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Identification of differentially expressed mRNAs in human fetal liver across gestation

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ABSTRACT

Differential gene expression, with its precise start and stop times, is believed to be critical for the programmed development of new cells and tissues. Within the developing fetus, one tissue of particular interest is fetal liver. This organ undergoes rapid changes in the pathway toward liver development *in utero* since it is also the major site of hematopoiesis, until bone marrow hematopoiesis predominates. Believing that patterns would emerge from the bi-weekly large-scale inspection of expressed genes in the fetal liver, we employed differential display reverse transcription–polymerase chain reaction (DDRT–PCR) as our primary inspection tool. Using DDRT–PCR, we isolated cDNAs differentially expressed throughout fetal liver development and in adult liver. We displayed ~25 000 cDNAs from 10 and 24 week fetal liver and adult liver. From this initial screen, we determined that ~0.1–1% of the mRNA population undergoes expression changes. We extracted, purified and sequenced 25 differentially displayed cDNA bands. Fourteen cDNAs had similarities to known genes, while 11 cDNAs were not similar to any characterized gene. The differentially expressed cDNAs from known genes present in fetal liver include α-fetoprotein, stem cell factor, erythroid α-spectrin, 2,3-bisphosphoglycerate mutase, insulin-like growth factor-2, porphobilinogen deaminase and Mac30. The differentially expressed cDNAs present in adult liver but not in 10 week fetal liver were nicotinamide deaminase, human fibrinogen-related protein and α-acid glycoprotein. The majority of differentially expressed genes found during this effort appear to be turned on during organogenesis, however, some genes were found that are apparently turned off completely.

INTRODUCTION

That human developmental processes are complex is underscored by the recalcitrance of these systems in yielding clues regarding the key steps guiding embryogenesis and fetal development. Certainly the precise turning on or off of genes at uncompromising time points is critical to the process of normal development. And while we know that certain genes are critical to normal development in the human fetus, the overall pattern of interacting genetic elements remains obscure. In addition, little is known of the genetic cascade that results in complex multicellular organizations, such as organ formation. Deciphering the pattern of interacting genes during development is an important step. Hematopoiesis is a process that undergoes tremendous change during fetal development and as such is an excellent system for analyzing the molecular processes that control growth and differentiation. This is a time when changes in gene expression should be very frequent and hence most obvious.

Erythroid cells are one of the distinct blood cell lineages generated from a small population of pluripotent hematopoietic stem cells that are established at multiple sites during vertebrate ontogeny. Fetal erythroid cell production occurs within the blood islands of the yolk sac, a phenomenon conserved from amphibians to mammals. This transient population of cells are nucleated in all vertebrates and termed ‘primitive’. The primary site of fetal erythropoiesis then switches to the liver. This organ generates a smaller, morphologically distinct ‘definitive’ population of erythroid cells that, in mammals, extrude their nuclei. In mammals, the final switch begins just prior to birth when definitive erythropoiesis becomes permanently established in the bone marrow (1). Thus, the identification and characterization of genes associated with growth and differentiation of the hematopoietic system are important.

Our goal is to identify differentially expressed genes at the early stages of hematopoietic development. We have employed the mRNA differential display technique first reported by Liang and Pardee (2) and further optimized by Bauer *et al.* (3) to screen for changes in gene expression during the transition between the cell division and cell expansion phases in developing liver.

Differential display reverse transcription–polymerase chain reaction (DDRT–PCR) facilitates the cloning of differentially expressed mRNAs, including rare mRNAs that may be difficult to clone with techniques such as the differential screening of cDNA libraries. This method has been successfully used to isolate

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genes differentially expressed in cancers (4–6), heart diseases (7,8), diabetes (9), embryogenesis (10), the brain (11,12) and growth factor stimulation (13), as well as in other situations. A similar method called arbitrarily primed RNA fingerprinting and differential gene expression by display of 3′-end restriction fragment of cDNAs has also been described (14,15). Recently, a subtraction enrichment protocol called SABRE (selective amplification via biotin and restriction-mediated enrichment) and differential substraction display have been used to identify differentially expressed genes (16,17).

Briefly, in mRNA differential display, a subset of mRNAs is reverse transcribed into the corresponding first strand cDNA. An arbitrary primer is added to the reaction and the cDNA is amplified by PCR and displayed on a sequencing gel. It is estimated that ~15 000–20 000 different mRNAs are expressed in each mammalian cell. We used two different primer sets (2,3) to display ~25 000 cDNAs. To confirm differences in mRNA abundance, the differentially expressed cDNAs were isolated, cloned, sequenced and used to probe northern blots and Southern blots of cDNA or as probes for RNase protection analysis (RPA). We report the identification of several known and unknown genes that show differential expression between 10 and 24 week fetal liver and between the fetal liver samples versus adult liver. We found that α-fetoprotein (AFP), stem cell factor (SCF), erythroid α-spectrin (ES), 2,3-bisphosphoglycerate mutase (EBGM), Mac30, porphobilinogen deaminase (PBD) and insulin-like growth factor-2 (IGF-2) mRNAs are highly expressed in fetal liver but only weakly or not at all in adult liver. We also demonstrate that nicotinamide deaminase (ND), human fibrinogen-related protein (HFREP) and α-acid glycoprotein (AGP) are expressed in adult liver but not fetal liver. Several genes of unknown function were also found to be differentially expressed between fetal and adult liver.

MATERIALS AND METHODS

Materials

Human fetal and adult liver, bone marrow and cord blood samples were obtained from a non-profit research tissue bank (Anatomic Gift Foundation, Woodbine, GA or Advanced Bioscience Resources Inc., Alameda, CA) in accordance with the guidelines of the Department of Health and Human Services regulations for the protection of human subjects, the National Organ Transplant Act and the Uniform Anatomical Gift Acts as well as other federal and state regulations.

