



Published as: *Dev Biol.* 1999 August 15; 212(2): 323–336.

Vertebrate Homologs of *Drosophila* *Suppressor of Fused* Interact with the Gli Family of Transcriptional Regulators

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Abstract

The hedgehog (Hh) signaling pathway is crucial for pattern formation during metazoan development. Although originally characterized in *Drosophila*, vertebrate homologs have been identified for several, but not all, genes in the pathway. Analysis of mutants in *Drosophila* demonstrates that *Suppressor of fused* [*Su(fu)*] interacts genetically with genes encoding proteins in the Hh signal transduction pathway, and its protein product physically interacts with two of the proteins in the Hh pathway. We report here the molecular cloning and characterization of chicken and mouse homologs of *Su(fu)*. The chick and mouse proteins are 27% identical and 53% similar at the amino acid level to the *Drosophila melanogaster* and *Drosophila virilis* proteins. Vertebrate *Su(fu)* is widely expressed in the developing embryo with higher levels in tissues that are known to be patterned by Hh signaling. The chick *Su(fu)* protein can physically interact with factors known to function in Hh signal transduction including the *Drosophila* serine/threonine kinase, Fused, and the vertebrate transcriptional regulators Gli1 and Gli3. This interaction may be significant for transcriptional regulation, as recombinant *Su(fu)* enhances the ability of Gli proteins to bind DNA in electrophoretic mobility shift assays.

INTRODUCTION

The vertebrate *hedgehog* genes, *Sonic (Shh)*, *Indian (Ihh)*, and *Desert hedgehog (Dhh)*, encode secreted signaling molecules that are critical for the patterning of many tissues during development and for regulation of cell growth and homeostasis in adult organisms. For example, during development *Shh* is expressed in the zone of polarizing activity (ZPA) of the limb bud, the notochord, the floor plate of the neural tube, regions of the ventral diencephalon, the branchial arches, the gut, and hair follicles in the skin (Echelard *et al.*, 1993; Riddle *et al.*, 1993; Bitgood and Mc-Mahon, 1995). *Ihh* is expressed predominantly in the proliferating and prehypertrophic chondrocytes of the developing bones (Bitgood and McMahon, 1995; Vortkamp *et al.*, 1996) and in the endoderm of the midgut (Bitgood and McMahon, 1995). *Dhh* is expressed in the cranial ganglia and in the Sertoli cells of the testes (Bitgood *et al.*, 1996). Although each of the vertebrate hedgehog genes is expressed in a unique pattern, all three appear to act through the same signal transduction pathway.

Despite the importance of this signal transduction pathway in vertebrates (reviewed in Hammerschmidt *et al.*, 1997; Goodrich and Scott, 1998), most of our knowledge of the molecular mechanism of vertebrate hedgehog (Hh) signaling is inferred from studies in *Drosophila* (reviewed in Ingham, 1998). The *Drosophila hh* gene was originally identified in a screen for genetic mutations that affect patterning in larvae (Nüsslein-Volhard and Wieschaus, 1980). A number of other genes functionally related to the *hh* gene emerged from this screen. Several of these have been shown to encode members of the Hh signal transduction pathway including the following: the 12-transmembrane domain protein Patched (Ptc), which can physically interact with Hh protein (Hooper and Scott, 1989; Nakano *et al.*, 1989; Marigo *et al.*, 1996a; Stone *et al.*, 1996); the 7-transmembrane domain protein Smoothed (Smo), which is a member of the serpentine G-protein-coupled receptor superfamily (Alcedo *et al.*, 1996; van den Heuvel and Ingham, 1996); the serine/threonine (ser/thr) kinase Fused (Fu; Preat *et al.*, 1990); the kinesin-like cytoplasmic protein Costal-2 (Cos2; Sisson *et al.*, 1997); and the zinc-finger class transcriptional regulator Cubitus interruptus (Ci; Orenic *et al.*, 1990). Of these, Smo, Fu, full-length Ci, and Hh itself are necessary to activate target gene transcription, while Ptc, Cos2, and a proteolytically processed form of Ci repress target gene transcription.

In *Drosophila*, the earliest detectable event occurring after Hh binding to Ptc is the phosphorylation of Fu by an unknown factor, presumably resulting in activation of Fu (Therond *et al.*, 1996). Fu activity is critical for Hh signaling as null mutations of *fu* severely inhibit Hh signal transduction (Ingham, 1993; Preat *et al.*, 1993). Based upon early genetic dissections (Preat *et al.*, 1993) and recent biochemical studies (Robbins *et al.*, 1997), Fu has been subdivided into two major domains. The amino-terminus (~292 amino acids) contains a domain with homology to all known ser/thr kinases, while the longer carboxy-terminus (~580 amino acids) has been labeled the extracatalytic or regulatory domain.

One key tool for the analysis of Fu function came with the identification of a semidominant suppressor of the *fu* mutant phenotype, *Suppressor of fused [Su(fu)]* (Preat, 1992). While *Su(fu)* encodes a protein with no obvious homologies to known proteins (Pham *et al.*, 1995), it does contain a putative PEST sequence, a feature thought to be involved in rapid protein degradation (Rogers *et al.*, 1986). Paradoxically, flies lacking all *Su(fu)* function are viable, fertile, and display only very subtle developmental defects (Preat, 1992; Ohlmeyer and Kalderon, 1998), yet are phenotypically resistant to loss of *fu* function (Preat, 1992). This suggests that the *Su(fu)* gene product functions as an inhibitor to Hh signaling and that the *fu* gene product is required to bypass the inhibition. Consistent with this interpretation, extra copies of the *Su(fu)* gene enhance the phenotype of a weak *fu* allele (Pham *et al.*, 1995).

While many *fu* alleles are fully suppressed by loss of *Su(fu)* function, mutations that alter only the carboxy-terminal regulatory domain of Fu in a *Su(fu)* null background result in a phenotype similar to *cos2*, i.e., equivalent to eliminating transcriptional repression of *hh* target genes (Preat *et al.*, 1993). Furthermore, *Su(fu)* acts as a dominant enhancer of *cos2* mutations. For example, the *cos2*³ allele is heterozygous viable, with no obvious phenotype, while the loss of *Su(fu)* function in a (*cos2*^{3/+}) background results in larval lethality (Preat *et al.*, 1993).

In addition to the genetic interactions between *Su(fu)*, *fu*, and *cos2*, biochemical studies have shown that *Drosophila* Su(fu) protein can physically interact with Fu and Ci proteins both in the yeast two-hybrid system and in the GST pull-down assay (Monnier *et al.*, 1998). The *cos2* gene encodes a kinesin-like protein that is found in a complex with Fu and Ci proteins in *Drosophila* embryonic extracts (Aza-Blanc *et al.*, 1997; Robbins *et al.*, 1997; Sisson *et al.*, 1997). These genetic and biochemical observations for *Su(fu)*, *fu*, and *cos2* are consistent with the model that their three protein products interact to regulate signaling by Hh, with Fu acting as an activator of Hh target gene transcription and both Su(fu) and Cos2 acting as repressors.

In addition to vertebrate homologs described for *Drosophila* Hedgehog (Fietz *et al.*, 1994), vertebrate homologs have also been identified for the Ptc and Smo receptors (Marigo *et al.*, 1996a; Stone *et al.*, 1996; Akiyama *et al.*, 1997; Carpenter *et al.*, 1998; Motoyama *et al.*, 1998) and for the transcriptional regulator Ci (Kinzler *et al.*, 1987; Ruppert *et al.*, 1990; Aruga *et al.*, 1994, 1996a, 1996b; Hui *et al.*, 1994; Nagai *et al.*, 1997; Nakata *et al.*, 1998). However, vertebrate homologs of the intermediate cytoplasmic factors involved in the transduction of the Hh signal from the cell surface to the nucleus [Fu, Su(fu), and Cos2] have not been described.

Although the mechanism of Hh signal transduction in *Drosophila* and that in vertebrates appear to be very similar, it is clear that some differences do exist. In *Drosophila* both the positive regulation and the negative regulation of target genes by Hh appear to occur predominantly through the Ci protein, while in vertebrates, there appears to be a complex interplay between the various members of the Gli and Zic families of transcriptional regulators (Brewster *et al.*, 1998; Brown *et al.*, 1998). Since this pathway plays such critical roles in vertebrate embryonic development as well as in cellular homeostasis in the adult, it is important to more clearly understand the molecular nature of the vertebrate signaling pathway. To this end, we have cloned and completed an initial characterization of *Su(fu)* from the chicken and mouse.

