Short Contribution

Identification of *elf1*, a β-spectrin, in early mouse liver development

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ABSTRACT **β–spectrins play essential roles in cell-cell interactions and in the maintenance of cell polarity. Our aim was to identify β-spectrin genes important for the establishment of hepatocyte polarity and differentiation. Using subtractive screening of cDNA libraries from early embryonic mouse livers (post-coital days 10,11, and 12), we have isolated *elf1* (embryonic liver fodrin 1), a differentially expressed β-spectrin or fodrin (β SpIIΣI). *Elf1* encodes a 220-amino acid protein with an NH_2 terminal actin-binding domain. *In situ* hybridization studies demonstrate *elf1* expression initially in day 10 embryonic heart tissue, then in day 11-11.5 hepatic tissue. These studies suggest that *elf1* may play a role in the emergence of hepatocyte polarity during liver development.**

KEY WORDS: *spectrin, liver development, in situ hybridization*

The establishment of cell polarity, inductive events, and intercellular communication are critical for growth and differentiation during development. However, the precise mechanisms by which these effect hepatocyte differentiation have yet to be elucidated. Our strategy to identify molecular markers and inductive transcripts for liver development was to construct three embryonic liver cDNA libraries, at e (embryonic days post coitus) 10,11,12, and perform subtraction hybridization (e11-e10 cDNA and e12-e11 cDNA). Clones obtained were analyzed for stage and tissue specificity, and inserts were sequenced (Mishra *et al.*, 1997a).

Sequencing and full-length cloning of one of these, sc32 revealed it to be a β-spectrin (or Fodrin), which we have termed *elf1* (**e**mbryonic **l**iver **f**odrin). Because spectrins are of pivotal importance in the assembly and maintenance of specialized domains on the cell surface (Hu *et al.*, 1995), the β-spectrin that we isolated potentially has an important function in the development of hepatocyte polarity. In this study we describe the characterization of *elf1*, and its expression in early liver development.

Characterization of cDNA libraries

The four stages in liver development, at e10, e11, and e12, are defined times marking the progression from undifferentiated endodermal cells to a well-differentiated fetal liver.

At e9-10, a change in cell polarity occurs with invasion and migration of endodermal cells into surrounding mesenchyme. At e11-12, cords of hepatocytes together with early sinusoids form into lobules, establishing a well-developed embryonic liver. cDNA libraries at these stages would therefore represent "captured" mRNA species expressed at crucial time periods for hepatocyte formation.

Qualitative and developmental profiles of the e10, e11, e12 cDNA liver libraries

cDNA e10, e11, e12 liver libraries containing $6.1x10_6$-$4.1x10^7$ independent clones were generated (Table 1). A library containing $5.0x10_5$ is considered to be a representative library, with a 99% probability that rare transcripts (fewer than ten copies per cell) are present (Sambrook *et al.*, 1989). Our libraries are therefore likely to be highly representative of their respective mRNA species for that stage.

Qualitative profiles of the e10, e11, e12 cDNA libraries were obtained using genes (such as *IGF1, IGF-II, IGFBP-2, HNF1/LFB1, C/EBP*) known to be expressed at different times in developing liver. The data in Table 2 demonstrate that *IGF-I* was not detected in the e11 or e12 cDNA libraries, while *IGF-II* was detected in the e10 and e12 libraries (Three at e10 and four at e12; Table 2). For *IGFBP-2*, one clone per 100,000 was detected at e10 and e11. This difference in the expression of *IGFBP-2* from IGF-II has been confirmed by *in situ* studies (Wood *et al.*, 1992). *HNF1/LFB1* was detected only in the e12 library, and *C/EBP* was detected

Abbreviations used in this paper: elf1, ELF1, **e**mbryonic **l**iver **f**odrin, mouse nonerythroid β-spectrin; β SpIIΣI, mouse brain β-spectrin; β SpIΣI, mouse erythroid β-spectrin; IGF-I, insulin-like growth factor-1; IGF-IR, IGF-I receptor; IGFBP-2, IGF binding protein-2; HNF, hepatocyte nuclear factor; p.c., post coitus; e, embryonic days post coitus.