Total RNA isolation and differential display

Average warm ischemia time for the liver tissue was between 8 and 15 min. Total cellular RNA was extracted from frozen liver tissue (10, 12, 14, 16, 18, 20, 22 and 24 week human fetal and adult liver) by using the RNeasy protocol (Qiagen, Chatsworth, CA). Human fetal liver samples from different gestational ages were collected in tissue culture medium. The fetal blood from liver cells (FBL) was isolated by mechanical mincing of fetal liver and filtered through a 74 μm filter to remove tissue clumps. The filtered cell suspension was pelleted by centrifugation at 1500 r.p.m. for 10 min at 4°C and snap frozen using liquid nitrogen. Total RNA was isolated from cell pellets using the same procedure as for fetal liver tissue. mRNA differential display was performed as described (2,3) with some modifications (18). Briefly, total RNA (50 μg) was treated with 20 U DNase I [Boehringer Mannheim Corporation (BMC), Indianapolis, IN], 40 U human placental RNase inhibitor (RNasin; Promega Corp., Madison, WI), 10 mM Tris–HCl, pH 7.4, 1.5 mM $MgCl_2$, incubated at 37°C for 30 min and heated at 65°C for 10 min to destroy the enzyme. The RNA was then repurified using the Qiagen RNeasy protocol. First-strand cDNA was synthesized using oligo(dT) primers that had two anchored nucleotides at the 3′-end. The nine primer sequences used were T11VA, T11VC and T11VG (where V may be either A, C or G). In some reactions, oligo(dT) primers with a single anchored nucleotide at the 3′-end were used (2). First-strand cDNA template was synthesized by mixing 5.6 μl DEPC-treated water, 3 μl of DNased-RNA (200 ng) and 2.6 μl of the oligo(dT) primer. The reactions were heated for 5 min at 65°C and placed on ice to cool. Then 4 μl of 5× first-strand synthesis buffer [Gibco BRL (BRL), Gaithersburg, MD] 2 μl of 0.1 M DTT, 0.8 μl of 0.5 mM dNTPs and 0.5 μl of RNasin were added into the reaction mixture and incubated at room temperature for 2 min. The synthesis reaction was started by adding 1.5 μl (300 U) of MMLV reverse transcriptase (BRL) to each tube and the tubes were further incubated at 37°C for an additional 90 min. The tubes were then heated for 5 min at 95°C, centrifuged briefly, aliquoted and stored at –20°C for the subsequent PCR reaction.

The 3′ anchored primer was labeled as follows (19). A 120 pmol solution of T11VV or H-T11V was radiolabeled in a volume of 120 μl containing 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 250 μCi [γ-^{33}P]dATP and 10 U T4 polynucleotide kinase (BMC) and incubated at 37°C for 1 h with a subsequent incubation at 65°C for 10 min to destroy the enzyme. The unincorporated nucleotides were removed using a Centricon 3 column (Pharmacia Biotech, Piscataway, NJ). For PCR amplification of a given subgroup, 9.9 μl water, 2 μl 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl), 1.2 μl of a 100 μM solution containing a mix of each dNTP, 2 μl of an arbitrary primer (2 μM), 4 μl of 1 μM ^{33}P-end-labeled downstream primer, 0.4 μl of *Taq* DNA polymerase and 0.5 μl of reverse transcriptase reaction were mixed. A mix of all of these reagents, excluding the primers and template, was made first and aliquoted into each tube for more uniform results. The first denaturation was done at 94°C for 5 min and then 30 rounds of PCR were done at 95°C for 30 s, 40°C for 2 min and 72°C for 30 s. A final cycle at 72°C for 10 min was done to ensure completion of the final extension.

The amplified cDNA was then separated on a 6% DNA sequencing gel containing 7 M urea. The gel was dried without fixing and autoradiographed on Kodak Biomax film.

Retrieval and cloning of differential display products

Differences in cDNA patterns between 10 and 24 week fetal liver and adult liver differential display reactions were identified and the bands corresponding to these cDNAs were excised from the dried gels. Each gel slice was incubated in 100 μl water at room temperature for 10 min then boiled for 10 min to release the PCR products. The cDNA was recovered by ethanol precipitation in the presence of 0.3 M sodium acetate and 5 μl 10 mg/ml glycogen. The DNA pellet was redissolved in 20 μl water. The eluted products were subjected to PCR in a reaction mixture containing 5 μl of the gel slice eluate, 1× PCR reaction buffer (BMC), 2.5 mM $MgCl_2$, 50 μM dNTPs, 1 μM oligo(dT) and 5′ primers and 2 U of *Taq* DNA polymerase. The first denaturation was done

at 95°C for 4.5 min, followed by 40 cycles at 94°C for 30 s, 40°C for 2 min and 72°C for 30 s. A final cycle of 72°C for 10 min was done to ensure completion of the final extension. A portion of the PCR product was electrophoresed on a 1.5% agarose gel to confirm a clean single band reamplification of the DNA fragment. PCR bands of the expected size were purified with a Qiagen PCR purification kit and ligated into the TA cloning vector. The DNAs from each of five colonies for each band were sequenced. The sequences were aligned to each other using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). Those DNAs with different sequences were then used as probes for northern blots, RPAs and RT–PCR Southern blots.