MATERIALS AND METHODS

Cloning of mouse and chicken *Su(fu)*

Electronic screening of the NCBI expressed sequence tag (EST) database revealed a clone with homology to *Drosophila Su(fu)*. The EST clone ID 513730 (GenBank Accession No. AA061391) was obtained from Genome Systems, Inc. This clone was originally isolated from a mouse testis cDNA library and contains an insert of approximately 950 bp. The chicken *Su(fu)* cDNA clone was obtained using the *Su(fu)* homologous region of the mouse EST clone to probe a cDNA library under high-stringency conditions (random primed Lambda ZAP II library from stage 18–24 chicken limb bud RNA). This screen yielded 87 positive clones of varying intensity. Of these positives, 10 were carried to plaque purity and analyzed. The longest clone isolated from the chicken library contained a 3.0-kb insert with a 1452-bp open reading frame, a 35-bp 5'UTR, and a 1.5-kb 3'UTR. The mouse partial cDNA was obtained by high-stringency screening of an 8.5-dpc whole mouse embryo λ -gt10 library (a gift from Dr. B. Hogan, Vanderbilt University, Nashville, TN) with the radiolabeled (Prime-It II, Stratagene) 420-bp *EcoRI* fragment from the mouse *Su(fu)* EST.

The 5' end of the 1.4-kb insert is equivalent to base 236 in the published sequence of the mouse *Su(fu)* EST. 5' RACE (Gibco) was performed according to the manufacturers directions using 17.5-dpc mouse skin RNA. Primer sequences used for RACE were as follows: 5'-TCT-GGAGGAAAGTCACTACCCC-3', 5'-TGCAGACACCAACAATC-TGG-3', and 5'-TCCACTGTTGGGCTGAATG-3'. RACE products were cloned using the pCR2.1 TA cloning kit (Invitrogen). All sequence was obtained by ABI automated sequencing.

Expression analysis

For whole-mount *in situ* hybridizations of chicken embryos, we generated a probe for *Su(fu)* expression by subcloning an 800-bp 5' fragment (from *EcoRI* in the multiple cloning sequence to the *SalI* site in the open reading frame) into pBS SK(-). The template for the probe was generated by amplifying the insert of this *SalI* 800-bp clone by PCR using M13 forward and reverse universal primers and the AdvantageTaq polymerase system (Stratagene) (PCR cycle consists of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min ×20 cycles). The resulting PCR product was gel purified and 1.0 µg of template was transcribed in the presence of digoxigenin-11-UTP and T7 RNA polymerase to generate a digoxigenin antisense riboprobe. The integrity of riboprobes was checked by analysis on a 1.5% agarose gel in 0.5× Tris-Borate EDTA (TBE) buffer. The probe was added to hybridization buffer (hybridization buffer: 50% formamide, 5× SSC, pH 4.5, 50 µg/ml yeast tRNA, 1% SDS, 50 µg/ml heparin).

Chick embryos were obtained by incubating fertilized eggs from white Leghorn chickens (Spafas) at 37°C in a rotating egg incubator (Petersime). Eggs were removed from incubation at varying times and staged according to described criteria (Hamburger and Hamilton, 1992). Embryos were dissected, rinsed, fixed with 4% paraformaldehyde, and subsequently permeabilized with 10 µg/ml proteinase K for 15 min at room temperature, with the exception of stage 30–32 embryos, which were permeabilized with 60 µg/ml proteinase K for 15 min. Subsequent steps were performed according to a described protocol (Riddle *et al.*, 1993).

For mouse whole-mount *in situ* hybridization, the 420-bp *EcoRI* fragment from the mouse EST was used in run-off transcription reactions to generate digoxigenin-11-UTP-labeled riboprobes (Boehringer Mannheim). Digoxigenin incorporation was tested by dot-blotting probes on nitrocellulose, probing with anti-digoxigenin alkaline phosphatase-coupled antibodies, and analyzing for alkaline phosphatase activity using NBT and BCIP. Probes were dissolved in 100 µl DEPC-treated water and added to hybridization buffer at 5 µl/ml.

Mouse embryos were obtained from B6/CBA F1 females mated to B6/CBA F1 males. Embryos were staged by defining noon on the day following copulation as 0.5 dpc. Mouse whole-mount *in situ* hybridizations were performed as described (Wilkinson, 1992). Proteinase K treatment was varied with age (15 min for 10.5 dpc, 7 min for 9.5 dpc, and 5 min for 8.75 dpc). After alkaline phosphatase activity was visualized with NBT and BCIP for 1 h, embryos were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in methanol, and rehydrated before storing in PBT. For Northern analysis, a mouse multiple-tissue RNA blot (Clontech) was probed with the radiolabeled approximately 950-bp *XhoI*/

PstI fragment from the mouse EST. As a loading control, the blot was subsequently probed with the human β -actin probe supplied by the manufacturer. Hybridization and wash conditions were as described by Clontech.

GST protein:protein interaction assays

The GST-Su(fu) construct was generated by using the bacterial expression vector pGEX-KG to fuse glutathione *S*-transferase in-frame with a 2.0-kb *NcoI* chick Su(fu) fragment extending from the initial methionine (the ATG in the 5' *NcoI* site) to 600 bp past the stop codon. Transformed DH5 α bacteria were grown to OD_{600nm} = 1.0 and induced with 3 mM isopropyl β -D-thiogalactopyranoside for 3 h. Expressing bacteria were pelleted and lysed by sonication in lysis buffer (0.4 M NaCl, 50 mM Tris, pH 7.6, 1 mM EDTA, 1 mM DTT, 10% glycerol, and the Boehringer Complete mini protease inhibitor cocktail tablets).

Recombinant protein was bound to glutathione agarose beads in lysis buffer and washed. Protein purity was assessed on SDS-PAGE gels. By staining the SDS-PAGE gels with Coomassie brilliant blue it is estimated that all proteins used in these assays were greater than 90% pure. Prior to the interaction assay the protein bound glutathione beads were equilibrated with protein:protein interaction buffer (PPI: 20 mM Tris, pH 7.9, 200 mM NaCl, 1 mM EDTA, 4 mM MgCl₂, 0.02% NP-40, 10% glycerol, protease inhibitors, and 1.0 mg/ml BSA). Constructs for producing labeled binding proteins were generated by standard subcloning techniques and *in vitro* translated in either reticulocyte lysate or wheat germ extract using the TnT-coupled transcription/ translation system (Promega) in the presence of Redivue [³⁵S]methionine (Amersham). The r1-only, Zn-only, *NcoI*-Zn3', and *Drosophila* Fu-reg (aa 306–805) translation constructs were generated by PCR using oligonucleotides that flank the domains of interest and adding an initial methionine and Kozak sequence to the 5' end of each domain. All TnT translated proteins were analyzed for protein integrity by SDS-PAGE and normalized with other reactions based on TCA precipitable counts.

To test interactions, 50,000 TCA precipitable cpm of ³⁵S-labeled protein were added to 50 μ l of glutathione agarose beads (50:50 slurry in PPI buffer +BSA) bound to either an excess of control (GST) or experimental GST-Su(fu) fusion proteins. The reaction was incubated at 25°C for 20 min (vortexed every 5 min). Beads were washed four to six times with 1.0 ml of PPI buffer. Any ³⁵S-labeled protein bound to the beads after the washes was eluted by boiling in 25 μ l of 2 \times protein loading buffer (2% SDS, 10% glycerol, 100 mM Tris, pH 6.8, 0.7 M β -mercaptoethanol, 0.002% bromophenol blue) for 1–2 min. After boiling, 20 μ l of denatured eluate was loaded onto a 12% SDS-PAGE polyacrylamide protein gel for analysis. For quantitative analysis, 2 μ l (10%) of the eluate was spotted onto a glass fiber filter and counted in a liquid scintillation counter.

