Tail gut endoderm and gut/genitourinary/tail development: a new tissue-specific role for *Hoxa13*

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**SUMMARY**

*Hoxa13* is expressed early in the caudal mesoderm and endoderm of the developing hindgut. The tissue-specific roles of *Hoxa13* function have not been described. Hand-foot-genital syndrome, a rare dominantly inherited human malformation syndrome characterized by distal extremity and genitourinary anomalies, is caused by mutations in the *HOXA13* gene. We show evidence that one specific *HOXA13* mutation likely acts as a dominant negative in vivo. When chick *HFGa13* is overexpressed in the chick caudal endoderm early in development, caudal structural malformations occur. The phenotype is specific to *HFGa13* expression in the posterior endoderm, and includes taillessness and severe gut/genitourinary (GGU) malformations. Finally, we show that chick *HFGa13* negatively regulates expression of *Hoxd13* and antagonizes functions of both endogenous *Hoxa13* and *Hoxd13* proteins. We suggest a fundamental role for epithelial specific expression of *Hoxa13* in the epithelial-mesenchymal interaction necessary for tail growth and posterior GGU patterning.

Key words: *Hoxa13*, Tail, Endoderm, Gut, HFG, Chick

**INTRODUCTION**

Vertebrate gastrulation events form the three germ layers and pattern the early anterior body region. Although disputed, the posterior region probably forms via a separate secondary event at the undifferentiated mesenchyme of the tailbud to form the tail somites, distal neural tube, notochord and tailgut (Catala et al., 1995; Gajovic et al., 1993; Griffith et al., 1992; Holmdahl, 1925; Knezevic et al., 1998; Pasteels, 1943; Schoenwolf, 1977; Schoenwolf, 1979). A crucial early event in patterning the posterior embryo is the formation of the caudal intestinal portal (CIP), which initiates the development of the hindgut and tail, and forms the cloaca [the common gut/genitourinary (GGU) chamber]. The cloaca is maintained throughout life in avian and some other vertebrate species. In mammals, the cloaca exists as an embryonic structure that undergoes septation to become distinct urethral, anal, and genital orifices. Abnormal development of the cloaca causes severe congenital malformations in vertebrates, including humans (Kluth et al., 1995; Martinez-Frias et al., 2000).

Tail growth is a function of a specialized region of ectoderm, the ventral ectodermal ridge (VER), which acts analogously to the apical ectodermal ridge (AER) of the limb bud. Both the AER and VER signal adjacent mesodermal tissues to maintain an undifferentiated state, facilitating growth and elongation (Goldman et al., 2000; Saunders, 1948). The VER forms before morphological tail development [stage 18 in chick (Mills and Bellairs, 1989) and E10 or 35+ somites in the mouse (Gruneberg, 1956)] from the cloacal membrane posterior to the tailbud where ectoderm and endoderm are juxtaposed (Gruneberg, 1956). During the ensuing gastrulation events, the tailgut develops anterior to the cloacal membrane and elongates in close association with the tail and in close proximity to its ectoderm. Apoptotic degeneration of the tailgut endoderm and VER heralds completion of tail growth (Fallon and Simandl, 1978; Miller and Briglin, 1996; Mills and Bellairs, 1989).

Despite common tail formation in early vertebrate development, tail growth ceases at different developmental time points in a species specific manner. Tail length is a characteristic phenotype among species, yet its molecular controls are unknown. Molecular candidates include the Hox genes [homeobox-containing transcription factors that function in many aspects of pattern formation during development (Krumlauf, 1994)]. In vertebrates, the Hox genes are expressed in a characteristic spatial and temporal pattern mirroring their physical location in the chromosome. The most 5′ Hox genes are expressed in the posterior body region including the posterior mesodermal regions of the gut (Roberts et al., 1995; Yokouchi et al., 1995b). These play an important role in patterning the gut along the anteroposterior axis (AP) (Roberts et al., 1995; Roberts et al., 1998; Warot et al., 1997). *Hoxa13* and *Hoxd13* are expressed specifically in the cloacal mesoderm and also uniquely in the hindgut and cloacal endoderm (Roberts et al., 1995; Yokouchi et al., 1995b). Their endodermal function is unknown.
Mutations in Hoxa13, both spontaneous and transgenic, exist in mice (Goodman and Scambler, 2001). The mutant phenotypes show both limb and GU anomalies. Spontaneous murine Hoxa13 mutant, hypodactyly (hd), has a 50 base deletion within the first exon of Hoxa13, resulting in a mutant fusion protein (Mortlock et al., 1996). Although these mice suffer a high perinatal mortality, some homozygotes survive but are infertile because of hypoplasia of distal reproductive structures (Warot et al., 1997). As the Hoxa13-null mice are early perinatal lethal (Fromental-Ramain et al., 1996), Hoxa13 protein may act as a gain-of-function mutant.