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GCGCTGCTCTGTGAGCTGGAGCACAGCGTGCTTAGAGTTGGCCATATTTAAAATATTTTCCAATAGGATCCTGCGTCCTTCCTCCTTTTC   90
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GGTGGTAACTCCACCAGGCTAGGTTGGACTCTGCAGCCAACTTCCTATCAGATCACCCTGCACCTATTTCCGACCCGACCGGAATGCGAC  270
TGGCTTGAGGTCCAGCCCTTTCGCCTGGGCGGGAGCAGAGCCGCGGAAGCTGCTTGGAGTTGGATGGGGGTAGGAAGGGGCTGGAGCGGG  360
AATCCTACGGTGCAACTGGCCTGGGCCTAAGGTTGGGCATAATGGAGTTGCAGAGGACATCCAGCATTTCAGGGCCGCTGTCGCCGGCCT  450
                                  M  E  L  Q  R  T  S  S  I  S  G  P  L  S  P  A  Y              17
ACACCGGGCAGGTGCCTTACAACTACAACCAACTGGAAGGAAGATTCAAACAGCTCCAAGATGAGCGTGAAGCTGTACAGAAGAAGACCT  540
  T  G  Q  V  P  Y  N  Y  N  Q  L  E  G  R  F  K  Q  L  Q  D  E  R  E  A  V  Q  K  K  T  F   47
TCACCAAGTGGGTCAATTCCCACCTTGCGAGAGTGTCCTGCCGAATCACAGACCTGTACACGGACCTTCGAGATGGACGGATGCTCATCA  630
  T  K  W  V  N  S  H  L  A  R  V  S  C  R  I  T  D  L  Y  T  D  L  R  D  G  R  M  L  I  K   77
AGCTACTGGAGGTCCTCTCTGGAGAGAGGCTGCCTAAACCCACTAAGGGACGGATGCGGATCCACTGTCTGGAGAATGTCGACAAGGCTC  720
  L  L  E  V  L  S  G  E  R  L  P  K  P  T  K  G  R  M  R  I  H  C  L  E  N  V  D  K  A  L  107
TTCAATTCCTGAAAGAGCAGAGAGTCCATCTTGAGAACATGGGCTCCCATGACATTGTGGATGGAAACCACCGGCTGACAACGTTGGAGC  810
  Q  F  L  K  E  Q  R  V  H  L  E  N  M  G  S  H  D  I  V  D  G  N  H  R  L  T  T  L  E  L  137
TACTGGAAGTGCGCAGACAGCAAGAGGAAGAAGAAAGAAAGAGGCGGCCACCTTCTCCGGACCCAAACACGAAGGTTTCAGAGGAGGCTG  900
  L  E  V  R  R  Q  Q  E  E  E  E  R  K  R  R  P  P  S  P  D  P  N  T  K  V  S  E  E  A  E  167
AGTCCCAGCAATGGGATACTTCAAAAGGAGACCAAGTTTCCCAGAATGGTTTGCCGGCTGAGCAGGGATCTCCACGGGTTAGTTACCGCT  990
  S  Q  Q  W  D  T  S  K  G  D  Q  V  S  Q  N  G  L  P  A  E  Q  G  S  P  R  V  S  Y  R  S  197
CTCAAACGTACCAAAACTACAAAAACTTTAATAGCAGACGGACAGCCAGTGACCATTCATGGTCTGGAATGTGAAGTTCACTACCATTTG 1080
  Q  T  Y  Q  N  Y  K  N  F  N  S  R  R  T  A  S  D  H  S  W  S  G  M                        227
TCAAGAACCACTCTGTCCACATCCTTTGACCTTTTGGCTTCCACGTCACCCAGAGTGTTAAAATTTTTACTTAATTCATAGCTGTCCTTG 1170
ATTTCATATTTGTTTGCATTTAATTTATGTTTCTTTGGATCCTCATTGCCTCAAAGCAGCATACTTAATTTTTGTTTATTTATTGTGAGC 1260
TTTTTACTTTAAGATTTTACATGAGTAATCAAAATTAAATTATAGCATAATG 1312
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Fig. 1. Nucleotide and predicted amino acid sequence of *elf1*. *The nucleotide sequence of the constructed cDNA for elf1 is shown on the first line, and the predicted amino acid sequence is shown on the second line. Important features of the nucleotide sequence are in boldface type and underlined. They are: the upstream stop sign codon at nt 276; the initiator codon at nt 402; and the stop codon at nt 1062. The underlined highlighted amino acid residues 36-55 indicate the actin-binding domain. The highlighted amino acid residues 147-220 indicate the*

at day 11 and 12 in low abundance (2 clones per 100,000 at e11 and 5 at e12). All libraries had similar β-actin frequencies (between 120-300 per 100,000 clones), considered representative of such embryonic libraries (Weng *et al.*, 1989).