Two-hybrid analysis

Protein:protein interactions were detected using plasmids from the MATCHMAKER GAL4 System 2 (Clontech). pAS2-1 is the “bait” plasmid in this system and allows expression of the protein of interest as a fusion with the GAL4 DNA binding domain. pACT2 is the “prey” plasmid in this system and allows expression of the protein of interest as a fusion with the transcriptional activation domain of GAL4. The regulatory domain of *Drosophila* Fu (Fu-reg) (amino acids 306–805) containing an *EcoRI* linker was excised from LexA-Fu-reg

(Monnier *et al.*, 1998) and subcloned into the *EcoRI* site of pAS2-1. The 2-kb *NcoI* chick Su(fu) fragment extending from the initial methionine (the ATG in the 5' *NcoI* site) to 600 bp past the stop codon was cloned into the *NcoI* site of pACT2. pVA3-1 and pTD1-1 (Clontech) were used as control plasmids and contain amino acids 72–390 of p53 in pAS2-1 and amino acids 84–708 of the SV40 large T antigen in pACT2, respectively.

Yeast were transfected by a modified version (Gietz *et al.*, 1992) of the lithium acetate method as described in the Hybrid Hunter instruction manual (Invitrogen). Yeast strain PJ69-4A (James *et al.*, 1996) was chosen to detect protein:protein interactions because it contains three reporters: *ADE2* under control of the *GAL2* promoter, *HIS3* under control of the *GAL1* promoter, and β -galactosidase under control of the *GAL7* promoter. Yeast were cotransfected with the following pairs of plasmids: pVA3-1 plus pTD1-1; pAS2-1 Fu-reg plus pTD1-1; pAS2-1 Fu-reg plus pACT2 Su(fu); and pVA3-1 plus pACT2 Su(fu). After transformation, yeast were plated onto trp-, leu-deficient (trp-leu) medium for several days to select for the presence of the pAS2-1 and pACT2 derivatives. Selected yeast were replica-plated onto medium lacking ade, trp, and leu to test for reporter activation and allowed to grow for 5 days. Yeast grown on -trp, -leu were then assayed by the X-gal colony filter lift method for β -galactosidase activity as described by Clontech (Breedon and Nasmyth, 1985).

Band shift assays

Two Gli DNA binding sites were synthesized that correspond to the natural human Gli-B and Gli-C binding sites (Kinzler and Vogelstein, 1990). Band shifts with the Gli-B binding site are shown, but indistinguishable results were obtained with the Gli-C site. The sequence of one strand of the Gli-B site is

5'AACCAGACGCGTGG**ACCACCCA**AAGACGAAATTCACACTC-3' and the Gli-C binding site is 5'-AGGCACACAGATAG**ACCACCC**AGCTTCAGGTGGGGGACC-3'.

The residues in boldface type are the core binding sequence in each case. The two strands of the binding site were annealed, radiolabeled by phosphorylation with polynucleotide kinase in the presence of [γ - 32 P]ATP, and purified with a 6% nondenaturing polyacrylamide gel or over a Sephadex G25 spin column. Band shift binding buffer (1 \times) contained 10% glycerol, 10 mM Tris, pH 7.5, 50 mM KCl, 10 μ M ZnCl₂, 2 mM spermidine, 0.5 mg/ml ssDNA, 1 mM DTT, 0.5 mg/ml BSA. Five microliters of nuclear or cytoplasmic extracts or of nonradiolabeled, *in vitro* translated human Gli1 or Gli3 was added to the binding buffer together with 1–5 μ l of either GST or GST–Su(fu) bacterially expressed fusion proteins (total volume of the binding reaction was 20 μ l). The proteins were incubated at 37°C for 30 min prior to adding 50,000 cpm of the 32 P labeled, purified, Gli-B binding site. After incubation for 20 min at room temperature with the binding site, complexes were analyzed by electrophoresis through a 4.5% nondenaturing polyacrylamide gel in 0.25 \times TBE buffer (Fig. 5A and 5C) or over a 6% nondenaturing polyacrylamide gel in 0.5 \times TBE buffer (Fig. 5B).

Western blot analysis

Rat polyclonal antisera were prepared against *Drosophila melanogaster* Su(fu). Bases 22–1015 of *D. melanogaster* Su(fu) were cloned into the pATH10 vector (Rimm and Pollard, 1989) that results in the fusion of amino acids 8–338 of Su(fu) with Trp E. The Su(fu)–Trp

E fusion was expressed in DH5 α cells. The fusion protein was isolated from cell extracts by cutting a preparative SDS-PAGE gel and was used to immunize rats (Josman Labs). Nuclear and cytoplasmic extracts were made from either dechorionated *Drosophila* embryos or stage 30–31 chick embryonic tissue by dissociating embryos into single cells in a dounce homogenizer and extracting cytoplasmic and nuclear protein according to a previously described protocol (Schreiber *et al.*, 1989). Extracts were denatured and separated over a 10 or a 4–12% gradient SDS-PAGE gel and blotted onto nitrocellulose membrane. Membranes were blocked and subsequently probed with the anti-*Drosophila* Su(fu) antibody described above (SF57) or the control antibodies monoclonal mouse anti-Isl-1 or anti-tubulin antibody. Antibodies were detected using HRP anti-rat or HRP anti-mouse antibodies and were developed with ECL detection reagents (Amersham).

RESULTS AND DISCUSSION

Isolation and Expression of Vertebrate Homologs of *Drosophila* Suppressor of Fused

Scanning of the NCBI EST database revealed a mouse sequence annotated as bearing homology to *Drosophila* Su(fu). A cDNA fragment containing the Su(fu) EST was used to screen a random-primed stage 18–22 chicken limb-bud library yielding a 3-kb clone that encodes a protein whose closest relative is *Drosophila* Su(fu). Because the mouse EST does not contain a complete cDNA [it does not contain an initiation ATG, a translational stop, or a poly(A) tract], a mouse 8.5-dpc whole embryo λ gt10 library was screened with the Su(fu) homologous region of the EST. The mouse clone identified in this screen begins at base 236 of the published sequence of the EST. 5' RACE was used to obtain 41 additional bases of sequence 5' to the start of the EST, but failed to reach the ATG. The 3' end of the cDNA does contain a translational stop followed by additional stop codons in every frame.

The chicken and mouse proteins are 27% identical and 53% similar at the amino acid level to both *D. melanogaster* and *Drosophila virilis* Su(fu) proteins (Fig. 1). The similarity is strongest between the amino-terminal regions of the predicted *Drosophila* and vertebrate proteins. The only notable feature of *Drosophila* Su(fu) is a possible PEST sequence that resides in the carboxy-terminal portion of the protein. PEST sequences have been identified in proteins such as Cactus and Notch and are believed to be involved in rapid protein degradation (Rogers *et al.*, 1986). We analyzed the *Drosophila* and vertebrate Su(fu) proteins using the computer algorithm PESTfind (<http://www.at.embnet.org/htbin/embnet/PESTfind>) for potential PEST sequences. The algorithm searches proteins for potential PEST sequences and assigns a score of –50 to +50. A score greater than zero indicates a possible PEST sequence, while scores above 5 are considered more likely to function as bona fide PEST domains. Using PESTfind, amino acids 309–326 of *D. melanogaster* and the corresponding amino acids (313–330) of *D. virilis* Su(fu) both score +1.48. Interestingly, it is just prior to this potential PEST sequence that the *Drosophila* and vertebrate Su(fu) protein sequences diverge. When chick and mouse Su(fu) proteins were analyzed by PESTfind, amino acids 440–455 of cSu(fu) and the corresponding amino acids of mSu(fu) score +1.51 and +4.07, respectively. Although both vertebrate proteins contain possible PEST sequences, the actual sequences are totally different from the *Drosophila* Su(fu) putative PEST sequence and are located in a different region of the protein, so they do not

appear to be derived from a common ancestral domain. We therefore question whether the fly or vertebrate PEST-like sequences will actually affect Su(*fu*) protein stability.

To analyze *Su(fu)* expression in vertebrates, probes were hybridized *in situ* to chicken embryos of various stages. Although low levels of *Su(fu)* message appear to be in virtually all tissues, there are notable locations where *Su(fu)* appears to be expressed at higher levels. At stage 17 (29–32 somites), there is slightly higher expression in the neural tube and a significantly higher abundance of mRNA in the anterior tip of the developing forebrain (Fig. 2A). By stage 21, slightly higher expression can be seen in the limb buds, somites, and tail bud (Figs. 2B and 2C). By stage 30 we observe the most dramatic *Su(fu)* expression in a pattern similar to the induction of *Ptch1* in the perichondrium of the developing bones, adjacent to the domain where *Ihh* is expressed in the prehypertrophic chondrocytes (Vortkamp *et al.*, 1996). Additionally, while at stage 31 *Su(fu)* message appears to be highly abundant in many tissues, we can clearly see higher expression of *Su(fu)* in the developing feather buds (Fig. 2E). This is reminiscent of the previously described induction of *Ptch1* by *Shh* in the feather buds (Marigo and Tabin, 1996).