Murine Hoxd13+/− males show subtle GU anomalies (Dolle et al., 1993; Kondo et al., 1996; Podlasek et al., 1997) and have abnormalities of the lowest sacral vertebra (Dolle et al., 1993). Hoxd13+/− embryos crossed with Hoxa13-null heterozygotes, have more severe GU anomalies than the Hoxd13+/− alone, suggesting that cooperation and redundancy between Hoxa13 and Hoxd13 exists in their function in GU development (Warot et al., 1997).

Missense and nonsense mutations of one allele of HOXA13 cause hand-foot-genital (HFG) syndrome (Goodman and Scambler, 2001), a rare, dominantly inherited human disease (OMIM 140000). Affected individuals have mild, fully penetrant, symmetrical and bilateral hand and foot anomalies. Affected individuals also exhibit incompletely penetrant and variably severe GU tract abnormalities, including hypospadias in males and Müllerian duct fusion abnormalities in females. Both sexes show abnormalities in ureter/bladder placement. The variety of mutations described includes deletions, truncations of the protein, or amino acid substitutions within the conserved homeodomain. The resulting proteins are thought to act either as a gain-of-function mutation or as a possible competitor of the wild-type protein.

Mutations in human HOXD13 cause a severe distal limb phenotype termed syndactyly (SPD) (OMIM 186000). This rare, dominantly inherited syndrome is caused by mutations in a polyalanine repeat within the coding region of HOXD13, including expansions and intragenic deletions (Goodman and Scambler, 2001). Affected males often demonstrate GU abnormalities (Goodman and Scambler, 2001).

The manner in which these HOXA13 and HOXD13 mutations and nulls lead to these specific GU malformations is unknown and may be due to mesodermal and/or endodermal effect of the mutation. To date, murine models have failed to address their possible tissue-specific functions.

To study the role of Hoxa13 in GGU development we have used chick embryos. We have studied the specific endodermal role of Hoxa13 using the avian specific retroviral expression system and in ovo electroperoration to overexpress wild-type and mutant forms of Hoxa13 in the chick hindgut endoderm in ovo. We constructed a chick Hoxa13 homolog of one of the specific mutations that causes HFG (HFGa13). We chose the originally described Hoxa13 mutation in which a small deletion in the C-terminal domain results in truncation of the protein (Morlock and Innis, 1997). When the truncated protein is expressed in the chick posterior endoderm, a dramatic GU and tail malformation results. We show that this effect is specific to endodermal HFGa13 expression. We show that HFGa13 probably acts by interfering with the normal function of endogenous Hoxa13 and Hoxd13.

**MATERIALS AND METHODS**

**Embryos**

Timed fertilized white leghorn eggs (SPAFAS, CT) were incubated at 38°C in a humidified incubator (Kuhl, NJ) until used experimentally. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) or by embryonic day (E).

**Constructs**

Isolation of chicken Hoxa13 gene has been previously described (Nelson et al., 1996). Although this clone was thought to be a full-length CDNA, a recent manuscript described evolutionarily conserved 3’ sequence which was not present in our original clone (Mortlock et al., 2000). We isolated this N-terminal sequence by RT-PCR on E6 hindgut total RNA with a primer designed to amplify the conserved 3’ sequence (ATGTTTCTCTAGACACACG). After sequence verification and subcloning we verified the full-length chick Hoxa13 cDNA. By PCR, we mutated chick Hoxa13 to produce a truncation similar to a specific human mutation (HFGa13). The mutated reverse oligonucleotide (TCAGATTGTGACCTGTGCG) produced a premature stop codon as described by Mortlock and Innis (Mortlock and Innis, 1997).

Wild-type Hoxa13 and HFGa13 CDNAS RCAS(A) or RCAS(B) viruses, and a Hoxd13 RCAS(A) virus were produced as described (Morgan and Fekete, 1996). We found no difference in any of the experimental results described below using either the short or long form Hoxa13 or HFGa13, and therefore we have not distinguished between them. Similarly, 3’ long and short forms of RCAS(A)Hoxd13 acted equivalently in a chick limb bud overexpression study (Goff and Tabin, 1997). We found no difference in the infectivity of either A or B envelope coats of RCAS; both acted equivalently.

