Genomic organization and embryonic expression of *Suppressor of Fused*, a candidate gene for the split-hand/split-foot malformation type 3

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Abstract The genes for human and mouse *Suppressor of Fused* (*SU(FU)*/*Su(Fu)*) in the Hedgehog signaling pathway were characterized and found to contain 12 exons. Human *SU(FU)* localized on chromosome 10q24–25 between the markers D10S192 and AFM183XB12. We detected three additional SU(FU) isoforms, two of which have lost their ability to interact with the transcription factor GLI1. Expression analysis using whole mount in situ hybridization revealed strong expression of *Su(Fu)* in various mouse embryonic tissues. *SU(FU)* was considered a candidate gene for the split-hand/split-foot malformation type 3 (SHFM3). However, no alterations in the *SU(FU)* gene were found in SHFM3 patients.

Key words: Suppressor of Fused; Split-hand/split-foot malformation; Chromosome mapping; In situ hybridization

1. Introduction

The determination of the function of the Hedgehog signaling pathway in vertebrates is of considerable biological interest [1]. Recently, one additional component of this system, called the Suppressor of Fused (Su(Fu)) in *Drosophila* [2], has been identified in human [3,4], mouse [3,5,6], and chicken [7]. In *Drosophila*, Su(Fu) has been shown to form a complex with three other molecules, Fused (Fu) [8], Costal-2 (Cos2) and the transcription factor Cubitus interruptus (Ci) [9] that all are involved in the transduction of the Hedgehog signal. This complex associates with microtubules in the absence of Su(Fu) [10]. The human and chicken Su(Fu) are also able to bind to the Ci homolog GLI1 [3,7] and to the *Drosophila* Ci and Fu [5,7]. Furthermore, Su(Fu) has been shown to bind GLI1 and GLI3 in the nucleus and to enhance their binding to their recognition site on DNA [3,7]. The Hedgehog signaling pathway is further influenced by transcriptional regulation of Patched and Gli by Gli itself [11], and by the proteolytic generation of Ci/Gli isoforms with opposite transactivation properties [12–14].

Because of its chromosomal localization, biological function and expression pattern, *SU(FU)* is considered a candidate for the gene mutated in the hereditary disorder split-hand/split-foot malformation type 3 (SHFM3, OMIM #600095). This rare condition is transmitted as an autosomal dominant trait and characterized by an enlargement of the median cleft and degeneration and fusion of the middle digits. The chromosomal locus for SHFM3 is 10q24–25 [15–17]. The human SHFM3 is mirrored by a mouse condition called dactylaplasia (Dac) [18] that results in a similar phenotype. The *Dac* gene is mapped to chromosome 19 [19], which is syntenic to chromosome 10 in human, and it has been proposed that SHFM3 and Dac might be caused by mutations in corresponding genes [19]. Recently, the Dac mutation was identified as an alteration in the previously unknown F-Box/WD40 gene *Dactylin* [20]. At the same time, the human homolog for *Dactylin* was also published [21] but for the human *DACTYLIN* a link to SHFM3 is not yet demonstrated.

In order to gain insight into the expression and regulation of *SU(FU)*/*Su(Fu)*, we have determined the genomic organization and chromosomal localization of the human and mouse genes, and analyzed the expression pattern in human tissues and during mouse embryogenesis. We have also analyzed SHFM3 patients for mutations in *SU(FU)*.

2. Materials and methods

2.1. Biological materials

Timely mated NMRI mice were from Charles River Laboratories. The partially overlapping bacterial artificial chromosome (BAC) clones RPCI-11-170J3 containing the 5′-end and 243I14 containing the 3′-end of the human *SU(FU)* gene were from Research Genetics. The mouse *Su(Fu)* BAC clones 17985 and 17986 were from Incyte Genomics, Inc. The mouse *Su(Fu)* EST clones 513730, 1195307, 1224813, and 963990 from the IMAGE consortium were from the UK HGMP Resource Centre, Hinxton, Cambridge, UK.

2.2. Patients

Patients with SHFM3 were members of families previously linked to chromosome 10q24–25 [16].