Although the higher expression of *Su(fu)* in the developing bones and feather buds appears similar to the induction of *Ptch1* by *Ihh* and *Shh*, respectively, *Su(fu)* is not expressed at an elevated level in other cells that receive Hh signals. For example, the expression of *Su(fu)* is no higher in the posterior limb bud mesenchyme, which is exposed to endogenous *Shh*, than it is in the anterior mesenchyme. Moreover, *Shh*-soaked beads are unable to induce higher expression of *Su(fu)* in the anterior wing bud (data not shown). In the early developing mouse, *Su(fu)* mRNA is also widespread. The mRNA is ubiquitous at 8.75 dpc (post-turning) and 9.5 dpc (Figs. 2F and 2G). However, as in the chick, by 10.5 dpc, both the neural epithelium and the limb buds express higher levels of *Su(fu)* (Fig. 2H).

The widespread distribution of *Su(fu)* mRNA in the developing vertebrate is not surprising, because in *Drosophila*, *Su(fu)* mRNA is ubiquitous throughout embryonic development (Pham *et al.*, 1995). Additionally, *cos2* and *fu* mRNA expression patterns also contrast with that of *hh* itself, which is spatially controlled, and *ptc*, which is transcriptionally induced in response to Hh signaling (Hidalgo and Ingham, 1990; Lee *et al.*, 1992; Taylor *et al.*, 1993; Tabata and Kornberg, 1994; Sisson *et al.*, 1997).

To determine whether *Su(fu)* mRNA is produced in adult tissues, a mouse multiple-tissue RNA blot was probed with the mouse *Su(fu)* EST clone. Identical results were obtained by probing the blot with the 1.2-kb *EcoRI* fragment from the mouse *Su(fu)* clone identified in the λ library (data not shown). Two transcripts were detected. The larger, 5.4-kb transcript was present at low levels in testis, kidney, heart, liver, and brain and at very low levels in lung (Fig. 2I). The smaller, more abundant 2.0-kb transcript was testis specific. It is possible that the high *Su(fu)* expression in the testis relates to the presence of *Dhh* signaling in Sertoli/Leydig cell communication (Bitgood *et al.*, 1996; Carpenter *et al.*, 1998). However, at the present time it is not known how the testis-specific transcript differs from the larger transcript. Expression of *Su(fu)* in adult tissue is not unexpected, as *Ptch1* mRNA has been detected (at least at low levels) in all adult tissues examined (Goodrich *et al.*, 1996). It is also known that vertebrate Hh signaling plays a role in cellular homeostasis, as constitutive

activation of vertebrate Hh signaling in the adult can lead to certain types of tumors (Chidambaram *et al.*, 1996; Hahn *et al.*, 1996; Uden *et al.*, 1996; Dahmane *et al.*, 1997; Fan *et al.*, 1997; Oro *et al.*, 1997; Raffel *et al.*, 1997; Vorechovsky *et al.*, 1997; Xie *et al.*, 1998). Therefore, it is possible that *Su(fu)* also plays a role in cellular homeostasis in the adult.

Protein:Protein Interactions between *Su(fu)* and *Gli1*, *Gli3*, and *Fu*

If the vertebrate and *Drosophila* *Su(fu)* proteins play similar roles in regulating Hh signal transduction, we would expect them to exhibit similar biochemical interactions. Insight into the *Drosophila* Hh signaling pathway came with the observation that the *Fu*, *Ci*, and *Cos2* proteins exist in a large cytoplasmic complex that is bound to microtubules in the absence of Hh signaling (Robbins *et al.*, 1997; Sisson *et al.*, 1997). Upon binding of Hh to *Ptc*, this cytoplasmic complex is released from the microtubules but appears to remain otherwise intact. Although it has not been shown biochemically that *Su(fu)* is present in such complexes, it has been shown that *Su(fu)* interacts physically with both the carboxy-terminal regulatory domain of *Fu* and the amino-terminus of *Ci* (Monnier *et al.*, 1998). To determine whether the vertebrate *Su(fu)* protein exhibits similar biochemical interactions we investigated the ability of chick *Su(fu)* to physically interact with the vertebrate homologs of *Ci* and with *Drosophila* *Fu*, since a vertebrate homolog of *Fu* has not yet been identified.

The closest vertebrate relatives of *Ci* are the *Gli* family of Zn finger transcriptional regulators. *Gli1* was originally identified based on its amplification in glioblastoma cell lines (Kinzler *et al.*, 1987). *Gli2* and *Gli3* genes were subsequently isolated by their homology to human *Gli1* (Ruppert *et al.*, 1990; Grindley *et al.*, 1997; Hughes *et al.*, 1997; Mo *et al.*, 1997). In the chick, *Gli1* expression is induced in the anterior limb bud mesenchyme in response to Shh-soaked beads while *Gli3* is repressed (Marigo *et al.*, 1996b), indicating that *Gli1* may be an activator of Shh target genes while *Gli3* may repress genes in the absence of Shh. This hypothesis is further supported by mutations of *Gli3* in mice, which result in a preaxial polydactyly due to ectopic Shh expression in the anterior limb bud (Hui and Joyner, 1993; Buscher *et al.*, 1997). However, it has recently been reported that *Gli3* is able to bind the transcriptional coactivator CBP and that *Gli3* may function as both a positive and a negative regulator of transcription (Dai *et al.*, 1999).

The notion that *Gli* proteins may both positively and negatively regulate Shh target genes is supported by studies in *Drosophila*. In the absence of Hh signaling, *Ci* protein is proteolytically processed into a repressor form (*Ci*⁷⁵) that contains only the amino-terminal and DNA binding domains (Aza-Blanc *et al.*, 1997). Although it has been recently reported that in mouse embryos *Gli3*, and not *Gli1*, is proteolytically processed (Dai *et al.*, 1999), it is not clear whether this processing results in a conversion from an activator to a repressor form of *Gli3* or whether each *Gli* factor exerts a distinct positive or negative influence on the signaling pathway. Based on the similarity of *Gli* proteins to *Ci*, it seems likely that *Gli* proteins will exert both positive and negative control over gene expression by some mechanism. Recent genetic data indicate that *Zic2*, a Zn finger transcriptional regulator that is more distantly related to *Ci* than the *Gli* genes, may also play an important role in Shh signal transduction (Brewster *et al.*, 1998; Brown *et al.*, 1998).

To investigate the ability of Su(fu) to interact directly with Gli proteins, we performed *in vitro* GST protein: protein interaction assays using a chick GST–Su(fu) fusion and ³⁵S-labeled human Gli1 and Gli3. Both Gli1 and Gli3 interact with Su(fu) by this assay (Fig. 3A). To map the domain of interaction on Gli3 we made a deletion series of human Gli3 protein for use in the GST pull-down assay (Fig. 3B). Gli1 and Gli3 share seven regions of similarity to each other (Ruppert *et al.*, 1990). Among these domains, region 2 (the Zn finger DNA binding domain) and region 1 (r1), have the highest similarity to the equivalent regions in the *Drosophila* Ci protein. Since *Drosophila* Su(fu) interacts with the amino-terminal part of Ci (without the Zn finger or the carboxy-terminus), we predicted that Su(fu) would interact specifically with the r1 domain in Gli1 and Gli3. Indeed, while Su(fu) interacts with either full-length Gli3 or Gli3 lacking either the carboxy-terminus or the Zn finger domain, the interaction is lost when the protein is truncated just aminoterminal to the Gli3 r1 domain. A small radiolabeled peptide containing only the Gli3 r1 domain is also sufficient for the interaction with Su(fu). Gli3 deletion proteins containing only the sequence carboxy-terminal to the r1 domain interact only very weakly, the signal being slightly above background (Figs. 3B–3D). Thus, r1, a domain that is conserved between *Drosophila* Ci and all known human Gli proteins, appears to be involved in protein: protein interactions with Su(fu) proteins.