Constructs for electroporation were prepared with wild-type Hoxa13 and HFGa13 cDNAs cloned into pcDNA3 (Invitrogen). An N-Flag epitope oligonucleotide was inserted in frame with the GAL4 DNA-binding domain (Sadowski and Ptashne, 1989). All sequences were confirmed before use in experiments.

**Transfection studies and western blots**

Plasmids used for transfections were purified using the maxiprep reagent system (Qiagen). COS-7 cells at 60-80% confluence were washed twice with serum-free medium and then co-transfected with 100 ng of reporter plasmid [pG5Luc containing 5 GAL4-binding sites (Promega)], 10 ng of Renilla luciferase promoter vector (Promega) and 100 ng of different Hoxa13 plasmids with 3 μl of LipofectAMINE DNA-binding domain (Promega). Promoter activities were expressed as relative luciferase activity (units/Renilla units) and each value represents the mean of six separate wells. Relative expression of the GAL4 fusion proteins was assessed by western blot analysis of COS-7 extracts. Transfections with wild-type Hoxa13 and HFGa13 constructs fused to GAL4 DNA-binding domain (DBD) were as described previously (Sadowski and Ptashne, 1989). GAL4 DBD antibody (Santa Cruz Biotech) was used as described by the manufacturer.

Cells were fixed 24 hours after transfection for 30 minutes in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), permeabilized with PBS containing 0.02% Triton X-100, and incubated in 10% normal goat serum in PBS for 30 minutes at room temperature. Cells were then incubated with an anti-FLAG M5 monoclonal antibody (Kodak; 1:500) for 2 hours at room temperature, followed by incubation with secondary antibody. Images were collected and processed on a Microphot Nikon microscope.

**Viral infection**

This technique has been previously described (Morgan and Fekete,
Embryos at stage 8-10 were used for experiments of the posterior endoderm, stage 18 for experiments in the limb. Approximately 1-5 µl of freshly defrosed virus dyed with Fast Green was injected per embryo. For hindgut experiments, the virus was injected into the region lateral and posterior to the tailbud after a published fate map (Matsushita, 1999). Double injections were performed by mixing equal volumes of each viral aliquot before injection, as previously described (Bendall et al., 1999). In all cases, the HFGa13 virus was cloned in RCAS(B) and the wild-type viruses were in RCAS(A). For limb injections, the right hindlimb bud was viewed at stage 18. Approximately 1-5 µl of virus was injected, filling the entire limb bud. Eggs were then placed at 38°C until harvested. Injected viral constructs included short and long forms (see above) of the entire limb bud. Eggs were then placed at 38°C until harvested. Injected viral constructs included short and long forms (see above) of wild-type Hoxa13, HFGa13 and, as controls, wild-type Hoxd13 and GFP. More than 10 dozen embryos were injected with each construct.

**Electroporation**

This technique was adapted to a technique previously published (Grapin-Botton et al., 2001). E2.5 (stage 11-14) embryos were used. This technique was adapted to a technique previously published (Smith et al., 2000). Immunohistochemical stains were performed using standard techniques. Section in situ hybridization was performed using published techniques (Smith et al., 2000). Riboprobes were transcribed using Roche riboprobes synthesis kits as per the manufacturer’s directions. All riboprobes used herein have been previously published (Nielsen et al., 2001; Roberts et al., 1995; Roberts et al., 1998; Smith et al., 2000; Vogel et al., 1996).

**Accession numbers**

GenBank Accession Number for Gallus gallus Hoxa13 is AY030050.

**RESULTS**

**Spatiotemporal expression of Hoxa13 during posterior GGU/tail development**

Hoxa13 is first expressed early in the most posterior part of the embryo, adjacent to Hensen’s node in the area that will give rise to the CIP (stage 10) (Fig. 1A). Hoxa13 is expressed at stage 14 in the CIP (Fig. 1B). Later, Hoxa13 expression is restricted to the dorsal mesoderm of the tailbud, cloacal mesoderm, hindlimb bud mesoderm and caudal endoderm (Fig. 1C,D). Hoxa13 and its product are strongly expressed in the endoderm of the hindgut and cloaca through early development of the gut (Fig. 1E,F) (Roberts et al., 1995; Yokouchi et al., 1995b).