2.3. Chromosomal localization, mapping, and sequencing of the human *SU(FU)* and mouse *Su(Fu)* genes

The chromosomal localization of *SU(FU)* was determined by radiation hybrid mapping using the two primer pairs 5′-CAGTTGTGTCAACGAGATCTCC-3′/5′-CTGTGGCCTGTGCATGGCAC-3′ and

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Abbreviations: Su(Fu), Suppressor of Fused; Dac, dactylaplasia; BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; dpc, day post coitum; dpn, day post natum

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5′-CCGCTGGCTAAGCCTTGTGAC-3′/5′-CAGGGTCCAGAGTCAAACCTCA-3′ on the Genbridge G4 map (Research Genetics). The exon–intron borders were determined by sequencing BACs. Restriction enzyme digests of BAC DNA and subcloned DNA fragments were transferred to Hybond N^+ filters (Amersham) and hybridized with radiolabeled oligonucleotide probes for individual exons to assemble the *Su(Fu)* locus map.

2.4. Screening for mRNA variants

cDNAs from normal human tissues (human MTC panel II) or from xenografted human tumors (human tumor MTC panel) were from Clontech. For mouse embryo cDNA, organs were homogenized and RNA was extracted using RNAzol B (Tel-Test, Inc.). Subsequently, cDNA was prepared using oligo-(dT) and reverse transcriptase (RT; Clontech). Polymerase chain reaction (PCR) products were generated containing the entire coding region of *SU(FU)*/*Su(Fu)*. Subsequently, nested PCR products containing exons 2–3, 4–5, 6–7, 8–9, and 10–11, respectively, were obtained. After gel electrophoresis, variant transcripts were detected by size difference.

2.5. Whole mount in situ hybridization

Mouse embryos were cut sagitally and after fixation in 4% paraformaldehyde hybridized according to standard procedures [22]. Digoxin-labeled probes were prepared using the DIG RNA labeling kit and detected using the DIG Nucleic Acid Detection kit (Boehringer Mannheim). The template for the RNA probe was the mouse Su(Fu) EST cDNA clone 513730 (987 bp) that contains exons 1 through 9. Specificity of the reaction was monitored by comparison to samples hybridized to sense probes.

2.6. Epitope-tagged constructs

Myc-SU(FU) and HA-GLI1, both in pCMV-5, have been described [3]. The myc-tagged constructs for the transcription variants were generated by cloning cDNAs into the pGEM-T vector (Promega). In detail, a *Bst*EII (nucleotide 327)–*Sal*I (behind the STOP codon) fragment from near full-length SU(FU)-XL was cloned into myc-SU(FU), and *Eco*NI (nucleotide 751)–*Sal*I fragments of SU(FU)-Tt and SU(FU)-Lk were ligated into myc-SU(FU)-XL.

2.7. Transient transfections and Western blotting

10 μg each of the tagged GLI and SU(FU) constructs were transfected into a near confluent 15-cm culture dish with 293 kidney carcinoma cells using 75 μg of polyethyleneimine (Sigma Chemicals) as a transfecting agent in 12 ml serum-free medium. After 36 h cells were harvested and lysed in 1% Triton X-100. The lysates were precleared one time over protein A/G-agarose preincubated with goat serum and then precipitated with mouse anti-myc monoclonal antibody 9E10 or rabbit anti-mouse antiserum. After electrophoretic separation on a reducing 10% SDS–PAGE gel and blotting onto PVDF membrane, myc-tagged proteins were detected by rabbit anti-myc antiserum, followed by incubation with a peroxidase-conjugated secondary antibody.

2.8. Sequencing of the coding regions of SU(FU) in SHFM3 patients

mRNA was obtained from transformed SHFM3 patient B cell lines as described in Section 2.4. cDNA and subsequently PCR products of the coding regions were generated and sequenced. The first 49 nucleotides of the *SU(FU)* coding region were not included in the cDNA so that was sequenced from genomic DNA instead. At least 13 clones from each patient sample were analyzed. In addition, exon 1 from the patients was sequenced from genomic DNA. Single-strand conformational polymorphism (SSCP) analysis was performed on PCR fragments amplified from genomic DNA as previously described [23].

3. Results and discussion

3.1. Chromosomal localization and genomic organization of the human SU(FU) and mouse Su(Fu) genes

Previously, we identified the human and murine homologs of the *Drosophila* signal transducer molecule Su(Fu) [3]. To determine the chromosomal localization of the human *SU(FU)* gene, a radiation hybrid mapping panel was screened. The *SU(FU)* gene was localized to chromosome 10q24 between the markers AFM183XB12 and D10S192, with a distance of 0.10 cR from AFM183XB12 (lod > 3.0).