In light of the regulation of Ci by proteolytic processing, it has been proposed that some of the Gli proteins, specifically Gli3, may be similarly processed (Dai *et al.*, 1999). If, as in *Drosophila*, amino-terminal processed forms of the Gli proteins act as repressors, this could explain the dominant phenotypes that result from mutations producing truncated forms of Gli proteins. Three dominant human mutations of Gli3 result in truncations of the protein, causing varying degrees of polydactyly depending on the location of the mutation in heterozygous individuals (Vortkamp *et al.*, 1991; Radhakrishna *et al.*, 1997). Two of the mutations retain both the r1 domain and the Zn finger domain, while one of the mutations, identified in patients with Greig cephalopolysyndactyly syndrome (GCPS), results in a protein that contains only the amino-terminus without the Zn finger. In the GCPS mutant the only defined domain remaining in the Gli3 protein is r1. Although it is quite possible that other factors interact with the amino-terminus of Gli3 and perhaps even compete with Su(fu) for interaction with r1, it is tempting to speculate that the interaction between the truncated Gli3 (lacking a DNA binding domain) and Su(fu) may result in a dominant abnormal regulation of the Shh signaling pathway in these individuals. While we have not tested the ability of the Zic family of transcriptional regulators to interact with Su(fu), they do not contain any region similar to r1.

Drosophila Su(fu) was originally identified in a screen for suppressors of the *fused* phenotype and has since been shown to interact physically with the Fu regulatory domain (Preat, 1992; Preat *et al.*, 1993; Alves *et al.*, 1998; Monnier *et al.*, 1998; Ohlmeyer and Kalderon, 1998). As a vertebrate homolog of *fused* has yet to be identified, we investigated the ability of chick Su(fu) to interact with the regulatory domain (amino acids 306–805) of *Drosophila* Fu (Fu-reg) in the both the yeast two-hybrid system and the GST pulldown assay. Yeast strain PJ69-4A, a strain with two auxotrophic nutritional reporter genes, ADE and HIS, was used to test the Su(fu):Fu-reg interaction. In addition, the strain contains a β -

galactosidase reporter. In the absence of interacting proteins, the ADE reporter in this strain is fully repressed. Growth on ADE-deficient medium is readily detectable when interacting proteins are present (James *et al.*, 1996). We chose to assay for ADE and β -galactosidase reporter activity, and not for HIS function, due to the intrinsic low level of background activity of the HIS reporter.

The Fu-reg–DNA binding domain fusion was tested for its ability to interact with Su(fu)–activation domain fusions. The two proteins do interact, based on colony growth that is dependent upon transcription of the ADE reporter. In contrast, Fu-reg–DNA binding domain fusion did not interact with the control prey, the SV40 large T antigen– activation domain fusion. The Su(fu)–activation domain fusion did not interact with a control bait, the p53–DNA binding domain fusion. However, the well-documented interaction between SV40 large T antigen and p53 was readily detected, indicating that both proteins are expressed in yeast. Only the Fu-reg:Su(fu) and T antigen:p53 combinations were able to activate transcription of the β -galactosidase reporter, as detected by the X-gal filter lift assay (Fig. 4A).

To eliminate the possibility that a yeast protein mediates the detected Fu-reg:Su(fu) interaction, *in vitro* GST protein: protein interaction assays were performed. Fu-reg was *in vitro* translated in the presence of ^{35}S -labeled methionine and incubated with either GST alone as a control or GST–Su(fu). Only GST–Su(fu) was able to interact with Fu-reg in this assay (Fig. 4B), confirming the yeast two-hybrid data.

The ability of chick Su(fu) to interact with *Drosophila* Fu-reg indicates that the Fu-interacting domain has been functionally conserved over the wide evolutionary distance between *Drosophila* and vertebrates. Su(fu) can also interact with Gli1 and Gli3, so in vertebrates, as in flies, Su(fu) may link the cytoplasmic transducers of Hh signaling to nuclear transcription factors.

Localization of Su(fu) Protein

The *Drosophila* full-length Ci protein has been observed only in the cytoplasm, while a truncated repressor form of Ci is mostly nuclear. In vertebrates the Gli1 protein seems to be mostly nuclear, while there is evidence that Gli3 is mostly cytoplasmic (Kinzler and Vogelstein, 1990; Lee *et al.*, 1997). Since the Gli proteins, like Ci, seem to reside in different cellular compartments, we investigated Su(fu) localization. The high degree of similarity in the amino-terminal regions of vertebrate and *Drosophila* Su(fu) proteins allows an antibody raised against the amino-terminus of *Drosophila* Su(fu) (SF57) to cross-react with chick Su(fu). The antibody recognizes both bacterially expressed and *in vitro* translated chicken Su(fu) (data not shown). We made cytoplasmic and nuclear extracts of stage 31 chicken embryonic tissue and *Drosophila* embryos. To test the separation between nuclear and cytoplasmic proteins the extracts were probed with control antibodies that recognize known nuclear antigens (Isl-1 and Pax6) for the chick extracts or for known cytoplasmic antigens (tubulin and kinesin heavy chain) for *Drosophila* embryo extracts. These control antibodies recognize appropriately sized bands predominantly in the nuclear and cytoplasmic extracts, respectively (Figs. 5D and 5E; and data not shown). The Su(fu) antibody recognizes one major band of approximately 50 kDa [the predicted size of Su(fu)]

in both *Drosophila* and chick extracts. While in *Drosophila* this protein band is predominantly cytoplasmic, in chicken it is much more intense in the nuclear extract than in the cytoplasmic extract (Figs. 5D and 5E). While we cannot rule out the possibility that the SF57 antibody is cross-reacting in the chicken extracts with a non-Su(fu) 50-kDa protein in the nuclear extract, these results at face value show a contrast between the cytoplasmic *Drosophila* protein and the nuclear chick protein.

Su(fu) Protein Enhances the Ability of Gli and Gli3 to Bind Target DNA Sequences

Sequences within the human genome selected by their ability to bind Gli1 protein contain the core sequence 5'-GACCACCCA-3' (Kinzler and Vogelstein, 1990). Remarkably, this consensus DNA sequence is perfectly conserved between human Gli and *Drosophila* Ci binding sites. The promoter of the *Drosophila ptc* gene contains three exact matches of the Gli binding site that are required for activation of *ptc* by *hh* (Alexandre *et al.*, 1996). Since Su(fu) may act within the Shh signal transduction pathway by interacting with Gli1 and Gli3, and since chick Su(fu) protein appears to be in the nucleus where Gli proteins ultimately affect gene transcription, we tested whether Su(fu) can affect the binding of DNA by Gli proteins. We synthesized a site corresponding to a described natural Gli binding site within the human genome referred to as Gli-B (Kinzler and Vogelstein, 1990). This site contains the core 9-bp sequence as well as flanking sequence (see Materials and Methods for full sequence).

In vitro translated human Gli1 and Gli3 are both capable of binding to the Gli binding sites on their own. Strikingly, addition of bacterially expressed chicken Su(fu) protein results in a dose-dependent enhancement of the binding of both Gli1 and Gli3 (Fig. 5A). To further analyze this enhancement of DNA binding, we tested the ability of Su(fu) to enhance the binding of truncated Gli3 proteins. The Zn finger domain alone binds strongly to DNA and is unaffected by Su(fu). When the Zn finger domain is tethered to the r1-containing amino-terminal region (Gli3 *NcoI*-Zn3') it does not bind efficiently to DNA until Su(fu) is added, behaving similarly to full-length Gli3 (Fig. 5B). Thus, the amino-terminus contains a domain that inhibits DNA binding in this assay and the effect of this domain can be overcome by the addition of Su(fu). Under the gel shift conditions used in Fig. 5A (4.5% acrylamide, 0.25× TBE) we never see a change in migration of the Gli1:DNA or Gli3:DNA band caused by the addition of Su(fu). However, under the conditions used in the mapping of the Su(fu)-affected domain in Fig. 5B (6% acrylamide, 0.5× TBE), we do see a slower mobility when Su(fu) is added to the gel shift reaction. These data, combined with the protein:protein interaction between Gli3 and Su(fu), suggest that Gli proteins and Su(fu) might bind together on DNA. In some, though not all, cases where proteins bind together on DNA the identities of the proteins in the complex can be verified by “supershift” with specific antibodies. Attempts to supershift the Su(fu) enhanced band with anti-Su(fu) or anti-GST antibodies were unsuccessful.