**Human HFG and chick HFGa13 overexpressed embryos have similar phenotypes**

Overexpression of Hoxa13 in the posterior embryo failed to produce a tail or gut phenotype. We did see an epithelial transformation (from midgut to hindgut) when midgut mesodermal tissues expressed Hoxa13 (see Fig. 7H for comments) as was described with ectopic Hoxd13 expression in the midgut (Roberts et al., 1998).

To determine if expression of chick mutated Hoxa13 (HFGa13) results in a phenotype similar to that observed in the human HFG syndrome, we chose to construct a similar nonsense mutation as described by Mortlock and Innis (Mortlock and Innis, 1997). The C-terminal mutation leads to a mutated Hoxa13 protein with the last 20 last amino acids deleted. We expected that HFGa13 protein would be able to interfere with the endogenous Hoxa13.

In order to test our hypothesis, we first misexpressed HFGa13 in the hindlimb, and we were able to produce a severe morphological change reminiscent of the limb defect described in HFG syndrome (Fig. 2A). The HFGa13-expressing hindlimb showed a specific malformation characterized by a substantial reduction in limb size in both the anteroposterior and dorsoventral axes compared with the uninfected contralateral control limb. HFGa13 E6 infected hindlimbs revealed a specific skeletal hypoplasia of the fibula (without changes in the tibia) and a reduction of the entire autopod area.
This phenotype suggested a late disruption of the apical ectodermal ridge (AER). Consistent with this, Fgf8 mRNA expression (Vogel et al., 1996) was not detectable in the distal hindlimb AER of the HFGa13 hindlimbs (Fig. 2C). The distal fibular cartilage fails to properly develop and remains undifferentiated. Expression of Bapx1 was decreased only in the malformed fibula (Fig. 2D). No AER disruption or fibula maldevelopment were observed with the wild-type Hoxa13 overexpression (data not shown). These results suggest that HFGa13 interferes with the maintenance of the AER in the hindlimb and with formation of the fibula. We conclude that our construct functions to give a chick phenocopy of the human HFG syndrome.

**Endodermally expressed HFGa13 causes abnormal hindgut and tail development**

HFGa13 and control constructs were expressed in the chick in ovo by injection of virus, targeting the prospective hindgut in stage 8-10 embryos. We used this targeting technique previously to infect the midgut mesoderm (Roberts et al., 1998), but in the
phenotype involves maldevelopment of the cloaca (CL*), hindgut (HG*) and tail. Allantoic internalization is present (AL*) and ceca are unaffected (CE). (F) Anti-N-flag immunostaining demonstrating expression of the tagged-HFGa13 in the endoderm of the hindgut; the phenotype involves maldevelopment of the cloaca (CL*), hindgut (HG*) and tail. Allantoic internalization is present (AL*) and ceca are unaffected (CE). (F) Anti-N-flag immunostaining demonstrating expression of the tagged-HFGa13 in the endoderm of the hindgut; the mesoderm is not stained. Note that the electroporated endodermal cells appear undamaged and intact. Misexpression of HFGa13 and Hoxa13 constructs by electroporation show similar expression levels in the gut endoderm layer (data not shown).

Specific gross morphological defects were obtained only with HFGa13 infections and only when the infection included the posterior endoderm (Fig. 3A,B). Hoxa13, Shh, Bmp4 and GFP constructs always failed to produce this phenotype ever, even when expression was noted in the endoderm (data not shown). HFGa13 embryos infected specifically in the mesoderm only were phenotypically normal (Fig. 3C,D). Our survival rate was 50-80%, depending on time of incubation (survival to E3 better than to E18). Approximately 20% of HFGa13-infected embryos demonstrated the mutant phenotype. All HFGa13 embryos were analyzed for viral infection. All mutant embryos harbored posterior endodermal and mesodermal virus expression. Those without the phenotype showed no viral infection or only posterior mesodermal virus expression (no infection in the endoderm).

To determine if endodermal expression of HFGa13 is sufficient to obtain these posterior defects we used an in ovo electroporation technique (Grapin-Botton et al., 2001). Three groups of E2.5 (stage 11-16) embryos were electroporated: a control group with empty vector or a GFP-containing vectors, a group with the wild-type Hoxa13 construct and a group with HFGa13. In E5 survivors, posterior short tail/hindgut atresia was present only in the HFGa13 electroporated embryos, whereas electroporation of control constructs produced no defects (data not shown). The mutant phenotype was restricted to those embryos with hindgut epithelial expression of HFGa13 [electroporations restricted to the midgut endoderm failed to produce the posterior phenotype (data not shown)]. Survival was about 80% for all groups. The posterior endodermal HFGa13 expressing embryos show similar, albeit more severe, defects as those obtained using the injection/infection technique (Fig. 3E). Confirmation of tissue integration and endodermal tissue survival were performed by tag immunostaining on all embryos (Fig. 3F). Sections of experimental embryos confirmed an intact endoderm and a histologically normal neural tube and notochord (Fig. 4).