In order to investigate the gene structure, we identified BAC clones containing both the human *SU(FU)* and the murine *Su(Fu)* genes and determined the exon–intron organization. Both genes contain 12 exons with exon–intron boundaries at identical positions (Table 1). The mouse *Su(Fu)* gene was found to span approximately 100 kb (Fig. 1). The promoter region contained no consensus TATA- or CAAT-boxes immediately upstream of the putative transcriptional start site. However, using the Transcription Start Site Wingender database (TSSW) at UK HGMP Resource Centre (www.hgmp.mrc.ac.uk) a promoter in this region with an excellent prediction value (LDF = 20.80) was found. When sequencing upstream of both the putative human and murine promoters, the ATG-containing exon for the gene *actin-related protein 1* (*ARP1* [24] and *Arp1* [25] in human and mouse, respectively) was detected in opposite transcriptional orientation (Fig. 1). The two ATG initiation codons of the *ARP1*/*Arp1* and the *SU(FU)*/*Su(Fu)* genes are only 1.5 kb apart. In addition, sequencing of the end of BAC 17986 revealed an exon for the *ADP ribosylation factor-like protein 3* (*Arl3*) [26] downstream of the mouse *Su(Fu)* gene (Fig. 1). During the course of these studies, genomic organization of the mouse

Table 1
Exon–intron boundaries of the human *SU(FU)* and mouse *Su(Fu)* genes

Exon	Human exon	Human intron	Mouse exon	Mouse intron (length)
1	1-ATGGCG…GTACTG-182	gtatgc…ttgcag	1-ATGGCG…GTACTG-182	gtctgc…ttgcag (3.7 kb)[b]
2	183-GTTGGG…CCATGA-317	gtgaag…tttcag	183-GTTGGG…CCATGA-317	gtgagt…tttcag (24 kb)[b]
3	318-GTTTAC…AGTCAG-454	gtagga…ccacag	318-GTTTAC…AGTCAG-454	gtaata…ccacag (22 kb)[b]
4	455-AGAACA…CTCCAG-597	gtgagg…cctcag	455-AGAACA…CTCCAG-597	gtgagg…ccacag (607 bp)[a]
5	598-ATCGTT…GCCTAT-683	gtgagt…ccacag	598-ATTGTT…GCCCAT-683	gtgagt…ccacag (226 bp)[a]
6	684-TGCTGG…CTGCAA-756	gtatgt…*aagc*ag[c]	684-TGCTGG…CTGCAA-756	gtatgt…*aagc*ag[c] (2.3 kb)[b]
7	757-GAGAGA…GCAAAG-910	gtggga…ttgcag	757-GAGAGA…GCAAAG-910	gttgag…ttgcag (1419 bp)[a]
8	911-ACACAG…GGCCCC-1022	gtaagt…tcacag	911-ACACAG…GGCTCC-1022	gtaagt…tcacag (22 kb)[b]
9	1023-GAGCCG…CCTAAG-1157	gtgagc…ctccag	1023-GAGCCG…CCTAAG-1157	gtgagc…ccccag (1.6 kb)[b]
10	1158-GGGCAG…TTACAA-1296	gtgaga…tcacag	1158-GGGCAG…TTACAA-1296	gtgaga…tcccag (9.9 kb)[b]
11	1297-ATTCTGTTGA[d]…GAGGAA-1365	gtaagc…ccacag	1297-ATTCTG…GAGGAA-1365	gtgagc…tcacag (1.9 kb)[b]
12	1366-TTCAAA…		1366-TTTAAA…CTGCAG-4309	

The translational start site is numbered as nucleotide 1 since the transcriptional start site is not exactly defined. Exon 1 contains approximately an additional 150 bp 5′ of the translational start site.
[a]Fully sequenced.
[b]Partially sequenced.
[c]The italicized *ag* is that alternatively used in SU(FU)-XL.
[d]The double-underlined TGA is the STOP codon used in SU(FU)-Lk.