To rule out the possibility that the enhancement of Gli binding is an artifactual effect of Su(fu) in the reticulocyte lysate of the *in vitro* translation mixture, we transfected a chicken mesodermal cell line (DF-1) with a plasmid expressing Gli1 protein and isolated both cytoplasmic and nuclear extracts from these cells. Nuclear extracts from these cells bind

only very weakly to the Gli-B binding site. The addition of GST–Su(fu) fusion protein again causes a dramatic increase in the Gli binding activity of both cytoplasmic and nuclear extracts (Fig. 5C). Cells transfected with the pcDNA3 vector alone show no Gli-B DNA binding regardless of the presence of Su(fu) (Fig. 5C). Su(fu) alone displays no binding to the Gli site in any of these assays (data not shown) and the addition of GST protein without Su(fu) fails to alter Gli binding activity.

A recent paper proposed that in *Drosophila* full-length Ci protein must undergo an activation event to convert it into a labile transcriptional activator and that Su(fu) stabilizes full-length Ci in its preactivated state (Ohlmeyer and Kalderon, 1998). If Gli proteins are also stabilized by vertebrate Su(fu), then one interpretation of our band shift results is that labile Gli proteins in both the cellular extract and the reticulocyte lysate are rapidly degraded and Su(fu) results in enhanced binding due to Gli stabilization. However, we have followed the integrity of *in vitro* translated radiolabeled Gli proteins in the presence and in the absence of Su(fu) and see no significant effect of Su(fu) on the stability of Gli1 or Gli3 in the reticulocyte lysate environment (data not shown).

Either of two models could explain the observed effect of Su(fu) protein on Gli DNA binding. The r1 domain may inhibit DNA binding directly, and the binding of Su(fu) to this domain could release the inhibition by inducing a conformational change. This simple model is inconsistent with the role of *Su(fu)* in *Drosophila* as inferred from genetic analysis. Another explanation is that Su(fu) is naturally part of a ubiquitous repressor complex to which Gli proteins are tethered. The large excess of Su(fu) protein added to these band shift assays allows the possibility that exogenous Su(fu) may disrupt a naturally occurring complex within the protein mixtures. The disruption of this protein complex would then free Gli proteins for DNA binding. Since we cannot confirm that the slower migrating complexes in Fig. 5B actually contain Su(fu), Gli proteins may be reassociating with separate complexes that are permissive for DNA binding. While the effect of Su(fu) on Gli binding activity in the band shift experiments reflects a functional interaction between Su(fu) and Gli proteins, the biological significance of this interaction must be tested *in vivo*.

In summary, the two vertebrate homologs of the *Drosophila* gene *Su(fu)* are predicted to encode proteins highly similar to the *Drosophila* Su(fu) protein with the notable exception of the previously described PEST sequence. As in *Drosophila*, the vertebrate *Su(fu)* gene is widely expressed and does not appear to be induced in response to Hh signaling. Vertebrate Su(fu) seems to have retained some of the protein:protein interaction properties of its *Drosophila* counterpart, such as the interaction with Gli proteins *in vitro* and with *Drosophila* Fu protein both in yeast and *in vitro*. *Drosophila* Su(fu) protein appears to be predominantly cytoplasmic, while in chicken embryonic extracts an anti-*Drosophila*-Su(fu) antibody recognizes a single band that is enriched in nuclear fractions. Finally, the enhancement of the binding of Gli proteins to DNA by Su(fu) in electrophoretic mobility shift assays suggests that Su(fu) proteins may regulate Gli activity.

ACKNOWLEDGMENTS

We thank L. Goodrich, K. Higgins, L. Milenkovic, J. Jin, and J. Hartford for help with embryo collections, sequencing and *in situ* procedures; K. Suyama for the anti-Su(fu) antibody and for help with Western blotting; K.

Vogan for band shift help; and members of the Cepko, Scott, and Tabin labs for critically reading the manuscript. L.S.C. also thanks the Baxter foundation. L.S.C. is supported by a Howard Hughes Medical Institute predoctoral fellowship. R.V.P. is supported by a postdoctoral fellowship from the NIH. M.P.S. is an investigator with the Howard Hughes Medical Institute. C.J.T. is funded by a grant from the NIH.

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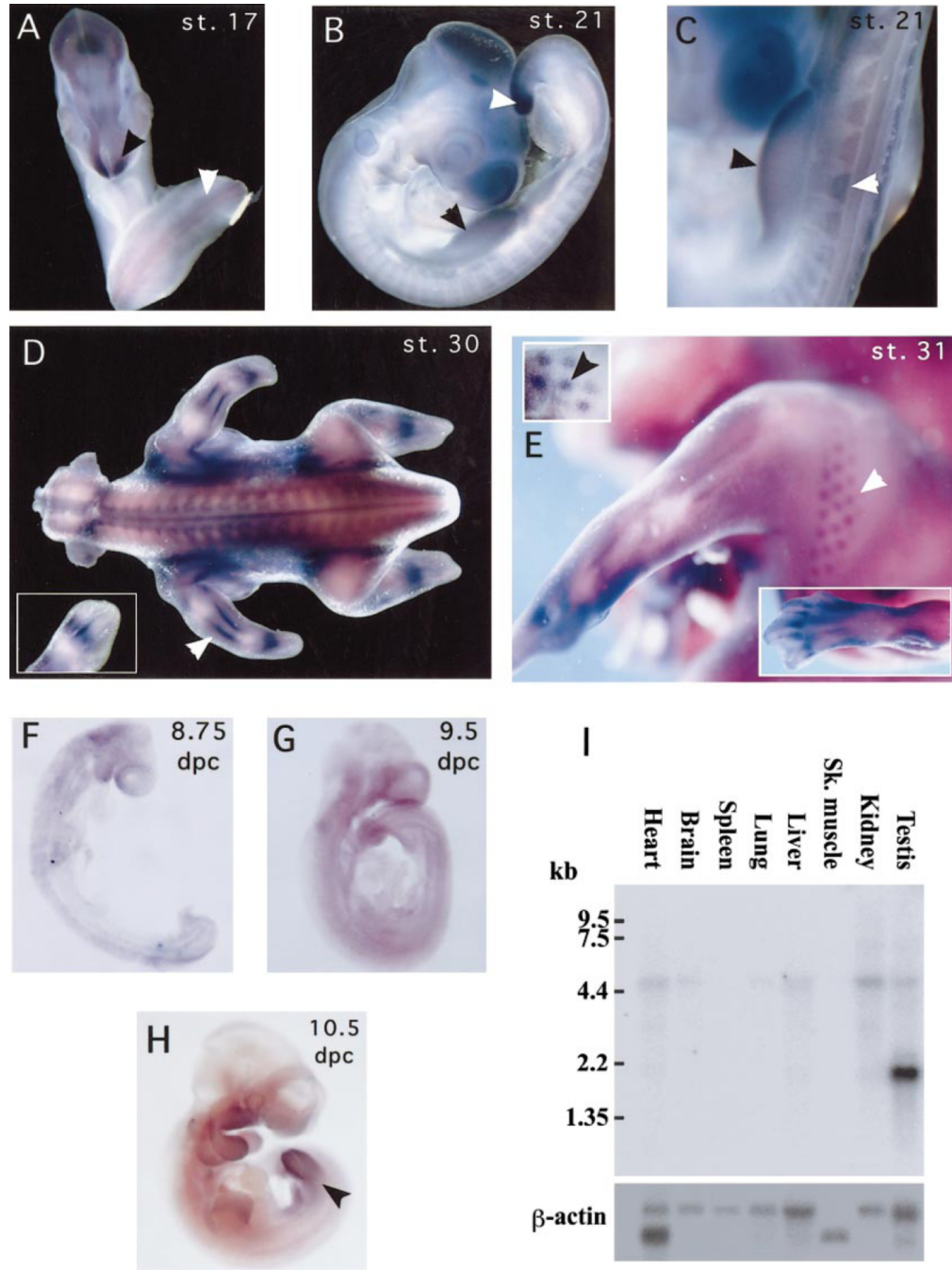


FIG. 2.