Approximately 10 embryos (all the survivors and all the embryos with documented expression in the hindgut endoderm) showed the phenotype that included malformations of the hindgut and tail (Fig. 4A,C,E,G), including maldevelopment of the tail somites and a short tail (compare Fig. 4A,C, respectively, with Fig. 4B,D). Hindgut defects included hindgut atresia anterior to the cloaca (Fig. 4E,F) and malformed/malpositioned cloacas (compare Fig. 4C,E, respectively, with Fig. 4D,F). Additional defects were noted in other cloaca-associated viscera, including cystic mesonephric maldevelopment with normal non-atretic ureters and atresia of the distal Mullerian ducts (Fig. 4G,H). No associated neural tube defects or malformations are present (as shown in Fig. 4). We occasionally obtained a severe phenotype, termed ourentery (Rabaud, 1900), in which the tail appears to have grown ventrally and internally to the remaining cloacal orifice, often accompanied with ventral internal malpositioning of tail structures into the hindgut and internalization of the allantois (compare Fig. 4A,C with Fig. 4B,D and also Fig. 3B with Fig. 3D).

HFGa13 affects expression of Hoxd13, Fgf8 and Bapx1

Molecular analyses of HFGa13 mutant embryos were made with specific dorsoventral (DV), anteroposterior (AP) and cytodifferentiation markers. Strong downregulation was observed with VER marker Fgf8 (ectoderm and mesoderm) and with AP marker Hoxd13 (mesoderm and endoderm) (Fig. 5A and Fig. 5D, respectively). Bapx1 shows a diminished expression in the short tail (Fig. 5B). No change was noted in the expression...
of ventral mesodermal markers Wnt5a (Fig. 5C) and Bmp4 (data not shown), and there was normal expression of Shh (Fig. 5E).

Caudal gut endodermal signals are needed for normal posterior gut and tail development

To test our hypothesis that posterior gut and tail development requires specific endodermal signals, we used whole embryo explant cultures in which we removed the endoderm at early (pre-CIP) stages (stage 9-11). Survival (90% of unmanipulated controls, 75% of manipulated embryos) after 2 days, to stage 20, allowed analysis of tail development. None of the control embryos had caudal defects (Fig. 6A). All embryos in which the caudal endoderm had been removed showed severe caudal defects of the tail and gut. Those embryos that survived to form hindlimb buds generally formed abnormal CIP and blunted tails similar to those produced in the HFGa13-injected or electroporated embryos (Fig. 6B). Ourentery was present in 50% of manipulated survivors (Fig. 6D). No neural tube defect was present in these embryos.

When examined histologically, we confirmed that the midline ventral endoderm overlying the tailbud was removed (data not shown). This endoderm is absent for the first 24 hours after dissection, then it appears that the adjacent endoderm occasionally re-grows over this defect (Fig. 6D). No defects are seen in the more anterior endoderm (Fig. 6C). To confirm that removal of the caudal endoderm was complete, we analyzed the normal and abnormal embryos for the presence of early endodermal markers. In the caudal ventral tissues of normal embryos, we found expression of Shh, CdxA (Fig. 6E), Hoxa13 (Fig. 6A) and Hoxd13 (data not shown). However, we could not detect expression of these markers in the caudal ventral tissues of abnormal embryos before 24 hours (Fig. 6B,F). Rescue experiments included transplanting donor endoderm (either anterior or posterior) harvested from stage 17 embryos to the embryos in which the CIP endoderm had been removed. Only transplanted posterior endoderm rescued the tail and gut defect (Fig. 6G). Anterior endodermal transplants failed to rescue blunted tail phenotype (Fig. 6H). Our results show that the posterior endoderm produces signals necessary for normal tail and hindgut development.

