of cells in the intestine, *in-situ* hybridization remains the most sensitive and specific way of identifying transcripts that are expressed primarily in Paneth cells. The efficiency of the technique may be further enhanced if relatively pure intestinal epithelial crypts or crypt cell preparations are used. Thus further application of the DD-PCR technique may result in the identification and characterization of other Paneth cell specific genes.

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