Expression patterns of chick and mouse *Su(fu)*. *Su(fu)* expression in the chick (A, B, C, D, E) and in mouse (F, G, H) embryos and in mouse adult tissues (I). (A) Hamburger–Hamilton stage 17 chick embryo showing *Su(fu)* expression at low levels in the neural tube (white arrow) with higher expression in the anterior tip of the neural epithelium (black arrow). (B) Stage 21 embryo showing slightly higher *Su(fu)* expression in the developing limb buds (black arrow) and in the tail bud (white arrow). (C) Higher magnification of limb bud (black arrow) and somite (white arrow) expression in stage 21 embryos. (D) Stage 30 chick embryo

showing high levels of *Su(fu)* expression in the regions of the developing perichondrium. White arrow shows staining in the developing stylopod (forearm) in the region of the perichondrium and inset shows staining in the developing autopod (wrist and hand). (E) Stage 31 embryo showing higher expression in the developing feather buds (white arrow). The inset (upper left) shows staining in the feather buds in epithelium that has been dissected away from muscle tissue to clarify the specificity of the staining. Second inset (lower right) shows strong staining in the developing bones of the hind limb autopod. (F) 8.75-dpc mouse embryo showing diffuse, ubiquitous *Su(fu)* mRNA (G) 9.5-dpc mouse embryo showing the persisting ubiquitous expression of *Su(fu)*. (H) 10.5-dpc mouse embryo shows slightly higher *Su(fu)* expression in limbs (I) Multiple mouse tissue Northern hybridization with mouse *Su(fu)* probe. One large transcript (5.4 kb) is seen at low levels in many adult tissues including heart, brain, liver, kidney, testis, and to a lesser extent, in lung. A smaller transcript (2.0 kb) is highly expressed in the testis.

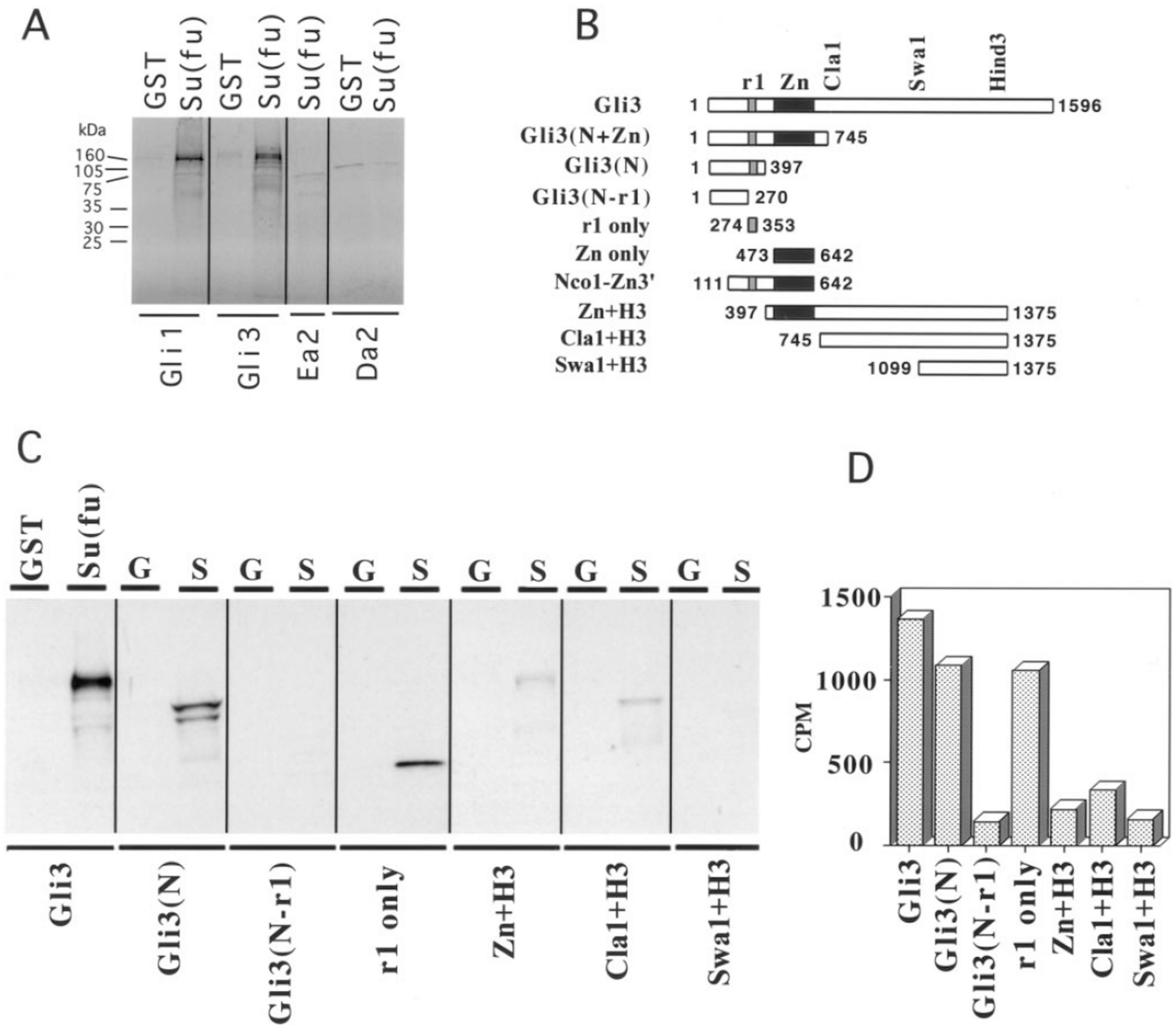
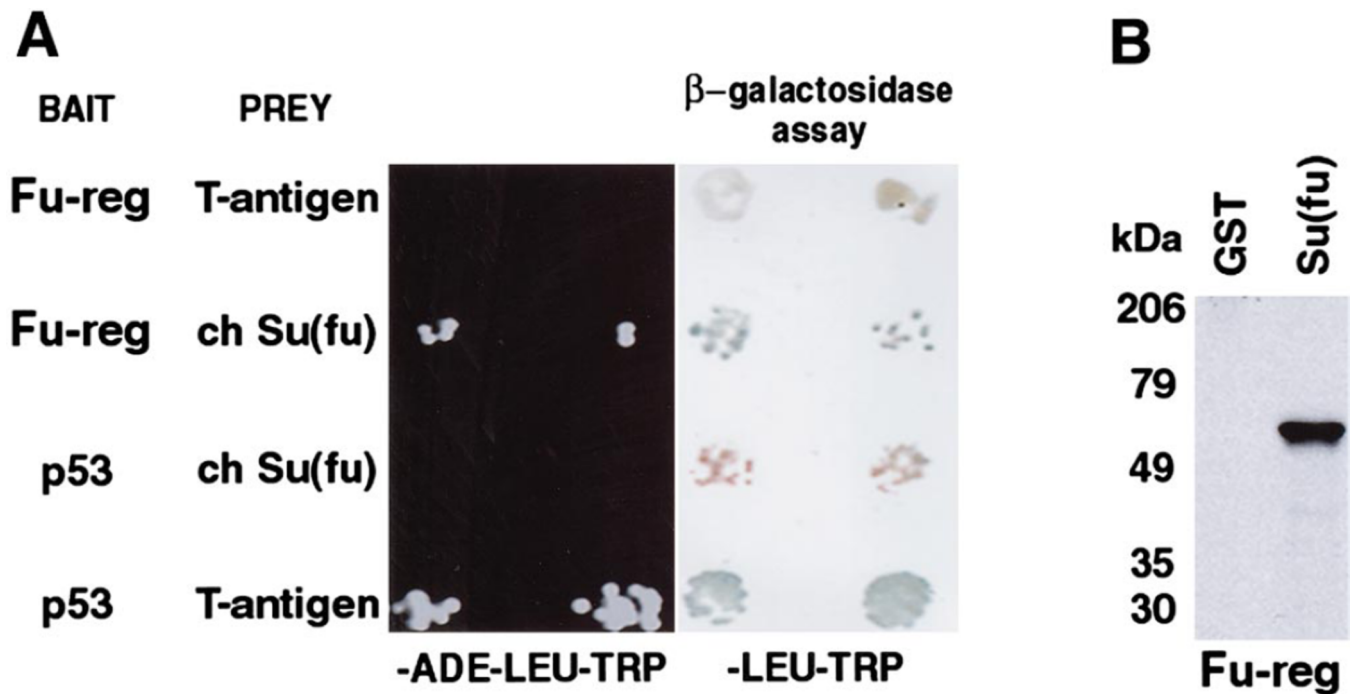


FIG. 3. Su(fu) interacts with the r1 domain of the Gli proteins. (A) GST protein:protein interaction assay showing interactions between GST–Su(fu) bound to glutathione agarose beads and ³⁵S-labeled *in vitro* translated proteins. Labels at the top of the lanes designate the glutathione agarose bound protein used in the assay: GST, GST alone; Su(fu), GST–Su(fu). Labels at the bottom of each lane indicate the *in vitro* translated radiolabeled protein. Radiolabeled Gli1 and Gli3 that remained bound to GST–Su(fu) bait are equivalent to 10% input (data not shown). Ea2 and Da2 are ³⁵S-labeled negative controls. (B) Illustration of all Gli3 deletion constructs used in this figure and in Fig. 5. r1 is one domain of homology between all Gli proteins and Ci; Zn is the Zn finger DNA binding domain; and Cla1, Swa1, and Hind3 mark the positions of restriction sites in human Gli3. Numbers flanking the schematics indicate the amino acid boundaries of each construct. (C) GST protein:protein interaction assays mapping the Su(fu) interaction domain on Gli3 to the r1 domain. The GST

fusion bait construct used in each assay is indicated at the top of each lane. GST or G, GST alone; Su(fu) or S, GST–Su(fu) fusion protein. ^{35}S -labeled prey constructs are labeled at the bottom of each lane and are schematized in B. (D) Quantitation of the interactions between GST–Su(fu) and each ^{35}S -labeled prey construct. The graph shows, on the Y-axis, 10% of the total cpm specifically binding to the GST–Su(fu) bait minus the cpm that nonspecifically bound to the GST-only control bait.