HFGa13 interferes with the cellular functions of Hoxa13 and Hoxd13 proteins

To investigate the molecular pathway by which HFG Hoxa13 mutation functions, transactivation activities of wild-type Hoxa13 and HFGa13 were first analyzed in heterologous COS-7 cell line by luciferase assay using the synthetic GAL4 reporter system. Constructions for transfection studies were prepared using the pSG424 vector that contains the GAL4 DNA-binding domain. Wild-type Hoxa13, HFGa13 and Hoxd13 cDNAs were subcloned into pSG424 vector in frame with the GAL4 DBD. We show that wild-type Hoxa13 protein...
fused to the GAL4 DNA-binding domain is able to activate transcription of this synthetic reporter (Fig. 7A). The HFGa13 construct is not able to activate the synthetic GAL4 promoter but we did notice a decrease in the basal activity using this construct. To verify expression and protein stability, we performed western blot analysis on whole COS-7 cell extracts of transfected cells using a specific GAL4 DBD antibody. We show that all these proteins were expressed at comparable levels, as assayed by western blotting, indicating that the inability to activate transcription is not linked to a lack of expression or instability of the HFGa13 proteins (data not shown). To determine whether a difference in intracellular localization of HFGa13 proteins could affect transcriptional activity, localization of N-flag-tagged proteins within transfected COS-7 cells was examined by immunostaining (Fig. 7C,D). Strong signals for wild-type Hoxa13 and HFGa13 proteins were observed in the nuclei of transfected COS-7 cells.

Using the same synthetic GAL4 reporter system, we investigated the possible interactions between Hoxa13 and HFGa13. We used the same conditions with a plasmid containing Hoxa13 and a GAL4 DBD, but added one plasmid containing HFGa13 cDNA without GAL4 DBD (unable to bind the 5 GAL4 DBD repeats). Using this competition assay with same molar ratio, we show that HFGa13 protein is able to decrease Hoxa13 transcriptional activity (Fig. 7B). Increased amounts of HFGa13 increase the strength of the repression (data not shown). Interestingly, HFGa13 protein is also able to act in a dominant negative fashion with Hoxd13 by repressing Hoxd13 transcriptional activity (Fig. 7B).

In order to determine if HFGa13 acts as a dominant negative in vivo we used a competitive assay taking advantage of an epithelial phenotype alteration induced by overexpression of the wild-type Hoxa13 in the midgut. At E18, epithelial differentiation is nearly complete. Midgut epithelium is characterized by long and thin villi (Fig. 7E), whereas hindgut epithelium shows wide and flat villi (Fig. 7F). We have previously shown that ectopic Hoxd13 expression in the midgut mesoderm causes the midgut epithelium to develop with a hindgut/cloacal phenotype (Roberts et al., 1998). We now show that the same epithelial transformation occurs with Hoxa13 misexpression in the midgut mesoderm (Fig. 7H,I). However, infection of the midgut with HFGa13 did not transform the epithelium (Fig. 7G). If our HFGa13 acts as a dominant negative, then it should be able to repress the midgut epithelial transformation induced by ectopic midgut mesodermal Hoxa13 or Hoxd13 expression. In order to test our hypothesis, we coinfect mesodermal midgut with either RCAS(A)-Hoxa13 or RCAS(B)-HFGa13 in equal titer/volume. We used the different RCAS envelope proteins to facilitate cellular co-infection as previously described (Morgan and Fekete 1996; Bendall et al., 1999). To verify co-infection, we checked viral expression in the midgut mesoderm by 3c2 immunostaining, and we deduced co-expression of either Hoxa13 or Hoxd13 and HFGa13 by in situ hybridization probing with the wild-type probes (Fig. 7L). Co-expression of HFGa13 with Hoxa13 (Fig. 7l) or with Hoxd13 (Fig. 7K) was sufficient to inhibit the action of Hoxa13 or Hoxd13. As we found wild-type epithelium phenotype in the co-infected mesoderm midguts, we can deduce that HFGa13 must have acted as a dominant negative.

These experiments indicate that this HFG nonsense mutation probably functions as a dominant negative. HFGa13 may compete with the endogenous function of wild-type Hoxa13 and/or Hoxd13 proteins in vivo as a dominant-negative, as observed with human heterozygous for this mutation, probably by interfering with protein partners and/or transcriptional machinery.