Cloning and DNA sequencing

Reamplified cDNAs were cloned into the pCR II vector using the TA cloning kit from Invitrogen (San Diego, CA) and digested with *Eco*RI or *Hin*dIII to confirm the insert. Prior to DNA sequencing, minipreparation of recombinant plasmids was performed using a Qiagen plasmid isolation kit. The plasmids were sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit with M13 primers on an ABI 373A DNA sequencing system (Applied Biosystems/Perkin-Elmer Corp., Foster City, CA). Sequences were compared with the National Center of Biotechnology Information non-redundant sequence database using the BLASTX and BLASTN programs (20,21). Significant sequence similarity at the nucleotide level was defined as at least 96% identity over the entire length of the clone.

Northern blot analysis

Total RNA was electrophoresed in a 1.2% agarose gel containing 6% formaldehyde and 1× MOPS buffer (1× MOPS buffer is 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA). The amounts of RNA for each preparation were roughly equivalent on the blot, as judged by ethidium bromide staining of rRNA. Transfer of RNA to Hybond nylon membranes was carried out with a TurboBlotter using a transfer buffer containing 3 M NaCl, 8 mM NaOH and 2 mM Sarkosyl (Schleicher & Schuell, Keene, NH). After transfer, the RNA was crosslinked to the membrane in a Stratalinker (Stratagene, La Jolla, CA). Some blots were then stained with methylene blue to check the efficiency of transfer. For analysis of RNAs, cDNA probes were labeled with [α-^{32}P]dCTP using a random prime labeling kit (BMC) and hybridized to the blot (10^6 c.p.m./ml hybridization solution) in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate and 100 µg/ml denatured salmon sperm DNA at 42°C for 16–24 h (22). After hybridization, the filters were washed twice in 2× SSC, 0.2% SDS for 10 min at room temperature and twice in 0.1× SSC, 0.1% SDS for 30 min at 55°C. Finally, the filters were washed with 2× SSC for 5 min at room temperature. Hybridization signals were analyzed using the PhosphorImager scanning system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For a loading control, some blots were stripped and reprobed with a GAPDH cDNA probe.

Antisense RNA probe preparation and RPAs

For some probes (IGF2, PBD and LIV-2), a PCR fragment was subcloned into the *Eco*RI site of the TA cloning vector. The recombinant plasmid was linearized with *Bam*HI restriction enzyme and a single-stranded, homogeneously radiolabeled antisense RNA probe was synthesized by T3 RNA polymerase in the presence of [α-^{32}P]UTP (23). A total of 10^5 c.p.m. of each antisense RNA probe was simultaneously hybridized overnight with 5 µg of total RNA in a 10 µl reaction volume containing 80% formamide, 0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA. The mixture was diluted in 50 µl of a solution containing 10 mM Tris–HCl, pH 7.5, 0.3 M NaCl and 10 mM $MgCl_2$ and treated with RNase H, RNase A and RNase T1 at 30°C for 30 min, followed by treatment with proteinase K (300 µg/ml) in 0.1% SDS at 37°C for 15 min. The mixture was phenol/chloroform extracted, ethanol precipitated, denatured at 80°C for 5 min and electrophoresed on a 6% polyacrylamide/bisacrylamide (19:1) 8.3 M urea-containing gel. After transfer to Whatman paper, the gel was dried and scanned by a PhosphorImager.

Southern blot analysis of cDNA products

Double-stranded cDNA was synthesized from 2 µg of mRNA from 10 week fetal and adult liver using a cDNA Synthesis kit, digested with *Rsa*I and ligated to an adapter (Clontech, Palo Alto, CA). The inserts were amplified by PCR with primers homologous to the adapter sequences and the products were fractionated on 1% agarose gel and transferred to Hybond membranes. Southern blot hybridization was performed according to standard procedures (24)

RESULTS AND DISCUSSION

Differential display and overview of the cDNAs

We displayed a broad spectrum of expressed genes from fetal and adult liver using optimized DDRT–PCR reaction conditions (18) and used northern blots, RPAs and Southern blot analysis of cDNA products to confirm that the isolated cDNA fragments came from differentially expressed mRNAs. The estimated number of different mRNAs in a eukaryotic cell is ~15 000, with most of the messages expressed at a low level (2,25). In total we have displayed ~25 000 cDNAs, which is more than the predicted number of expressed genes per cell. To minimize the occurrence of finding false positives, we used 5′-labeled primers, multiple gestational ages of fetal liver and stringent conditions (clear intensity difference between fetal and adult cDNA signal) to select cDNAs for subsequent analysis. Table 1 and 2 list the differentially expressed cDNAs between 10 and 24 week fetal liver and adult liver RNA. We show that 0.12% (19/15 000 expressed mRNAs) of the cDNAs analyzed show differential expression. This number is probably a 10-fold underestimate, as we extensively analyzed only ~10% of the cDNAs that showed differential expression on the original DDRT–PCR profile. Based on the total of 15 000 expressed mRNAs screened, ~0.1–1% of the mRNA population undergoes changes from fetal to adult liver on the basis of northern blots, RPAs and Southern analysis of RT–PCR products.

Table 1. Analysis of cDNA fragments identified by mRNA differential display

Clone no.	3′ Primer	5′ Primer	10	16	24	Adult	Sequence similarity	Accession no.
			Northern blot analysis					
LIV-1	T11CG	1	+	+	+	–	EST	AA887845
6	T11CG	10	+	+	+	–	SCL	M63589
1	T11CC	25	+	+	+	–	EBGM	M23068
K-5	T11GA	8	+	+	+	–	Mac30	L19183
K-10	T11CC	14	+	+	+	–	ES	M61877
T1	H-T11C	H-AP25	+	+	+	-	AFP	J00077
TA 5-1	H-T11C	H-AP29	+	+	+	–	IGF-2	S62621
A2	H-T11G	H-AP54	–	–	–	+	ND	U08021
A5-1	H-T11C	H-AP32	–	–	–	+	HFREP-1	D14446
D4	H-T11C	H-AP54	–	–	–	+	AGP	X02544
			Ribonuclease protection assay					
TA 21	H-T11A	H-AP28	+	+	+	–	PBD	X04217
LIV-2	H-T11C	H-AP56	+	+	+	–	EST	AA112480

10, 16, 24 are fetal livers of the indicated gestational week. Adult is adult liver. The sequences of the numbered 5′ primers are reported in Bauer *et al*. (3). The H-AP and H-T11N primers were obtained from GenHunter Corp. (Nashville, TN).