Identification of stage-specific clones by subtractive methods, full length cloning and sequence analysis of elf1.

Two subtracted libraries, comprising 64 clones (e12-e11 cDNA) and 174 clones (e11-e10 cDNA) were constructed. By Southern blot analysis, as well as sequencing, 34 clones were shown to be stage-specific and to contain no mitochondrial, ribosomal, or globin sequences. Further analysis was carried out on one of these clones, sc32, identified as a β-spectrin.

Screening of the e10 and e11 libraries revealed three overlapping clones to sc32 and included the sequence encoding *elf1* (Fig. 1). The first in-frame ATG is present at nt 402, and is preceded by an nt 276 upstream TAG codon. *Elf1* is predicted to encode a 220 amino-acid protein showing 57% overall identity to β-fodrin (β SpIIΣI, a non-erythroid β-spectrin). ELF1 is located at domains I and III of the β-spectrins, but does not include any of the domain II seen inβSpIIΣI. The NH_2 terminus of ELF1 is markedly similar to those of both βSpIIΣI and erythroid β-spectrin (β SpIΣI), indicating that ELF1 is capable of binding *f*-actin (Goodman *et al.*, 1995; Hu and Bennett, 1995). Domain III ofβSpIIΣI is a COOH terminus domain which contains varying numbers (52-265) of residues in alternatively spliced forms, giving rise to tissue-specific expression. The COOH terminus domains of ELF1 and βSpIIΣI are very similar. The terminus may be involved, together with the NH_2 terminus domain of the α-subunit, in spectrin dimer association.

Identification and developmental regulation of elf1 transcripts

Utilizing an ^{35}S-UTP -labeled insert representing *elf1*, northern blot analysis revealed a 2.4 Kb transcript with maximal expression of *elf1* in heart, then in liver tissues, specifically on day 11-11.5 (Fig. 2). *In situ* hybridization demonstrates *elf1* expression in cardiac

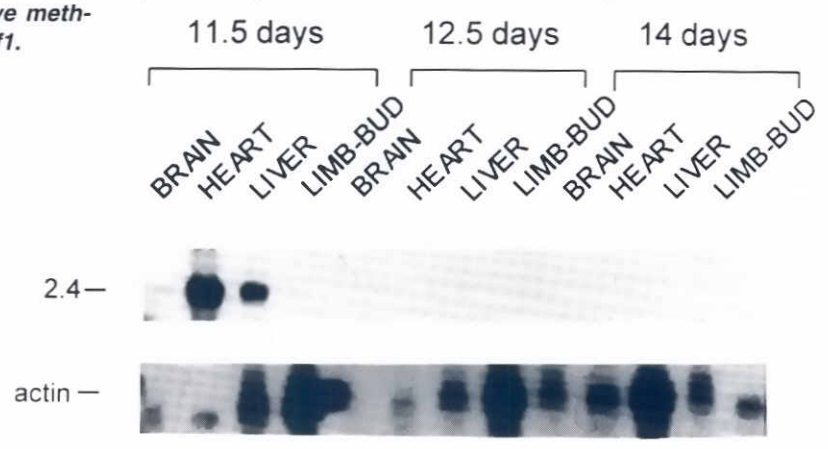

Fig. 2. Autoradiograph of northern blot analysis of *elf1* mRNA during midgestational development. *10 ug of poly A RNA from tissues at post coital developmental days 11.5, 12.5, and 14 were electrophoresed and the subsequent blot probed with elf1 and actin. The size of the elf1 transcript is 2.4 Kb.*