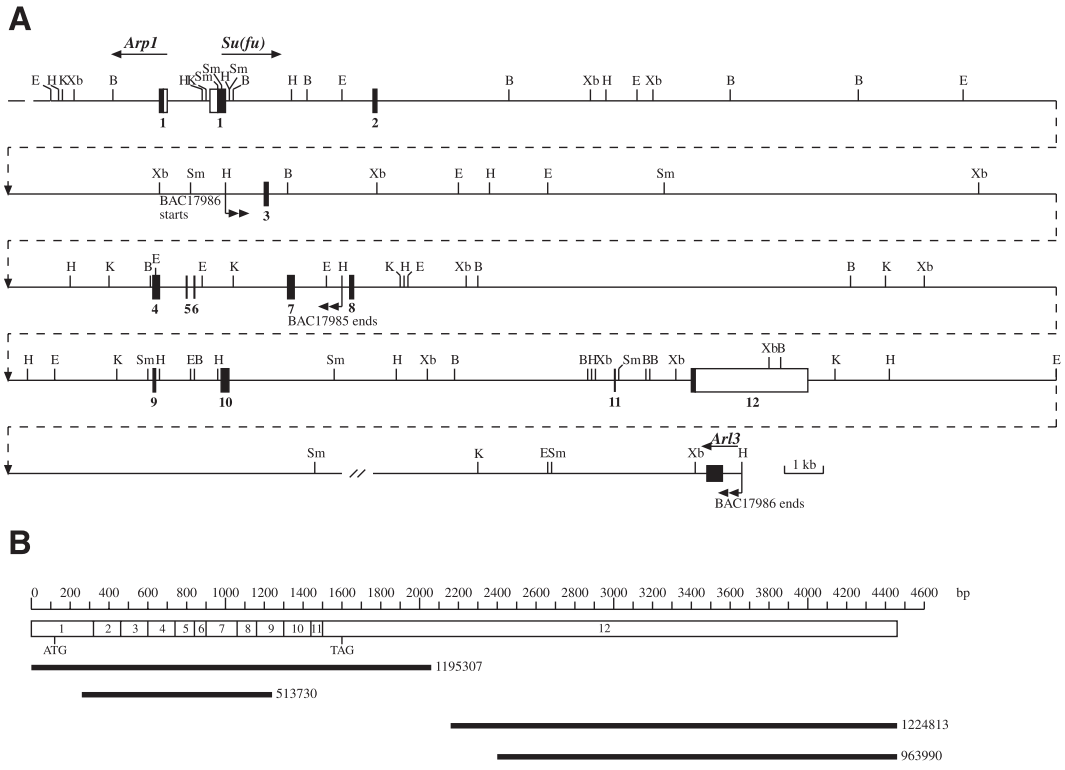

Fig. 1. Structure of the mouse *Su(Fu)* gene and cDNAs. A: The mouse *Su(Fu)* gene as assembled from mapping and partial sequencing of BAC clones 17985 and 17986. Boxes indicate position of exons. Filled and unfilled boxes indicate coding and non-coding regions, respectively. Arrows indicate direction of the transcriptional unit for *Su(Fu)* and the linked upstream gene for *Arp1* and the downstream gene for *Arl3*. Only the exon containing the ATG translational start of *Arp1* and the exon after the ATG-containing exon of *Arl3* have been mapped and sequenced. Double arrowheads indicate start and end of BAC clones. Not all sites of the indicated enzymes have been mapped. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; Xb, *Xba*I; Sm, *Sma*I. B: Structure of the sequenced mouse *Su(Fu)* EST cDNAs 1195307 (mammary gland), 513730 (testis), 1224813 (heart), and 963990 (mammary gland), from the IMAGE consortium collection. Numbers 1 through 12 indicate the *Su(Fu)* exons drawn to scale for a full-length mRNA transcript. ATG and TAG indicate the translational start and stop, respectively. The thick lines represent the structural composition of each of the four EST clones positioned relative to the hypothetical full-length transcript. The following EMBL/GenBank accession numbers have been assigned: AJ308627 (exon 1), AJ308628 (exon 2), AJ308629 (exon 3), AJ308630 (exon 4–6), AJ308631 (exon 7–8), AJ308632 (exon 9), AJ308633 (exon 10), AJ308634 (exon 11), AJ308635 (exon 12), AJ308626 (1195307), AJ308625 (513730), AJ308624 (1224813), and AJ308636 (963990).

Su(Fu) gene was published [27]. However, their published gene structure was not complete in that several intron sizes were not determined and that the last exon (exon 12) was not identified.