Table 2. Analysis of cDNA fragments by Southern blotting of RT–PCR products

Clone no.	3′ Primer	5′ Primer	Southern blot analysis		Sequence similarity	Accession no.
			10	Adult		
48	T11CG	10	ND	ND	Myeloblast	D13639
LIV-3	T11CG	13	+	–	New sequence	
LIV-4	T11CC	14	ND	ND	New sequence	
34-8	T11CA	24	+	+	βγ-Crystallin	U83115
K7-10	T11CG	11	+	–	HMGI-C phosphoprotein	U28754
LIV-5	T11GA	19	ND	ND	EST	AI018193
LIV-6	T11GG	1	+	+	New sequence	
LIV-7	T11GG	23	+	–	EST	AA858396
LIV-8	T11CG	22	+	–	EST	F22751
60-1	T11GA	8	+	–	CLK2 kinase	AF023268
LIV-9	T11GA	8	+	–	EST	T64870
LIV-10	T11CC	13	+	–	EST	AA913548
LIV-11	T11GA	8	+	+	EST	AA345778

10 is 10 week fetal liver. Adult is adult liver. ND, not detected; +, signal detected; –, signal not detected. The sequences of the numbered 5′ primers are reported in Bauer *et al*. (3).

To confirm that the cDNAs are differentially expressed, we isolated RNA from additional gestational ages of liver (12, 14, 16, 18 and 20 in addition to 10 and 24 week fetal liver) and repeated some of the DDRT–PCR reactions. The care taken to obtain undegraded cellular RNA is evidenced by the tremendous reproducibility of the cDNA banding pattern between lanes (see Fig. 1). Average warm ischemia times for the human tissue were between 8 and 15 min. Preliminarily, all cDNAs were used as probes on northern blots or in RPAs for detection of mRNA transcript in 10, 16 and 24 week fetal and adult liver. Twelve of 25 cDNAs detected mRNA in either fetal and/or adult liver using either northern blots or RPAs (Table 1). The remaining 13 cDNAs failed to give a northern blot signal even after multiple day exposures. We therefore screened these for differential expression in a Southern blot analysis of cDNA products. The presence or absence of cDNA product for each gene sequence is summarized in Table 2. Three of the cDNAs did not show an expression difference and an additional three could not be detected. All 25 cloned cDNA fragments were sequenced and a sequence similarity search was performed. A computer search against the GenBank, European Molecular Biology Laboratory (EMBL) and Expressed Sequence Tag (EST) databases revealed that

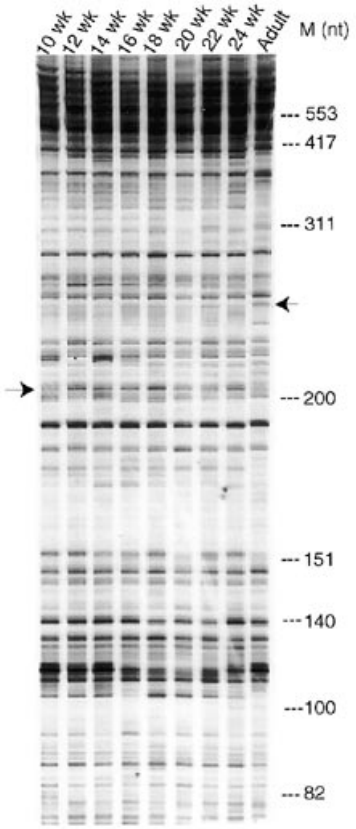

Figure 1. mRNA differential display of liver cDNA from 10, 12, 14, 16, 18, 20 and 24 week human fetal liver and adult liver. Each primer combination displayed ~100–150 bands. A putative differentially expressed cDNA is indicated with an arrow. The primer combinations used for the experiment were T11CA and 5′-TTT TGG CTC C-3′.

14 cDNAs had similarity to known genes, eight cDNAs showed similarity to ESTs and three cDNAs had no similarity to any registered DNA sequence. For all known sequences, we have used a cut-off value of >96% sequence identity to define a significant sequence similarity. We designate the unknown genes LIV-1–LIV-11; the nucleotide sequences of these novel genes are shown in Figure 2.

Identification of genes differentially expressed in fetal liver (erythropoietic genes)

We identified several genes from fetal liver which are known to be involved in erythropoiesis. For example, clone 1 is a partial sequence of an EBGM gene that is differentially expressed in fetal liver. Clone 1 detects a 1.8 kb transcript strongly expressed in fetal liver, as shown in Figure 3A, and only weakly expressed in adult liver. EBGM controls the metabolism of 2,3-diphosphate glycerate, the primary allosteric effector of hemoglobin. EBGM has been detected in several erythroid tissues, including fetal liver, bone marrow and spleen (26). Non-erythroid cells also contain a reduced amount of EBGM; this is consistent with the reduced level of mRNA in adult liver seen in Figure 3A.