**FIG. 4.**

Su(fu) interacts with the regulatory domain of *Drosophila* Fused in the yeast two-hybrid system. (A) Strain PJ 69-4A yeast were cotransformed with the indicated bait (in pAS2-1) and prey (in pACT2) constructs. Bait and prey constructs were maintained in yeast by selection on trp- and leu-deficient media (trp and -leu), respectively. After transformation, yeast were grown on -ade-leu-trp medium (left) to assay for *ADE* reporter activity and on -leu-trp medium (right) to assay for β -galactosidase activity. *Drosophila* Fu regulatory domain (Fu-reg) and chicken Su(fu) are able to interact and activate reporter gene transcription as demonstrated by growth on medium lacking ADE and by β -galactosidase activity. This interaction is specific as Fu-reg does not interact with the SV40 large T antigen and Su(fu) does not interact with p53. However, the well-documented interaction between p53 and the SV40 large T antigen is readily detected. Two representative transformants for each pair of bait and prey plasmids are shown. (B) GST-protein:protein interaction assay showing the direct interaction between chick Su(fu) and *Drosophila* Fu-reg. GST-Su(fu) but not GST alone binds 35 S-labeled protein *in vitro* translated Fu-reg. Size of molecular weight standards are indicated in kDa. *In vitro* translated Fu-reg is predicted to be 55 kDa.

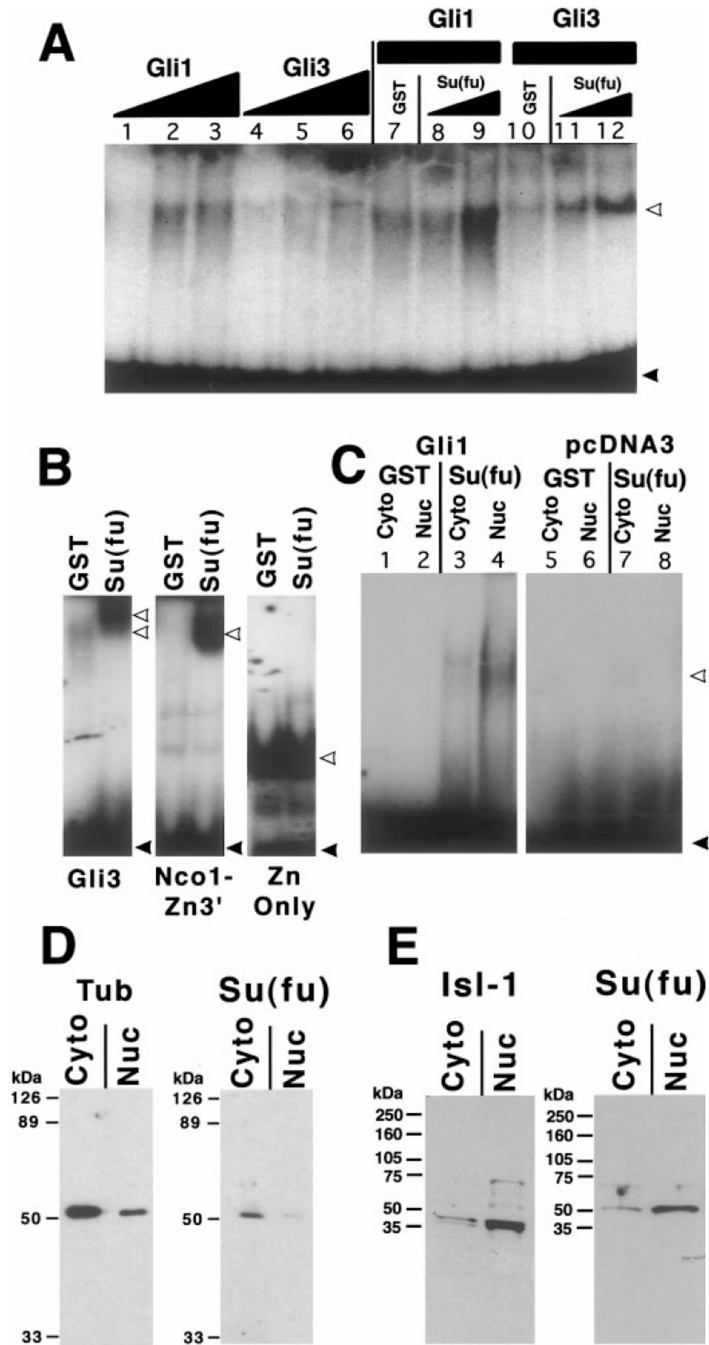


FIG. 5. Su(fu) protein enhances the binding of Gli proteins to their consensus DNA binding site. (A) Gel mobility shift assay shows the binding of unlabeled *in vitro* translated human Gli1 or Gli3 proteins to a ³²P-labeled Gli-B DNA binding site. Lanes 1–3 show the Gli binding activity in 1, 5, or 10 μl of pGli1 programmed reticulocyte lysate. Lanes 4–6 show the binding of 1, 5, or 10 μl of pGli3 programmed lysate. All reactions with less than 10 μl of programmed lysate were balanced up to 10 μl with unprogrammed lysate. Lanes 7–9 show the effects of adding GST alone (approx. 100 μg; lane 7) or of adding 1 or 5 μl of GST-

Su(fu) fusion protein (approx. 2 or 10 μg ; lanes 8–9, respectively) to 5 μl of pGli1 programmed lysate. Lanes 10–12 show the same reactions as in lanes 7–9 except that 5 μl of *in vitro* translated Gli3 was added to each binding reaction rather than Gli1. Gel shown in (A) is 4.5% acrylamide in 0.25 \times TBE running buffer. White arrows, probe protein complexes; black arrows, free probe. (B) Mapping of the DNA binding inhibitory domain on Gli3. Three versions of Gli3 were analyzed for the ability to bind DNA in the presence or in the absence of Su(fu) (Gli3, full length; NcoI-Zn3', Zn finger + portion of the amino terminus; and Zn only, DNA binding domain only; see Fig. 3B for schematics). All lanes were run on the same gel and are shown correctly proportioned to one another, but the Zn only lanes shown were exposed for a shorter period for clarity. Band shift conditions in B were 6% acrylamide in 0.5 \times TBE running buffer. (C) Gel mobility shift assay showing the binding activity in 5 μl of cytoplasmic or nuclear extract from DF-1 cells transfected with vector alone or with an expression construct of human Gli1. Lanes 1–4 show the effect of 5.0 μl of either eluted GST alone (lanes 1, 2) or GST-Su(fu) (lanes 3, 4) on the formation of the binding complex in this assay. (D, E) Western blot analysis of cytoplasmic or nuclear protein extracts from *Drosophila* embryos (D) or stage 31 chick embryos (E) probed with an antibody raised against the amino-terminus of *Drosophila* Su(fu) (SF57). Blots shows a predominant band of approximately 50 kDa that is stronger in cytoplasmic fractions from *Drosophila* embryos (D) and in nuclear fractions of chicken embryos. Fractionation controls (anti-tubulin for *Drosophila* extracts and anti-Isl-1 for chick extracts) illustrate the degree of separation of cytoplasmic and nuclear proteins in the respective extracts.