**DISCUSSION**

It has been known for some time that there is a close association between the development of the gut and the tail or its related structures (coccyx and sacral vertebrae). Human congenital malformations in one often affect the other systems. This association is seen in spontaneous and transgenic
malformations in many vertebrate species (Maatman et al., 1997; Warot et al., 1997). The GGU and tail tissues derive from the tailbud mesenchyme probably via secondary body formation (Griffith et al., 1992). The factors involved in this process, if mutated, may affect multiple caudal structures. There is evidence to support this, both experimentally and spontaneously produced, in many vertebrate systems. When regions of the tailbud are removed early in chick development, tail truncations and cloacal anomalies are common (Schoenwolf, 1978). In some specific spontaneous murine mutants with tail truncation anomalies, GGU malformations are common. For example, Danforth’s short tail mutant (sd) develops anal stenosis, rectal duplications and anal atresias in association with the characteristic short tail (Dunn et al., 1940). In humans, the relationship between sacral and coccygeal vertebral defects and hindgut defects has also been documented (van der Putte, 1986) in sporadic/isolated malformations [e.g. cloacal and bladder extrophy (Loder and Dayioglu, 1990; Martinez-Frias et al., 2000)] and syndromic malformations [e.g. VATERCL syndrome that includes anal atresia and hemivertebrae (Beals and Rolfe, 1989)]. Another example is the limb/pelvis-hypoplasia/aplasia syndrome that includes absent fibulae, Müllerian aplasia and sacral hypoplasia (Raas-Rothschild et al., 1988). The elucidation of the mechanism of this association has been difficult, probably owing to the complexity of the malformations. As the malformations often involve anomalies of all three germ layers, deciphering the primary from the secondary effects in a transgenic model is troublesome.

Tail development is universal among vertebrates, although the persistence of a tail is not. Tail length is a function of the developmental time point when tailgut and VER apoptosis occurs. We suggest a functional relationship between the caudal endoderm and the VER. Gruneberg suggested that the origin of the VER is from the cloacal membrane (Gruneberg, 1956). Other experimental evidence supports the association between caudal endoderm and the VER. VER ablation causes a displacement of the tailgut dorsally, by increasing the number of cells between the VER and tailgut (Goldman et al., 2000). In murine chimeras derived from wild-type and sd mutant ES cells, it was shown that the sd cells never populated the ventral hindgut or tailgut, suggesting a ventral signal from the gut endoderm is absent in the sd mutant mouse (Maatman et al., 1997). It may be that the failure of heterozygous and homozygous sd mutant cells to colonize the ventral hindgut endoderm is the earliest manifestation of the sd phenotype (Maatman et al., 1997).

The molecular controls of normal or abnormal tail/GGU are poorly understood, but VER function and signals have recently been described (Goldman et al., 2000). Signals from specialized ectoderm in the limb (AER) and the tail (VER) direct elongation of their respective subjacent structures. The AER and VER do not appear to be functionally equivalent. In mice, both the AER and VER express a fibroblast growth factor and a bone morphogenic protein (Dudley and Tabin, 2000; Maatman et al., 1997). Although exogenous application of FGF or BMP to the AER rescues the limb phenotype in AER...
Endodermal role for Hoxa13 ablated embryos (Zuniga et al., 1999), when these proteins were placed on VER ablated tails in vitro, they failed to rescue the blunted tail phenotype (Goldman et al., 2000). Transplanting the AER to VER ablated tails also fails in rescuing growth (Goldman et al., 2000). There are clearly other factors, either from the VER or other tail tissues, that are important in directing tail development. We conclude that signaling between the endoderm and ectoderm at this very early stage of development is critical and independent of notochord or neural tube related inductions. We suggest that one of these factors is Hoxa13 derived from the caudal endoderm.