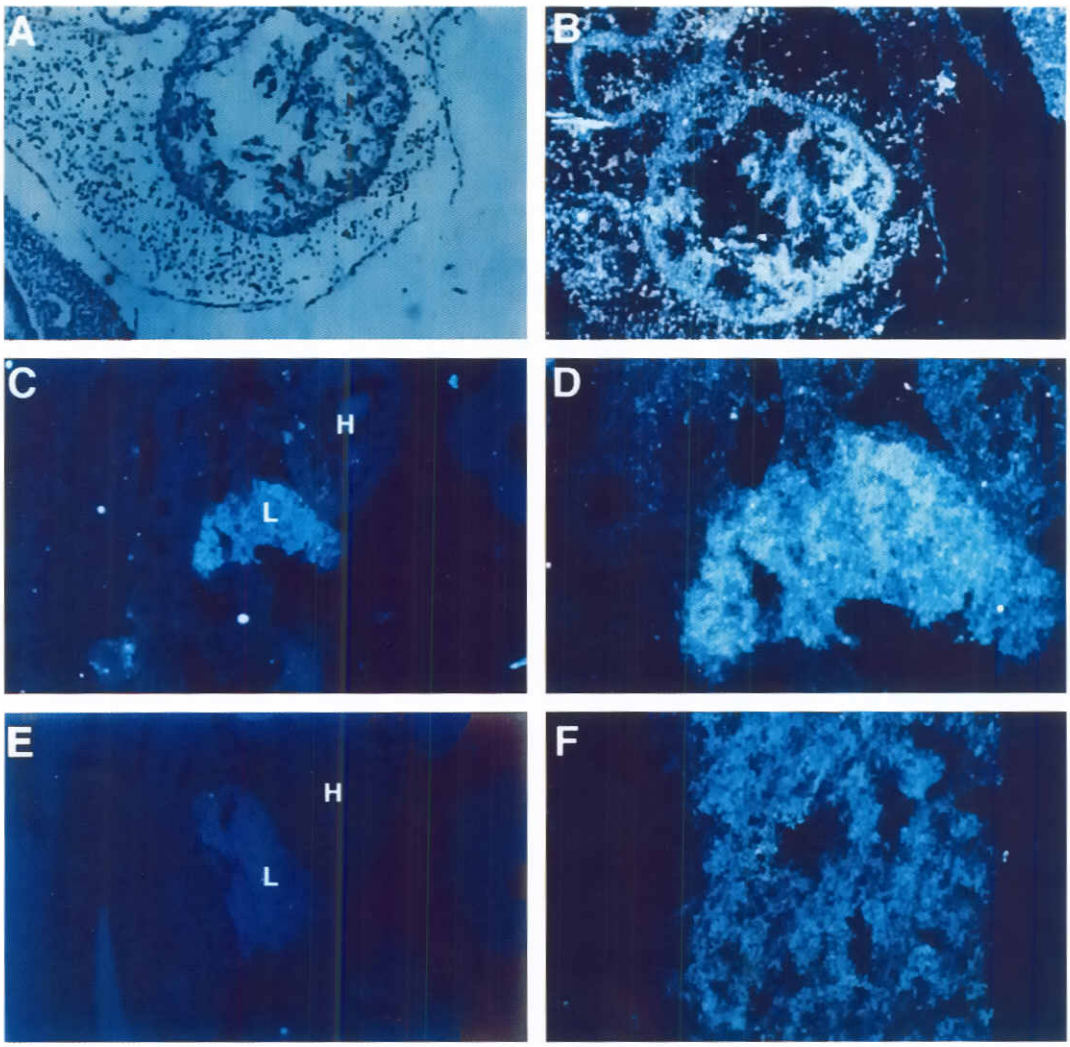

Fig. 3. Antisense *elf1* RNA probes hybridize specifically to mouse embryonic heart tissue at e10 (B) and liver tissue at e11 (C,D) in comparison to a-fetoprotein RNA probes which hybridize to e11 liver tissue (E,F). *B,C,D,E,F are darkfield sections. A is a brightfield section. Magnifications at: 10x (C,E); 20x (A,B,D); 40x (F). Abbreviations: L, liver; H, heart.*

tissue (Fig. 3B) at day 10. The caudal liver bud region, is devoid of silver grains (not shown). At the next stage, day 11 (Fig. 3C and D), silver grains highlight the developing liver (L). Control riboprobes to α-fetoprotein outline the developing liver at day 11 (Fig. 3E and F).

In chick embryos at the head process stage, liver differentiation is dependent on the presence of cardiac tissue. After the completion of gastrulation, the liver and heart areas partially segregate during the somitic stage. Tissue explant studies at later stages have also revealed that, normal liver development is entirely dependent upon the cardiac/septum transversum component (Le Douarin, 1975; Houssaint, 1980).