Clone 6 is a 426 bp cDNA that detects a 4.2 kb transcript that was observed in 10, 16 and 24 week fetal liver but not in adult liver (Fig. 3B). Clone 6 is 100% homologous to the 3′-untranslated region of the stem cell leukemia gene (SCL), also known as TCL5 or Tall (27,28). SCL encodes a bHLH (basic domain helix–loop–helix) motif and is expressed primarily in immature hematopoietic tissues and cell lines. The bHLH motif is similar to those found in a variety of eukaryotic transcription factors, each of which appears to play a role in cell type-specific growth or development. The SCL transcript has previously been identified in fetal liver and bone marrow (27).

Clone K10 is a 417 bp sequence contained within the ES gene. Northern blot analysis with K10 as a probe detected a 7.5 kb mRNA expressed only in fetal liver (Fig. 3C); this is in agreement with a previously published report (29). Spectrin is the most abundant protein of the erythrocyte skeleton and consists of two highly homologous chains called α and β. Spectrin subunits associate into dimers and the dimers self-associate into tetramers; the tetramers assemble with numerous other proteins to form the mature erythrocyte membrane skeleton (29). Spectrin synthesis begins early in erythropoiesis, before the colony-forming unit erythroid stage of RBC maturation.

Clone TA21 is part of the PBD gene. PBD is a cytosolic enzyme involved in heme biosynthesis (30). Expression was detected in 10, 16 and 24 week fetal liver but not in adult liver (Fig. 4A). Two isoforms of PBD, encoded by two mRNAs differing solely in their 5′-end, are known: one is found in all cells and the other is present only in erythroid cells (30).

Identification of genes differentially expressed in fetal liver (non-erythropoietic genes)

We also identified several other non-erythropoietic genes differentially expressed in fetal versus adult liver. cDNAs for AFP were identified independently several times; clone T1 is one of the cDNAs characterized (Fig. 3D). AFP is a major fetal serum protein, which is absent or present only in trace amounts in sera of healthy adults. The AFP gene codes for a 70 kDa glycoprotein that is involved in the transport and cellular delivery of fatty acids. It is produced mainly by the yolk sac and by the fetal liver and to a small extent by the fetal gastrointestinal tract and kidney (31). The expression of AFP mRNA is very strong in early gestation (10 week fetal liver) and gradually decreases during fetal development; it is not expressed in adult liver.

The differential display screen identified a partial cDNA sequence of a gene (Mac30) that was previously identified to be overexpressed in meningiomas. Meningiomas are the most common tumors of the central nervous system (32–34). Northern blot analysis from normal mouse tissues indicates that the Mac30 gene is expressed in a broad spectrum of tissues, such as brain, lung, heart, skeletal muscle, testes, ovary and pregnant uterus. Mac30 is a member of the insulin-like growth factor binding protein family and regulates IGF activity. The clone K5 encodes part of the Mac30 sequence (32). This 224 bp cDNA hybridizes to two transcripts of ~2.3 and 2.8 kb in 10, 16 and 24 week fetal liver but does not hybridize to mRNA in adult liver (Fig. 3E). This suggests that alternative splicing of a common mRNA precursor might generate the two mRNAs, by a gene duplication event or, less likely, by a common regulatory pattern for genes that share some homology. This is the first time that the Mac30 gene has been detected in fetal liver, suggesting its possible role in growth and differentiation.

Clone TA5-1 is part of the IGF-2 gene. IGF-2 encodes a 4.8 kb mRNA (Fig. 6) that is known to regulate growth and differentiation and is known to be present in several fetal tissues (35). Ribonuclease protection assays show that IGF-2 is expressed in

LIV-1

GAACATTTCTCAGGGCAAAGGAACCTGGCTCATTTGAAGATTAATGTTCCATGCCTTTGTGGTCAAACGGTCAGCACTTA
ACACAGGAAAAAACTAGGTGTTGTTTTGTTTTGTTATTTTGGACAACATAAAATTCAGGAATGTTTTATTTAGCCTTGGT
TTCTAGAAGGAAGGGAAATAATATTTCTTGAGCATTTACTAGGGTGTTGCGTGCTGTGCTAAGTAAATTTTAAGTCTTTC
AGTTTTATAGATACGGAAAACAAGGGTGACTCTTTACCACAGGATGAATAAAGAACTAAGTAATATGGGAATGCAGCAAT
TTCTGGACTAGCTGA

LIV-2

CGAAGATCAGTACTTTATTTTCTCTAGCTCCAGTGTTTTGCAACTGTAGCAGCATATCAGAAACATCCCCACACAAAAAC
ACACAATTCTCCCCTTCTTCAAAGAGCTGGCAACAATTGAGAGGCAGAAACAATAGTTACTACAGGCATTTGAGAAATTT
AAGAAATAACACTTGCTCACCCTTGAAACATACATTGTGCGTCTTGCAGGAAGCTTA

LIV-3

AGGCAGGAGAAGAAAAATAGCGTGTTTGGGAAAGCTCAAAAATTAGAGGAGAGCAAGGTCAGTCAGCTCTCCTGACCTGT
GTCCACCAAGTCTTCTCTCTTCAGAAGTATGCTTTGATGAGACAAGGTGGAGATGTCATTGCATCCCCCTCCTGGAAGAA
TGTATTTCCAGAAAGAATTTCATTCATCAATTTCTACCCTCCCCCCCACTCAGATGATGGTCTCATGTTTCTCCTGACAC
TTTAAGTTCATTCGGATACTTATAGTAAATCACACACATTTCCTGGTAGGCCAAGTTAAAACACATCCCATAAAAAATGG
CAGGAATGGAAATATTTGAGTTCCTAATAAGATCCAAGAGCTTGTCTTTACCATATCTCCTTCTGTTCAAATTTGC