Fig. 7. HFGα13 interferes with the cellular functions of Hoxa13 and Hoxd13. (A) Transcriptional transactivation by wild-type Hoxa13 and HFGα13 proteins in a GAL4-fusion assay in COS-7 cells. Relative luciferase activities were normalized to the empty GAL4 DNA-binding domain expression vector. Fusion protein of the GAL4 DNA-binding domain and Hoxa13 shows transcriptional activation of the synthetic reporter. By contrast, fusion protein of the GAL4 DNA-binding domain and HFGα13 fails to activate transcription of the same promoter and is able to decrease the basal activity. Luciferase assays were performed after two independent transfections, each done in triplicate (A,B). (B) Perturbation of the transcriptional transactivation of wild-type Hoxa13 and Hoxd13 by HFGα13 in a GAL4-fusion assay in COS-7 cells. In this assay, we monitored GAL4 transcriptional activity induced by GAL4 DBD fusion proteins without or with pcDNA3-HFGα13 construct. In a same molar ratio, the HFGα13 form specifically decreases Hoxa13 and Hoxd13 transcriptional activation. (C,D) Intracellular localization of Hoxa13 and HFGα13 proteins. Immunostaining of transfected N-flag tagged Hoxa13 (C) and HFGα13 (D) constructs in COS-7 cells with specific N-flag antibody shows that both have nuclear localization. Note an additive cytoplasmic signal with the HFGα13 construct. (E-K) Hematoxylin and Eosin stained sections of E18 control (E,F) or infected (G-K) guts. (E) Normal midgut with thin and long villi. (F) Normal hindgut with flat and short villi. (G) HFGα13 mesodermally infected midgut has wild-type midgut epithelium. Hoxa13 (H) and Hoxd13 (J) mesodermally infected midgut shows hindgut-like epithelial transformation (as shown by arrows). HFGα13 midguts co-infected with either Hoxa13 (I) or Hoxd13 (K) show rescue of the epithelial hindgut phenotype. (L) Hoxd13 and HFGα13 mesodermal midgut co-infection show presence of virus (detected by 3C2-Ab, L1), and ectopic HFGα13 (detected with Hoxa13 probe, L2) and Hoxd13 (L3) co-expression, which is associated with normal epithelial phenotype.
Clearly, there are multiple factors involved in tail development. Many different model systems (genetic, mechanical and toxic) can result in the phenotype of blunted tail and cloacal anomalies. Classical anatomic literature has examples of toxic or pharmacological induction of blunted tail and ourenteric malformations in chick, including exposure to insulin (Moseley, 1947), organophosphides (Wytenbach and Thompson, 1985) and retinoic acid (Griffith and Wiley, 1989). Mechanical disruptions by transection or extirpation of the notochord, tailbud and hindgut endoderm, shaking, or placement of a conductive glass tube all result in blunted tail and ourentery (Moseley, 1947; Hotary and Robinson, 1992). The transgenic data in mice shows that perturbations in many different pathways result in blunted tails including FGFR (Furthauer et al., 1997), BMP (Brunet et al., 1998), Wnt (Yamaguchi et al., 1999) and retinoic acid (Abu-Abed et al., 2001). What is common to these diverse ‘methods’ of producing the combination of caudal tail/vertebrae and gut defects? We suggest one possibility may be interruption of caudal endoderm signaling needed for normal tail development.

A spontaneous genetically dominant chicken mutation resembles the phenotype of our HFGa13 embryos. Dominant rumpless chicks develop a truncated tail and abnormal cloaca, and often show ourentery (Zwilling, 1942). It would be very interesting to determine if Hoxa13 is mutated or if abnormalities in this pathway are present in this strain. We are currently studying dominant rumpless chick embryos for Hoxa13 mutations.

In the human syndrome HFG, no sacral or coccygeal anomalies have been reported. In the murine counterpart, hd shows anal stenosis but not caudal vertebrae or tail defects (Post and Innis, 1999). Interestingly, caudal vertebral malformations due to mutations in the paralog Hoxd13, though, have been reported both in human SPD syndrome (Akarsu et al., 1996) and in homozygous Hoxd13 knockout mice (Dolle et al., 1993). We show that our HFGa13 construct interferes with the normal expression and function of Hoxd13 (Fig. 5, Fig. 7). Our HFGa13 phenotype may be in part an indirect phenomenon that is due to downregulation of Hoxd13.

It is curious that the human, chick and murine phenotypes differ given the same genetic alteration. It may be that there are subtle vertebral defects in human individuals with HFG not described to date. Similarly, subtle murine lumbosacral or tail abnormalities may have escaped observation in the hd or Hoxa13−/− mice. Our findings in chick may relate to the particular Hoxa13 mutation we constructed or to the relative levels of wild-type and mutant proteins in the ‘transgenic’ embryos. Or, it may be due to a different function of Hoxa13 in avian species in the posterior vertebrae compared with that of mouse and human.

A theory derived from our results suggests that the presence of a tail structure in a vertebrate species may be related to persistence of the tailgut during development. In humans and avians the tailbud undergoes apoptotic regression relatively early in development (Fallon and Simandl, 1978; Miller and Brigin, 1996), whereas in rodents the tailgut persists over a much longer relative developmental time period (Goldman et al., 2000). Although this study does not address the upstream controls of Hoxa13 expression in this caudal region, it follows that significant differences in this control should exists among species with different tail lengths.

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