Because our findings show that elf1 is expressed in early cardiac tissue, then in hepatic tissue, we believe it is a novel marker for early liver development. Sequence analysis has shown *elf1* to bear 57% identity to β-fodrin, a non-erythroid β-spectrin, βSpIIΣI. β-spectrins are required for the maintenance of both cell-surface polarity (Nelson and Hammerton, 1989) and cell-cell junctions (Luna and Hitt, 1992). β-spectrins contain binding sites for ankyrin and actin (Speicher and Marchesi, 1984). Disruption of the interaction of spectrin with actin results in loss of epithelial cell morphology (Hu and Bennett, 1995). Further studies are necessary to determine whether the actin-binding domain of ELF1 functions in a similar manner as that of β SpIIΣI in conforming cell polarity.

Smaller isoforms of β-spectrins have been well described; for instance, a 4.0 Kb muscle tissue transcript is thought to encode a β-spectrin important for the clustering of acetylcholine receptors (Bloch and Morrow, 1989). A potential function for ELF1 may be the assembly and maintenance of

TABLE 1

EARLY EMBRYONIC LIVER CDNA LIBRARIES

Initial size		Total RNA (RNA Per embryo μg)	Poly A RNA(μg)
e10	6.1×10^6	63 (1.04)	26 (4%)
e11	4.1×10^7	60 (6.7)	16.8g(3%)
e12	1.6×10^7	N.D.	40

TABLE 2

CLONE FREQUENCIES FOR DAY 10, 11 AND 12 LIBRARIES

Probe	e 10	e11	e 12
IGF I	N.D.	0	0
IGF II	3	0	4
IGFBP -2	1	1	0
LFB I	0	0	1
C/EBP	N.D.	2	5
Beta Actin	120	130	246

N.D. = Not Done
Positive cDNA clones per 100,000 poly A+ containing cDNA clones.

specific subclasses of proteins into discrete membrane domains, a fundamental step in establishing hepatocyte polarity and thus differentiation.

Experimental Procedures

Cloning and sequencing of elf1

Embryonic liver was obtained from matings of random-bred ICR mice (Harlan). The plug date was designated as Day 0 and embryos collected at days 10, 11 and 12 post conception (Theiler, 1989). To prepare cDNA libraries, RNA was isolated (Chomczynski and Sacchi, 1987) and One to 5 μg of poly(A)+RNA selected using oligo(dT)-cellulose (Collaborative Research Type 3). cDNA library construction of day 11 and 12 embryonic liver was carried out by conventional techniques (Gubler and Hoffman, 1983) and of day 10 embryonic liver using the Stratagene Unizap cDNA library kit.

Two subtracted libraries were then constructed (Schweinfest *et al.*, 1990), comprising 64 clones (e12-e11) and 174 clones (e11-e10). Purification of bacteriophages and preparation of DNA were carried out by the Stratagene *in vivo* excision protocol. Plasmid DNA was sequenced using T7 DNA polymerase (Sanger and Coulson, 1980).

Sequence analysis

The NCBI non-redundant (nr) databases as of December 16, 1997 were searched using the blastp2 and blastn2 programs, which permit gapped alignments (Altschul and Gish, 1996), with the default parameters and ELF1 protein or nucleotide sequences as queries.

RNA preparation and analysis

Embryonic livers for the specific stages were pooled and total RNA isolated (Chomczynski and Sacchi, 1987). 10 μg RNA were electrophoresed on a 1% formaldehyde gel and transferred onto Hi-bond nylon membrane (Amersham) using standard procedures (Sambrook *et al.*, 1989). Radioactive ^{32}P-labeled probes were synthesized by random primer methods (Feinberg and Vogelstein, 1984) and hybridized to the filters. Filters were washed at high stringency with a final wash in 0.2xSSC (30 mM NaCl, 3mM sodium citrate, pH 7.4) 0.5% sodium dodecyl sulfate at 65°C. for 60 min. Filters were then autoradiographed with intensifying screens at -70°C.

In situ analysis

In situ analysis was performed for *elf1* (Mishra et al., 1997b). The RNA probes were synthesized and labeled with ^{35}S-UTP (400 Ci/mmole) via the T7 or SP6 promoter for RNA polymerase. Sense or antisense probes were added to the appropriate sections, mounted, sealed with rubber cement, and incubated at 50°C. overnight. Exposure times were from three weeks to four months. The emulsion was developed according to manufacturer's directions.

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