LIV 4

CAAGATTATGACAACTCAGGCACAACATTGTCTTTCAAATGAGTTGTGCTAGGTCAATTTTTCTGCCACTTCTTCCTATC
TGGGGGCACT

LIV-5

CAATGAGAAAAAATTTTTTTTTTTGACGATCTTGAGCAGTATAAAACTCAGAAGCTCCACTGAGGTGAAGGAAACATGGAC
ATGATACTAAGCAAAGCCTAGTCTTTTCCATAAAATGAATAAGAAGTACATTTGGTGGAGTTTGAGACCAGCCTGGGCAA
CACAGTGAGACCCTGTCTCTAAAAGCATTAAAGCATTAATCCTCGCATTTCGATAG

LIV-6

GAGAGCAGATATCCCAGCTGATTGCAGGCTACATTGACATCATCCTGAAAAAGAAACAAAGTAAAGATCGATTTGGACTA
GAAGGTGATGAGGAGTCAACCATGTTAGAAGAGTCCGTTTCCC

LIV-7

GGGACAGGGGACAGTTTATTGTAAAATATGAAGCAGCAATGATTGAAGCAAGTGAATTTCTCCTTTATATTCC

LIV-8

GGGAAGCTGTGGAAACACATCAAGCACAAGTATGAGAACAAGTAGTTCCTTGGAGGCCCCCATCCAGGCCAGAAGGACCA
GGTCCACCCAGCAGCTGTTTGCCCAGAGCTGGAGCCTCAGCTTGAAGATGATGCTCAAGGTACTCTTCATGGACCACCAT
TCGCTGTTGGCAAGAAACGGCTTTACTTACAAAACAGACTCTTTACCTTCTGCTGTGTTTGAAGTATGTTTAGTCAGCAT
GCTCAGGAAATAAATGTGAATTGC

LIV-9

CGTTGTGAATACAGCTGTGTCTGCAGCGGTCCAAGCTGTTCGGGGCAGAGGGAGAGGAACTCTAACAAGGGGAGCTTTTG
TTGGGGCGACAGCTGCTCCTGGCTACATAGCTCCAGGCTATGGAACACCATATGGTTACAGCACAGCGCCCCTGCCTATG
GTTTACCCAAGAGAATGGTTCTGTTACCCGTTATGAAATTTCCAACATATCCTGTTC

LIV-10

GAAGGCAAGGAAAAAGCTGATGGTCCTGTCCAAAGGCTATTAGGCAGGAAGAATTCTCTTAGGGCGGAGTTAGCTCTTTT
GTTCTATTCAGGCCTTCAACTGATTCAGCAAGGCCCGCCCACATTTGGGAGGACAGTCTGCATTACTCAGTCTACTGATT
TGAATGTTAATGTCATTGAGAAACACCCTCACAGGAACACTCAGAATAATGTTTGACCAAATAGCTGGGCATCTTGTGAC
CCAGTTAAGTGGATACATAAGATTAACTATCACAGTGACTCAGTGGAATTTTTTGTTTGCTTTTGTACAGTTTTAAAATA
AAAGTTTGGTATTTGTGTTTTG

LIV-11

GAAGAAAAGAAGGGGAAGAAGATCAAAGAAGAAAAGAAGAAGGGGAAGAAAAGAAGGGGAAGAAGATCAAAACCCACCAT
GCCCCAGGCTCAACAGCGTGCTGATGGAAGTGGAAGAGCCTGAAGTCTTGCAGGACTCACTGGATAGATGTTATTCGACT
CCATCAATGTACTTTGAACTACCTGACTCATTCCAGCACTACAGAAGTGTGTTTTACTCATTTGAGGAACAGCACATCAC
CTTTG

Figure 2. Nucleotide sequences of 11 unknown cDNAs differentially expressed in fetal or adult liver. The nucleotide sequence data of these LIV-1–LIV-11 fragments will be deposited in the DNA Data Bank.

fetal livers (10, 16 and 24 week) but not in adult liver or adult bone marrow (Fig. 4B). In the RPA, there are two protected fragments of 120 and 95 nt in length. The 120 nt fragment represents full-length cDNA while the 95 nt fragment is either from a different allele or uncovers a site of alternative RNA processing. This protein is produced by osteoblasts and is an important paracrine and autocrine regulator of osteoblast proliferation and differentiation. IGF-2 is also known as a potent mitogen for mammalian epithelial cells (35).

We also identified two cDNAs differentially expressed in fetal liver with no similar sequence in the database. LIV-1 detected 4.6 kb mRNA transcripts in all four tissues but the expression was lower in adult liver (Fig. 3F). LIV-2 is a 233 bp cDNA, which protects a range of mRNAs from nucleotide 200–233 in an RPA gel (Fig. 4C).

Identification of genes differentially expressed in adult liver

In addition to cDNAs differentially expressed in fetal liver, we detected cDNAs expressed primarily in adult liver. Nicotinamide N-methyltransferase catalyzes the methylation of nicotinamide. We identified a cDNA (A2) corresponding to ND which detects