Mining the GenBank mouse EST database, several Su(Fu) EST clones were found demonstrating expression in a wide range of tissues: fertilized egg, embryos from day post coitum (dpc) 8, 10–11, and 13, head from day post natum (dpn) 6, skin from dpn 10, adult testis, heart, kidney, B cell, and mammary gland. Four mouse Su(Fu) ESTs were sequenced and their structures are shown in Fig. 1B. The combined EST cDNAs extend over 4400 bp, which fits well with the mRNA size seen on Northern blots (~4.5 kb, data not shown). The predicted AUG codon of the translational initiation fits the Kozak consensus sequence. The clones 1224813 and 963990 that extend the most 3′, as well as a number of other human and mouse ESTs in the GenBank end at the same position. However, no consensus AATAAA polyadenylation signal was found at the expected position. Possibly, an ATTAAA sequence about 15 bp upstream of the polyA tract, conserved in both human and mouse, is used instead.

3.2. SU(FU) mRNA isoforms

We screened a panel of cDNAs from various tissues of apparently healthy donors for expression of variants of *SU(FU)*. One *SU(FU)* variant found had a tri-nucleotide insertion after nucleotide 756 of the coding sequence resulting in an extra glutamine after amino acid 252 resulting in a protein with 485 amino acids. This insertion created a novel *Eco*NI cleavage site that allowed easy detection of this variant, which we called SU(FU)-XL. SU(FU)-XL was found to be expressed in five of seven tissues tested with a relative abundance varying from roughly 10% to 50% of total SU(FU) (Fig. 2). This variant is due to an AG di-nucleotide one nucleotide upstream of the standard AG splice acceptor site in the end of intron 6 (Table 1). Use of this AG as an alternative splice acceptor will introduce an extra CAG codon in the mRNA product as seen in SU(FU)-XL.

We found two additional splice variants (Fig. 2), one of them, SU(FU)-Lk, was expressed in peripheral leukocytes, the other one, SU(FU)-Tt, in testes. By DNA sequencing it was found that SU(FU)-Lk is lacking exon 10, and that the downstream sequence has a shifted reading frame resulting in a STOP codon after two amino acids. This results in a protein with 388 amino acids. SU(FU)-Tt has an extra exon after exon 8 (exon 8a), which encodes 19 amino acids followed by a STOP codon resulting in a protein containing 359 amino acids.

We also screened a panel of tumor cell xenograft cDNAs (two breast, two lung, two colon, one prostate and one pancreatic carcinoma sample) and cDNA samples from a panel of dpc 13.5 mouse embryonic tissues (lung, intestine, skeletal muscle, heart, liver, eye, brain, spinal cord, tongue, kidney, whole limb, skin) and we detected the normal SU(FU)/Su(Fu) transcripts but no variants except SU(FU)-XL (data not shown).

Functional analysis of these SU(FU) variants was performed by assessing their potential to co-precipitate GLI1, one of the physiological binding partners of SU(FU). N-terminally myc-tagged versions of all SU(FU) variants and a hemaglutinin-tagged version of GLI1 (HA-GLI) were transfected into 293 cells. With an antibody against HA we could co-precipitate myc-SU(FU) or myc-SU(FU)-XL, but not myc-SU(FU)-Lk or myc-SU(FU)-Tt as detected by an anti-myc antibody after Western blotting (Fig. 3). Thus, the two organ-specific expression variants SU(FU)-Lk and SU(FU)-Tt might provide additional regulatory mechanisms to Hedgehog signaling since these variants lack the ability of binding GLI1. Thereby, GLI1 could be uncoupled from one of its cytoplasmic retention mechanisms.

Another transcriptional variant has been described [4] that is expressed predominantly in testis and has the N-terminal 432 amino acids in common with the standard SU(FU) followed by a STOP codon.

3.3. Expression of Su(Fu) during mouse embryogenesis

We analyzed mouse embryos in the time range between dpc 8.5 and dpc 15.5 for expression of Su(Fu) by whole mount in situ hybridization. In particular, at dpc 10.5 and 11.5, we observed an intense ubiquitous staining where the neural tube and the forming CNS stained strongest. Only the liver appeared to be negative. For the other stages however, we found that certain tissues expressed Su(Fu) with considerably stronger intensity than the ubiquitous reactivity of the embryos. At dpc 8.5 and 9.5 we detected Su(Fu) in the neural

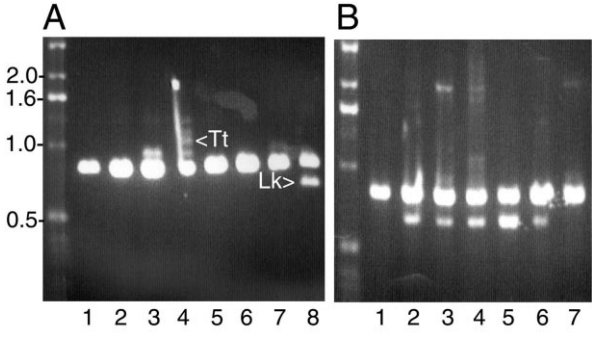

Fig. 2. Human SU(FU) variant transcripts. A: RT-PCR of a SU(FU) fragment containing exons 5 through 9 from various tissues. Tt and Lk indicate the respective variants. The other minor bands in lane 3 and lane 4 represent PCR products that are not related to SU(FU). B: Same as in A, after *Eco*NI digestion. The lower band represents the *Eco*NI susceptible SU(FU)-XL variant. Since the mobility of the *Eco*NI-digested fragment equals the mobility of the SU(FU)-Lk band, lane 8 was omitted here. Lane 1, thymus; 2, prostate; 3, spleen; 4, testis; 5, colon; 6, ovary; 7, small intestine; 8, peripheral leukocytes. C: Sequence of the intron 8a in SU(FU)-Tt. The italicized letters signify the flanking exons 8 and 9.

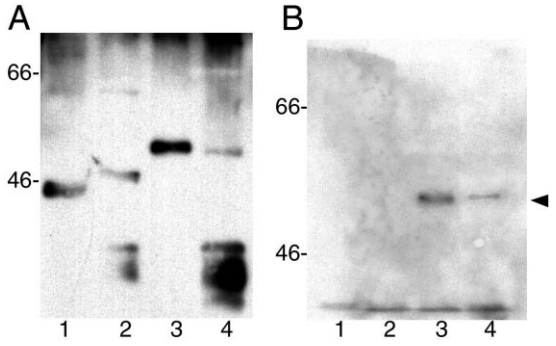

Fig. 3. Myc-SU(FU)-Lk and myc-SU(FU)-Tt cannot co-precipitate HA-GLI1. 10 μg each of HA-GLI1 and myc-SU(FU)-Lk (lane 1), myc-SU(FU)-Tt (lane 2), myc-SU(FU)-XL (lane 3) and myc-SU(FU) (lane 4) were transfected into 293 cells. Lysates were precipitated with anti-myc (A) and anti-HA (B) antibodies and blotted with anti-myc antiserum. Molecular weight markers (kDa) are to the left of the blots. The arrowhead next to blot B indicates the position of the co-precipitated myc-SU(FU)-XL and myc-SU(FU).