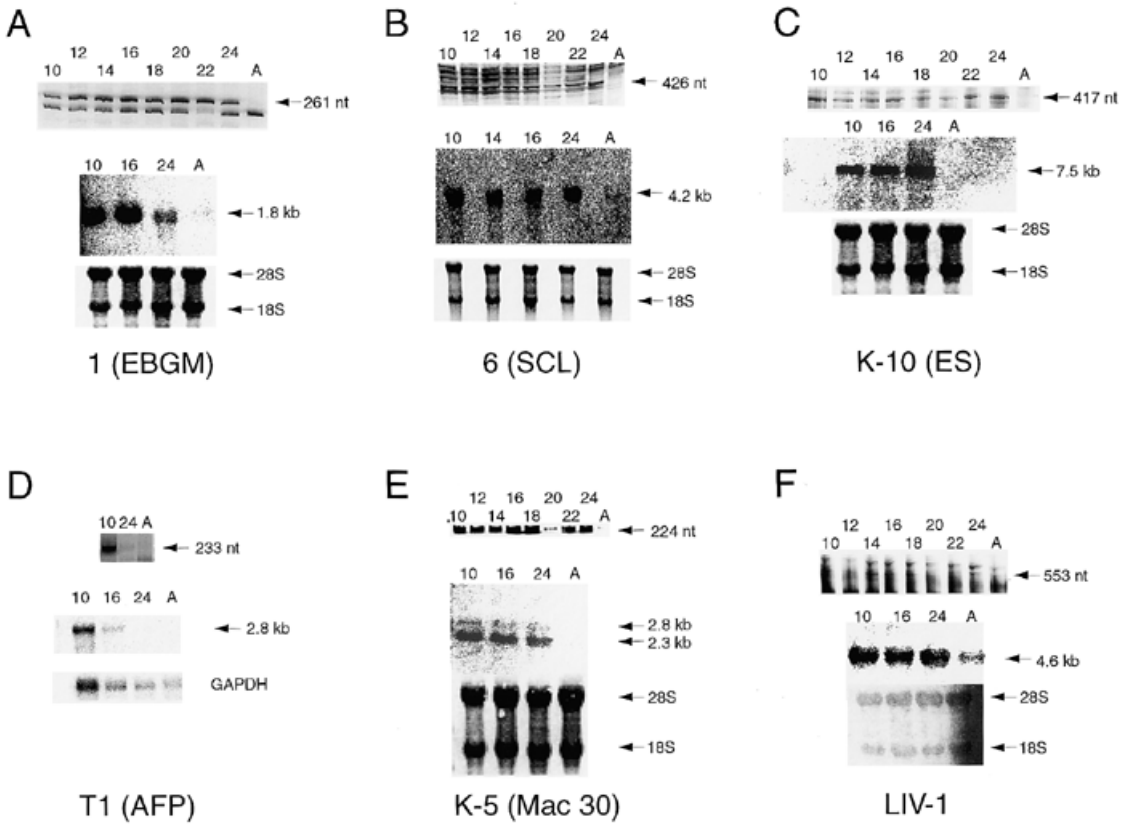

Figure 3. Confirmation of differentially expressed cDNAs by northern blots. The arrows on the right indicate the differentially expressed band (top) and, in the middle panel, northern blot analysis of each DDRT–PCR fragment. An aliquot of 20 µg of total RNA was loaded and the loading was verified by methylene blue staining after transfer onto Nytran membrane (bottom); alternatively, the blot was stripped and reprobed with a GAPDH probe as a loading control. 10, 12, 14, 16, 18, 20, 22 and 24 week fetal liver; A, adult liver.

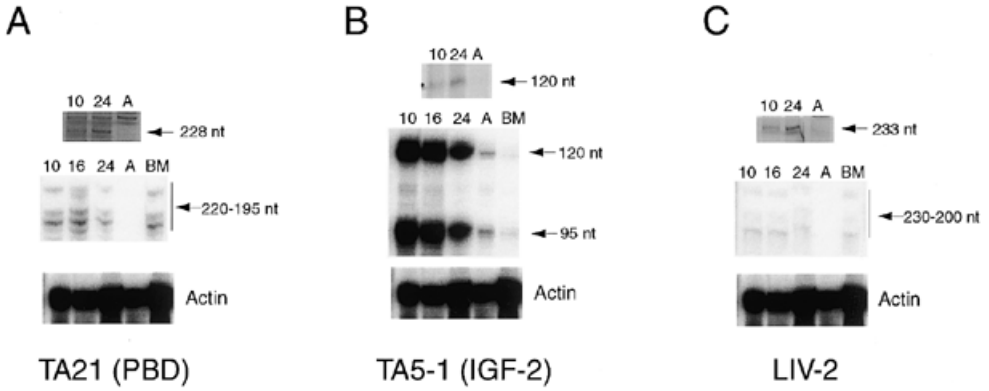

Figure 4. Confirmation of differentially expressed cDNAs by RNase protection assay. The top picture in each panel is a cut-out of the differential display gel. The middle picture is the RPA and an actin loading control is at the bottom. The arrows on the right indicate the differentially expressed cDNA or the protected fragment. The actin control shows that an equal mass of total RNA was used for each reaction. 10, 16 and 24 week fetal liver; A, adult liver; BM, bone marrow.

a 1.2 kb mRNA that is overexpressed in adult versus fetal liver (Fig. 5A). It was previously reported that ND is expressed in adult liver (36). Another clone, D4, is contained within the AGP mRNA. By northern blot analysis, we detected expression of the 0.8 kb message of this gene in adult liver but not in a 10, 16 or 24 week fetal liver (Fig. 5B). Human AGP, also known as orosomucoid, is a major acute phase plasma protein and is known to be expressed in adult liver and some hepatocarcinomas (37,38). Finally, A5-1 is a cDNA which detects a 1.3 kb transcript that is more abundant in adult liver than in fetal liver (Fig. 5C).