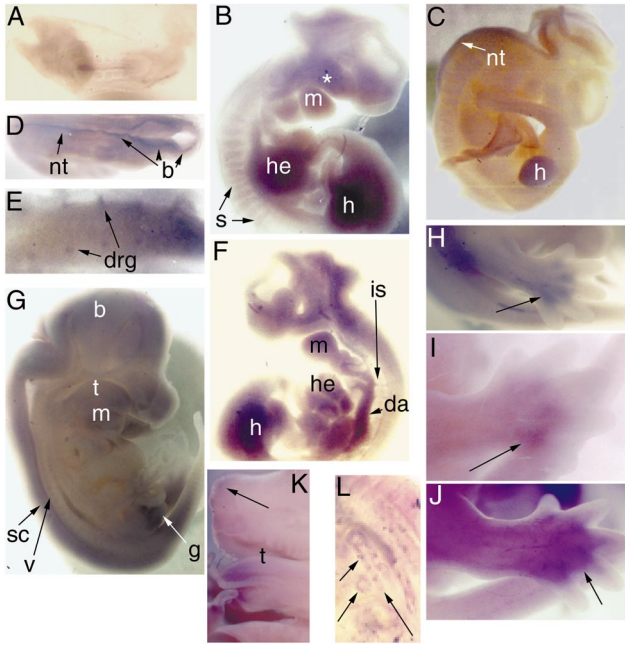

Fig. 4. Whole mount in situ hybridization of mouse embryos with a Su(Fu) antisense probe. A: dpc 8.5. B: dpc 9.5, lateral view. Hindlimb (h), heart (he), mandibular arch (m), somites (s), the mesenchyme surrounding the prospective mouth cavity (*). C: dpc 9.5, lateral view, prior to clearance with glycerol. Neural tube (nt). D: dpc 10.5, dorsal view. Brain vesicles (b). E: dpc 10.5, dorsal view of the tail after removal of the skin. Dorsal root ganglia (drg). F: dpc 9.5, sagittal view. Dorsal aorta (da), intersegmentary arteries (is). G: dpc 13.5, sagittal view. Spinal cord (sc), brain (b), mandible (m), tongue (t), genital tubercle (g), mesenchyme close to the vertebrae (v). H–J: Staining of the mesenchyme adjacent to the ossification zone of the digits in the hindlimb at dpc 13.5 (H), 14.5 (I), 15.5 (J). The arrows point to the Su(Fu) expressing areas that move distally with time. K: dpc 14.5 sagittal section of the snout. Tongue (t), most distal part of the snout (arrow). L: dpc 15.5, sagittal view of the dorsal part of the thorax. The arrows point to the tracheoles.

tube and in the somites (Fig. 4A–C). At dpc 9.5 Su(Fu) was expressed further in the heart and the dorsal artery as well as in the intersegmentary arteries (Fig. 4C,F), in the bud of the hindlimb (Fig. 4B,C), in the branchial arches (Fig. 4B,F), and in the mesenchyme of the trunk and of the head surrounding the prospective mouth cavity (Fig. 4B). Su(Fu) expression in the neural tissues persisted through the entire observed period. At dpc 10.5 Su(Fu) was expressed in all brain vesicles (Fig. 4D) and in the dorsal root ganglia (Fig. 4E) as detected after removal of the skin. At all later stages, the brain and the spinal cord were stained with high intensity (Fig. 4G). The expression of Su(Fu) in the somites persisted as well; however, from dpc 10.5 only the sclerotome and the sclerotome-derived tissues were stained. The mesenchyme surrounding the ossifying part of the vertebrae expressed Su(Fu) from dpc 12.5 (Fig. 4G and [3]) and of the ribs from dpc 13.5 [7]. Like in the somite-derived skeletal parts and even in the digits, the expression of Su(Fu) was observed to parallel the progress of ossification as the area that expresses Su(Fu) was migrating distally during the course of development (Fig. 4H–J). The expression of Su(Fu) in the branchial arches was continuing within the maxilla, mandible and in the tongue and disappearing from dpc 12.5 onwards when the mouth cavity was closed, with at dpc 15.5 only tongue being stained (Fig. 4K). Through the entire observed period, Su(Fu) was expressed in the genital tubercle (Fig. 4G) and from dpc 13.5 in the mesenchyme surrounding the developing tracheoles of the lung (Fig. 4L).

The sites of expression grossly coincide with that of other members of the Hedgehog signaling pathway, in particular with the expression of Gli3 [3]. However, in detail, this overlap is not found in all cases. Three other reports have investigated Su(Fu) expression in the mouse embryo [4,7,27] and our data extend and confirm these studies.

3.4. Examination of DNA and RNA from SHFM3 patients for mutations in the SU(FU) gene

The human *SU(FU)* gene is located in a region on 10q24 where the locus for the genetic disorder SHFM3 has been mapped.

To investigate the hypothesis that *SU(FU)* would be the gene involved in this syndrome, we initially screened five unrelated SHFM3 patients by Southern analysis with four restriction enzymes (*Eco*RI, *Hin*dIII, *Bgl*II, and, *Pst*I). No signs of gross alterations of the *SU(FU)* locus were seen (data not shown). For a more detailed analysis, we sequenced a number of cDNA clones of *SU(FU)* from B cell lines derived from two unrelated heterozygous SHFM3 patients. No differences from the normal sequence were observed over the entire coding region (data not shown). In addition, SSCP analysis was conducted on exons 2, 4, and 8–12 on the two probands from the linked families as well as six other unrelated individuals. Again, no alterations were observed (data not shown). With our approach, however, mutations in the promoter or in introns that would affect the transcriptional regulation or splicing of *SU(FU)* would not have been detected. Therefore, although the possibility has decreased, the *SU(FU)* gene can at this point not formally be excluded as a candidate gene for the SHFM3 syndrome and additional studies are needed.

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