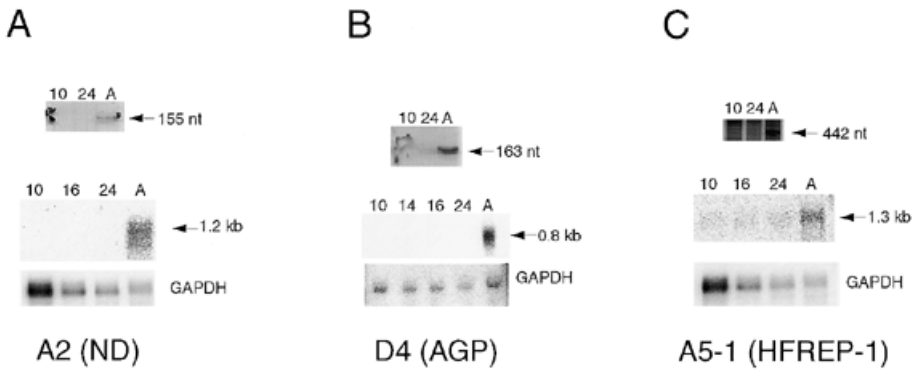

Figure 5. Northern blots of cDNAs differentially expressed in adult versus fetal liver. The top picture in each panel is a cut-out of the differential display gel. The middle picture is the northern blot. The blots were stripped and reprobed with GAPDH for a loading control (bottom). 10, 16 and 24 week fetal liver; A, adult liver; BM, bone marrow.

Clone A5-1 has 100% sequence similarity to HFREP. This protein was found to be specific to liver and hepatocellular carcinoma cell lines (39).

Gene expression profiles in developing tissue

During human ontogeny, primordial hematopoietic development occurs in the yolk sac and then hematopoietic stem cells migrate to the developing mid-gestational fetal liver. The fetal liver remains the major site of hematopoiesis until 16 weeks, whereupon it migrates to the bone marrow, which remains the major site of hematopoiesis throughout adult life. In an effort to extend the expression analysis of EBGM, SCL, Mac30 and IGF-2 genes in hematopoietic cells during embryonic development, northern blot analysis was performed first on 10–12, 13–15, 16–18 and 22–24 week FBL [which contain ~60–80% nucleated red blood cells (nRBC) and possess the same cell lineages and progenitor populations as adult bone marrow (ABM)], 16 week cord blood (which contain 10–25% reticulocytes, 2–4% nRBC), adult peripheral blood (APB) (which contain 0–1% reticulocytes and 0% nRBC) and ABM. Northern blot analysis showed that EBGM and SCL were abundantly expressed in 10–24 week FBL and weakly expressed in ABM. The low signal observed could be due to a small population of cells in ABM. Expression of EBGM was also detected in 16 week cord blood while no expression of SCL was detected in cord blood and APB. Very interestingly, IGF-2 and Mac30 mRNA were abundantly expressed only in 10–12 week FBL and weakly expressed in 13–24 week FBL and not in APB or ABM. IGF-2 shows high expression in cord blood and Mac30 mRNA is weakly expressed in cord blood and might be a liver- or hematopoietic cell-specific gene (Fig. 6). The data presented here clearly demonstrate that the expression of these genes is developmentally regulated and are one of a larger repertoire of proteins that are expressed only early in hematopoietic development.

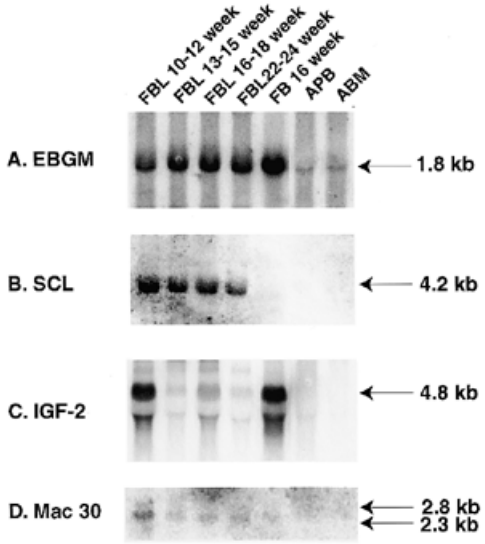

Figure 6. Developmentally regulated expression of EBGM (**A**), SCL (**B**), IGF-2 (**C**) and Mac30 (**D**) mRNA in fetal blood from liver cells (FBL), fetal blood (FB), adult peripheral blood (APB) and adult bone marrow (ABM), as detected by northern blot analysis. An aliquot of 20 µg of total RNA was loaded in each lane.

CONCLUSION

Our data substantiate that differential display is a powerful and fast technique to analyze gene expression and to identify genes differentially expressed during erythropoiesis and liver development. We used 5′-end-labeled primers and applied stringent conditions for cDNA candidate selection from the DDRT–PCR gel profile. This approach resulted in a very low rate (~15%) of false positives, consistent with data reported by others (19). Since not all of the cDNAs detected a mRNA on northern blots, we used more sensitive methods such as Southern blot analysis of amplified cDNA and RPAs to interrogate the putative differentially expressed candidates. Our data suggest that differential display identifies both rare and moderately abundant mRNAs.

Interestingly, our data suggest that ~99% of genes expressed during development are not differentially regulated at the transcription level (Fig. 1). Most transcripts show only minor changes in spite of the multiple developmental programs that are occurring simultaneously. This further suggests that the development process involves very few expressed genes that interact to program developmental changes. That ~1% of genes are

differentially expressed between fetal and adult liver is quite unexpected considering the divergent function of the two organ systems. While adult liver is nearly free of hematopoietic function, the 10 week fetal liver is comprised of ~80% hematopoietic cells. It appears that only the actions of a few genes are required to markedly distinguish different tissues. Since many of the differentially expressed genes are actually a part of the hemopoietic gene class, the true number of genes directing liver development could be quite small indeed. Perhaps transcripts that were not identified herein are so rare that they are simply below the sensitivity of DDRT–PCR as a method. Or perhaps the results of other methods, when combined with the results of differential display, may yield a more complete picture of liver development during this critical fetal period. This continues to be an active topic of investigation by this laboratory.

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